

3D BIOHYBRID MUSCLE ELECTRONICS SYSTEMS

BY

YONGDEOK KIM

DISSERTATION

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Doctoral Committee:

Professor Rashid Bashir, Chair
Associate Professor Cecilia Leal
Associate Professor Qing Cao
Assistant Professor Hua Wang

ABSTRACT

Biohybrid systems consisting of biological components and artificial materials facilitate a better understanding of biological systems by recapitulating the functions and structures of native tissues. Biological machines using biohybrid systems with muscle cells or tissues and soft materials, can sense, respond, and adapt to environmental cues dynamically with biological/biomedical implications, useful for soft robotics and tissue-on-a-chip (TOC) applications. Integrating bioelectronics with such systems has a great potential to enhance its functionality such as electrical actuation of muscle, sensing of strain, and wireless communications. This thesis focuses on the integration of bioelectronics with *in-vitro* three-dimensional (3D) engineered skeletal muscle systems for muscle-on-a-chip and soft robotics applications.

Given their similarity to biological systems in structure and function, 3D biohybrid tissue systems are more attractive than two-dimensional (2D) cell sheets-based models. However, 3D integration of electronics and soft tissue is challenging due to mechanical mismatch and planar geometry of conventional electronics systems. To overcome these limitations, novel materials fabrication approaches are introduced in the thesis. Graphene offers significant mechanical flexibility, electrical characteristics, and biocompatibility. A soft polyethylene glycol diacrylate (PEGDA) substrate was used to make a conformal integration of the graphene sheet with a millimeter-scale of 3D printed soft hydrogel structure instead of using a rigid poly (methyl methacrylate) (PMMA), a conventional supportive layer for graphene wet transfer. Such a 3D graphene electrode was integrated with 3D engineered skeletal muscle tissue, showing

biocompatibility and muscular functionality with electrical stimulations via the graphene electrode.

For electrical muscle strain sensing platform, a 3D framework capable of electrical strain sensing is introduced to integrate with 3D engineered optogenetic skeletal muscle tissue. The compliant 3D framework was fabricated using a polyimide backbone with metal trace patterns on a pre-strained soft elastomer. The 3D engineered optogenetic muscle tissue was integrated with the two pillars of the 3D framework that come out after the buckling process. The full Wheatstone bridge circuits integrated into each post enabled the electrical measurement and quantification of the muscle active contractile forces supported by quantitative simulations. Electrical sensing using the 3D framework instrument provided higher accuracy and temporal resolution than the conventional optical microscopy tracking method for measuring muscle force. Furthermore, the biohybrid muscle electronics system showed long-term sensing stability and biocompatibility when monitored over a month. In addition, its sensing capability enabled the drug and chemical dose-response studies of skeletal muscle tissue, demonstrating the potential for drug screening systems, disease modeling, and medical countermeasures.

While the above two platforms are tethered for muscle-on-a-chip application, an untethered, muscle-driven wireless optoelectronics integrated robot is introduced in the following chapter. Integrating onboard electronics with biological machines can broaden a variety of novel applications across the fields of engineering, biology, and medicine. The hybrid bioelectronic muscle-driven robots were equipped with battery-free and micro-inorganic light-emitting diodes (μ -ILEDs) for wireless control and real-time communication. Additive manufacturing with 3D printing allowed the biohybrid robot design to host onboard optoelectronics, and its design and locomotion were optimized by computational evolutionary algorithm. These centimeter-scale bio-

inspired bipedal walking robots utilized an onboard microcontroller with dual panels of μ -ILEDs to independently stimulate individual optogenetic skeletal muscle actuators, allowing for remote-control of advanced robotic functions including switching, walking, turning, plowing, transport, and manipulation of multiple robots. This work paves the way for the realization of advanced functionalities using wireless optogenetic control of advanced biohybrid robotics.

Implementing biohybrid systems is highly interdisciplinary with materials science, bioengineering, mechanical and electrical engineering, and computational modeling. Through multidisciplinary approaches and active collaborations, 3D biohybrid muscle electronics systems have been introduced for advanced TOC and soft robotics applications. Integrating electronics and biohybrid systems can extend capabilities not only be shown in this thesis (ex. actuation, sensing, and wireless communication). In addition, the proposed platforms can be utilized with other types of tissue or multicellular systems for biological studies or biomedical applications. For example, integrating motor neurons with these systems would be an interesting future direction for developing neuromuscular junction (NMJ) driven autonomous biological machines and onboard neural computing.

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TABLE OF CONTENTS

CHAPTER 1: INTRODUCTION	1
1.1. Background and Motivation	1
1.2. Thesis Overview	4
1.3. References	5
CHAPTER 2: 3D GRAPHENE ELECTRODE INTEGRATED WITH MUSCLE.....	7
2.1. Introduction.....	7
2.2. Materials and Methods.....	9
2.3. Results and Discussion	13
2.4. Conclusions.....	18
2.5. Figures.....	19
2.6. References.....	26
CHAPTER 3: 3D FRAMEWORKS STRAIN SENSORS INTEGRATED WITH MUSCLE	28
3.1. Introduction.....	28
3.2. Materials and Methods.....	30
3.3. Results and Discussion	37
3.4. Conclusions.....	47
3.5. Figures.....	49
3.6. Table	76
3.7. References.....	77
CHAPTER 4: MUSCLE-DRIVEN ROBOTS WITH WIRELESS OPTOELECTRONICS	79
4.1. Introduction.....	79

4.2. Materials and Methods.....	81
4.3. Results and Discussion	91
4.4. Conclusions.....	96
4.5. Figures.....	97
4.6. Table	130
4.7. References	131
CHAPTER 5: CONCLUSIONS AND FUTURE DIRECTIONS	133
5.1. Summary and Conclusions	133
5.2. Future Directions	135
5.3. Figures.....	139
5.4. References	142

CHAPTER 1: INTRODUCTION

1.1. Background and Motivation

Biohybrid systems consist of biological components (ex. living cells or tissues) and artificial materials such as biomaterials and bioelectronics to recapitulate biological structures and functions. [1] Such systems can be very useful as tools for better understanding biological systems and for many biomedical applications such as implantable devices [2-4], regenerative medicine [4, 5], tissue-on-a-chip (TOC) [6-14], and soft robotics [15-30]. Biohybrid implants coupled with cells and biomaterials improve interfaces with native tissues and reduce foreign body reactions, so *in-vivo* biohybrid systems such as implantable devices and regenerative medicine have been developed and studied. [4] In addition, *in-vitro* biohybrid systems including TOC and soft robotics are emerging as new technologies that can have many biological or biomedical implications. [6, 15, 30] Especially, TOC, also known as organ-on-a-chip or microphysiological systems, can monitor and control *in-vitro* tissues, and show very promising potential in biomedical fields to replace animal models for disease and pharmaceutical studies. Recently, various types of TOC have been reported including lung, liver, heart, skeletal muscle, and brain. [6-14]

Among them, skeletal muscle is an attractive target, serving as the primary actuators in animals and the most abundant tissue in the human body consisting of $\sim 40\%$ of the total mass. [31] Although some skeletal muscle TOC platforms have been reported for relevant disease studies (e.g. Duchenne muscular dystrophy), most of those are limited to planar geometry. [11, 12] Given their biological relevance in structure and functions, 3D TOC platforms are more important than 2D cell sheet models. Advancements in additive manufacturing and biomaterials have enabled bio-

fabrications of 3D *in-vitro* tissues, including skeletal muscles and neurons for diverse designs. [21-26, 32]

In addition, biohybrid actuators using skeletal muscles or cardiomyocytes have emerged as a new class of soft robotics capable of dynamically sensing, responding, and adapting to environmental cues in real-time. [15-29] Although the recent advancements in materials science and artificial muscles have enabled the precise control of soft actuators, there are still many limitations in replicating the sophisticated motions of the biological musculoskeletal system. [33, 34] Meanwhile, biohybrid design strategies using cells or tissues as living actuators have demonstrated animal-like locomotion such as walking, swimming, and pumping. [15-29] In addition, the recently developed non-invasive optogenetic method enables spatiotemporally precise muscle actuations without invasive electrodes immersed in a conductive medium. [18-22] For example, biohybrid robotic stingray using optogenetic cardiomyocytes for swimming with maneuver turning and photo guidance, [18] and swimming fish with biomimicking of human cardiac muscles and autonomous swimming with body caudal fin propulsion have been demonstrated [19]. Like these, 3D skeletal muscle-based bio-hybrid machines have been reported with various types of soft robots, such as walkers, swimmers, and grippers, offering a wider range of design customizations with 3D geometry and better controllability compared to 2D spontaneously actuated cardiomyocytes sheets [20-28].

Additive manufacturing and bio-fabrication techniques can enable the fabrication of 3D-engineered skeletal muscle-based biological machines with mm to cm scales. [20-25] The 3D printing technologies using stereolithography apparatus or digital light processing can allow for design customizations and fabrications of biocompatible hydrogel structures with polyethylene glycol diacrylate (PEGDA) through photopolymerization. Mechanical properties of 3D printed

hydrogel skeleton can be easily tuned by changing the molecular weight of PEGDA, concentrations of PEGDA and photoinitiator and exposed light energy dose. The 3D engineered muscle tissue was fabricated by seeding myoblasts with extracellular matrices (ECM) mixture including Matrigel, fibrinogen, and thrombin onto the seeding mold. The thrombus reaction between fibrinogen and thrombin polymerized from cell-ECM solution into the cell-embedded fibrin gel. After muscle compaction and differentiation from myoblasts to myotubes, 3D engineered skeletal muscle tissue can generate the actuation from electrical stimulations and cause the deformation of soft hydrogel structure, resulting in the motion of a biological machine. With optogenetically transfected myoblast enabled muscle actuation and robot control using the light stimulations.

Integrating electronics with such *in-vitro* biohybrid systems can be highly synergistic to improve its functionality and applicability for advanced TOC and biohybrid robotics. Recent advances in bioelectronics have enabled various functions in biomedical applications such as transient electronics, neuromodulation or sensing with optogenetics, and bionics. [35-40] Electronics integrated TOC systems can monitor and control biological and physiological processes across a wide range of electrical, biochemical, and mechanical signals. [8, 9, 13, 14, 41, 42] However, integrating electronics systems with 3D tissue models for bioelectrical application remains challenging due to mechanical and geometrical mismatches. Almost all conventional electronics adopt simple planar geometry and rigid substrates because of their limitations in building functional 3D structures as scaffolds and electrically functional interfaces. [13, 14] Therefore, novel materials and fabrication approaches are required to achieve 3D electronics integrated TOC platforms. Not only TOC platforms, but the integration of onboard electronics

systems and wireless communications with the biohybrid robots will also be an attractive option to enhance functionality and controllability.

In this regard, this thesis focuses on the 3D biohybrid skeletal muscle system integrated with electronics for electrical muscle actuation, strain sensing, and wireless communications, eventually for advanced TOC and biohybrid robotics applications.

1.2. Thesis Overview

This thesis focuses on integrating 3D engineered skeletal muscle tissue and electronics to enable electrical muscle actuation (Chapter 2), muscle strain sensing (Chapter 3), and wirelessly controlled local optical stimulations to actuate muscle and biohybrid robot control (Chapter 4). Chapter 2 introduces how graphene electrodes were integrated with 3D hydrogel structure and engineered muscle tissue for electrical actuation. In Chapter 3, buckled strain sensors were utilized for electrical sensing of optogenetic muscle actuation from optical and chemical stimuli as potential drug screening systems, disease modeling, and medical countermeasures. Chapter 4 introduces biohybrid electronics robots integrating wireless optoelectronics with muscle-driven biological machines for remote control and advanced functions. Finally, Chapter 5 discusses conclusions and future directions such as neuromuscular junction (NMJ)-driven biological machines by adding optogenetic motor neurons.

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CHAPTER 2: 3D GRAPHENE ELECTRODE INTEGRATED WITH MUSCLE

2.1. Introduction

In this chapter, the goal is to integrate the 3D electrode integration with a soft hydrogel-based biological machine to actuate the muscle tissue. However, conventional electronics system is limited as they mainly adopt 2D planar geometry and rigid substrates. Biocompatibility also needs to be considered for muscle tissue integration.

In this regard, graphene is a good candidate for electrode integration onto the 3D soft hydrogel structures and muscle tissue. Graphene, a 2D monolayer of sp^2 -bonded carbon with a honeycomb lattice structure, is a promising nanomaterial for applications such as bioelectronics, imaging, drug delivery, and tissue engineering because of its unique optical, electrical, mechanical, and biological characteristics. [1-3] Graphene and its derivatives have also found many applications in tissue engineering and regenerative medicine. [4, 5] Among its tissue engineering applications, the enhanced adhesion, proliferation, and myogenesis of skeletal muscle cells on graphene have been reported on 2D substrates. [6-8] Although 3D structured graphene systems have been reported as a tissue scaffold using 3D printing or dry spinning with graphene derivatives such as reduced graphene oxide or graphene oxide, they have a limited electrical conductivity due to the presence of an insulating additive polymer and/or defect functional groups. [2, 9-12] CVD synthesized

*This chapter has been adapted from the following publication and the copyright owner has granted permission to reprint (Appendix A): Yongdeok Kim, Gelson Pagan-Diaz, Lauren Gapinske, Yerim Kim, Judy Suh, Emilia Solomon, Jennifer Foster Harris, SungWoo Nam, Rashid Bashir. "Integration of Graphene Electrodes with Three Dimensional (3D) Skeletal Muscle Tissue Models" *Adv. Healthcare Mater.* 9, 1901137 (2020).

graphene, on the other hand, is known to be more uniform and compatible with large area synthesis. [13, 14]

Previous works have been reported on 3D integration using CVD-grown graphene. [15-18] Lanza et al. and Shim et al. integrated graphene onto pyramidal atomic force microscopy tips with a thin PMMA film as a supportive layer. [15, 16] Choi et al. used a 3D patterned swollen polydimethylsiloxane (PDMS) to transfer PMMA-coated graphene onto various 3D features such as pyramids, domes, and pillars ranging from 3.5 to 50 μm . [17] They used a thinner PMMA as a supportive layer for 3D graphene transfer to reduce flexural rigidity. However, the rigid PMMA is still limited for 3D integration with other polymeric substrates or at larger scales. Other than PMMA, Morin et al. used PDMS and polyimide film as a supportive layer and applied vacuum to result in conformal integration of graphene onto the 3D printed implant. [18] However, this platform had variable coverage of graphene ranging from 74 to 95 % and no electrical application of 3D graphene was demonstrated. Realizing electrical contacts to 3D tissue models can result in new ways to interrogate the function of these biological tissues.

Skeletal muscles, serving as the primary actuator in animals, have also been used as biohybrid actuators in soft robotics. [19-21] Integration of 3D engineered skeletal muscle embedded in extracellular matrix (ECM) on 3D printed hydrogel skeleton has been shown for walking or pumping functions using electrical or optical stimulation. [22-24] If these biohybrid machines can be integrated with electronics, a suite of applications can be possible for electrical actuation control and sensing.

Here, we report a biocompatible 3D graphene transfer method using a PEGDA supportive layer and its integration with a 3D skeletal muscle ring. A softer PEGDA layer enabled the conformal integration of graphene with millimeter-scale 3D printed PEGDA pillars. Considering

that PEG is an FDA-approved biocompatible material, it is well suited to 3D biological applications with integrated electronics. As an electrical application of our 3D electrode integrated tissue system, a 3D skeletal muscle ring on the integrated structure was actuated electrically with different voltages and frequencies via the transferred graphene contacts to the muscle ring. This platform can provide electrical contact with 3D in-vitro tissue-on-chip models for drug screening and applications in soft robotics.

2.2. Materials and Methods

2.2.1. Synthesis of graphene and PEGDA sheet

The graphene was grown on Cu foil (Alfa Aesar, 0.001 inches) using chemical vapor deposition (CVD) with CH₄, H₂, and Ar gases. After loading the Cu foil in the quartz chamber, it was heated to 1050 °C with 25 °C min⁻¹ of ramp rate under 5 sccm of H₂ and 100 sccm of Ar gases flow. After ramping to 1050 °C, the sample was annealed for an additional 1 hour, then 10 sccm of CH₄ flowed for 1 hour at the same temperature. Then, the chamber was cooled to room temperature under Ar flow. To make a polyethylene glycol diacrylate (PEGDA) layer, a 30 % (w v⁻¹) of PEGDA 3400 g mol⁻¹ (Laysan Bio) and a 5 % (w v⁻¹) of Irgacure 2959 (Ciba) as a photoinitiator in dimethylformamide, was prepared separately. After mixing them with a 99:1 (PEGDA 3400: Iracure) ratio, the solution was filtered using 0.2 um of the filter. Then, it was spin-coated on the CVD-grown graphene on copper at 1000 rpm for 90 seconds and polymerized in 500 mJ cm⁻² of UV exposure. [25] The thickness of coated PEGDA layer was measured by the ellipsometer.

2.2.2. 3D Printing of mold and pillars

Stereolithography (SLA) was used to print 3D structures. Figure 2.1 (c) shows the CAD design for the two pillars structure and the ring mold was used from the previous design. [22] For the pillars, a 20 % (v v⁻¹) of PEGDA 700 g mol⁻¹ (Sigma Aldrich) with 0.5 % (w v⁻¹) of the Irgacure 2959 was polymerized by a 380 nm laser. A 20 % (v v⁻¹) of polyethylene glycol diacrymethyleate (PEGDMA) was used. Fabricated parts were sterilized in 70 % ethanol for 1 hour and stored in PBS before use.

2.2.3. Integration of a graphene / PEGDA sheet with printed pillars

The backside of graphene on copper was removed using oxygen plasma. Then, it was flipped to face the PEGDA layer on the bottom of the copper etchant (Sigma Aldrich) and the graphene side on the top after copper removal (Figure 2.1 (a)). After etching the copper layer, it was transferred from etchant to distilled water and rinsed three times through repeated transfers in distilled water. Finally, the graphene/PEGDA sheet on the water was transferred onto the 3D printed pillars as shown in the side and top views in Figure 2.1 (f) and (g).

2.2.4. Scanning electron microscopy (SEM) imaging

The graphene/PEGDA sheet on the water was transferred onto the Silicone substrate. The sample was imaged with FEI Quanta FEG 450 ESEM.

2.2.5. Muscle ring fabrication

Skeletal muscle C2C12 was cultured using Dulbecco Modified Eagle Medium (Gibco) with L-glutamine and pyruvate containing 10% (v v⁻¹) fetal bovine serum (ThermoFisher), 1% (v

v⁻¹) external L-glutamine, and 1% penicillin and streptomycin (Lonza). Figure 2.2 shows how ECM embedded muscle ring was formed by thrombus reaction with thrombin and fibrinogen. They were seeded on a printed PEGDMA ring mold after mixing with 30% Matrigel (ThermoFisher), thrombin (Sigma Aldrich), and fibrinogen (Sigma Aldrich). A cell media with 1 mg ml⁻¹ aminocaproic acid (ACA) was added to the ring mold 1.5 hours after seeding cells to prevent proteolytic degradation. [26] Three days later after seeding, the rings were transferred to a graphene sheet on the pillar structure. Then, differentiation media was added. The differentiation media consists of DMEM including 10 % Horse serum (Thermo Fisher), 1% L-glutamine, 1% penicillin and streptomycin, 1 mg ml⁻¹ of ACA, and 5 ug ml⁻¹ of insulin growth factor (Sigma Aldrich).

2.2.6. Raman Spectroscopy

Renishaw Raman/PL Micro-spectroscopy was used with the excitation wavelength of 633 nm, 10 seconds accumulation, 50X objective lens, and 1800/mm grating.

2.2.7. Calculation for passive tension

The passive tension for muscle ring on pillars was calculated using the Euler-Bernoulli beam-bending theory as per the below equation.

$$F_p = \frac{8EI\delta}{l^3}$$

Where F_p is the passive tension and δ is the measured deflection displacement. E represents the Young's Modulus, which was measured to be 150 kPa. The other constant values were calculated for the pillar dimension, where I is the moment of Inertia, and l is the height of the pillar.

2.2.8. Cell viability test

For the cell viability test, the MTS assay was used by combining MTS solution (CellTiter 96 Aqueous One Solution Cell Proliferation Assay, Promega) with warm DMEM without phenol red with a 1:5 ratio. Muscle rings were rinsed with PBS and incubated with 360 μ l of solution for 4 hours at 37 °C. Then, absorbance at 490 nm was used to measure cell viability.

2.2.9. Electrical stimulation and recording of muscle contraction

For electrical stimulation, a custom-built electrical stimulation setup was used. [27] The pulse output from a function generator was amplified by an AD797 inverting amplifier. A capacitor was added in series to minimize the electrolysis of the media by converting the square pulse to a biphasic pulse.

2.2.10. Immunofluorescence imaging

Muscle tissue was rinsed with PBS and fixed in 4% ($v v^{-1}$) paraformaldehyde for 20 minutes. The 0.25% ($v v^{-1}$) of Triton-X was used to permeabilize the tissue membrane for 20 minutes. Then, the sample was blocked and stored in 1% ($w v^{-1}$) of bovine serum albumin (Sigma Aldrich) at 4°C overnight. The primary antibodies, mouse anti-myosin heavy chain (MF-20) and rabbit anti α -actinin, were used to stain for myosin and the sarcomere, respectively, with a 1:500 and 1:200 dilution ratio, then, incubated overnight at 4°C. The secondary antibodies, AlexaFluor-488 anti-rabbit and AlexaFour-564 anti-mouse (ThermoFisher), were used to stain α -actinin and MF-20 antibodies, respectively, and incubated overnight with DAPI to stain the nucleus at 4°C. Between each step, the sample was washed with PBS three times for 5 minutes. The Multiphoton Confocal Microscope Zeiss 710 was used for fluorescence imaging.

2.2.11. Muscle creatine kinase assay

Muscle rings on 3D graphene/pillars were stimulated electrically with 1 Hz and 20 V of amplitude every 20 minutes from day 4 to day 7 after ring transfer, while control rings on pillars without graphene were not stimulated. On day 7, muscle rings were digested using RIPA buffer (Thermo Scientific) with sonication and centrifugation. Next, protein extract supernatant was collected and used to determine muscle creatine kinase (MCK) production with a Liquid Creatine Kinase Regent Set (Pointe Scientific). Then, absorbance at 340 nm was measured for MCK activity.

2.3. Results and Discussion

A PEGDA layer was used as a support layer on CVD-grown graphene for 3D conformal integration onto millimeter-scale 3D printed pillars. The flexural rigidity, defined as the resistance offered by a structure while undergoing bending, is an important factor for 3D integration. [17] A lower flexural rigidity is beneficial for 3D integration because the film can be more easily deformed under bending. Equation (1) shows that the flexural rigidity is proportional to Young's modulus, where D is the flexural rigidity, E is Young's modulus, h is the film thickness, and ν is the Poisson's ratio.

$$D = \frac{Eh^3}{12(1-\nu^2)}$$

Hence, a softer PEGDA support layer with a few tens of kPa of E is more beneficial for bending than a few GPa range of E for PMMA, which is typically used as a graphene support layer. The E is also larger than the MPa range of PDMS which Morin et al. used for 3D graphene integration.¹⁸ Although the spin-coated PEGDA layer has a thickness of about a micron and is much thicker than the usual PMMA coating layer on graphene, the large difference in Young's modulus dominates the lower flexural rigidity of the PEGDA layer than that of PMMA. In

addition, a thick PEGDA layer can provide mechanical stability to prevent the graphene layer from being torn off during the 3D transfer.

Figure 2.1 shows the fabrication steps to integrate a graphene sheet on 3D printed pillars and a muscle tissue ring. A PEGDA support layer on CVD-grown graphene on copper foil was polymerized by spin coating and UV exposure via the addition of a photoinitiator. The copper foil was etched in ferric chloride-based copper etchant after turning PEGDA-coated graphene upside down, with the graphene layer facing the top and PEGDA at the bottom as shown in Figure 2.1 (a). Figure 2.1 (b) shows the graphene/PEGDA sheet on water after etching of copper. Figure 2.3 shows the microstructure in the SEM images of the graphene/PEGDA sheet. Figure 2.1 (c), (d), and (e) show the CAD design for a printed pillar structure with dimensions to integrate with graphene and muscle, and the side and top images of the structure printed by stereolithography apparatus (SLA), respectively. After the graphene / PEGDA sheet was transferred onto printed pillars, the conformal integration of the sheet and pillar structure can be seen in Figure 2.1 (f) and (g). The muscle ring was then prepared with the printed mold shown in Figure 2.1 (h). The specific method for the creation of the 3D muscle ring is described in Figure 2.2. After the integration of the muscle ring, the graphene / PEGDA sheet is shown in Figure 2.1 (i). The detailed fabrication steps and Experimental methods are described in the Supporting Information.

Figure 2.1 (j)-(l) shows the graphene characterization after graphene transfer on 3D printed pillars. In Figure 2.1 (j), D, G, and 2D bands of graphene's Raman spectra are observed on the top of the graphene-transferred pillar structure. Significantly higher 2D band intensity compared to the G band implies that the synthesized graphene is a monolayer. Raman shift peaks of PEG are due to the PEGDA supportive layer beneath the graphene. The shifts for 2897 cm^{-1} and 2843 cm^{-1} are caused by the symmetric stretching vibration of the methylene group in PEG. [28] Also, the peak

for 1470 cm^{-1} of $\text{CH}_2\text{-CH}_2$ bending vibration from PEG is also shown. [29] The differences in intensity of PEG are similar to other references in that the peaks from symmetric stretching vibration of the methylene group are larger than those for $\text{CH}_2\text{-CH}_2$ bending vibration. [28, 29] The Raman measurement clearly shows that the graphene and PEG supportive layer was successfully transferred onto the top of the pillars.

For many applications, the electrical conductivity of the transferred graphene electrode is an important characteristic. To measure the electrical conductivity of the 3D transferred graphene sheet, silver paint was applied on the 4 spots on the graphene sheets shown in Figure 2.1 (k). I-V curves were measured by contacting probes on the corresponding painted spots. The difference in resistances between different spots is reasonable because the distance between two points for the left and right bottoms is longer than those for the other two sets, which have a similar range of sheet resistance from 77 k to $120\text{ k } \Omega\text{ sq}^{-1}$. Considering around $800\text{ } \Omega\text{ sq}^{-1}$ of graphene sheet resistance and the distance between silver paints, the 3D transferred graphene has a higher resistance than common planar graphene. [13, 30, 31] This is because the 3D graphene was supported by a PEGDA hydrogel, which was swollen due to wet transfer and could have resulted in defects in the graphene layer as shown in Figure 2.4 (on top of pillar after 3D transfer). Figure 2.4 (a) (same as Figure 2.1 (j)) indicates that the red spot in Figure 2.4 (b) has Raman peaks for D, G, and 2D bands of graphene and PEG, while the blue circle contains only Raman shifts for PEG and not graphene, as shown in Figure 2.4 (c). Although the 3D transferred graphene has a higher resistance than the planar one, they showed the linear I-V curves and expected Raman shifts.

Figure 2.5 shows the muscle ring characterization on the 3D graphene integrated with printed pillars. Images of a muscle ring on the structure before ring transfer and on days 1, 3, 5, 7, and 10 after ring transfer are shown. The muscle ring had been formed and cultured in a

differentiation medium for 10 days as described in earlier reports. [22, 23] Graphene sheet 10 days after the ring transfer is shown in Figure 2.5 (f). Figure 2.6 shows immunofluorescence images for the aligned longitudinal myotubes in the muscle ring on day 7 after ring transfer onto the 3D graphene integrated structure. As the C2C12 skeletal muscle differentiates from myoblasts to myotubes, the compaction of the muscle ring decreased the distance between the pillars as shown in Figure 2.5 (g). The passive tension calculated based on the Euler-Bernoulli beam-bending theory, [22] was 0.67 ± 0.28 mN right after the ring transfer and increased to 1.87 ± 0.35 mN on day 10 after ring transfer and is in a similar range as previous studies. [22] The cell viability in the muscle ring was determined by MTS cell proliferation assays. Cells in muscle rings cultured on a graphene sheet showed about a 7% higher viability level than those on pillars without the graphene sheet in Figure 2.5 (h). This is expected, as graphene is known to promote proliferation and differentiation of the skeletal muscles. [6, 8] Based on the passive tension results and the cell viability results, it can be concluded that the 3D graphene can be integrated with the tissue in a biocompatible manner.

Next, electrical stimulation was applied to the muscle ring with the transferred graphene electrode, as shown in Figure 2.7 (a). To make a connection with the graphene sheet, silver paint and copper tapes were used for both ends outside the cell culture well as shown in Figure 2.7 (b). Figure 2.7 (c) shows the graphene sheet extended from the muscle ring to the well. When a 5V signal was applied, no actuation was observed in the pillars. However, increasing the voltage amplitude to 10V and 20V resulted in a noticeable displacement change as shown in Figure 2.7 (e) and (f), respectively. The average displacements of the pillars were 3.9 ± 1.0 μm for 10V and 8.2 ± 1.3 μm for 20V, respectively. Figure 2.8 (a) shows the circuit of electrical stimulations set up using the 3D graphene electrode. The bias from the graphene electrode located between the muscle

ring and pillar could stimulate muscle tissue to depolarize membrane potential and actuate it. Figure 2.8 (b) represents the electrical circuit at the cellular level. The bias voltage between the intracellular and extracellular areas originating from graphene electrodes on pillars or cell media needs to be larger than the threshold voltage of 25 mV to depolarize the skeletal muscle cell membrane. In applied 5V, lower bias in the region cannot depolarize the myotubes, while a larger bias than 25 mV between cell membrane resulted in the 10V and 20V stimulations.

Various pulse frequencies of a given voltage through the graphene electrode also resulted in pillar displacement caused by muscle actuation. When a 0.5 Hz, 20V pulse was applied, the pillars moved with 0.5 Hz as shown the Figure 2.7 (g). In Figure 2.7 (h), a 2 Hz stimulation also matched with the movement from pillars, although the displacement did not fully recover to zero because of the viscoelastic properties of the printed PEGDA pillars. Figure 2.9 indicates that the amplitudes and frequencies of the pillar displacement caused by muscle twitching are proportional to the applied frequencies and voltages for electrical stimulation through the graphene electrode.

Furthermore, muscle creatine kinase (MCK) activity was measured for muscle ring with electrical stimulation through a 3D graphene structure to observe the effect of electrical stimulation on muscle tissue, as shown in Figure 2.7 (i). Although muscle rings with electrical stimulation on 3D graphene appear to show a slightly higher level of MCK activity than the control group without graphene and electrical stimulation, the p-value is 0.1165 considered to be not statistically significant. Our results show that the 3D transferred graphene electrode can actuate the integrated muscle rings and the availability of the platform for the 3D muscle tissue model with electrical stimulation.

To confirm that the electric signals go through the graphene and not through the fluid medium, we designed another electrical stimulation experiment as shown in Figure 2.7 (j). Two

pieces of graphene sheets were transferred to the glass substrate with the printed pillars such that they were not touching with pillars. The integrated muscle ring on the pillars did not contact the graphene sheets as shown in Figure 2.7 (k). When a 1Hz, 20V electrical pulse was applied to the graphene on the substrate, no change in pillar displacement was observed as shown in Figure 2.7 (l) confirming that the electrical contacts do indeed function as they were designed to.

2.4. Conclusions

We report a biocompatible CVD graphene transfer method using a PEGDA supportive layer. The soft PEGDA layer enabled a 3D conformal integration of graphene with a millimeter-scaled 3D printed pillar and a muscle tissue ring. After the 3D transfer, Raman spectroscopy and electrical measurement results demonstrated a conductive graphene electrode on the 3D structure. The integrated muscle tissue ring also showed similar compaction during cell differentiation and subsequent cell viability as compared to control rings without the transferred graphene sheet. The integrated muscle ring responded to the electrical stimulation through the transferred graphene electrode with various applied voltages and frequencies. Our results demonstrate a new method for 3D graphene electrode integration as a potential platform for electrical applications for 3D biological tissue systems. However, its tethered electrode system can be limited to apply to untethered biohybrid robotics. Incorporating a wireless communication system will be the next direction to transfer the electrical field to 3D integrated electrodes and stimulate the muscle actuator.

2.5. Figures

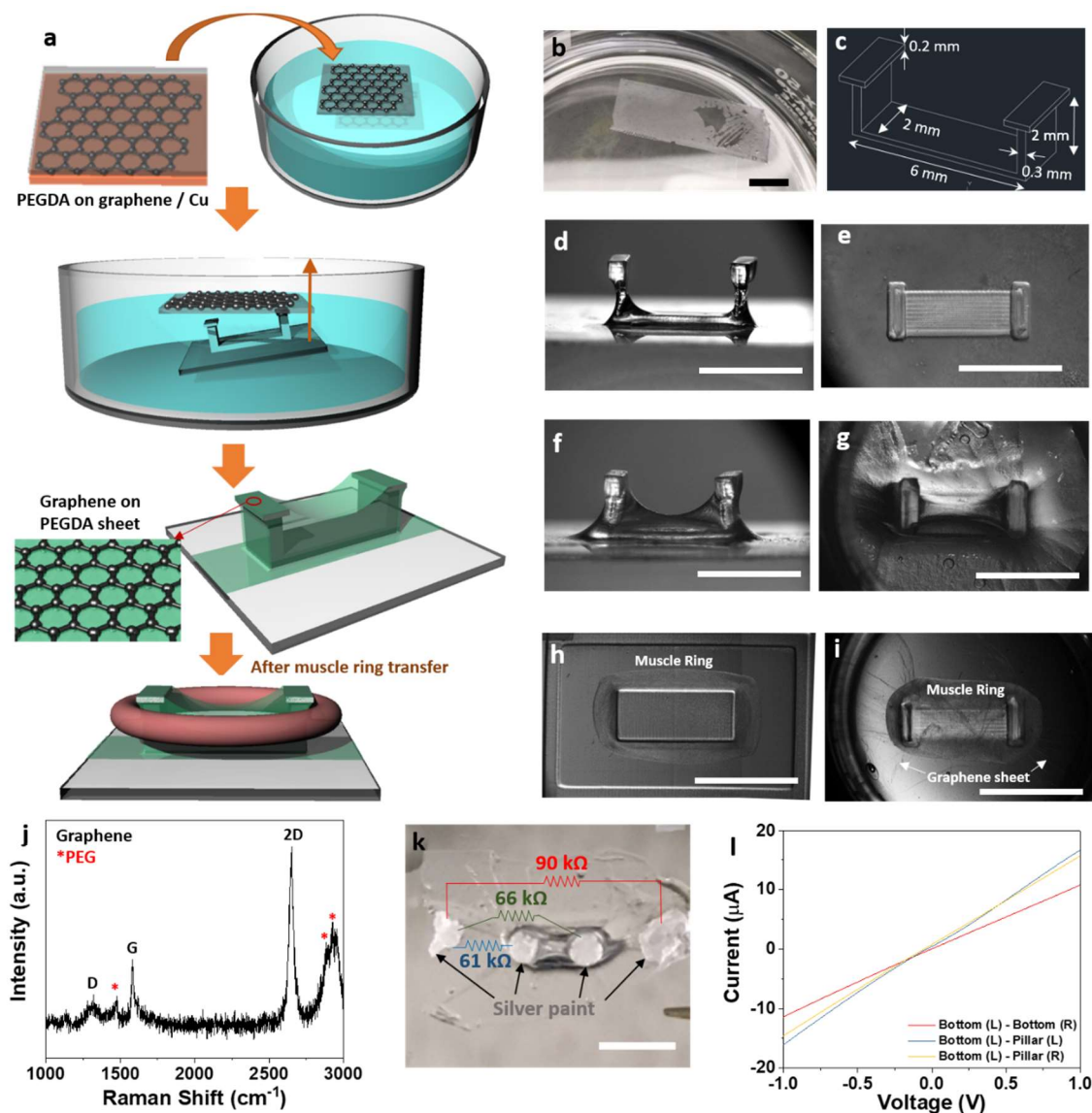


Figure 2.1. Fabrication for 3D graphene and muscle ring transfer onto the 3D printed PEGDA pillars. (a) Schematic of how to fabricate the 3D graphene with muscle ring. (b) Image of graphene / PEG sheet in DI water after Cu removal. (c) 3D CAD image and dimensions of printed PEGDA pillars. (d) Side and (e) top views of 3D PEG printed pillars. (f) Side and (g) top views of pillars after graphene transfer. Images for muscle ring (h) before transfer in the ring mold and (i) on the graphene sheet / PEG pillars after ring transfer. (j) Raman signal of graphene / PEG sheet on the top of pillar after graphene transfer. (k) The image of silver paints and graphene / PEG sheet on the pillars for measurement of electrical resistance between silver-painted electrodes. (l) I-V curves between left outside and right outside, left outside and on the left pillar, and left outside and on the right pillar. Scale bars: 5 mm.

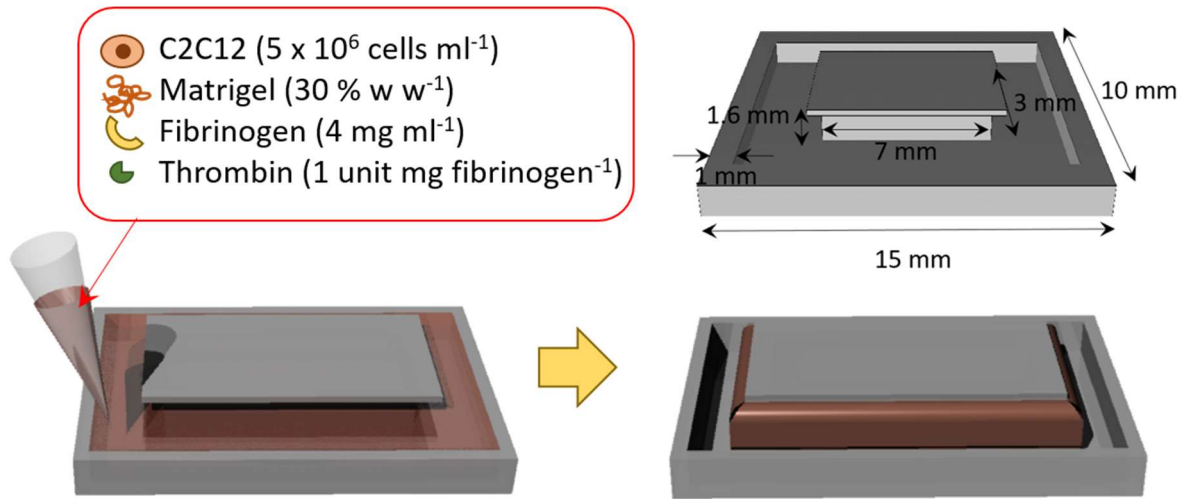


Figure 2.2. Schematic of fabrication of 3D muscle ring embedded extracellular matrix on printed ring mold. A C2C12 cell solution in media with a cell concentration of $5 \times 10^6 \text{ ml}^{-1}$ is mixed with 30 % w w^{-1} of Matrigel, 4 mg ml^{-1} of fibrinogen, and 1 unit mg of fibrinogen $^{-1}$ of thrombin. Then, 100 μl of the mixture was seeded on the printed ring mold. The liquid solution was solidified by a thrombus reaction in 1 hour.

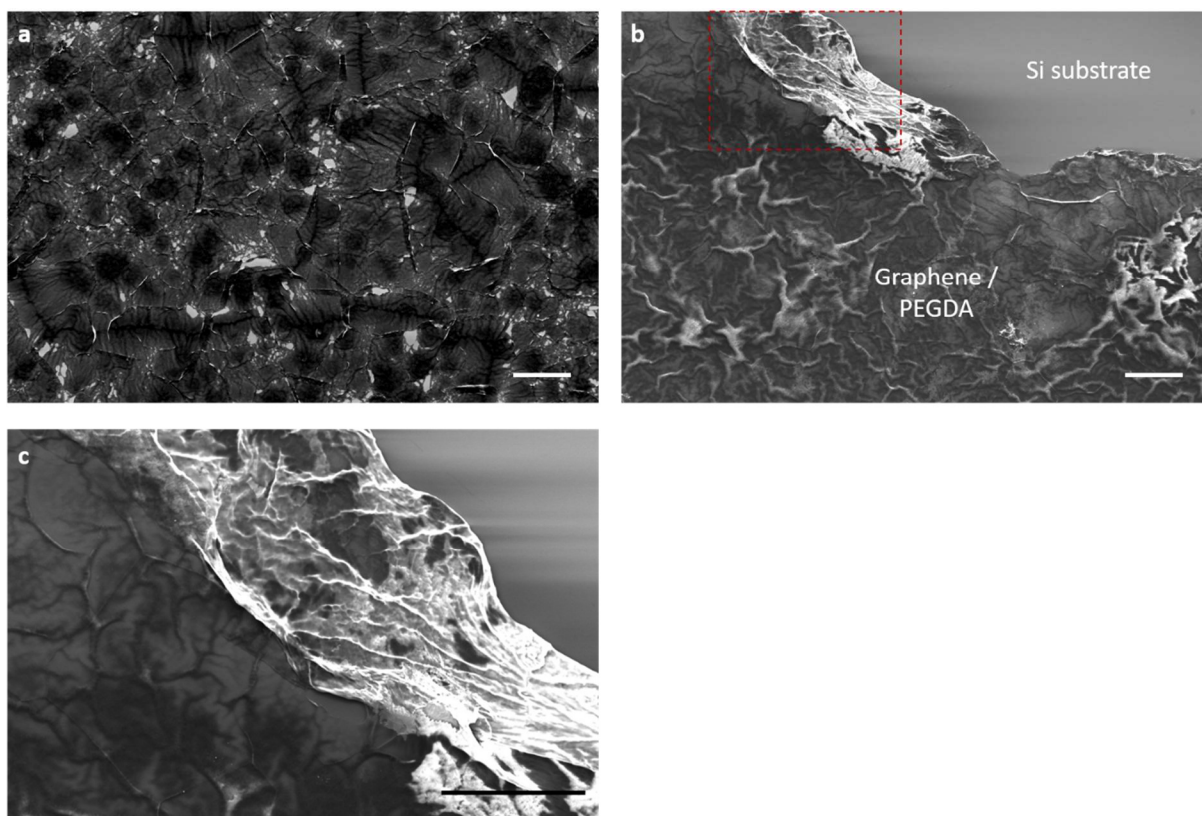


Figure 2.3. SEM images for graphene / PEGDA sheet on Silicone substrate. SEM images for (a) inside of the sheet, (b) edge of the sheet with the substrate, and (c) enlarged image from the red dotted square in (b). The different morphology between Si substrate and graphene / PEGDA sheet in (b), while the electron charging with white was observed on the edge of the sheet because the PEGDA was exposed to the top in the (b) and (c). The graphene / PEGDA sheet looks wrinkled because shrinkage of the PEGDA support layer occurred during the sample preparation for SEM. Scale bars: 100 μm .

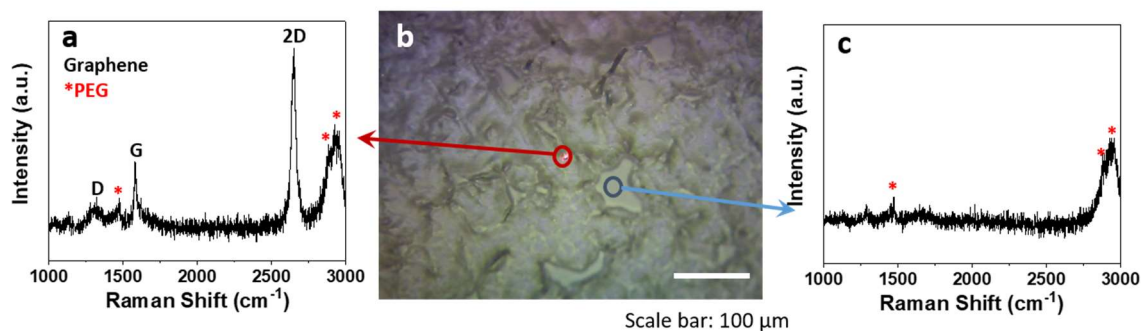


Figure 2.4. Raman signals from graphene electrode after 3D transfer. (a) Raman signal of the red spot in the image (b). (b) Optical image of graphene / PEG sheet on the top of pillar after was graphene transfer. Scale bar: 100 μm . (c) Raman signal of the blue spot in the image.

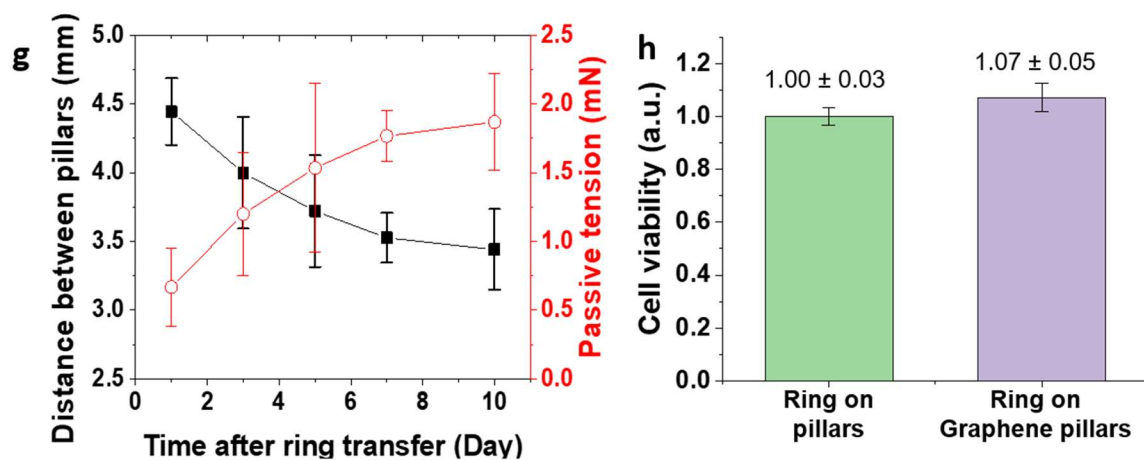
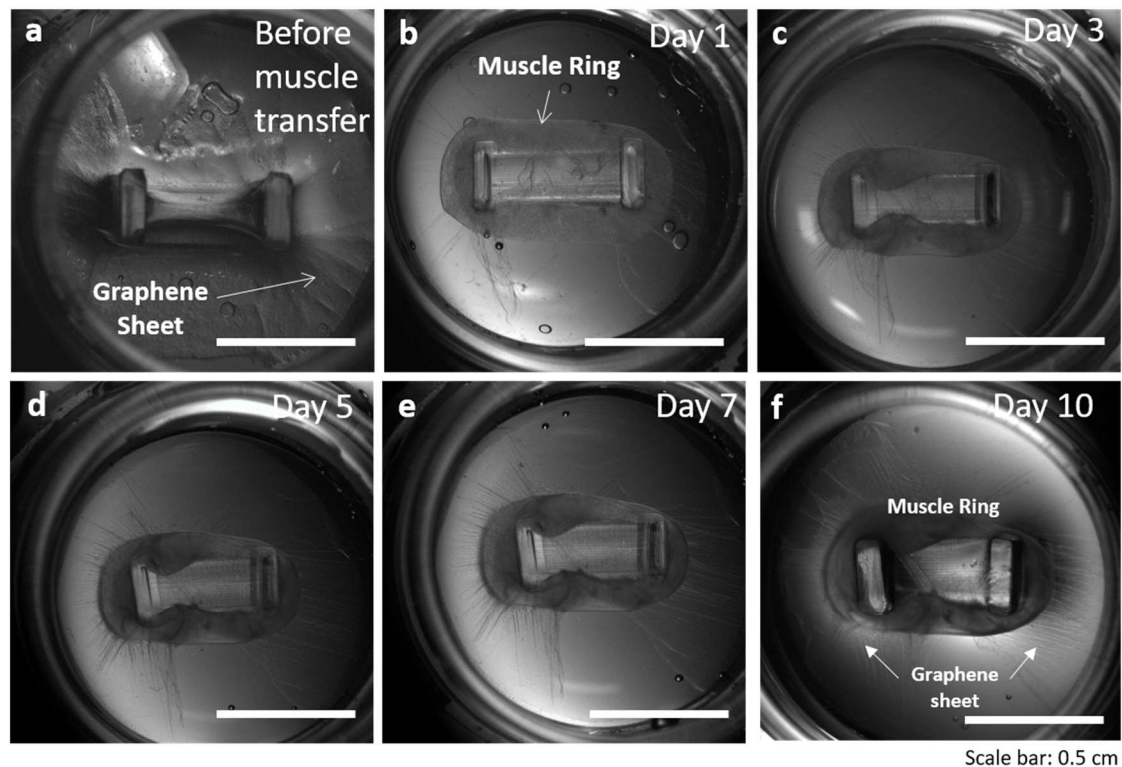


Figure 2.5. The interaction of muscle ring with 3D graphene / PEG pillars. Optical images of 3D graphene / PEG pillars (a) before muscle ring transfer, (b) day 1, (c) day 3, (d) day 5, (e) day 7, and (f) day 10, after muscle ring transfer. (g) The distance between pillars and passive tension with different days after muscle ring transfer. (n=3) (h) Cell viability was determined by MTS cell proliferation assay of muscle ring on the graphene / PEGDA pillars and PEGDA pillars without graphene on day 5 after ring transfer.

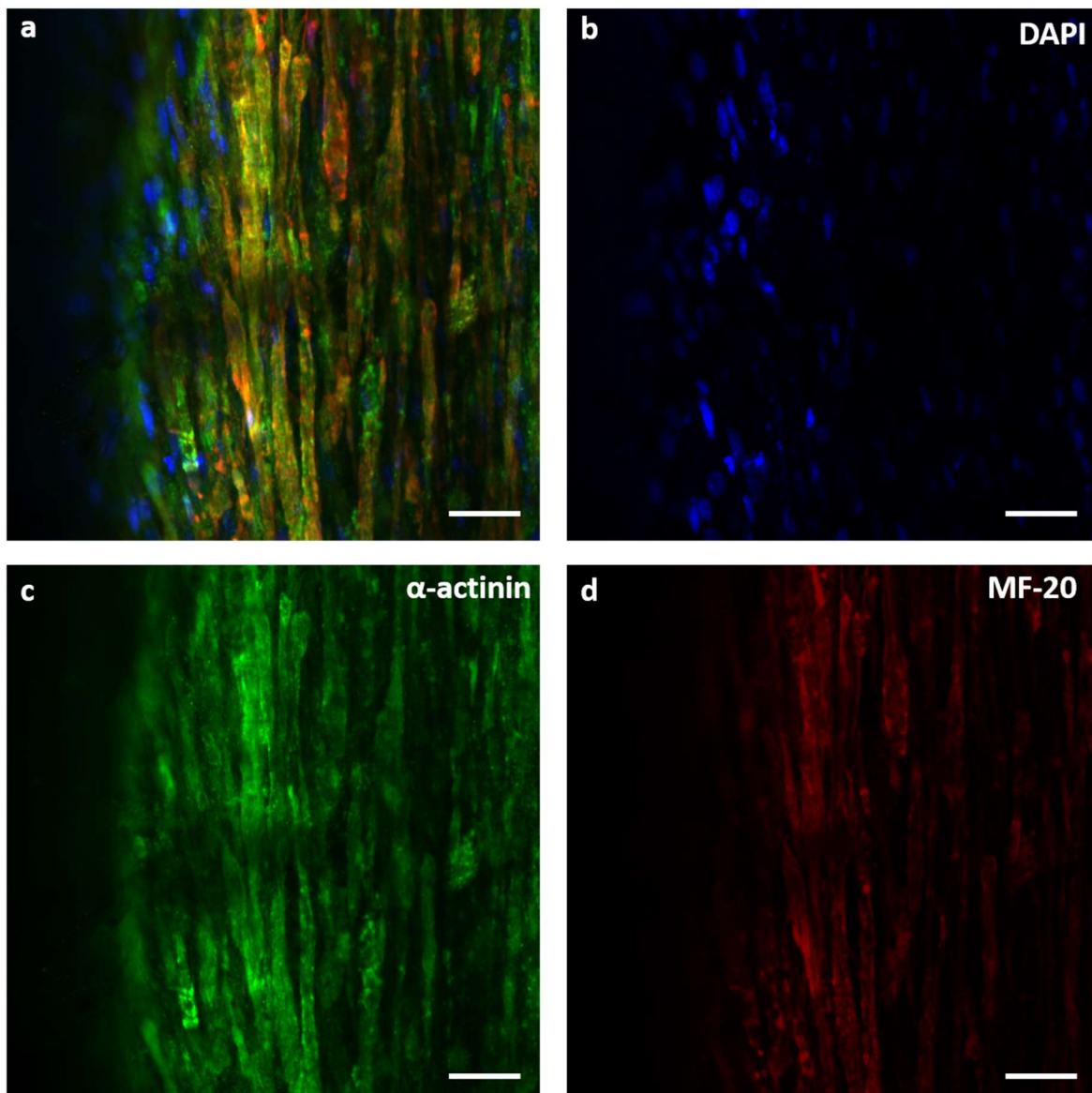


Figure 2.6. Immunofluorescence images for muscle ring with 3D graphene / PEG pillars on day 7 after ring transfer. The ring was electrically stimulated through a graphene electrode with 1 Hz and 20 V for every 20 minutes from day 4 to 7. (a) Combined image with staining by (b) DAPI for blue, (c) α -actinin for green, and (d) MF-20 for red. Scale bars: 50 μ m.

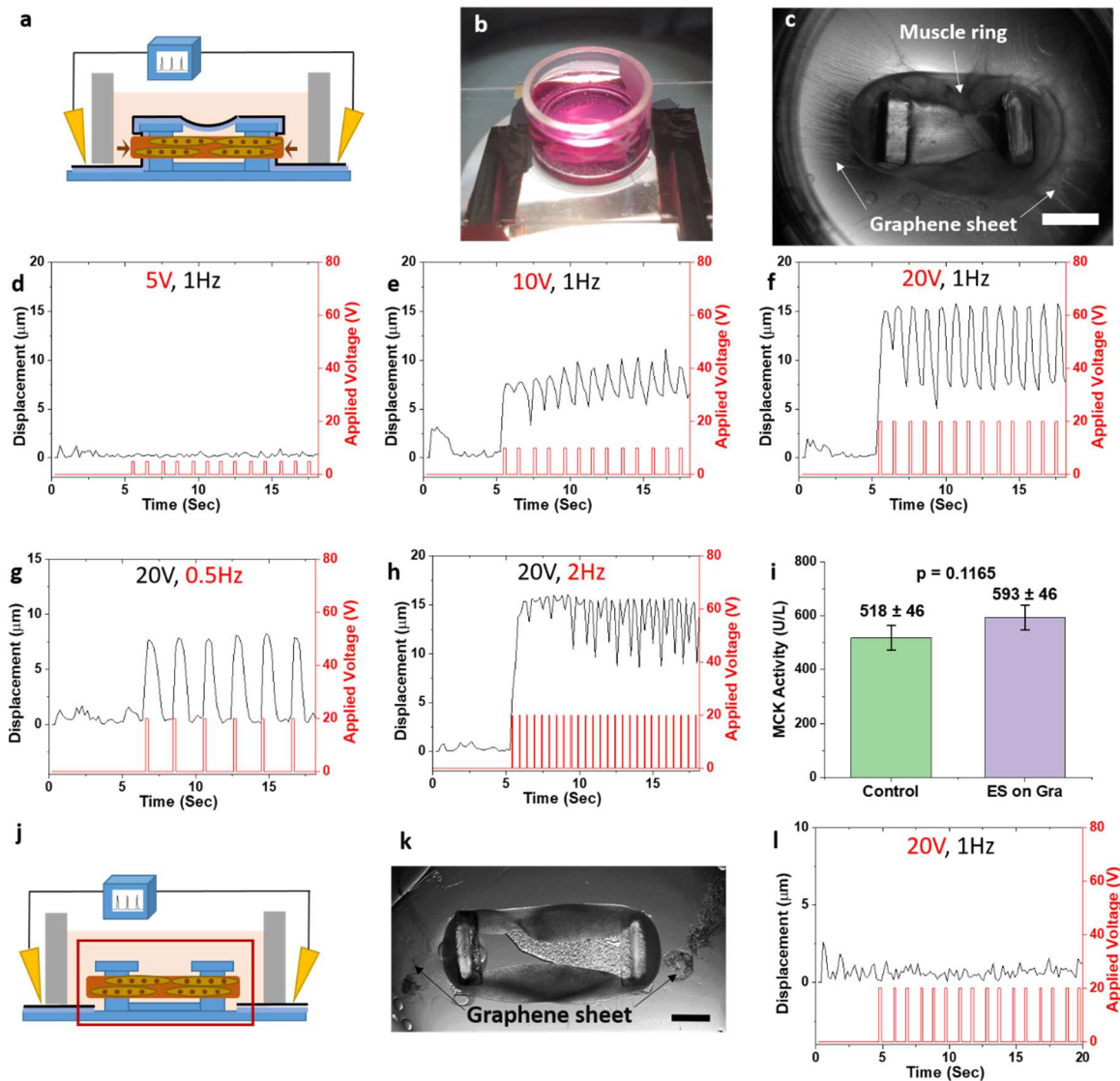


Figure 2.7. Electrical stimulation to the muscle ring through conformal graphene electrode. (a) Schematic of the system and (b) image of the device for electrical stimulation. (c) Optical image of the muscle ring on the graphene / PEG pillars. Scale bar: 2 mm. Displacement of pillars – time curves when graphene electrode was electrically stimulated by voltage amplitude of (d) 5, (e) 10, and (f) 20 V, with 1 Hz and frequencies of (g) 0.5 and (h) 2 Hz at 20 V of voltage amplitude. (i) Muscle creatine kinase (MCK) activities for muscle rings on pillars without graphene as a control group and graphene / PEG structure with electrical stimulation on day 7 after differentiation (n=3). Control experiment with graphene sheet only in contact with media separated from muscle ring. (j) Schematic and (k) optical image for electrical stimulation to muscle ring with graphene sheet not touching the muscle. (l) No displacement of pillars is measured with time when graphene is not touching the muscle ring. Scale bars: 1 mm.

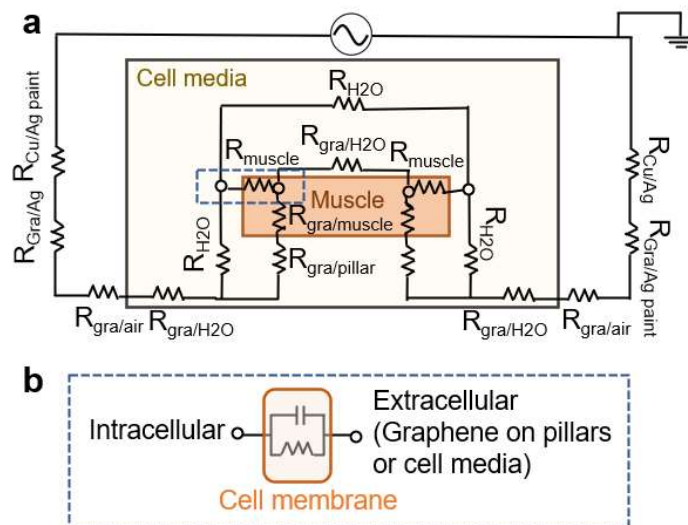


Figure 2.8. Electrical circuit of 3D graphene stimulations set up for (a) whole system and (b) cellular scale of blue dotted square area in (a).

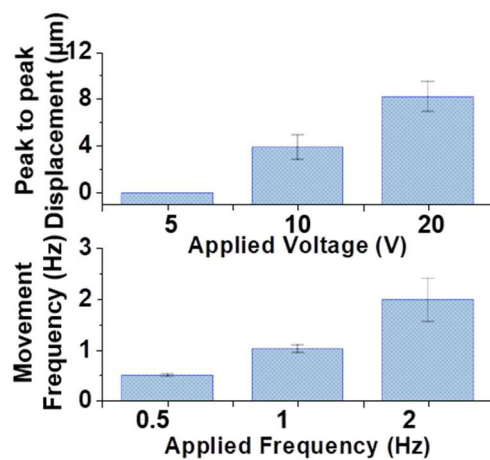


Figure 2.9. Peak to peak pillar displacement for different applied voltages from Figure 2.7 (d), (e), and (f), and pillar movement frequencies for different applied frequencies from Figure 2.7 (f), (g), and (h).

2.6. References

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CHAPTER 3: 3D FRAMEWORKS STRAIN SENSORS INTEGRATED WITH MUSCLE

3.1 Introduction

In the previous Chapter, the 3D integrated graphene allowed the electrical stimulations to actuate muscle tissue. For muscle functionality characterizations, the movement of hydrogel pillars was recorded using optical microscopy and tracked to calculate the muscle forces. However, the optical tracking method highly depends on the microscopy set up such as frame rate and image resolutions. In this Chapter, a 3D electrical strain sensor platform will be introduced to characterize the contractile forces from 3D-engineered muscle tissue. This work was performed in collaboration with Prof. John Rogers group at Northwestern University.

Tissue-on-chip (TOC) technologies, also known as organ-on-chip or microphysiological systems, can recapitulate native tissue environments in vitro. These engineered platforms can be used for research in drug discovery, disease development, and regenerative medicine, as alternatives to animal and traditional cell culture models. [1] Recent reports describe TOC platforms that capture certain essential features of the lung, heart, liver, and brain. [2-7] A frontier is in the development of architectures that mimic the 3D layouts of native tissues, with the ability to monitor and control natural biological and physiological processes, across a broad range of electrical, biochemical, and mechanical cues. [8, 9] 3D engineered tissues can reproduce essential

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structural and functional features of natural biological systems, with much greater relevance to living organisms than traditional 2D cell cultures. Most existing TOC platforms adopt simple planar geometries, due partly to the limited ability to build functional 3D architectures as scaffolds and functional interfaces. While advances in flexible electronics have allowed for conformal integration of electronic scaffolds in the form of patches [4, 10] or meshes [11-13] that can roll or fold to interface with surfaces of engineered tissues, challenges remain in creating deterministic 3D electronic scaffolds as interfaces to engineered thick tissues in a controlled, 3D spatiotemporal manner. Specific challenges are in precisely controlling the 3D locations and/or spatial arrangements of functional electronics for accurate monitoring and regulation of tissue functions. Recent efforts towards these goals include the development of 3D multifunctional electronic scaffolds formed by the mechanically guided assembly, as demonstrated in the recording of extracellular potentials of small dorsal root ganglion neuron networks and cardiac cell cultures formed on 3D scaffolds, [14, 15] and in electrical stimulation and applications [15].

These technologies offer promising avenues for biological research, but they are not, however, designed for interfaces to grown 3D tissues, nor do they provide mechanical support or sensing of forces or displacements of relevance to smooth, skeletal or cardiac muscle constructs. Skeletal muscle, in particular, is an interesting target as the most abundant tissue in the human body, rising approximately 40% of the total mass. [16] Skeletal muscle-on-chip platforms are important in the study of Duchenne muscular dystrophy disease, and they offer the potential for drug screening in this and other contexts. [17-19] In addition, 3D engineered skeletal muscles under electrical and optical control can serve as biohybrid actuators in soft robotics for walking, swimming, or pumping functions. [20-22]

Here we introduce a class of microfabricated 3D frameworks that can be created in precisely engineered shapes and geometries as mechanical interfaces to 3D skeletal muscle tissues for precise measurements of the spatial and temporal contractile forces associated with optical stimulation under a wide range of conditions. These systems exploit recent advances in mechanically guided assembly techniques to form compliant, 3D frameworks instrumented with strain sensors that connect to external control electronics. A key feature of the engineering approach is that it is compatible with well-developed planar electronic, optoelectronic, photonic, and microelectromechanical systems. The shape-matched 3D geometries and mechanically compliant layouts of these frameworks enable intimate contact and stable mechanical coupling to the tissues. Measurements of the contractile forces generated by 3D optogenetically active muscle rings with comparisons to results of conventional optical microscopy demonstrate the utility of these technologies in 3D tissue engineering.

3.2. Materials and Methods

3.2.1. Fabrication of the 3D frameworks instrumented with strain sensors

Fabrication of the instrumented 3D frameworks began with the spin coating (3,000 rpm for 50 s) of a layer of polydimethylsiloxane (PDMS, 10:1 mixing ratio) on a glass slide. Partial curing (90 °C for 60 s) of the PDMS, followed by lamination of a film of polyimide (PI; 12.5 µm in thickness, Fralock) on top and complete curing (110 °C for 3 min) allowed further processing. A lift-off process defined a patterned metal layer (Cr/Au, 10 nm/100 nm in thickness) on the surface of the PI. Deposition of a layer of parylene C (5 µm) and subsequent patterning using oxygen plasma etching (March RIE) formed a top encapsulation layer. Laser ablation (LPKF ProtoLaser R) defined the outline of the 2D precursor in Figure 3.1 (a), followed by the transfer of the 2D

precursor from the PDMS-coated glass to a water-soluble tape (polyvinyl alcohol, PVA). Deposition of Ti/SiO₂ (10 nm/50 nm in thickness) on the back side of the 2D precursor through a shadow mask defined the bonding sites. A sheet of PDMS (45:1 mixing ratio, 3.5 mm in thickness) served as the platform for mechanically guided assembly. UV ozone treatment of the 2D precursor and the PDMS created hydroxyl groups on the surfaces to allow covalent surface reactions between the SiO₂ and the PDMS. Pre-stretching the PDMS to a uniaxial strain of 40%, laminating the 2D precursor/PVA tape on top, and heating the system (70 °C for 10 min) led to strong adhesion at the bonding sites. Dissolving the PVA tape with water and releasing the pre-stretch transformed the 2D precursor into a 3D framework by controlled buckling.

3.2.2. Data acquisition and signal processing

Anisotropic conductive film (ACF) connected the leads of the instrumented 3D frameworks to a printed circuit board (PCB) for electrical measurements. A PowerLab computer interface (ADInstruments, Model 8/35) recorded the voltage output signal at a sampling rate of 100 kS/s from the Wheatstone bridge at an applied excitation voltage $V_e = 0.5$ V. For 1 Hz optical stimulation, a digital bandpass filter of 0.1-5 Hz eliminated high-frequency noise and smoothed the signal. Studies with different stimulation frequencies used a digital bandpass filter of 0.1-20 Hz for similar purposes. A custom MATLAB (MathWorks) code calculated the peak amplitudes from the data.

3.2.3. Gauge factor measurement

A planar strain sensor fabricated using procedures similar to those used to form the 2D precursors served as a structure for determining the calibration factor, with the 12.5 μ m PI film

replaced by a thicker (127 μm in thickness) PI substrate to minimize spontaneous curling and bowing of the film. A PowerLab computer interface recorded the change in resistance of the strain sensor upon application of a triangular cyclic tensile strain measured (10 S/s sampling rate) by a dynamic mechanical analyzer (DMA, TA Instruments RSA-G2).

3.2.4. Indentation test

An indenter system (Bruker Hysitron BioSoft Indenter) with a vertical indenter (400 μm in diameter) provided force-displacement recordings on an instrumented 3D framework rotated by 90°. During the approach of the indenter, a force threshold of 20 μN defined the point of contact between the indenter and the sidewall of a post. Repeating an approach (1 $\mu\text{m/s}$ for 5 s)-hold (5 s) step before reaching a maximum indentation displacement of 50 μm followed by repeated retraction (1 $\mu\text{m/s}$ for 5 s)-hold (5 s) yielded force-displacement curves at a sampling rate of 60 Hz. Synchronized recording (400 S/s sampling rate with 0.5 Hz lowpass digital filtering; excitation voltage: 3 V) of the 3D framework signal via a PowerLab computer interface correlated the mechanical deformation of the 3D framework to electrical readout.

3.2.5. Temperature calibration.

The PowerLab interface and a custom circuit enabled the measurement of the resistance of the temperature sensor at different temperatures from 30 °C to 50 °C, determined simultaneously by an infrared camera (FLIR A655sc). The slope of the $\Delta R/R$ vs. temperature defined by linear least-squares fitting yielded the temperature coefficient of resistance (TCR).

3.2.6. *Optogenetic muscle ring fabrication*

The fabrication process started with infecting skeletal muscle C2C12 myoblasts with pLenti2-EF1 α -ChR2[H134R]-tdTomato-WPRE plasmid to enable the expression of a mutated variant of the light-sensitive ion channel, Channelrhodopsin (ChR2[H134R]). Cells were cultured in the growth media using Dulbecco Modified Eagle Medium (DMEM) (Gibco) with L-glutamine and pyruvate containing 10% (v·v-1) fetal bovine serum (ThermoFisher), 1% (v·v-1) external L-glutamine, and 1% penicillin and streptomycin (Lonza). Figure 3.2 (a) illustrates the formation of an ECM-embedded muscle ring by thrombus reaction with thrombin and fibrinogen. Seeding occurred in a PDMS mold (5 mm/6.6 mm for inner/outer diameters) after mixing with 30% Matrigel (ThermoFisher), thrombin (Sigma Aldrich), and fibrinogen (Sigma Aldrich). [24] The growth media with 1 mg·ml⁻¹ aminocaproic acid (ACA) added to the ring mold 90 min after seeding prevented proteolytic degradation. [27] PDMS (1:10 mixing ratio) and SmoothCast 320 (Smooth-On) were used for the mold. The CAD design and an image of the mold appear in Figure 3.3. Three days after seeding, the media was switched to the differentiation media of DMEM including 10% Horse serum (ThermoFisher), 1% L-glutamine, 1% penicillin, and streptomycin, 1 mg·ml⁻¹ of ACA, and 5 μ g·ml⁻¹ of insulin growth factor (Sigma Aldrich). On day 5 after differentiation, the muscle rings were transferred from the mold and integrated onto the 3D frameworks manually using tweezers. The muscle rings were incubated at 37 °C and the media was changed every other day before integration and every day after integration.

3.2.7. *Cell viability test*

Preparation of the MTS assay began with combining MTS solution (CellTiter 96 Aqueous One Solution Cell Proliferation Assay, Promega) with warm DMEM without phenol red at a 1:5

ratio. Muscle rings were cultured and differentiated on the 3D frameworks and in the PDMS mold. On selected days after muscle ring integration shown in Figure 3.4 (e.g. 3 days, 1 week, 3, and 5 weeks), muscle rings were removed from the 3D frameworks and the mold and transferred to 24 well plates. The next step involved rinsing with PBS and incubating with the 360 μ L of solution for 4 hours at 37 °C. Measurements of absorbance at 490 nm defined the cell viability.

3.2.8. Immunofluorescence imaging

The muscle ring was rinsed in PBS, fixed in 4% paraformaldehyde (Electron Microscopy Sciences) for 30 min, permeabilized with 0.25% (v·v-1) Triton X-100 (Sigma Aldrich) for 20 min, and incubated overnight in blocking solution with 1 % (w·v-1) bovine serum albumin (Sigma Aldrich) in PBS at 4 °C. The primary antibodies, mouse anti-myosin heavy chain (MF-20) and rabbit anti α -actinin, were used to stain for myosin and the sarcomere respectively with 1:500 and 1:200 dilution ratios, followed by incubation overnight at 4 °C. The secondary antibodies, AlexaFluor-488 anti-rabbit and AlexaFour-564 anti-mouse (ThermoFisher) were used to stain α -actinin and MF-20 antibodies, respectively, followed by incubation overnight with DAPI to stain the nucleus at 4 °C. The muscle ring on the 3D framework was mounted with Vectashield antifade mounting media (Vector Laboratories). Between each step, the sample was washed with PBS three times for 5 min. The Multiphoton Confocal Microscope Zeiss 710 and Zeiss Stereo Discovery V20 Microscope were used for fluorescence imaging. The Zeiss Stereo Discovery V20 Microscope provides relatively a larger depth of focus for 3D perspective views (Figure 3.2 (e)) with a higher fluorescence background, as compared with the Multiphoton Confocal Microscope Zeiss 710.

3.2.9. SEM imaging

Samples were rinsed in PBS, fixed in 4% paraformaldehyde, and dehydrated using a series of ethanol solutions with concentrations of 33, 67, 95, and 100% (three times) for 10 min. Gold/palladium was deposited on the dried sample for 70 sec with a sputter coater (Desk II TSC, Denton Vacuum). FEI Quanta FEG 450 environmental SEM (ESEM) was used to acquire images.

3.2.10. Finite element analysis

Simulations for the compressive buckling of 3D frameworks were performed using the commercial software ABAQUS. Four-node finite strain shell elements were adopted, with refined mesh (~100,000 elements) smaller than the width of the metal trace to ensure accuracy. The indentation was modeled by applying a concentrated force on the buckled 3D framework, at the contact position with the indenter. The muscle ring tension was modeled by applying a uniform pressure on the 3D framework, in the area where the muscle ring attaches to the framework. All materials are linear elastic, except that a double linear plasticity model is adopted for Au. The Young's moduli of polyimide (PI), parylene, Au and Cr are $E_{PI} = 2.5$ GPa, $E_{Parylene} = 2.76$ GPa, $E_{Au} = 78$ GPa, and $E_{Cr} = 279$ GPa, respectively; the Poisson's ratios are $\nu_{PI} = 0.34$, $\nu_{Parylene} = 0.40$, $\nu_{Au} = 0.44$, and $\nu_{Cr} = 0.21$, respectively.

3.2.11. Optical stimulation and tracking

Stimulation used an optical fiber and 465-nm LED module driver (Doric). The driver and Doric Studio software were used to control the frequency, light intensity, and pulse width. Electrical measurements and optical stimulation were performed in an environmental chamber at 37 °C, as in Figure 3.5. Movements of muscle rings due to optical stimulation were tracked and

recorded using an Olympus IX81 inverted microscope (Olympus) at a frame rate of 14.9 fps and processed using the software Tracker (<http://physlets.org/tracker>). For frequency studies, 0.5, 1, 2, 4, and 6 Hz pulses with $11.5 \text{ mW}\cdot\text{cm}^{-2}$, and 50 ms of pulse width were applied for 20 seconds for each frequency value. For intensity studies, 0.26, 0.3, 0.4, 0.6, 0.8, 1.0, 1.5, 2, 2.5, 5, 7.5, 10, and $11.5 \text{ mW}\cdot\text{cm}^{-2}$ for 1 Hz pulses with 50 ms widths were applied for 20 seconds for each intensity value. For pulse width studies, 1, 2, 3, 5, 10, 20, 30, and 50 ms of pulses with 1 Hz and $11.5 \text{ mW}\cdot\text{cm}^{-2}$ were applied for 20 seconds for each pulse width value. The light intensity was measured with an optical power meter (Thorlabs).

3.2.12. Long-term studies

Experiments involved measurements during optical stimulation (with pulses at $11.5 \text{ mW}\cdot\text{cm}^{-2}$, 1 Hz, and 50 ms) every day until day 22 after muscle ring integration, followed by measurements every 2 days until day 32, then on day 35 and day 37. The samples were fed with the differentiation media every day.

3.2.13. Drug studies

Acetylcholine chloride (Sigma Aldrich), caffeine (Sigma Aldrich), and dantrolene sodium (Sigma Aldrich) were used for drug studies. Acetylcholine chloride and caffeine were dissolved in PBS and diluted for a series of different concentrations. 5 μL solution was added to the 1 mL of media that contained the muscle ring on the 3D framework, increasing the drug concentration every minute from low to high concentrations in the optical stimulation with 1 Hz, $11.5 \text{ mW}\cdot\text{cm}^{-2}$, and 50 ms of light pulses. The working concentrations of acetylcholine chloride and caffeine were diluted by approximately 200 times of the Bolus concentrations as shown in Table 3.1.

Dantrolene sodium was initially dissolved in 20 mM dimethyl sulfoxide (Sigma Aldrich) and diluted in PBS due to its poor solubility in water. [28] Light pulses with 1 Hz, $11.5 \text{ mW}\cdot\text{cm}^{-2}$, and 50 ms were applied for 1 min and measured electrically before adding the dantrolene solution and in 5, 10, 20, 30, 40, 50, and 60 min after adding it. Paired t-tests are used to calculate p values for active tension forces between before and after the addition of chemicals. Unpaired t-tests are used to calculate p values for active tension forces between the addition of 10 and 100 μM of dantrolene.

3.3. Results and Discussion

3.3.1. Design, fabrication, and characterization of 3D frameworks instrumented with strain sensors

The design and fabrication approaches exploit recent advances in mechanically guided assembly methods that enable the geometric transformation of functional materials from planar layouts into 3D architectures with well-defined layouts and wide-ranging options in designs. [29-31] The process starts with the formation of a 2D precursor structure that includes a polymer substrate (polyimide, 12.5 μm in thickness), a thin lithographically patterned film of metal (chromium/gold, 10/100 nm in thickness), and a top encapsulation layer (parylene C, 5 μm in thickness; a basic design appears in Figure 3.1 (a)). The metal pattern defines a collection of resistive strain sensors and a set of electrical interconnects. Specifically, narrow (5 μm in width) serpentine traces form sensing elements of strain sensors arranged in two groups located on the two sides of the symmetric 2D precursor. Each group forms an independent sensing unit that consists of 4 strain sensors (R_1 - R_4) with two on each side of a mesoscale ‘post’ structure that rises out of the plane. A laser ablation step defines the shape of this structure and other lateral features of the 2D precursor. Deposition of a bilayer of Ti/ SiO_2 on the backside of the 2D precursor through

a shadow mask forms sites for bonding to a pre-strained elastomer substrate (polydimethylsiloxane; PDMS). Releasing the pre-strain initiates the buckling process that transforms the 2D precursor into a 3D configuration that consists of two buckled posts each surrounded by 4 strain sensors. The designs of these posts facilitate integration with 3D engineered muscle tissues, as described subsequently. Details of the fabrication and assembly processes appear in Figure 3.6 and Methods. Figure 3.7 shows the layout of the 2D precursor. This overall scheme provides access to a wide range of complex 3D architectures that embed various functional components. Figure 3.1 (b) shows a complex, spider-like 3D configuration (2D precursor layout shown in Figure 3.8) with sensing functionality similar to that of the basic design in Figure 3.1 (a), highlighting the design versatility. Figure 3.9 illustrates the process of 2D to 3D shape transformation simulated by finite element analysis (FEA). The overall similarity of experimental and computational shapes and the good agreement between key dimensional parameters (Figure 3.10) validate the use of FEA as a tool to guide the structure design.

The locations of the 4 resistive strain sensors that surround the buckled 3D posts serve critical roles in the sensing process. Figure 3.1 (c) highlights two strain sensors strategically located on one side of the 3D post. Roughly half of each strain sensor resides within the corresponding bonding site, stably adhered to the substrate by covalent bonds, while the other half delaminates upon buckling as a result of the comparatively weak van der Waals interactions. The result defines a curved profile that supports a flat, roof-like region. All of the strain sensors are in a state of compressive strain (absolute value $-\varepsilon_{t,0}$, see Figure 3.11 (a)) along with their longitudinal directions because the metal layer lies above the neutral mechanical plane and the local curvature is negative (concave). The maximum equivalent strain (see Figure 3.11 (b)) in the gold layer that arises from the buckling process is within its fracture limit. [32, 33] The peak strain in the gold

appears close to the boundary of bonded/delaminated regions. While this peak value may slightly exceed the elastic threshold of gold thin films, [34] a low level of plastic deformation in these local regions has negligible influence on the ability to measure contractile forces accurately.

In this 3D geometry, the strain sensors are sensitive to small forces applied horizontally to the buckled posts due to their small bending stiffness. Figure 3.1 (d) shows the computed change in the strain of the metal layer ($\Delta \varepsilon = \varepsilon_l - \varepsilon_{l,0}$, approximately 0.01%) induced by a horizontal force (0.18 mN) applied from the left side of the post. This force results in a slight bending of the post toward the right, causing a decrease in the absolute values of the compressive strains of R_1 and R_3 , and an increase in the absolute values of the compressive strains of R_2 and R_4 (Figure 3.1 (d)). A full Wheatstone bridge circuit converts the changes in the resistances of R_1 - R_4 into a voltage V_o (Figure 3.1 (e)) according to $V_o = (\frac{R_3}{R_3 + R_4} - \frac{R_2}{R_1 + R_2})V_e$, where V_e is the excitation voltage.

Establishing the quantitative correlation between V_o/V_e and the force applied to the posts involves measurement of the gauge factors of the strain sensors through tensile tests on planar devices (parylene/metal/PI) fabricated using the methods described above (Figure 3.1 (f)). For uniaxial tensile strains between 0.1% to 0.5%, these sensors exhibit linear responses to strain, as expected for gold thin films under strains less than 1%, [35] with gauge factors of 2.68 ± 0.04 (Figure 3.12). The use of this gauge factor in mechanical models yields a relationship between structural deformations and the electrical readouts. Indentation tests on 3D frameworks instrumented with strain sensors provide direct comparisons of the results of FEA computations and experiments. The measurements involve force-displacement curves from the indenter and synchronized readout of voltage responses from the strain sensors (Figure 3.13). The data show a linear dependence between both the force and the change in output voltage with indentation

displacement across a range of 30 μm (Figure 3.1 (g)). The measurements match the results of FEA, thereby validating the use of FEA models for this system.

3.3.2. Integration of skeletal muscle rings with compliant, instrumented 3D frameworks

The instrumented 3D frameworks described above adopt features tailored for interaction with millimeter-scale ring structures formed using mouse skeletal muscle tissues according to procedures described elsewhere. [24, 36] The steps for preparing such rings and integrating them onto the 3D frameworks appear schematically in Figure 3.2 (a). The process starts with the delivery of optogenetic myoblasts seeded in an extracellular material solution into a soft mold of PDMS (Figure 3.3). Transfection of blue light-sensitive ion channel Channelrhodopsin-2 (ChR2), namely ChR2 (H134R), to C2C12 murine myoblasts follows lentiviral transduction protocols for optogenetically active skeletal muscle tissues. [24, 36] The seeded cell/gel solution solidifies through a thrombus reaction to form a muscle ring inside the mold. After differentiation of the myoblasts into myotubes for 5 days, manual transfer of the muscle ring onto the 3D framework completes the integration. The muscle ring wraps around the two posts (2.85 mm in spacing) in contact with the sidewalls (Figure 3.2 (b) and (c)). The top surfaces of the posts include two pairs of microscale ‘flaps’ (480 μm in length, 450 μm in width) designed to retain the muscle ring during periods of spontaneous contractions. Additional images and FEA results appear in Figure 3.14.

The posts provide a uniaxial constraint to facilitate cell alignment during the compaction of the muscle ring. In this way, the myotubes mature in a macroenvironment that leads to the native organization of functional skeletal muscle. Confocal images of the muscle ring integrated with the 3D framework highlight the longitudinal alignment of the myotubes with the muscle ring wrapped around the posts, visualized with an antibody against α -actinin protein (red), nuclei

(DAPI; blue), and mature myosin (MF-20; green) in Figure 3.2 (d) and Figure 3.15. Scanning electron microscopy (SEM) images of a dehydrated muscle ring on this same platform also show a high degree of myofiber alignment (Figure 3.16). Bright field and fluorescent images in the 3D perspective view highlight the integration (Figure 3.2 (e) and Figure 3.17).

3.3.3. Optical stimulation of optogenetic muscle rings integrated onto 3D frameworks

Muscle rings formed in this manner contract in response to optical ($\lambda \approx 470$ nm) stimulation, to generate active contractions. FEA models validated by the indentation tests can yield accurate relationships between the contractile force, the contractile displacement, and the electrical readout of the sensors. A passive tension force develops in the muscle ring upon integration of the muscle ring onto the sensor due to the expected effects of mechanical coupling. Optical stimulation induces additional, dynamic active tension forces as a result of the muscle responsivity to optical stimulation. These responses represent the primary focus of studies reported here. FEA calculations of the active and passive components of the forces appear in Figure 3.18. Figure 3.19 shows the change in the strain of the metal sensing layer induced by muscle contraction with a contractile displacement of 10 μm on each side of the posts toward the center. The computed active tension forces and electrical readout both exhibit linear relationships with the contractile displacement (Figure 3.19 (b)) within distances of up to 20 μm . These results enable quantitative conversion of sensor responses to contractile displacements and active tension forces.

A custom experimental setup inside a temperature-controlled (37°C) chamber supports optical stimulation with controlled intensities and time dependencies via an optical fiber (Figure 3.5). An inverted microscope allows for optical imaging through the transparent PDMS substrate that supports the device. A comparison of the measured active tension forces with and without a

muscle ring integrated onto a representative 3D framework appears in Figure 3.19 (c) and (d). The sensor readout V_o for this study, after digitally filtering (5 Hz low-pass filter) out high-frequency noise appears in Figure 3.20. Applying an additional high-pass filter (0.1 Hz) eliminates baseline drift caused by fluctuations of the external voltage supply for purposes of determining the peak amplitudes, as measurements of the active tension forces induced by muscle contractions. The computed amplitudes of peaks from the sensor without the muscle ring remain within 10 μN , largely invariant with increasing optical intensity. By contrast, sensors in 3D frameworks with muscle rings show increasing active tension forces with 1 Hz peaks synchronized to the stimulation frequency. The active tension forces increase with the strength of muscle contraction and corresponding increases in the optical intensity. The data indicate that noise from photothermal and ambient effects is negligible. A resistive temperature sensor integrated into a planar region of the 3D framework captures changes in temperature adjacent to (~ 1.8 mm distance) the strain sensors. The results provide estimates of photothermal effects that could arise during optical stimulation, as discussed in Figure 3.21.

A comparison of results obtained in the manner described above with those captured by optical microscopy highlights the key differences. Here, electrical sensor readout occurs simultaneously with video recording through an inverted microscope. FEA of the mechanics of the 3D frameworks converts measured contractile displacements to active tension forces. The trends in forces and motions correlate with optical intensity from 1.5-11.5 mW/cm^2 , with similar peak amplitudes (Figure 3.22). A direct comparison involves contractile displacements computed based on the readout of the instrumented 3D framework and measured by optical microscopy. The results are consistent for this range of optical intensities (Figure 3.23), thereby establishing the validity of the approaches in 3D mechanical sensing introduced here. Close inspection of the

measured active tension forces in Figure 3.22 (b) highlights important distinctions between these two schemes: the instrumented 3D framework records continuous and consistent peaks in the active tension forces due to high sampling rates (100 kS/s); by contrast, optical microscopy yields inconsistent results due to comparatively low frame rates (14.9 fps) of the standard video recording setups used here.

These advantages in measurement speed are particularly important for monitoring high frequency muscle contractions. Figure 3.22 (c) shows the amplitudes of active tension forces of an optogenetic muscle ring (1 day after integration) measured by an instrumented 3D framework and by optical microscopy for optical stimulation at frequencies from 0.5 Hz to 6 Hz with a fixed optical intensity ($11.5 \text{ mW} \cdot \text{cm}^{-2}$) and pulse width (50 ms). At stimulation frequencies of 0.5 Hz and 1 Hz, both sensor readout and optical microscopy capture all of the peaks and show similar amplitudes. Discrepancies may result from slight deviations in the shapes and contact areas of the muscle ring and in uncertainties in imaging and post-image processing. Large errors in the amplitudes (measurement period in the 20s) start to appear in optical microscopy at and above frequencies of 2 Hz. The peak frequencies also begin to deviate from the stimulation frequencies for values above 4 Hz. These issues do not appear in data from the sensor (Figure 3.22 (d)). Forces measured by both methods decrease as the stimulation frequency increases (Figure 3.22 (e)), consistent with incomplete muscle relaxation between adjacent stimuli. [36] Measurements obtained with the 3D framework are consistently larger than those with optical microscopy, again due mainly to the limited frame rate. Figure 3.22 (f) and Figure 3.24 show magnified waveforms of the active tension forces measured by the two methods, clearly demonstrating the advantages of electronic sensing.

The duration of optical stimulation also influences the level of muscle contraction. Figure 3.22 (g) presents the active tension forces of an optogenetic muscle ring (1 day after integration) as a function of pulse width for fixed intensity ($11.5 \text{ mW} \cdot \text{cm}^{-2}$) and frequency (1 Hz). The forces increase significantly with increased pulse width from $t_w = 1\text{-}20 \text{ ms}$ before reaching a plateau. This behavior suggests that the time threshold for activating full muscle contraction is roughly 20 ms, consistent with previous reports on optogenetic stimulation of skeletal muscles from transgenic mice expressing the ChR2. [37] Detailed comparisons appear in Figure 3.25.

3.3.4. Long-term studies

3D frameworks are well suited for long-term monitoring. Figure 3.26 (a) presents results recorded over 37 days after muscle ring integration. Both sides of the system can measure forces associated with contractile displacements on the corresponding side. The results agree reasonably well, limited by some positional asymmetry of the muscle ring. Upon integration, the active tension force increases to an average peak value of $120 \text{ } \mu\text{N}$ within 4 days (on day 5 after differentiation) as a result of maturation for myotubes during differentiation. Subsequently, the magnitude gradually decays over a period of approximately 4 weeks caused by the steady degradation of 3D tissues due to proteases secreted by the cells. [27, 36] Figure 3.27 shows a series of optical images before integration, after selected days of integration, and after removal of the muscle ring. After removal, the posts return to their original shapes. The distances between the posts before integration and after removal are 2.86 mm and 2.81 mm, respectively, consistent with an elastic, reversible response. Tests of the viability of the muscle ring on the instrumented 3D framework, determined by an MTS assay, show results identical to those for a ring cultured in a PDMS mold, for durations up to 5 weeks after integration onto the 3D framework (Figure 3.4).

3.3.5. Drug studies

The open architecture of 3D frameworks here creates additional opportunities such as those in dose-response studies of drugs that influence muscle contractile strength. Experiments with acetylcholine chloride, caffeine, and dantrolene demonstrate some possibilities. Acetylcholine is a representative neurotransmitter and caffeine is a chemical agent. Both interact with skeletal muscle. [39] Dantrolene is a commercial drug that prevents malignant hyperthermia as a skeletal muscle relaxant, by binding to the RYR1 receptor and inhibiting Ca^{2+} release from the sarcoplasmic reticulum. [28]

Figure 3.26 (b) shows the active tension forces of an optogenetic muscle ring under 1 Hz pulsed optical stimulation ($11.5 \text{ mW} \cdot \text{cm}^{-2}$ intensity and 50 ms pulse width) during the addition of acetylcholine at 100 nM-1 mM working concentrations. Figure 3.26 (c) presents the normalized active tension force, defined as the ratio of the active tension force after adding acetylcholine with each concentration to the initial active tension force before adding acetylcholine. The results indicate that acetylcholine with a concentration higher than $1 \text{ } \mu\text{M}$ causes larger decreases in active tension forces. Statistical significances appear in active tension forces after the addition of $100 \text{ } \mu\text{M}$ and 1 mM with 0.0045 and 0.0004 of p values compared with those before adding acetylcholine. A conclusion from these studies is that $\sim 10\text{-}100 \text{ } \mu\text{M}$ is likely the threshold acetylcholine concentration for effectively preventing muscle relaxation, corresponding to inhibition of periodic muscle contractions caused by optical stimulation. Acetylcholine is a neurotransmitter secreted by motor neurons through the neuromuscular junction to cause skeletal muscle contraction. After muscle contraction, excess acetylcholine that remains at the neuromuscular junction can be hydrolyzed by the acetylcholinesterase enzyme to relax the muscle. High-concentration acetylcholine overwhelming the capacity to hydrolyze the neurotransmitter by

acetylcholinesterase in this system leads to the accumulation of acetylcholine in the skeletal muscle, thereby paralyzing muscle contraction.

Caffeine triggers the release of calcium ions from the sarcoplasmic reticulum, to increase skeletal muscle contraction and overcome muscle fatigue. [39, 40] As Figure 3.26 (d) shows, the normalized active tension force in optogenetic muscle rings stimulated by pulsed light increases with additions of caffeine, while it saturates in 100 μM to 40% of increase compared with that before the addition of caffeine. Statistical significance appears in the active tension force after additions of higher than 10 nM of caffeine with p values below 0.05. The saturation in 100 μM may be explained by the overdose of caffeine. Overdose of caffeine is known to cause rhabdomyolysis due to an excessive concentration of intracellular calcium; 400-500 μM (80-100 $\mu\text{g/ml}$) of caffeine in vivo is considered lethal. [41, 42]

Experiments with dantrolene involve a modification of the measurement procedure to accommodate the poor solubility of dantrolene in water and the slow rates of diffusion. Here, recordings of muscle responses at different times occur after changing the cell media with 100 μM of dantrolene. Figure 3.26 (e) indicates that the normalized active tension force decreases after the addition of 100 μM dantrolene before it saturates after 30 min. Statistical significance in the active tension force with p values lower than 0.05 appears longer than 20 min after the addition of the drug. Figure 3.28 shows that 10 μM of dantrolene causes a significant decrease in the normalized active tension force 30 min after addition. While the average normalized active tension forces in 100 μM are lower than those in 10 μM , no statistical significance appears between the two concentrations. These results indicate that it may take 20-30 min for the dantrolene to cause a significant decrease in the active tension force, likely due to the poor solubility and slow diffusion rates of dantrolene in water-based media. These drug studies show the potential utility of the

system introduced here for sensitive measurements of responses of skeletal muscle tissues to various drugs, of relevance in screening, disease model studies, and medical countermeasures.

3.4. Conclusions

The 3D frameworks reported here exploit mechanically guided assembly processes to form systems that provide millimeter-scale mechanical supports with integrated microscale strain sensors for measurements of tissue contractility in 3D engineered skeletal muscle tissues. These compliant 3D architectures enable intimate contact and robust device-tissue coupling, for precise electronic sensing of mechanical responses under optical and chemical stimulation, with key advantages over traditional microscopy methods. Further improvement in the sensitivity is achievable via the use of strain sensing materials with larger gauge factors such as monocrystalline silicon. [31] High sensitivity measurements of engineered muscle tissue contraction are potentially useful for muscular disease studies or biohybrid robotic systems. The design and fabrication schemes presented in this study have additional potential in multifunctional sensing modalities for measurements of electrical potential, temperature, and electrochemistry with distributed collections of electronic, piezoelectric, optical, and optoelectronic components for advanced modulation of tissue functions in a wide range of applications in studies of other 3D tissue constructs based on cardiomyocytes, neurons, and others. [8, 9] In a complementary set of possibilities these constructs may represent interesting architectures for the actuation and control of biohybrid robots. [21, 36, 38] The ideas presented here also scale naturally to arrayed configurations for high throughput sensing in tissue engineering for drug screening and disease model studies. Not only TOC applications, integrating the wireless communication systems will

allow advanced functionality of untethered biohybrid robot applications with wireless data collection and transmission.

3.5. Figures

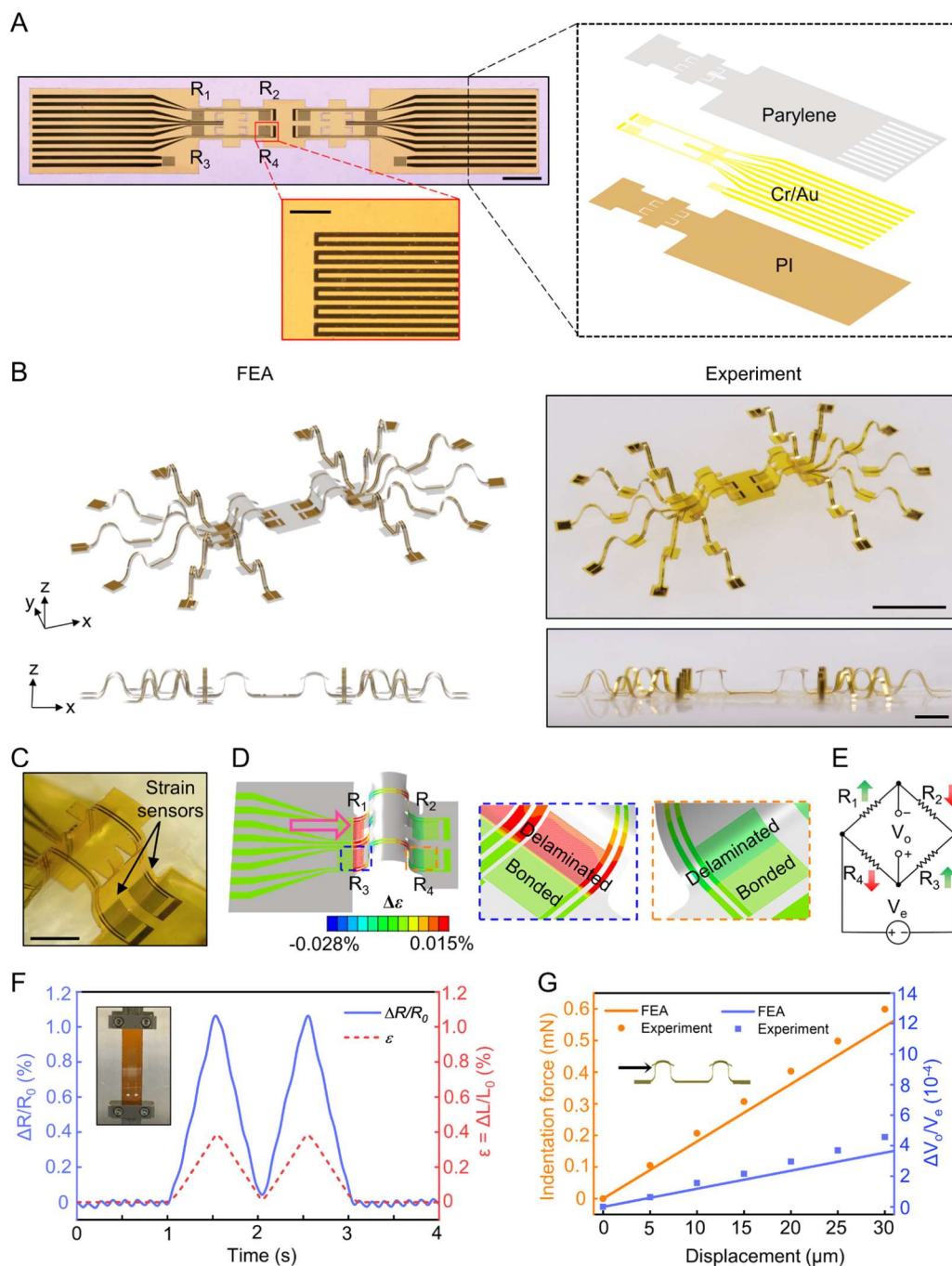


Figure 3.1. Design, sensing mechanism, and characterization of compliant 3D frameworks instrumented with strain sensors. (a) Optical image of a 2D precursor used for assembly of this 3D system, consisting of three layers (parylene, Cr/Au, and polyimide, right inset) with embedded strain sensors (bottom inset) in the metal layer. (b) FEA results and experimental images shown in perspective view (top) and side view (bottom) for a complex 3D framework designed to highlight the versatility of the assembly approach.

Figure 3.1. (Cont.) (c) Optical image highlighting the strain sensors near the base of a post. (d) Computed change in strain in response to an indentation displacement of $10\text{ }\mu\text{m}$ from one side of a post, featuring two strain sensors (resistors R3 and R4) on opposite sides. (e) Schematic illustration of a Wheatstone bridge circuit consisting of 4 strain sensors. (f) Relative change in the resistance of a parylene/gold/polyimide strain sensor upon cyclic stretching (inset: experimental setup of the tensile test) to 0.4% uniaxial strain. The results define the gauge factor. (g) Computed and experimental results for the indentation force and the corresponding change in output voltage as functions of the indentation displacement. Scale bars: 2 mm in a ($50\text{ }\mu\text{m}$ in inset), 5 mm (top) and 2 mm (bottom) in (b), 1 mm in (c). The Figure set was produced by Hangbo Zhao from the Rogers group.

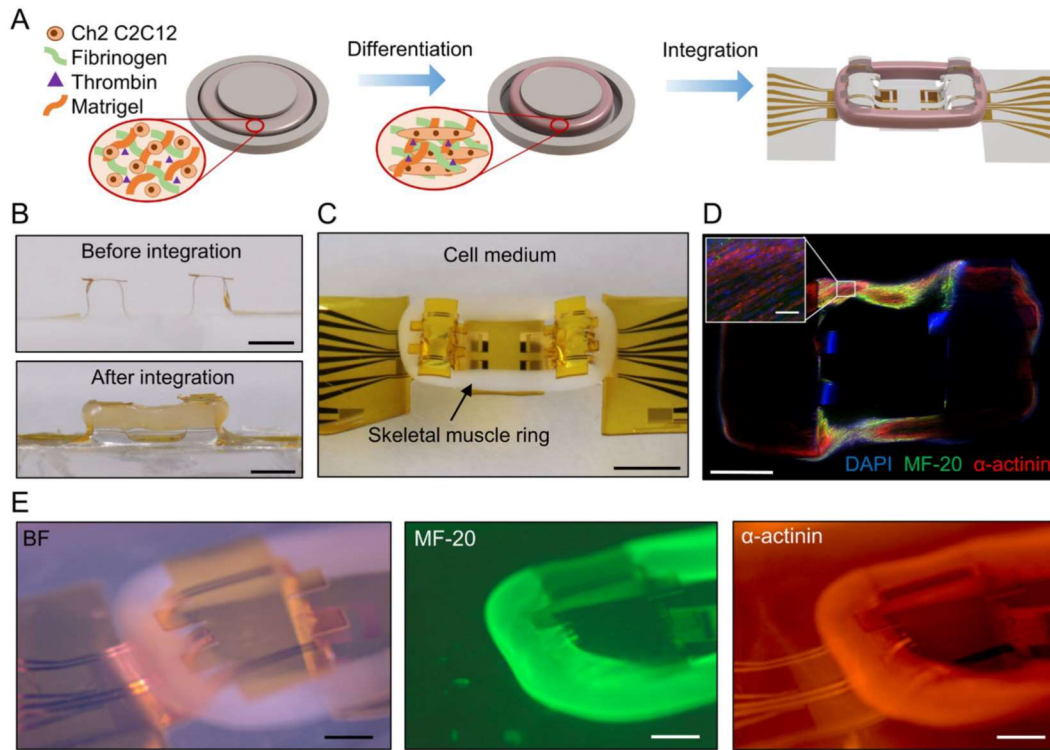


Figure 3.2. Integration of an engineered skeletal muscle ring onto a compliant 3D framework instrumented with strain sensors. (a) Schematic illustration of the process for forming an optogenetic skeletal muscle ring by molding of Ch2 C2C12 myoblasts and for integrating the resulting ring onto a 3D framework for sensing. (b) Optical images (side view) showing the 3D framework before (top) and after (bottom) integration with a muscle ring. (c) Optical image of the integration of a skeletal muscle ring with the 3D framework. (d) Confocal image of an optogenetic muscle ring integrated with the 3D framework. The results highlight alignment of the myotubes (inset), visualized with an antibody against nuclei (DAPI; blue), mature myosin (MF-20; green), and α -actinin protein (red). (e) Perspective view bright field (BF, left) and fluorescence images (middle and right) after staining to highlight the integration of an optogenetic muscle ring with a 3D framework at a location near one end of a post. Scale bars: 2 mm in (b) and (c), 1 mm in (d) (50 μ m in inset), 500 μ m in (e).

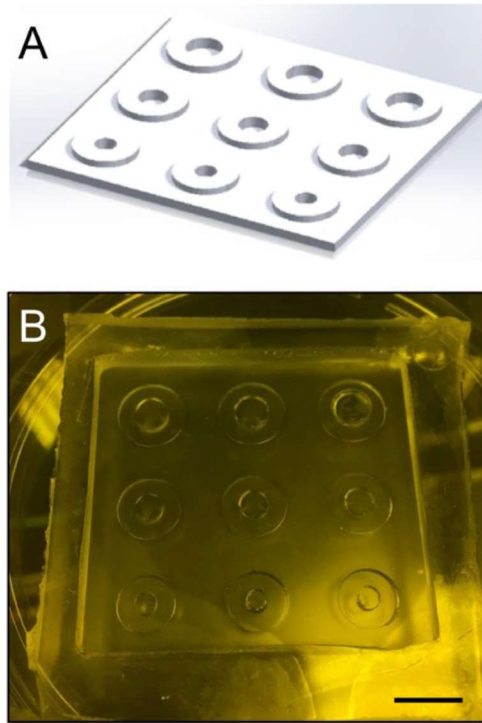


Figure 3.3. Molds for the fabrication of optogenetic muscle rings. (a) Computer-aided design for ring molds. (b) Optical image of PDMS ring molds. Scale bar: 1 cm.

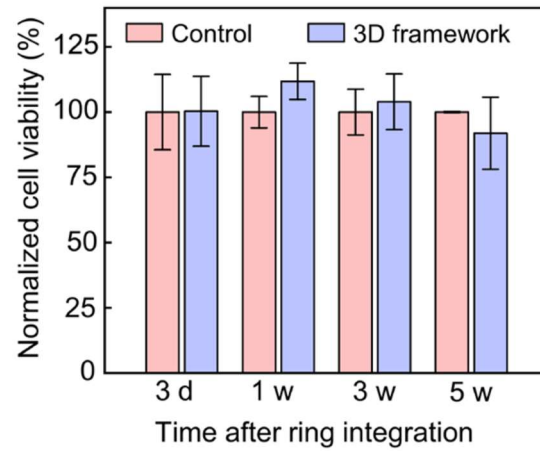


Figure 3.4. Cell viability on an instrumented 3D framework normalized to that on PDMS (control) determined by an MTS assay. Columns and error bars represent mean values $\pm$ standard deviations.

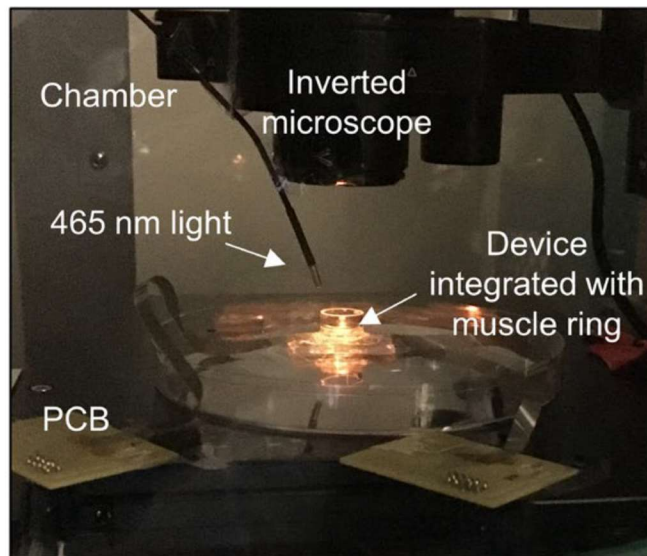


Figure 3.5. Optical image of the experimental setup for optical stimulation.

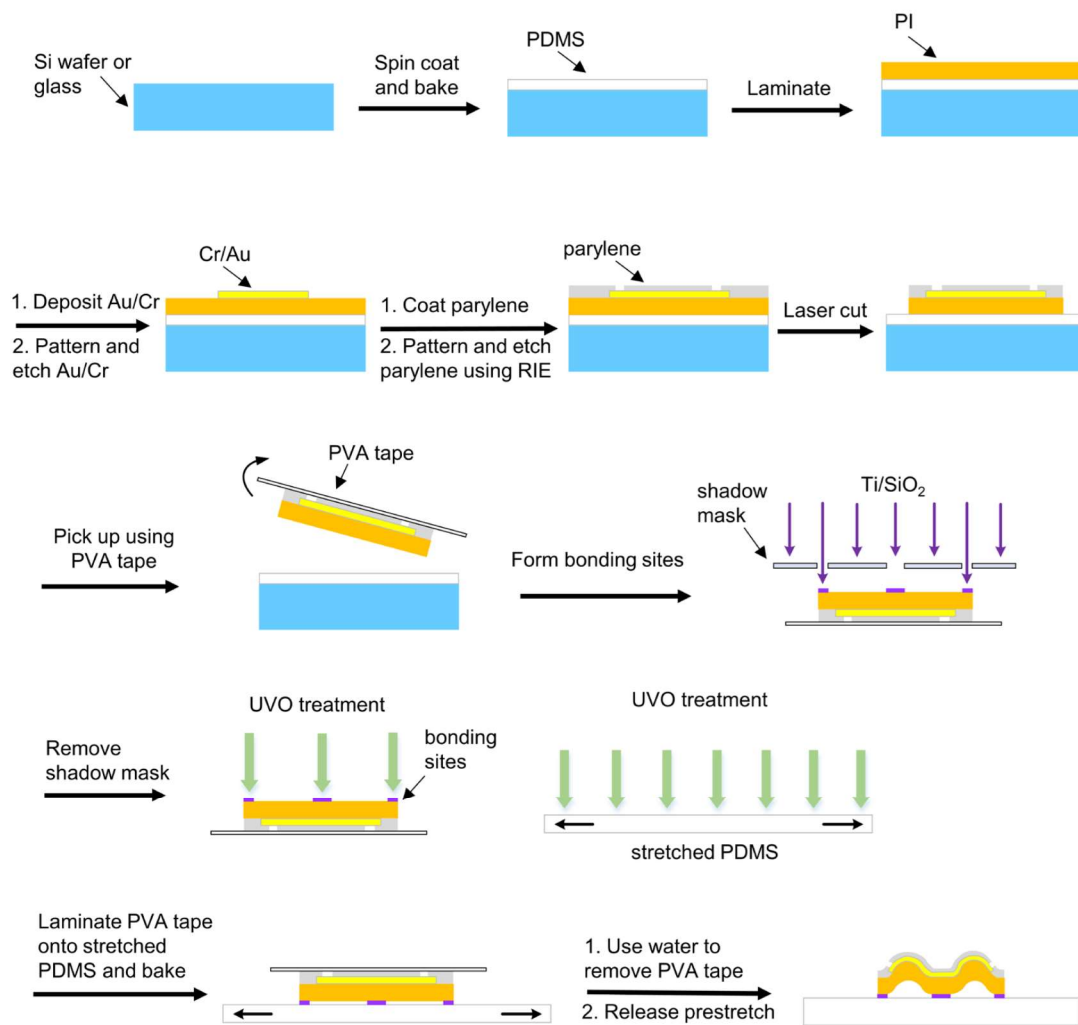


Figure 3.6. Schematic illustration of steps for fabricating compliant 3D frameworks instrumented with strain sensors. The Figure set was produced by Hangbo Zhao from the Rogers group.

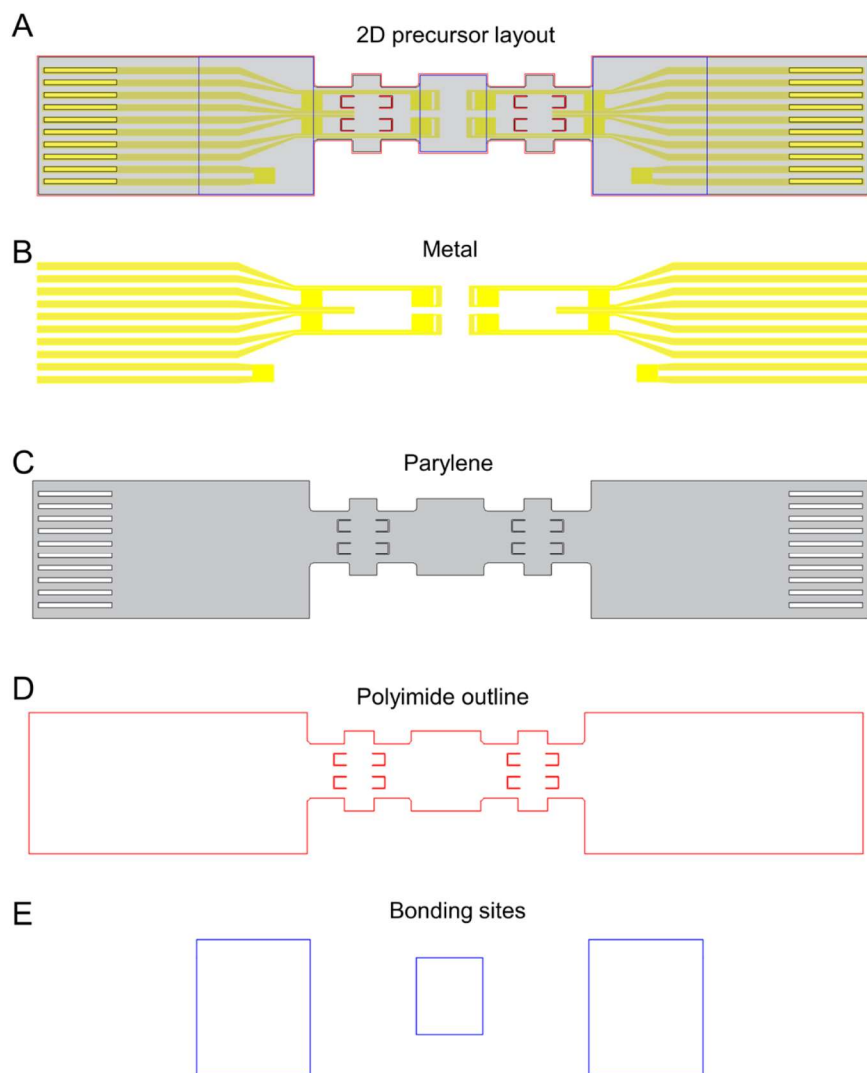


Figure 3.7. 2D layouts of the basic instrumented 3D framework design. (a) Overview of the stacked 2D precursor layouts. (b) Metal layer. (c) Parylene layer. (d) 2D polyimide outline created by laser ablation. (e) Shadow mask openings for defining bonding sites for mechanically guided assembly. The Figure set was produced by Hangbo Zhao from the Rogers group.

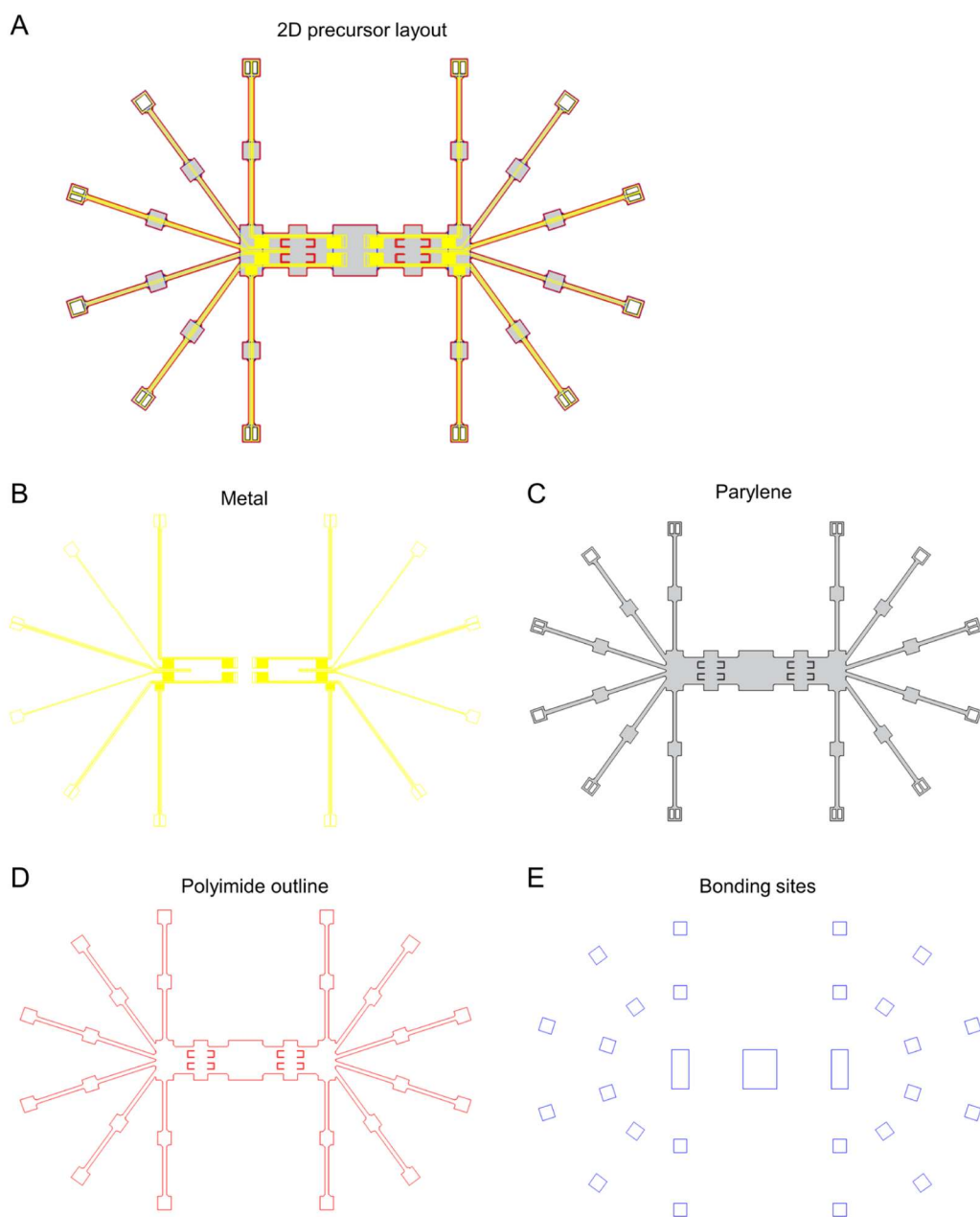


Figure 3.8. 2D layouts of an instrumented 3D framework in a complex 3D shape. (a) Overview of the stacked 2D precursor. (b) Metal layer. (c) Parylene layer. (d) 2D polyimide outline created by laser ablation. (e) Shadow mask openings for defining bonding sites for mechanically guided assembly. The Figure set was produced by Hangbo Zhao from the Rogers group.

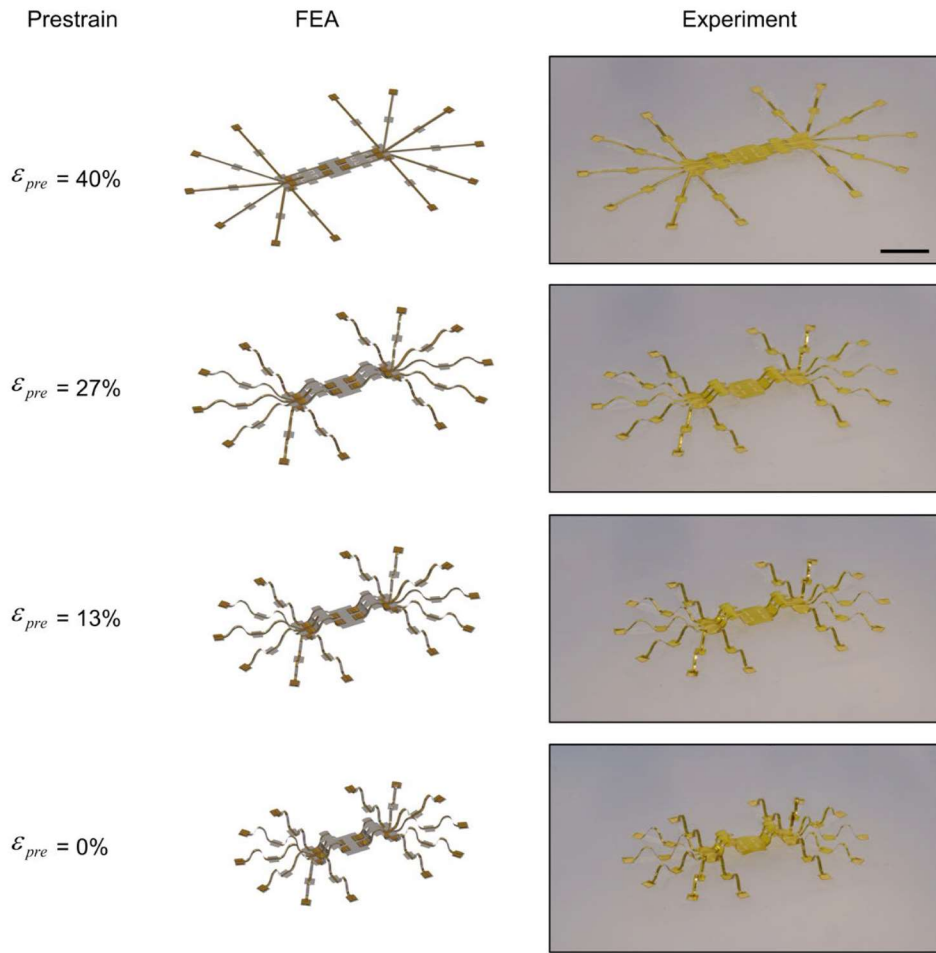


Figure 3.9. Comparison of FEA and experimental images of a compliant 3D framework in a complex 3D shape during the buckling process. Scale bar: 5 mm. The Figure set was produced by Hangbo Zhao from the Rogers group.

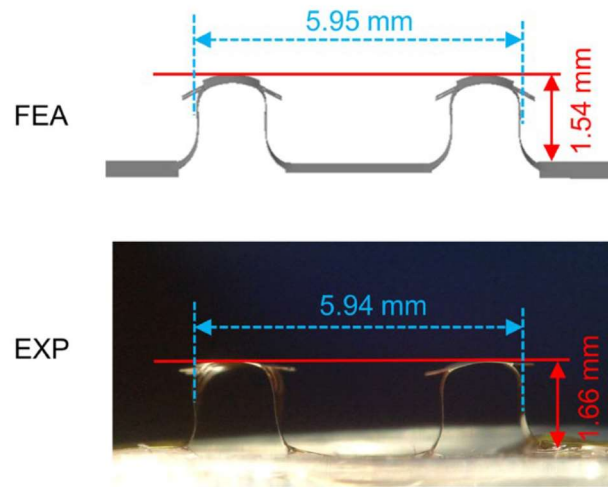


Figure 3.10. Comparison of key dimensions of a 3D framework in FEA and experiments. The Figure set was produced by Hangbo Zhao from the Rogers group.

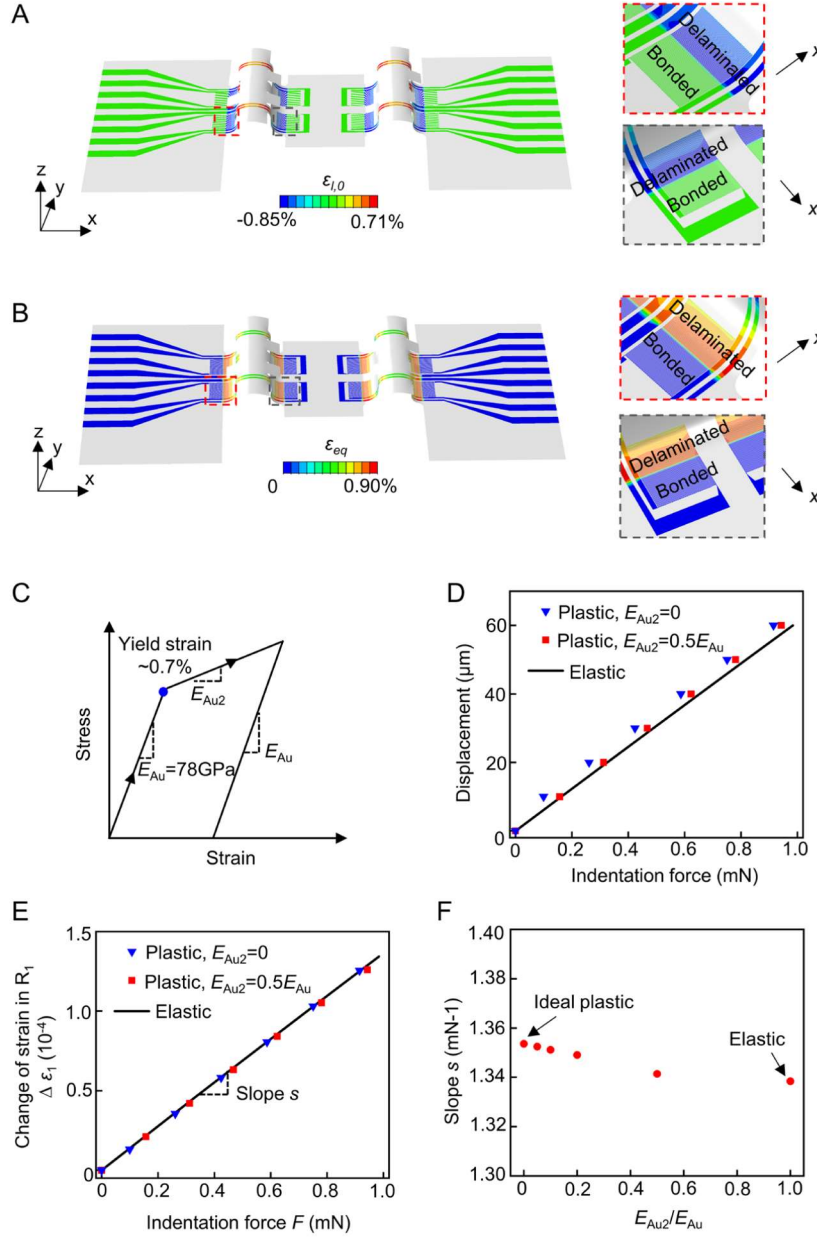


Figure 3.11. Strains in the metal strain sensors and effect of metal plasticity. (a) Computed strain distribution along the x-direction (longitudinal direction of the strain sensors) in the gold layer after mechanically guided assembly, with insets featuring two strain sensors on opposite sides of a buckled post. (b) Computed maximum equivalent strain distribution in the gold layer after mechanically guided assembly, with insets featuring two strain sensors on opposite sides of a buckled post. (c) Schematic stress-strain curve of gold modeled by a double liner plasticity model showing the transition from elastic deformation to plastic deformation after a yield strain of $\sim 0.7\%$. (d) Computed displacement as a function of the indentation force for the indentation test shown in Figure 3.1 (d), in the elastic regime and plastic regime with different moduli of gold. (e) Computed change of strain in resistor R_1 as a function of the indentation force for the indentation test shown in Figure 3.1 (d), in the elastic regime and plastic regime with different moduli of gold. (f) Computed slope of the change of strain vs. different ratios of E_{Au2}/E_{Au} . The Figure set was produced by Hangbo Zhao from the Rogers group.

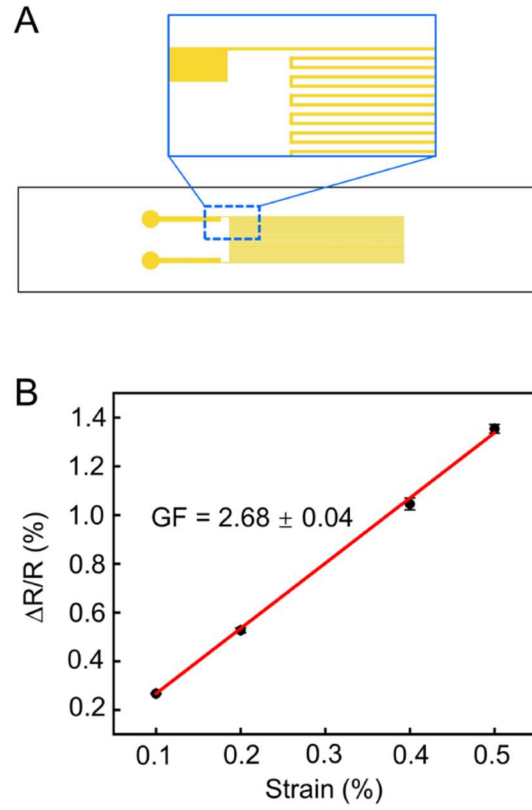


Figure 3.12. Measurement of gauge factors of strain sensors. (a) Layout of a planar strain sensor used for measuring the gauge factors via tensile testing. (b) Relative resistance change of the strain sensor as a function of the applied axial strain in tensile testing. The red line shows the linear least-squares fitting. The Figure set was produced by Hangbo Zhao from the Rogers group.

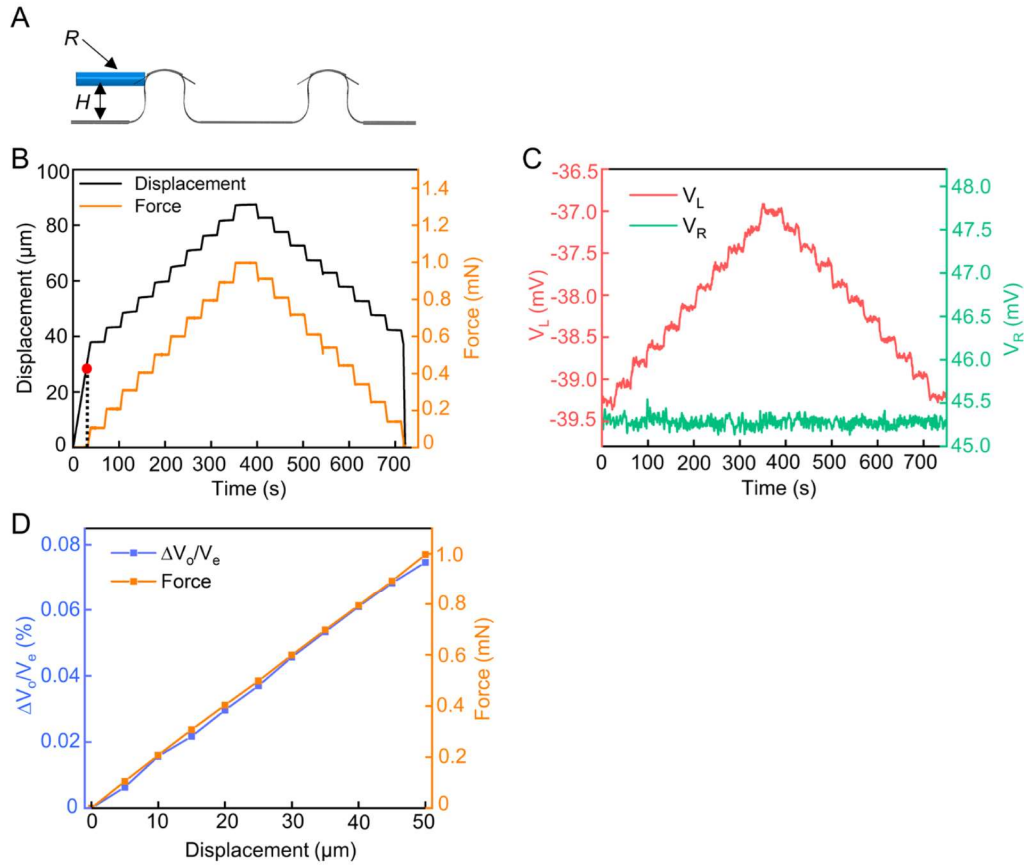


Figure 3.13. Characterization of instrumented 3D frameworks by indentation. (a) Schematic illustration of the indentation setup on an instrumented 3D framework. The indenter radius is $R = 0.2$ mm and the indentation height $H \approx 1.26$ mm. (b) Displacement (left axis) and the corresponding indentation force (right axis) recorded from the indentation test. The red dot indicates the point of contact between the indenter and the 3D framework. (c) Output voltages from the left (left axis) and right (right axis) Wheatstone bridges embedded in the 3D framework recorded simultaneously during the indentation test. (d) Relative output voltage change of the left Wheatstone bridge (left axis) and the indentation force (right axis) as a function of the indentation displacement. The Figure set was produced by Hangbo Zhao from the Rogers group.

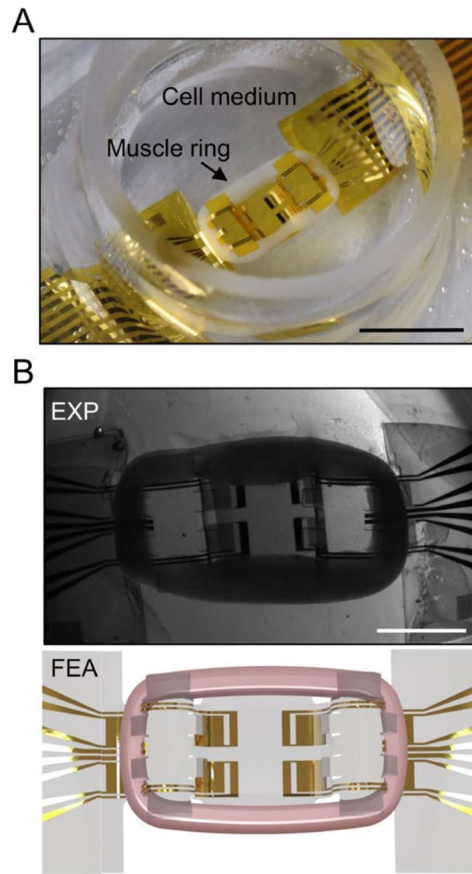


Figure 3.14. Optical and FEA images of a skeletal muscle ring integrated with an instrumented 3D framework. (a) Angled view (optical) of an engineering skeletal muscle ring integrated with a compliant 3D framework inside a glass well filled with clear cell medium. (b) Experimental inverted microscopic (top) and FEA (bottom) images showing the top-down view of the ring integrated with the 3D framework during measurements of the muscle ring contractions. Scale bars: 5 mm in (a) and 2 mm in (b).

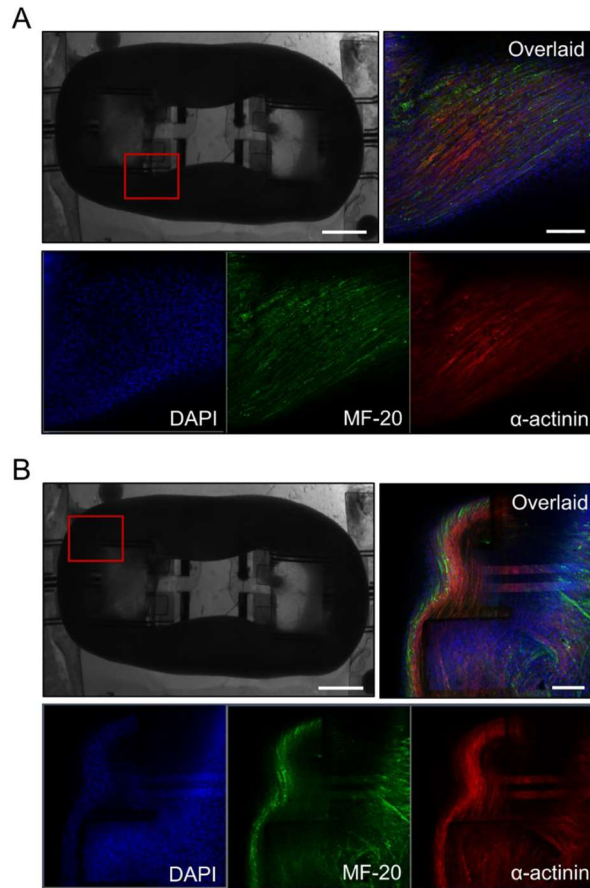


Figure 3.15. Top-down view of microscopic optical (top, left) and confocal imaging of a muscle ring integrated with a 3D framework. Confocal images highlight the alignment of the myofibers, visualized with an antibody against α -actinin protein (red), nuclei (DAPI; blue), and mature myosin (MF-20; green). Overlaid images are shown on the top, right and separate images are in the bottom panel. (a) and (b) highlight two different regions in the muscle ring indicated by the red boxes. Scale bars: 1 mm in the optical images and 200 μ m in the confocal images.

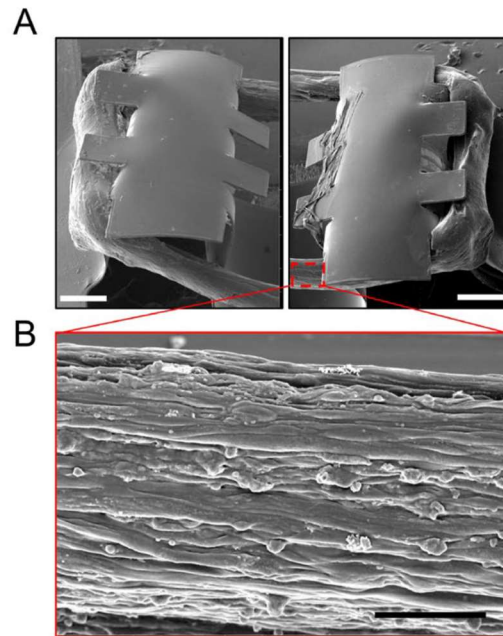


Figure 3.16. SEM images of an engineered muscle ring integrated with a compliant 3D framework. (a) Muscle ring on the two ends of the buckled posts. (b) Close-up image showing the alignment of the myofibers. Scale bars: 500 μm in (a) and 50 μm in (b).

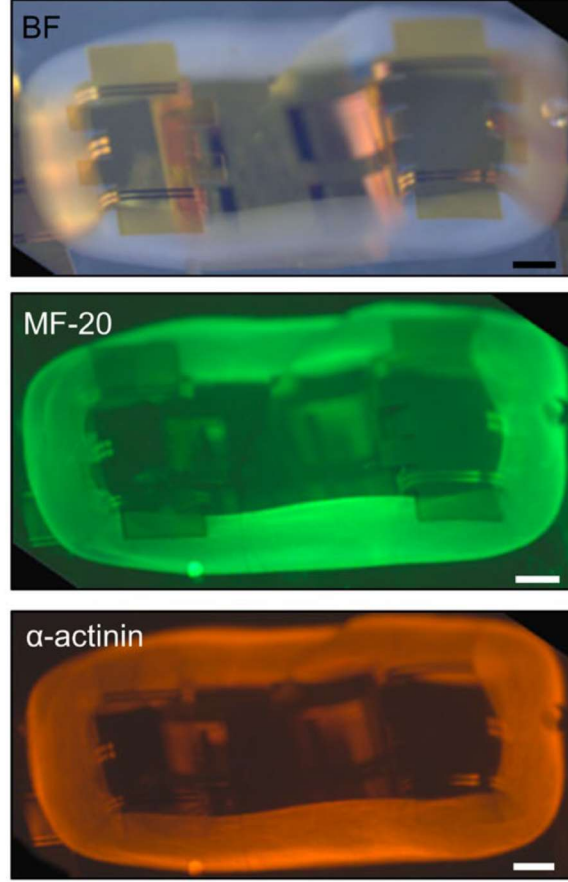


Figure 3.17. Top-down view of the bright field (BF) and fluorescence images visualized with an antibody against mature myosin (MF-20) and α -actinin protein. Scale bars: 500 μm .

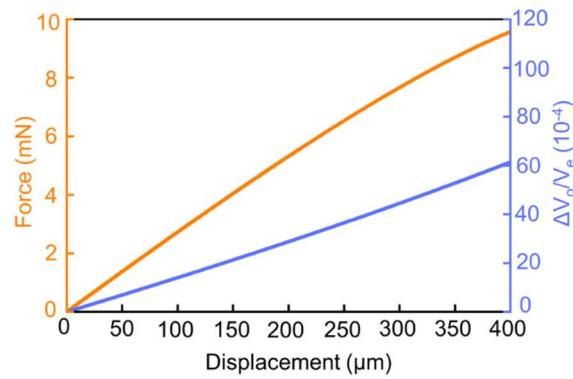


Figure 3.18. Computed tension force of the muscle ring and the corresponding displacement of the posts and output voltage of the 3D framework over a large deformation range. Zero displacement corresponds to no muscle ring on the as-buckled 3D framework. The Figure set was produced by Hangbo Zhao from the Rogers group.

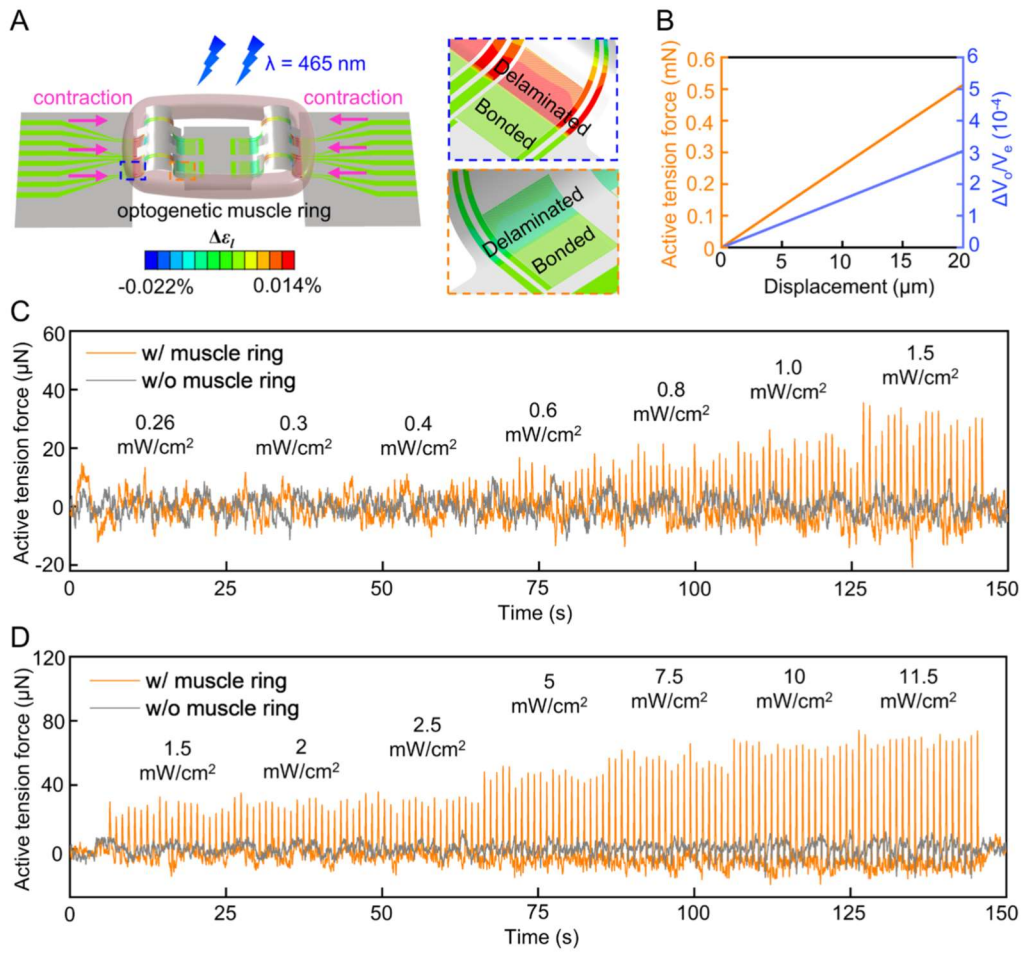


Figure 3.19. Measurements of active tension forces produced by an optogenetic muscle ring in response to optical stimulation. (a) Computed change in strain of the 3D framework in response to optical stimulation (contractile displacement: 10 μm), featuring two strain sensors on opposite sides of a post (inside dashed lines). (b) Computed active tension forces of the muscle ring and the corresponding relative output voltage of the strain sensor as functions of the contractile displacement. (c) Active tension forces with and without integration with an optogenetic skeletal muscle ring, in response to optical stimulation at intensities of 0.26, 0.3, 0.4, 0.6, 0.8, 1.0, and 1.5 $\text{mW}\cdot\text{cm}^{-2}$ with 1 Hz pulse frequency and 50 ms pulse width. (d) Active tension forces with and without integration with an optogenetic skeletal muscle ring, in response to optical stimulation at intensities of 1.5, 2, 2.5, 5, 7.5, 10, and 11.5 $\text{mW}\cdot\text{cm}^{-2}$ with 1 Hz pulse frequency and 50 ms pulse width.

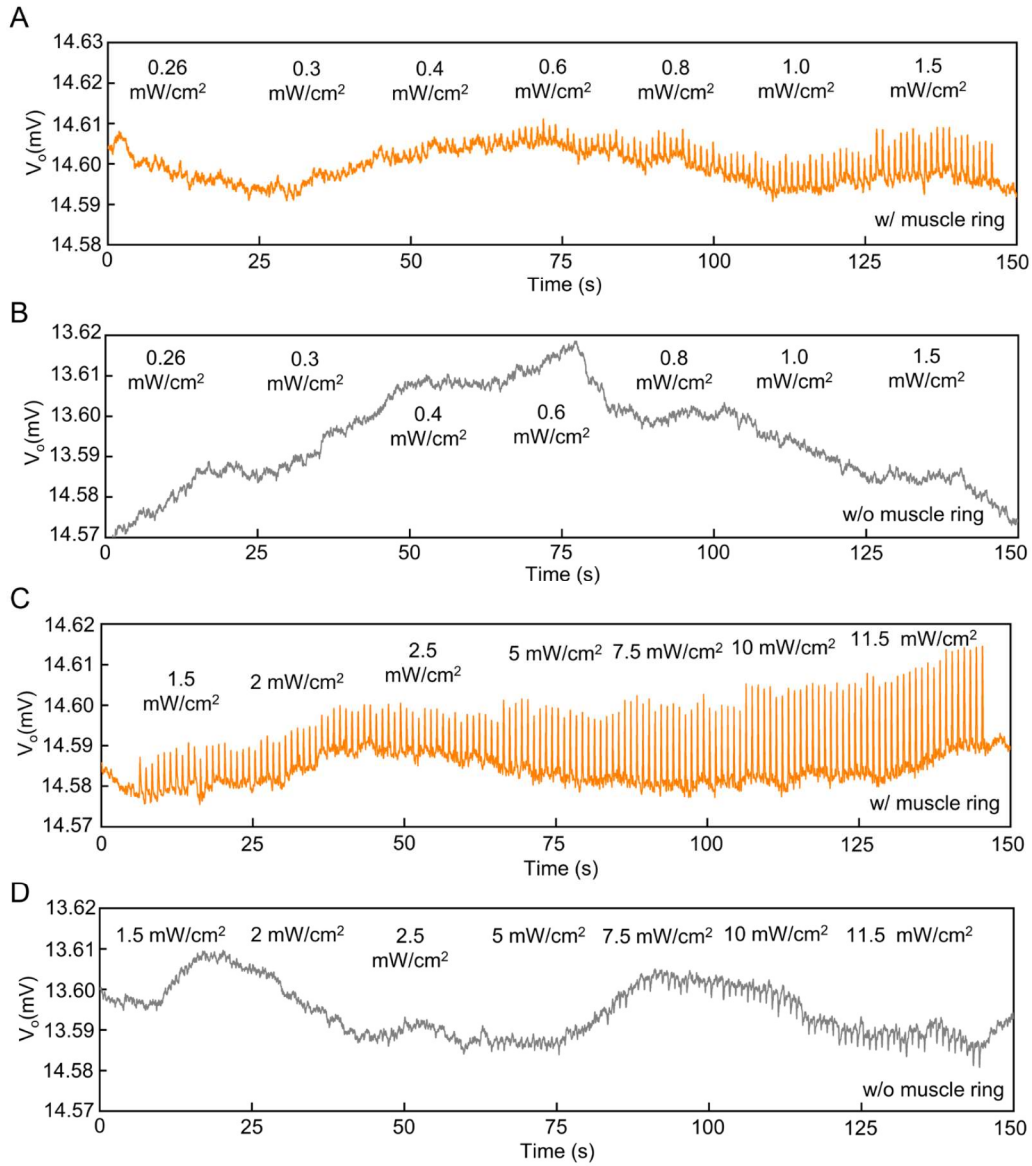


Figure 3.20. Raw data of the voltage output of a 3D framework with and without integration of an optogenetic muscle ring, in response to optical stimulation with different intensities. (a)-(b) Wheatstone bridge voltage output for optical intensities of 0.26, 0.3, 0.4, 0.6, 0.8, 1.0, and 1.5 $\text{mW}\cdot\text{cm}^{-2}$ with 1 Hz pulse frequency and 50 ms pulse width. (c)-(d) Wheatstone bridge voltage output for optical intensities of 1.5, 2, 2.5, 5, 7.5, 10, and 11.5 $\text{mW}\cdot\text{cm}^{-2}$ with 1 Hz pulse frequency and 50 ms pulse width.

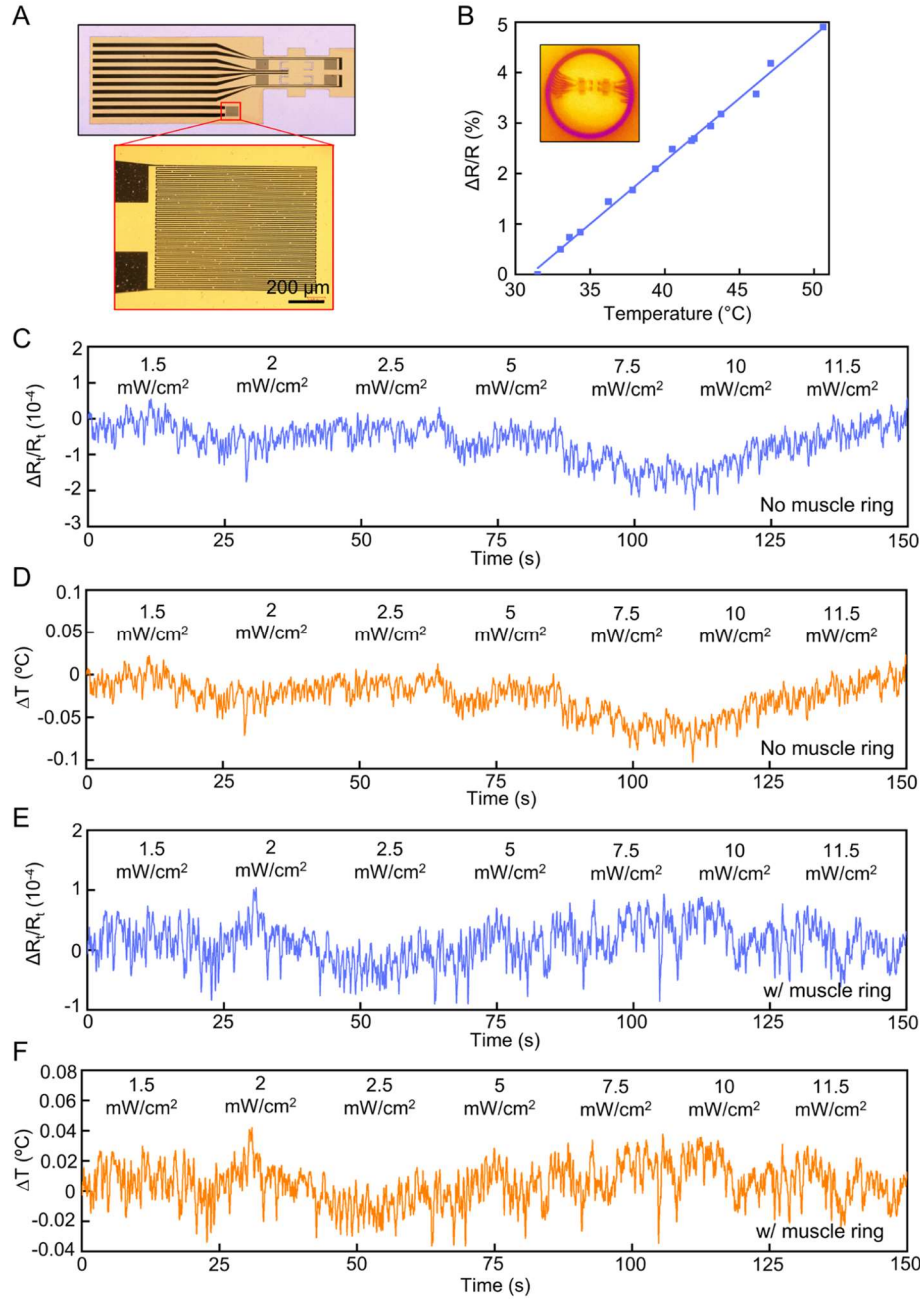


Figure 3.21. Characterization of the influence of temperature on the resistance change of strain sensors. (a) Optical images of the resistive temperature sensor on the 2D precursor. (b) Measured relative resistance change of a representative strain sensor in a temperature range of 30-50 $^{\circ}\text{C}$. Inset shows an infrared image of the instrumented 3D framework used in the temperature measurement of the selected strain sensor. (c) Relative resistance change and the corresponding calculated temperature (d) measured from the temperature sensor in an instrumented 3D framework under optical simulation at different light intensities without integration with a muscle ring. (e) Relative resistance change and the corresponding calculated temperature (f) measured from the temperature sensor in the 3D framework integrated with a muscle ring under optical simulation at different light intensities.

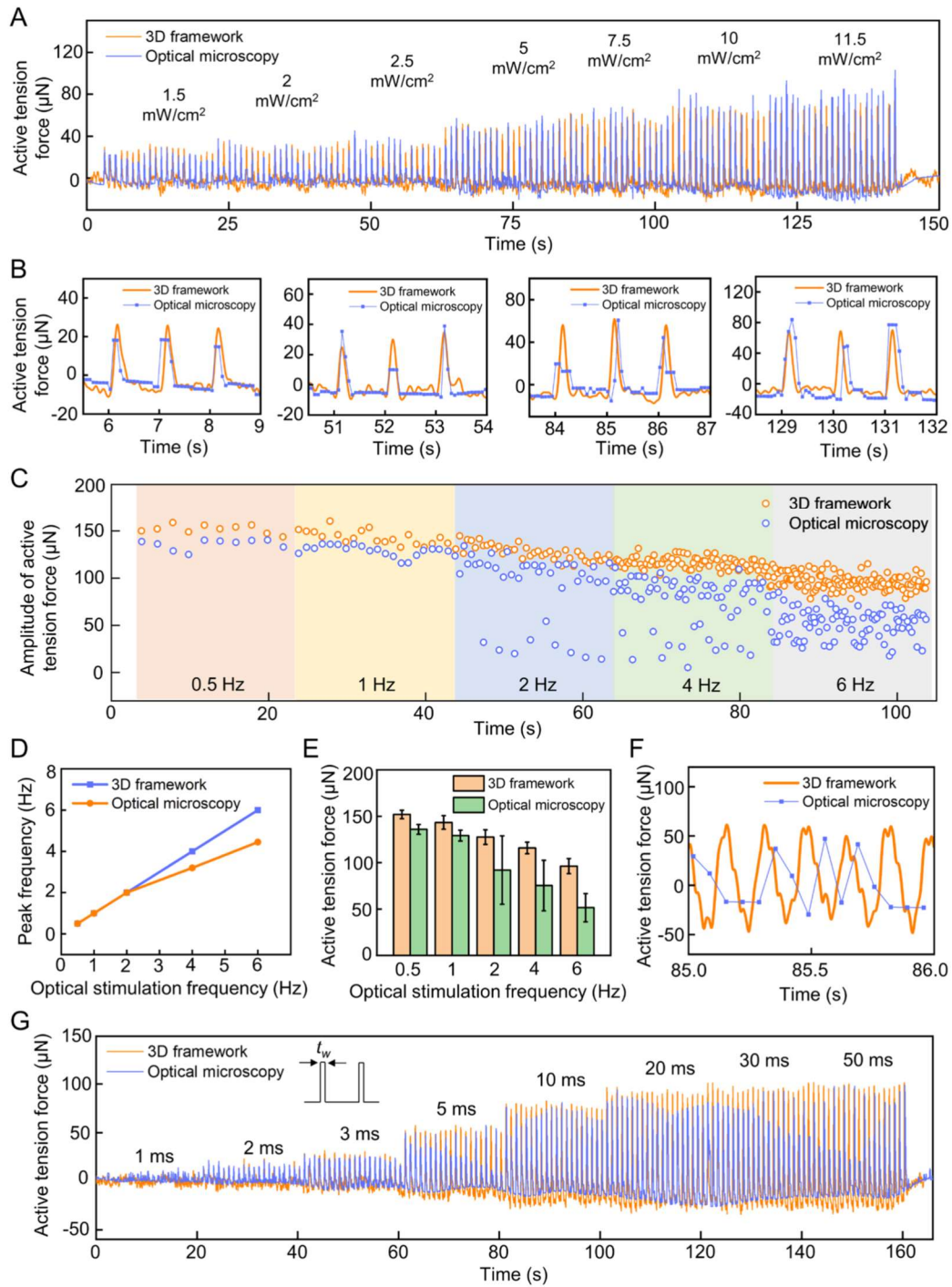


Figure 3.22. Comparison of active tension force measurements by instrumented 3D frameworks and by optical microscopy. (a) Comparison of the active tension forces of an optogenetic skeletal muscle ring measured by an instrumented 3D framework and by optical microscopy, in response to optical stimulation at intensities of 1.5, 2, 2.5, 5, 7.5, 10, and 11.5 $\text{mW}\cdot\text{cm}^{-2}$ with 1 Hz pulse frequency and 50 ms pulse width. (b) Selected time frames of the active tension forces measured in (a).

Figure 3.22. (Cont.) (c) Amplitudes of all the active tension peaks measured by an instrumented 3D framework and optical microscopy under optical stimulation with frequencies of 0.5, 1, 2, 4, and 6 Hz ($11.5 \text{ mW} \cdot \text{cm}^{-2}$ optical intensity and 50 ms pulse width). (d) Comparison of the measured peak frequency from the 3D framework and optical microscopy as a function of the optical stimulation frequency. (e) Comparison of measured amplitudes of active tension forces from the 3D framework and optical microscopy as a function of the optical stimulation frequency. (f) Active tension forces measured by the 3D framework and optical microscopy at a stimulation frequency of 6 Hz showing the advantages of the 3D framework in measurement accuracy as compared to optical microscopy. (g) Comparison of active tension forces of an optogenetic skeletal muscle ring measured by a 3D framework and optical microscopy, in response to optical stimulation with pulse widths of 1, 2, 3, 5, 10, 20, 30, and 50 ms ($11.5 \text{ mW} \cdot \text{cm}^{-2}$ optical intensity and 1 Hz pulse frequency).

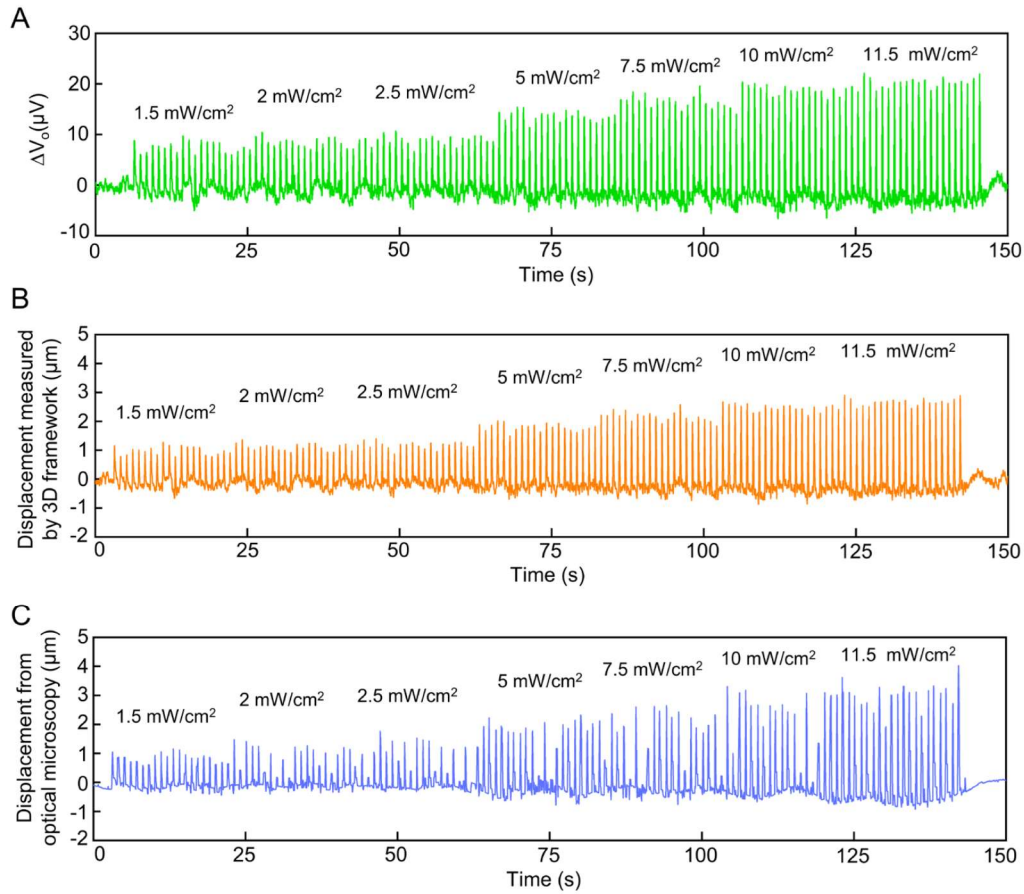


Figure 3.23. Wheatstone bridge output voltage and displacement measurements from 3D frameworks and optical microscopy. (a) Change of the Wheatstone bridge output voltage of a 3D framework integrated with an optogenetic muscle ring after applying a 5 Hz high-pass filter, in response to optical stimulation at intensities of 1.5, 2, 2.5, 5, 7.5, 10, and 11.5 $mW \cdot cm^{-2}$ with 1 Hz pulse frequency and 50 ms pulse width. (b) Muscle contractile displacements measured by the 3D framework from the sensor output voltage in (a). (c) Muscle contractile displacements measured simultaneously by optical microscopy.

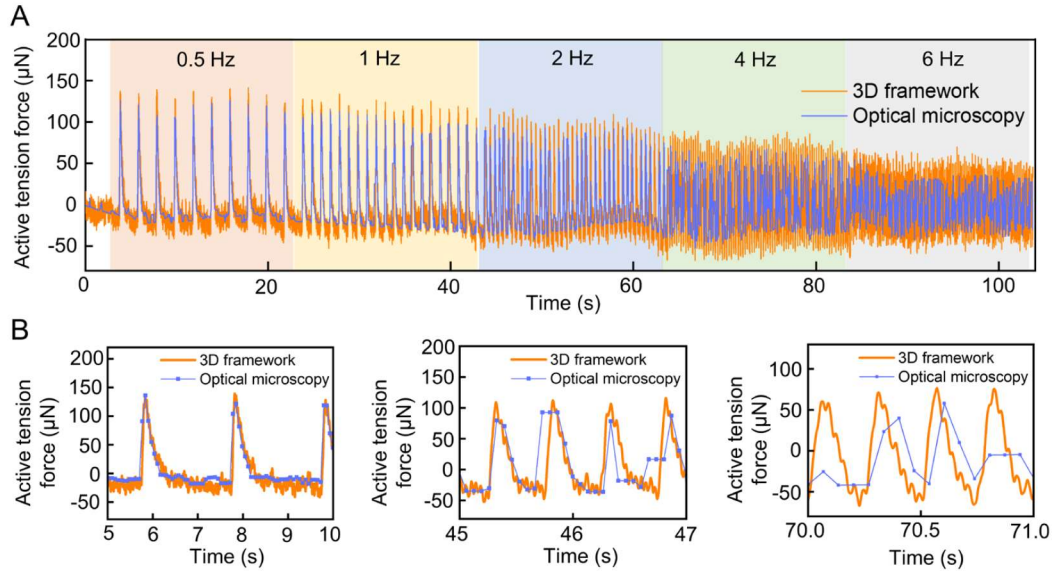


Figure 3.24. Comparison of active tension forces measured by a 3D framework and optical microscopy under optical stimulation with different frequencies. (a) Comparison of a 3D framework and optical measurements in the full frequency range. (b) Selected time frames of the active tension forces measured in (a) with corresponding stimulation frequencies of 0.5 Hz, 2 Hz, and 4 Hz. A digital bandpass filter of 0.1-20 Hz filtered high-frequency noise and smoothed the signal.

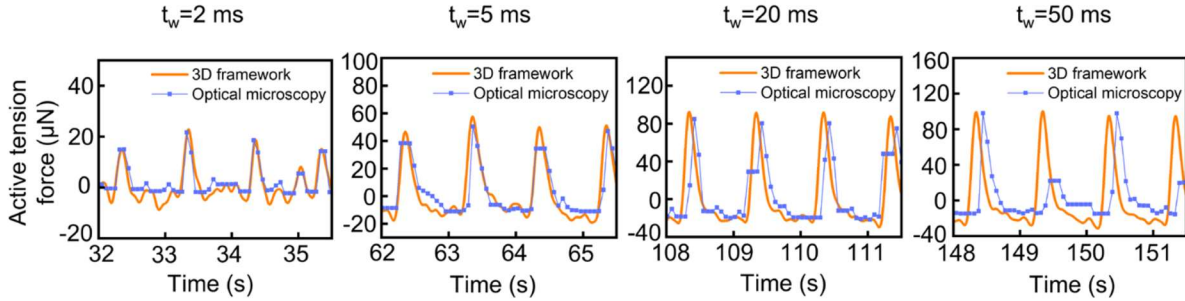


Figure 3.25. Selected time frames of the active tension forces with corresponding stimulation frequencies of 0.5 Hz, 2 Hz, and 4 Hz. A digital bandpass filter of 0.1-20 Hz filtered high-frequency noise and smoothed the signal. Selected time frames of the active tension forces measured in Figure 3.22 (g) with different optical stimulation pulse widths. The stimulation intensity is $11.5 \text{ mW} \cdot \text{cm}^{-2}$ with a 1 Hz pulse frequency.

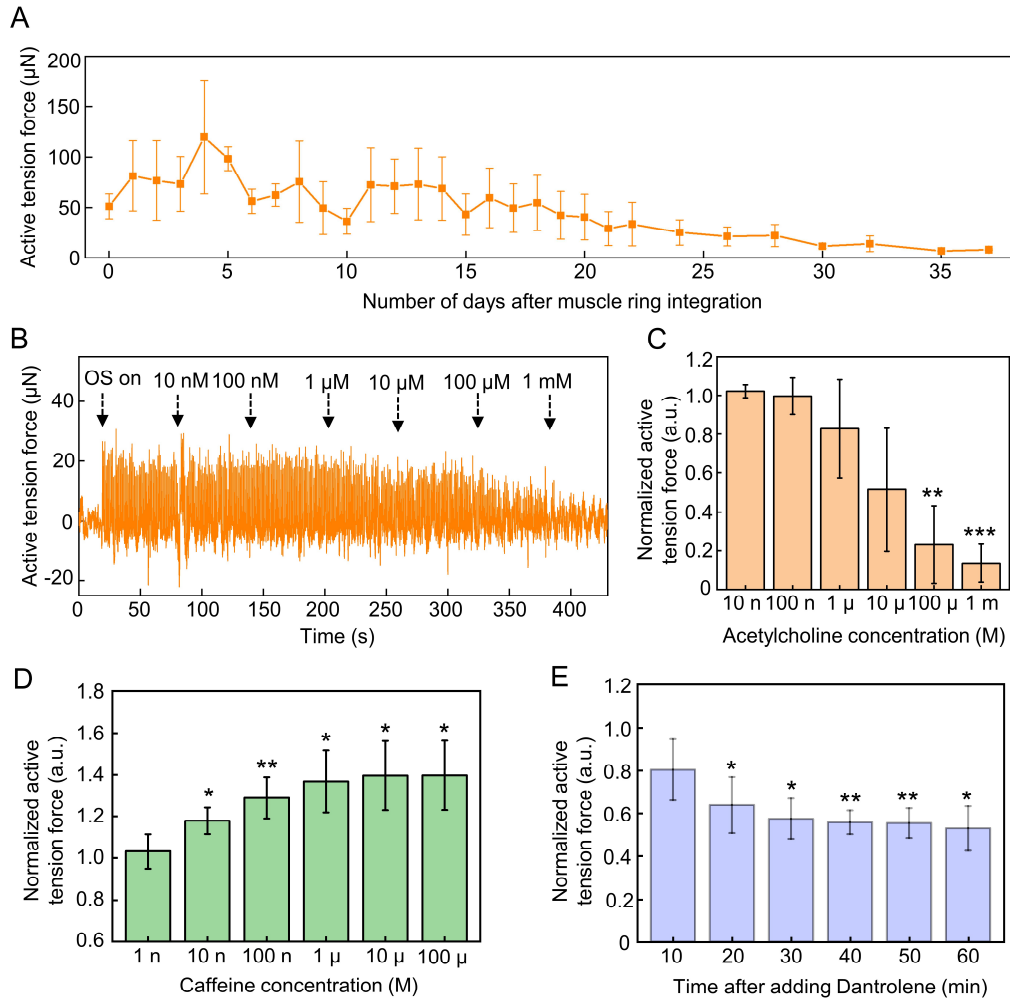


Figure 3.26. Development of active tension forces over time and responses to the introduction of drugs. (a) Development of the active tension forces under optical stimulation ($11.5 \text{ mW} \cdot \text{cm}^{-2}$ intensity, 1 Hz frequency, and 50 ms of pulse width) measured by optogenetic muscle rings integrated with compliant 3D frameworks instrumented with strain sensors over a period of 37 days after integration. Error bars correspond to the calculated standard deviation from three measurements. (b) Active tension forces measured by a representative optogenetic muscle ring in response to optical stimulation with simultaneous addition of acetylcholine chloride at different working concentrations. (c) Normalized active tension forces of optogenetic muscle rings in response to optical stimulation with simultaneous addition of caffeine at different working concentrations. Error bars correspond to the calculated standard deviation from four measurements. (d) Normalized active tension forces from optogenetic muscle rings in response to optical stimulation with simultaneous addition of caffeine at different working concentrations. Error bars correspond to the calculated standard deviation from four measurements. (e) Change of normalized active tension forces of optogenetic muscle rings in response to optical stimulation with the addition of $100 \text{ } \mu\text{M}$ working concentration of dantrolene. Error bars correspond to the calculated standard deviation from three measurements. Statistical significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

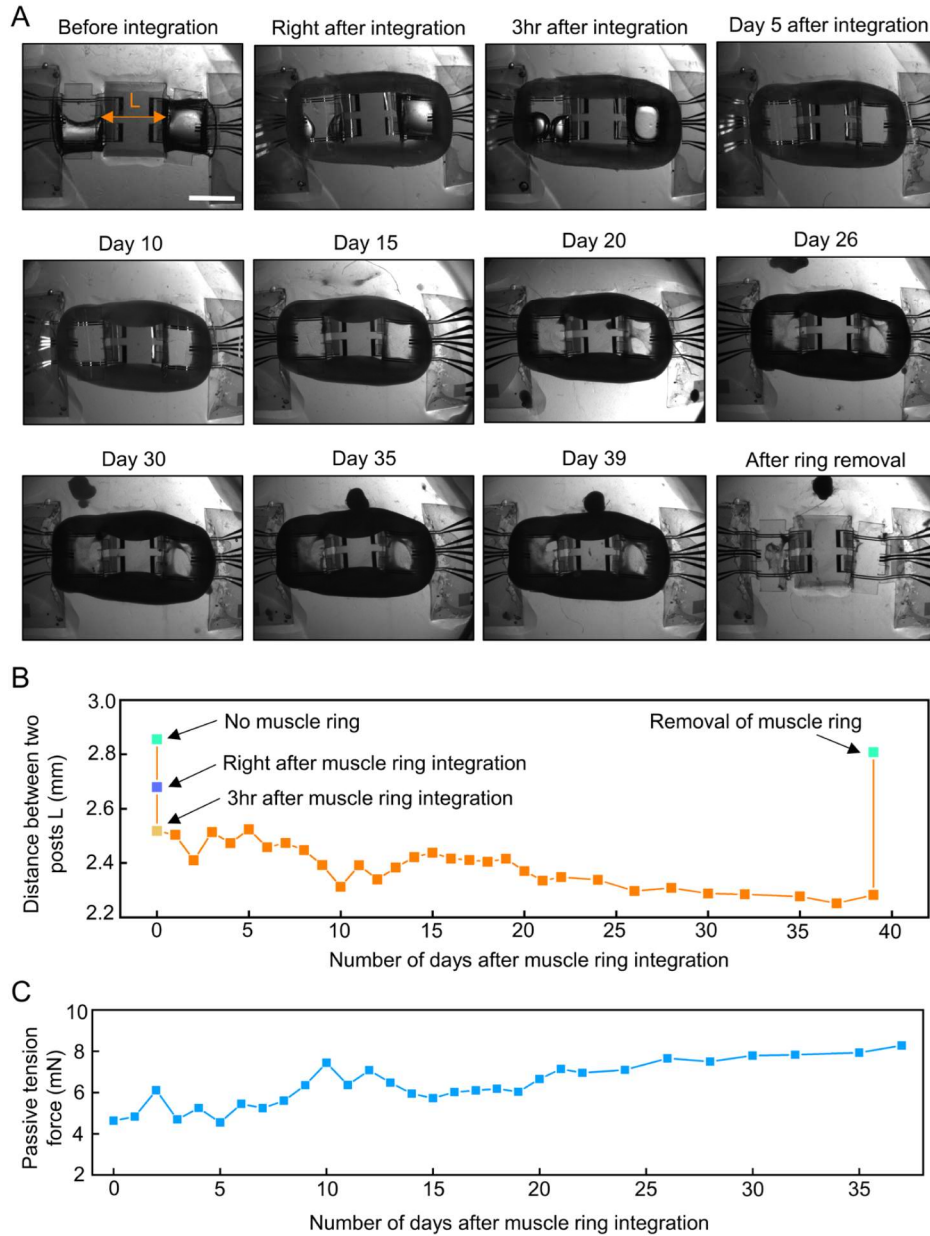


Figure 3.27. Tracking of structural deformations of the 3D framework and passive tension forces of the muscle ring over 39 days. (a) Microscopic images of a top-down view of a 3D framework before muscle ring integration, at different times after muscle ring integration, and after muscle ring removal. (b) Recorded distance between the two buckled posts in the 3D framework before muscle ring integration, at different times after muscle ring integration, and after muscle ring removal. (c) Passive tension force before and after muscle ring integration during the long-term studies calculated from the displacements of the 3D posts measured in (b). Scale bar: 2 mm.

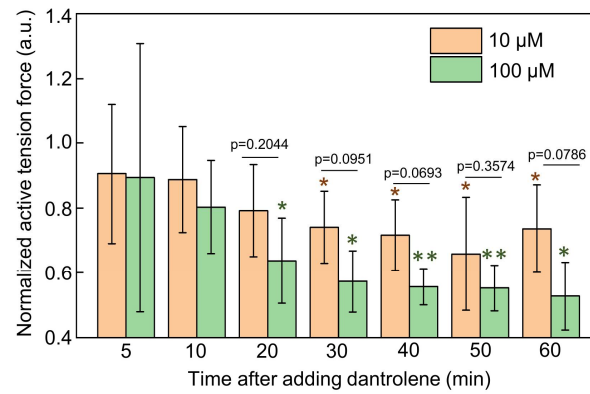


Figure 3.28. Change of active tension forces of optogenetic muscle rings in response to optical stimulation with the addition of 10 μ M and 100 μ M working concentrations of dantrolene. Error bars correspond to the calculated standard deviation from three measurements. Asterisks represent statistical significance compared with the active tension force before adding dantrolene (* $p < 0.05$, ** $p < 0.01$); the underlined p values represent the statistical significance between 10 μ M and 100 μ M of dantrolene at the same time after adding dantrolene.

3.6. Table

Table 3.1. Experimental procedure (drug concentration and timeline) for the addition of acetylcholine and caffeine for the study of drug responses.

Time		0-1 min	1 min	2 min	3 min	4 min	5 min	6 min
Acetylcholine	Bolus conc.	Not added	2 μ M	20 μ M	200 μ M	2 mM	20 mM	200 mM
	Working conc.		10 nM	100 nM	1 μ M	10 μ M	100 μ M	1 mM
Caffeine	Bolus conc.	Not added	200 nM	2 μ M	20 μ M	200 μ M	2 mM	20 mM
	Working conc.		1 nM	10 nM	100 nM	1 μ M	10 μ M	100 μ M

3.7. References

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CHAPTER 4: MUSCLE-DRIVEN ROBOTS WITH WIRELESS OPTOELECTRONICS

4.1 Introduction

In previous Chapters 2 and 3, electrical actuation and strain sensing of 3D skeletal muscle tissue were demonstrated through 3D electronics platforms. However, their tethered electronics systems are limited to tethered biohybrid robotic applications. In this Chapter, wireless optoelectronics integrated muscle-driven robots will be introduced for the advanced biohybrid soft robotics applications.

Biohybrid systems consisting of living and artificial components that recapitulate biological architectures and functions can enhance our understanding of biological systems and foster new applications in implantable devices, [1] regenerative medicine, [1] organ-on-a-chip, [2, 3] and soft robotics. [4-15] Recently, biohybrid robots consisting of cells and soft materials have demonstrated the ability to sense, respond and adapt to environmental cues in real-time,⁴ and achieving life-like functions such as swimming, walking, and pumping. [5-15] Demonstrations range from biohybrid rays [5] and fish [6] based on sheets of optogenetic (i.e., responsive to light) cardiomyocytes and neuromuscular junction driven micro-swimmers [7] to walkers, grippers, swimmers, and pumps that employ 3D skeletal muscle tissue, [8-13] activated either electrically or by light.

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While collectively, these prototypes offer a variety of solutions aimed at improving performance and controllability, severe limitations persist in terms of coordination of advanced functionalities. Unlike electric stimulation that is either invasive (implanted electrodes) or poorly selective (electrodes immersed in liquid baths where robots operate), optogenetics has indeed provided a powerful paradigm, enabling both non-invasive and localized stimulation. [5-8] Nonetheless, optogenetic-based control requires light sources placed near the muscles of interest and high intensities to activate transfected opsins, rendering manually-directed light stimulation – the only approach so far demonstrated – as cumbersome and of limited applicability.

In this regard, the integration of onboard lightweight, miniaturized, and flexible electronics to command and power multiple light points distributed at precise locations, without the hindering motion, becomes an attractive option. The integration of bioelectronics has great potential for advanced robotic functions also demonstrated by recent advances in neuromodulation and sensing *in-vivo*, [16-20] as well as electrical modulation or recording of muscle actuations of tethered, non-motile systems *in-vitro*. [2, 3, 13, 14] Here, we present the first wirelessly controlled biohybrid electronic robot (eBiobot) driven by a battery-free optoelectronic device with wireless communications, allowing remote control of switching, steering, and other more sophisticated functions. The eBiobot inherits the structural concept from previously demonstrated walking robots, [8-10] where 3D-engineered skeletal muscle tissue forms around an asymmetric hydrogel scaffold. Responding to external stimuli, muscles perform cyclic contractions, deform the scaffold, and propel the robot through asymmetric friction generated at the scaffold-substrate interface.

4.2. Materials and Methods

4.2.1. Fabrication process for wireless optogenetic devices

Figure 4.1 shows the schematics of the fabrication process for wireless optogenetic devices. A tri-layer stack Cu (18 μm)/PI (75 μm)/Cu (18 μm) (Dupont, Pyralux AP8535R) served as the fabrication substrate for the flexible printed circuit board (fPCB). The laser ablation process (LPKF Laser & Electronics, ProtoLaser U4) defined the layout of the device including receiver coil, circuits, and soldering pads. The components were mounted and soldered on the fPCB using low temperature tin-bismuth-silver alloy solder paste (ChipQuik) and hot air soldering processing. Finally, chemical vapor deposition (CVD, Special Coating Systems) formed a conformal waterproof parylene coating (14 μm) that encapsulates the devices. In this paper, we designed two device configurations: one with passive operation and the other with NFC active operation. For the passive operation, we used either one single $\mu\text{-ILED}$ or five $\mu\text{-ILEDs}$ electrically connected in parallel distributed at center and inner corners to satisfy the illumination requirements. For active operation, we designed a device with two bilateral pads, each containing five $\mu\text{-ILEDs}$. These active devices contain an RF accessible memory (M24LR04E, STMicroelectronics) and a low power 8-bit microcontroller (Attiny84, Atmel) to implement remote control of the $\mu\text{-ILED}$ arrays in two modes of operation: 1) synchronous and, 2) asynchronous operation, both with user programmable frequency and pulse widths.

4.2.2. Design and Fabrications of 3D printed hydrogel

A digital light processing (DLP) 3D printer (PICO2, ASIGA) was used to fabricate hydrogel parts designed in Solidworks. The printing resin solution composed of 20 (v/v) % polyethylene glycol diacrylate (PEGDA) MW 700 g/mol (Sigma-Aldrich) in distilled water

containing 1 mg/ml of lithium phenyl-2,4,6-trimethylbenzoylphosphinate (Sigma-Aldrich) as a photoinitiator, and 0.4 mg/ml of sunset yellow dye (Sigma-Aldrich) for the printing resolution by decreasing light scattering effect. [10] The fabricated structures were soaked in 10 (v/v) % bleach for 30 minutes to remove out the dye from 3D printed structures, followed by sterilization in 70 (v/v) % of isopropyl alcohol overnight and rinsing with phosphate-buffered saline (PBS) in 4 °C for at least 4 days before tissue seeding.

4.2.3. Cell culture and tissue fabrications

Optogenetic C2C12 myoblasts transfected with blue light sensitive ion channel Channelrhodopsin-2 tagged with tdTomato, namely ChR2 (H134R), were cultured in the growth media composed of Dulbecco's modified Eagle medium (DMEM) (Corning) with 10 (v/v) % of FBS (Lonza), 1 (v/v) % of L-glutamine (Corning), and 1 (v/v) % of penicillin-streptomycin (Lonza). A 5 M cells/ml of ChR2 C2C12s was seeded in 3D printed mold after mixing with 30 (v/v) % Matrigel (Corning), 4 mg/ml fibrinogen (Sigma-Aldrich), and thrombin (Sigma-Aldrich) with 0.5 unit/mg of fibrinogen. [21] In 1st and 2nd generations of eBiobots, the cell/gel solution was seeded on 3D printed mold in Figure 4.2 (c). In 3rd and 4th generations of eBiobots and bipedal eBiobots, it was seeded on assembled structure with skeleton and mold in Figure 4.3 (c)-i, Figure 4.4, and Figure 4.5. The growth media containing 1 mg/ml of aminocaproic acid (Sigma-Aldrich) was added to the seeded tissues with 3D printed structures after 90 minutes of seeding, followed by 3 days incubation for the compaction of cell/gel mixture with the same media in 37 °C. 3 days after seeding, the media was switched into the differentiation media, DMEM containing 10 (v/v) % horse serum (ThermoFisher), 1 (v/v) % penicillin/streptomycin, 1 (v/v) % L-glutamine, 1 mg/ml

of aminocaproic acid, and 1 ng/ml of insulin-like growth factor-1 (Sigma-Aldrich) to differentiate myoblasts to myotubes in muscle actuator.

4.2.4. Integration of battery free wireless optogenetic devices with eBiobot

In the 1st and 2nd generations of eBiobots, the wireless optogenetic device with μ -ILEDs as localized stimulators was attached on the 3D printed stage inside of skeleton beam as the red arrow in Figure 4.2 (a) and (b). The Kwik-Sil Adhesive (World Precision Instruments) was applied on the stage to glue with the backside of the wireless optogenetic device. Then, muscle actuator was transferred from the mold in Figure 4.2 (c) on day 9 after differentiation using tweezers. Figure 4.2 (d) and (e) show the wireless optogenetic device on the stage of beam located between muscle actuator and skeleton beam. In the 3rd and 4th generations, the wireless optogenetic devices were attached on 8 mm diameter of circular shape of PDMS using Kwik-Sil Adhesive, prior to assembling the device / PDMS on the hole of skeleton beam as indicated by the red arrows in Figure 4.2 (f) and (g). Stoppers were added on the skeleton in 4th generation to fit with and hold the device / PDMS stably. Wireless optogenetic device with two separate pads of μ -ILEDs was plugged in and out the 3D printed central stage of bipedal skeletons structure without using adhesive. All electronics and PDMS parts were sterilized with ultraviolet (UV) for at least 30 minutes before and after integrating with eBiobots.

4.2.5. Experimental implementation for wireless communication with the optogenetic devices

The power distribution box (PDB, Neurolux), in conjunction with an RF impedance matching for efficient power transmission and communication (Neurolux), drives a double parallel loop antenna with a 4.8 cm vertical distance that wraps around the 15 cm diameter circular

container for wireless power transmission to the battery-free wireless optogenetic devices at 13.56 MHz. To operate passive devices (single eBiobot device), the PDB is configured with an RF switch to deliver target operational pulses (e.g. 1 Hz pulses for 200 ms pulse widths) to the devices within the experimental arena. To operate active devices (bipedal eBiobot device), a customized graphic user interface (GUI) implemented in MATLAB (The MathWorks, Inc.) was used to establish communication with the devices via ISO 15693 NFC protocol supported by the PDB. This implementation together with the specialized firmware in the microcontroller allows to control the operation modality of each individual device such as On/Off, synchronous/asynchronous operation, and frequency/pulse widths reconfiguration in real-time. For the measurement of eBiobots, DMEM medium without phenol red (Corning) was used to prevent light absorption induced by phenol red.

4.2.6. Characterizations of optical irradiance of wireless optogenetic devices

Optical irradiance characterization was performed in two complementary steps using an external power supply. First, the I-V characteristic of the μ -ILED (460 nm; SunLED) was obtained using a semiconductor device analyzer (Keysight 1500A) connected to an electrical probe station (Signatone 1160) (Figure 4.6 (a)). This characterization yields the electrical power of the μ -ILED with respect to go through current. Then, the optical output power was obtained with an integrating sphere (FOIS-1, Ocean Optics), calibrated by a standard diffusive light source (HL-3 Plus, Ocean Optics) for different input currents, driven by the semiconductor device analyzer (Figure 4.6 (b) and (c)). This optical power, in conjunction with respective electrical power, yields the efficiency of the μ -ILEDs (Figure 4.6 (d)).

The second step involved measurements of go through the current of individual μ -ILED for both passive and active optogenetic devices during operation with respect to transmission antenna power, tilt angle, and locations. The outcome results, combined with obtained I-V characteristics and efficiency, yield the electrical power and the respective optical irradiance of the individual μ -ILED during operation. Results are included in Figure 4.6, Figure 4.7, and Figure 4.8.

4.2.7. Immunostaining and fluorescence imaging

Muscle tissues were removed from the skeletons and rinsed with PBS and fixed in 4 (v/v) % of paraformaldehyde (Electron Microscopy Sciences) for 20 minutes at room temperature. For cryosectioning of tissues, fixed samples were treated sequentially with sucrose solutions (10, 20, 30 (w/v) %) for 30 minutes a room temperature. After an overnight with 30 (w/v) % sucrose solution at 4 °C and washing with PBS, tissues were embedded in OCT solution (Tissue-Tek) and snap-frozen on dry ice prior to storing at -80 °C. Frozen tissue molds were sectioned with 50 μ m thickness onto the silane-treated glass slides (Electron Microscopy Sciences) using the cryostat (Leica CM3050S). Sectioned tissue slices were rinsed with PBS to wash out OCT after 1 hour of thawing of OCT at room temperature. Samples were bordered with a water repellent barrier using a Pap pen (Abcam) and blocked with 1 (w/v) % of bovine serum albumin (Sigma-Aldrich) in PBS containing 0.1% of Tween-20 detergent (PBST) at 4 °C overnight. The primary antibodies, mouse anti-myosin heavy chain, MF-20 (ThermoFisher), and rabbit anti α -actinin (Abcam) were diluted with a 1:400 ratio in PBST and incubated with tissue samples overnight at 4°C. After washing with PBS three times, the secondary antibodies, AlexaFluor-488 anti-mouse and AlexaFour-647 anti-rabbit (ThermoFisher) with 1:500 dilution ratio in PBST were added to stain MF-20 and α -actinin

antibodies, respectively, followed by incubation overnight with 1:5000 dilution ratio of DAPI to stain the nucleus at 4°C. After washing with PBS three times for at least 5 minutes, samples were mounted after adding the Vectashield Antifade Mounting Medium (Vector Laboratories) under a sealed coverslip. The Multiphoton Confocal Microscope Zeiss 710 was used for confocal fluorescence imaging.

4.2.8. Muscle force characterizations and locomotion analysis

Muscle actuation was recorded by the Dino-lite digital microscope camera (Dino-Lite) after flipping over the eBiobot to make pillars facing to the camera at a frame rate of 20 per second. Muscle forces were calculated using the Euler-Bernoulli beam-bending theory [8-10, 21, 22]

$$F = \frac{8EI\delta}{L^2l}$$

Where I is the moment of inertia, δ is the deflection on the beam, L is the length of the skeleton beam, and l is the moment arm from the point of applied force of the actuator to the beam. E represents Young's modulus of skeleton beam, which was measured to be 270 kPa. The Cannon EOS Rebel T5i was used for recording walking and turning for bipedal eBiobots at a frame rate of 29.87 per second. The movement of pillars and locomotion of eBiobots were tracked using the software Tracker (<https://physlets.org/tracker>).

4.2.9. Myotube alignments and surface radiosity analysis

To predict the locomotion of eBiobot corresponding to the different configurations of 5 μ -ILEDs, the superposition of myotube alignment and μ -ILED radiosity to myotubes were analyzed. ImageJ software (Orientation-J plugin) was used for the polar histogram, Fast Fourier Transform (FFT) analysis, and the determination of the vector field of the alignment of myotubes

from the confocal microscope image. The vector field was detected on myotubes in 10 pixels, and the distances of x- (dX) and y-axis (dY) of myotubes in each 10 pixels were analyzed (resolution of 4.07×4.07 mm confocal image is 6552×6552 pixels). A surface-to-surface radiation module (COMSOL Multiphysics 6.0) was selected to simulate the radiosity of a single and 5 μ -ILEDs with different angles to myotubes which are integrated on the skeleton based on the light intensities. The diffuse irradiance of each μ -ILEDs is 1900 W/m² for 5 μ -ILEDs and 10366 W/m² for a single μ -ILED, and the emitted irradiation direction is faced to myotubes. The distance between μ -ILEDs and the surface of the muscle actuator was known as 2.2 mm (1.24 mm in Figure 4.4 plus about 1 mm for passive strain of beam) the displacement and dimension of each μ -ILED are referred from the design of Figure 4.3 (a)-iii and Figure 4.4. The transparent refractive index of cell media is assumed as 1.345 [23], and the ambient emissivity of the skeleton and internal myotubes were set as “0”.

4.2.10. Friction study

The angle of repose method was used to determine the static friction coefficient by inclining the angle of the plane until the eBiobot skeleton started sliding using the customized equipment. The friction coefficient was calculated using: $\mu = \tan \theta$ (measured angle). The surface of PE film (McMaster) was functionalized with different surface chemistries. Oxygen plasma (Pico Diener) was treated for 1 minute with 100 W to make the surface hydrophilic. To tune hydrophobicity, the PE surface was treated with (3-Aminopropyl)triethoxysilane (APTES) (Sigma-Aldrich) after oxygen plasma treatment. 0.2 (v/v) % APTES in ethanol was used to treat the surface for 1 minute, followed by rinsing with PBS and this step was repeated 3 times. For further hydrophobic functionalization, the PE film was incubated in 5 (v/v) % APTES in ethanol

for 2 hours at room temperature, then was rinsed with PBS. The contact angle images were taken by the Dino-lite digital microscope after adding a drop of 2ul distilled water on surfaces.

4.2.11. Computational modeling of eBiobot

The eBiobot is computationally modeled using the solver *Elastica*, following a similar approach that has been developed and leveraged for modeling bio-hybrid robots [7, 9, 10, 24]. *Elastica* is an open-source software package for simulating assemblies of soft slender bodies using Cosserat rod theory. Cosserat rod is a mathematical description that captures the 3D dynamics of 1D slender bodies, while accounting for all modes of deformation (bending, twisting, stretching, and shearing). The eBiobot is then modeled as an assembly of Cosserat rods specialized to represent the muscle tissues and the scaffold. This assembly of rods is constructed via appropriate boundary conditions to account for structural connectivity and dynamic interactions among rods. We also take into account the dynamic effects induced by the onboard micro-electronics, by combining their masses into the rod representing the scaffold. Furthermore, environmental effects such as gravity, buoyancy, surface friction, and hydrodynamics are also characterized and implemented in simulation. The numerical solver *Elastica* is publicly available at <https://www.cosseratrods.org>.

4.2.12. Evolutionary design optimizations

With a computational eBiobot model in hand, we then tackle the problem of optimizing the scaffold to maximize its forward walking speed. To do this, we couple *Elastica* with the Covariance Matrix Adaptation-Evolution Strategy algorithm (CMA-ES, 24), a method specialized for dealing with non-linear, non-convex continuous optimization problems. Coupled with *Elastica*,

CMA-ES has been demonstrated as an efficient design tool for a range of engineering and bio-hybrid applications. [24, 25]

A critical aspect of designing bio-hybrid systems, in particular is that the characteristics of the biological actuator cannot always be precisely predicted and controlled. Therefore, an optimal solution obtained assuming a specified actuation may fail when the actuator performs differently. Here, to overcome this issue, our optimization process of the eBiobot takes into account the uncertainties due to the biological muscle variations. We first identify three key scaffold parameters (beam thickness, longer leg length, and leg difference) that are most influential on the walking speed, and manually pick initial values of these parameters as the input of CMA-ES. Then, the CMA-ES will evolve these parameters over a number of generations to improve the speed of the bot. In each generation, CMA-ES deploys N ($N=50\sim80$) parallel *Elastica* simulations, with each simulation taking a unique design parameter triple sampled from a multivariate Gaussian distribution. To evaluate the robustness of each scaffold design, every *Elastica* simulation creates 8 exact eBiobots, and simulates each one with a different muscle force sampled from the experimentally characterized range. The resulting speeds (defined as the fitness) of these 8 bots are then averaged and fed back into CMA-ES to inform the parameter selection of the next generation. An optimal solution is obtained when both the design parameters and the fitness start to converge.

4.2.13. Efficiency analysis through the cost of transport

The walking efficiency of the eBiobot is evaluated using the cost of transport (COT), a dimensionless number that quantifies the energy consumption of transporting an object over a

certain distance. As it is non-dimensional, this number enables a meaningful comparison against previously demonstrated bio-hybrid walking machines with different body scales. The COT is generally defined as

$$\text{COT} = \frac{E}{mgd},$$

where E is the overall energy spent to transport a body of weight mg over the distance d . As the metabolic efficiency of our system is not readily available, we estimate $E \cong E_c N$, where E_c is the muscle energy output provided during each contraction and N is the number of contractions stimulated over a certain time period. For consistency, the distance d is also measured over the same time period. E_c is then quantified through the work

$$E_c = F_{act} C_{act} / 2,$$

where F_{act} is the measured active force of the muscle and C_{act} is the active muscle contraction estimated from the leg deflection data recorded experimentally. We assumed that the muscle force increases linearly from rest before reaching its maximum value, hence the factor 1/2.

4.2.14. *Wireless control of eBiobots with the console system*

The multi-unit dynamic control of individual eBiobots was implemented with an application-specific hardware console equipped with an Arduino Uno board. This console interfaces asynchronously via USB to the host computer that runs the GUI that communicates with the devices via NFC as described previously. Six independent controllers, that pair with six different eBiobots, produce four command events triggered by two push-buttons (on+synchronous mode, on+asynchronous-left mode, on+asynchronous-right mode, off command), which are registered with the Arduino Uno board at a refresh rate of 50 ms. These events are stored in memory until retrieved by the GUI. The GUI, at a rate of 5 s, retrieves the collection of cumulative

command events from the console and stacks them in a dynamic queue for relaying them into action commands to the corresponding eBiobot on an individual basis. To account for communication errors with the eBiobots the GUI implements a persistent communication strategy that assures the commands are received and implemented.

4.3. Results and Discussion

The battery-free, wireless optogenetic devices exploit resonant magnetic induction between paired transmission (at the circumference of the experimental enclosure) and receiving (at the perimeter of the device) antenna system at radio frequency (RF, 13.56 MHz) to power μ -ILEDs to actuate the optogenetic muscles (Figure 4.1, Figure 4.3 (a), Figure 4.6, Figure 4.7, Figure 4.8, Figure 4.9). Amplitude modulation of the RF power produces on-off patterns of operation at programmable frequencies and pulse widths relevant for the optical stimulation of eBiobots contained within the experimental antenna cage, with a diameter of 15 cm (Figure 4.3 (a)-i). Constituent layers for the wireless electronic module include a thin dual-sided flexible circuit board composed of rolled annealed copper layers (18 μ m) separated by a polyimide film (75 μ m) and a conformal encapsulation layer of parylene ($\sim$ 14 μ m) to isolate the device from the cell culture medium (Figure 4.3 (a)-ii). Five μ -ILEDs electrically connected in parallel and distributed at the center and inner corners, produce the illumination spatial profiles used to trigger muscle contractions (Figure 4.3 (a)-iii). Overall device flexibility, lightweight construction ($\sim$ 7.3 mg), wireless power transfer, and control capability allow for robust remote operation of eBiobots.

While onboard optoelectronics provide the ability to command individual muscles, the overall walking performance of the eBiobot is determined by a complex interaction between active forces of muscle contraction and passive tension (Figure 4.10), [9, 10] components integration,

interfacial mechanics, and fluid drag. Therefore, a quantitative identification and optimization of robust designs are crucial. Here, we demonstrate the eBiobot through a synergistic, iterative design approach involving multiple stages of evolutionary design optimization, fabrication, and testing (Figure 4.3 (b-d)). We start off by computationally modeling the biohybrid walker as a heterogeneous assembly of slender elastic, passive (scaffold), and active (muscles) elements. To this end, we employ the solver *Elastica* (Figure 4.10), extensively validated and demonstrated in a variety of biophysical settings, including biohybrid robotics. [24, 25] The geometric characteristics of beam thickness, front leg length, and legs length mismatch are numerically identified as key to the eBiobot's walking abilities (Figure 4.3 (b)-i). We then couple *Elastica* with an evolutionary algorithm to optimize these parameters for fast forward speed. [26] Importantly, the optimization process accounts for variations in biological muscle characteristics and actuation, ensuring the robust performance of the final design (Figure 4.3 (b)-ii, Figure 4.10).

The design blueprint is then realized through bio-fabrication and additive manufacturing, where an *in-vitro* engineered skeletal muscle tissue forms around a 3D printed hydrogel scaffold (Figure 4.2, Figure 4.3 (c), Figure 4.4). The eBiobot is then deployed in the antenna arena for testing its mobility and controllability (Figure 4.3 (d)). To systematically improve the eBiobot's performance, we characterize the variation of muscle forces in each batch of experiments, feeding that information into the next design iteration. In the following, we expand on key design considerations and present the characterization and performance of the eBiobot.

A challenge to achieve locomotion lies in effectively stimulating the optogenetic muscles, so as to generate sufficiently strong contractions using μ -ILEDs as localized illumination sources. Our designs address this challenge in the following ways (Figure 4.11 (a)). First, the 3D printed mold is designed to match the shape of the muscle tissue with the spatial arrangement of μ -ILEDs

(Figure 4.11 (a)-i, Figure 4.4). Second, we distribute five μ -ILEDs at the center and corners of a circle defined by the inner radius of the receiver antenna. This design choice maximizes the illumination surface area, albeit the cost of lower μ -ILEDs' individual illumination intensity, when compared to the use of a single, centrally located μ -ILED (Figure 4.11 (a)-ii). As can be seen in Figure 4.11 (a)-iii, Figure 4.12, and Figure 4.13, the chosen design is found to induce larger muscle contraction forces across a range of stimulation frequencies.

After establishing the integration of μ -ILEDs and muscles, a proof-of-concept single actuator unidirectional eBiobot prototype is fabricated to characterize and validate our computational model. The performance is found (Figure 4.10) to be in good agreement with observed eBiobot legs deformation, across a range of actuation frequencies (Figure 4.11 (a)-iv). This sets the stage for the optimization of walking performance. In each optimization campaign, hundreds of different scaffolds are generated and numerically evaluated, employing muscle forces sampled from the experimentally characterized range (Figure 4.11 (b)-i). Subsequently, the obtained optimal solution is fabricated, characterized, and tested. The newly acquired muscle responses are aggregated to the initial set, thus improving our data-driven muscle model in concert with the evolution of the scaffold design. Over three campaigns, a 10X improvement in speed is achieved relative to our initial design (Figure 4.11 (b)-i, Figure 4.14). We also note that, relative to previous biohybrid walker instantiations, our eBiobot exhibits comparable efficiency evaluated through cost-of-transport (Figure 4.11 (b)-ii).

The walking performance of the final eBiobot design is comprehensively characterized in Figure 4.11 (c)-i. A walking speed of 0.83 mm/sec is achieved when stimulated at 4 Hz, 50 ms pulse width, and 10 W RF antenna power, setting the speed record among all skeletal muscle-based bio-hybrid robots (Table 4.1). This speed is attributed to the combination of applied

frequencies, optimized spatial μ -ILEDs arrangement, and surface friction. First, we note that walking speeds increase with applied frequencies, as illustrated in the linear displacement-time curves of Figure 4.11 (c)-ii, corroborating computational predictions. Second, while the use of 5 μ -ILEDs was found to provide wider illumination, favor muscle contractions (as previously discussed), and faster walking speed (Figure 4.15), we further optimize their orientation to match the myotubes alignments (Figure 4.16, Figure 4.17, Figure 4.18, Figure 4.19, and Figure 4.20). Finally, surface friction was also optimized by exploring different substrate chemistry as the walking depends on friction coefficient (Figure 4.21). [15, 27]

In addition to walking, robotic functionality is demonstrated using tool attachments inspired by the Lego® block assembly (Figure 4.11 (d)). A 3D printed accessory such as a plow or collector is plugged into a pair of 3D printed pillars at the scaffold ends, with stud and anti-stud coupling, allowing a skeleton to switch tools (Figure 4.11 (d)-i-iii, Figure 4.22). In the first demonstration, a plow attached to the eBiobot illustrates the removal of 3D printed objects littering the arena (Figure 4.11 (d)-iv). In a second demonstration, a collector is attached to the robot, so as to capture and displace objects like a courier (Figure 4.11 (d)-v).

In a step towards enhanced maneuverability, a second eBiobot inspired by bipedal biological architectures (Figure 4.23 (a)), is designed to entail two muscle actuators in combination with a central microcontroller (μ C), which extends laterally to bring two islands of 5 μ -ILEDs in intimate proximity with the muscle actuators (Figure 4.23 (a)-i, ii, Figure 4.24, Figure 4.25). The combination of standardized nearfield communication (NFC) protocols, low-power electronics, and μ C with specialized firmware allows for the implementation of wireless real-time control on illumination parameters (frequencies and pulse widths) as well as illumination profiles delivered to any arbitrary combination of μ -ILEDs (Figure 4.23 (a)-iii, Figure 4.26).

Once again, we numerically synthesize this bipedal eBibot to anticipate its motion characteristics under different stimulation protocols, whereby muscles can be either symmetrically (both 4 Hz) or differentially (4 and 1 Hz) stimulated (Figure 4.23 (b)-i). To ensure robustness across locomotory modes, muscle forces are allowed to vary within the characterized range (Figure 4.11 (b)-i). As shown in Figure 4.23 (b)-ii, by symmetric or differential stimulation of both muscles, the *in-silico* bipedal eBibot exhibits robust forward and bidirectional steering motions (Figure 4.27). Subsequently, this design is realized in experiments, successfully demonstrating the predicted locomotory modes, under user-programmable remote control (Figure 4.23 (b)-ii, iii, Figure 4.28). To further highlight the eBibot ability to maneuver, we challenge it to traverse an obstacle course. Figure 4.23 (c) shows a clockwise half turn through obstacles, over ~ 9.5 minutes, under 4 (Left) and 1 (Right) Hz of stimulations. Moreover, by switching the stimulation frequencies on both legs, we demonstrate in Figure 4.23 (d) its capability of bidirectional turning in one continuous run commanded via wireless remote control (Figure 4.29).

Further, we illustrate a console system connected with multiple gaming controllers for the simultaneous, but individual manipulation of a multitude of eBibots (Figure 4.23 (e)-i, Figure 4.30, Figure 4.31). The onboard μ -ILEDs panels are activated according to a preset stimulation pattern, matched to clicker buttons on a joypad (Figure 4.23 (e)-ii), through our customized software (Figure 4.30). Three bipedal eBibots are then manipulated by 3 gaming controllers to make an “I” (Figure 4.23 (e)-iii, iv, Figure 4.32). This biohybrid system may be further enhanced to incorporate onboard sensors and advanced feedback, to establish a fully automated, closed-loop robotic swarm system, with potential applications in environment, defense, and biomedicine.

4.4. Conclusions

In conclusion, we present the first biohybrid electronic robots powered by optogenetic skeletal muscles and commanded by wireless optoelectronics. The robot architecture and locomotion performance are computationally optimized through a staged iterative process, incorporating fabrication and experimental testing, for robustness. Obtained designs along with 3D printing additive manufacturing allow seamless integration of onboard electronics, multiple muscle actuators, and Lego[®]-like attachments. The result is a suite of advanced programmable functionalities such as remote-control switching, steering, plowing, and transport, both for individual and multiple robots. Considering that additional functionalities enabled by updated electronic systems can be added conveniently in the future, this platform paves the way to a new era of biohybrid electronics miniature robots, with applications in environment, defense, biomedical engineering, clinical, and manufacturing. Future integration of *in-vitro* optogenetic neural tissue and innervation with skeletal muscles can be synergistic with embedded optoelectronics for neural training, realizing a new breed of autonomous biohybrid robots equipped with cybernetic communication between biological and artificial intelligence.

4.5. Figures

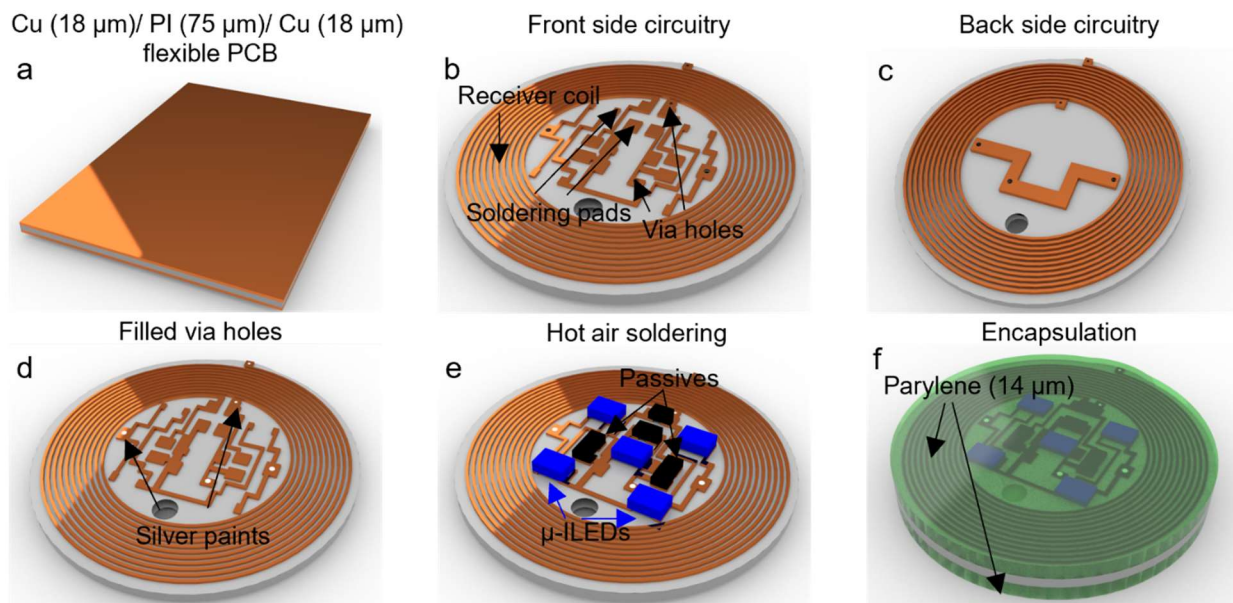


Figure 4.1. Fabrication process for passive wireless optogenetic devices. (a) Prepare a tri-layer flexible printed circuit board (fPCB) composed of Cu (18 μm) – polyimide (PI, 75 μm) – Cu (18 μm) as fabrication substrate. (b)-(c) fPCB preparation, front (b) and back (c), via laser ablation process to define receiver coil, soldering pads, and via holes. (d) Fill via holes with silver paint to connect the top and bottom electric traces. (e) Solder electronic components and $\mu\text{-ILEDs}$ using a low-temperature hot air soldering process. The number of $\mu\text{-ILEDs}$ can be further adjusted based on experimental requirements. (f) Encapsulate the entire device with chemical vapor deposited parylene (14 μm). The Figure set was produced by Yiyuan Yang from the Rogers group.

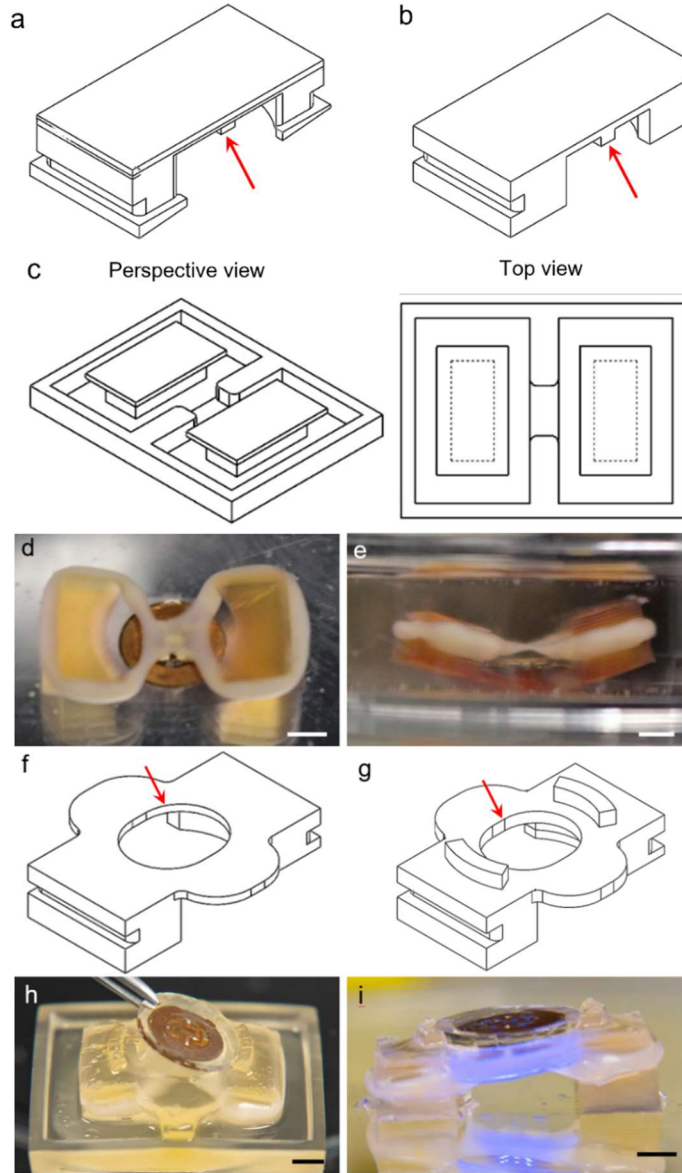


Figure 4.2. Design iteration of eBiobot. CAD of skeletons for (a) 1st and (b) 2nd generations and (c) tissue seeding mold with dimensions for the 1st and 2nd generations. (d) Angled and (e) side view images of eBiobot integrated with wireless optogenetic devices in the 2nd generation. CAD of (f) 3rd and (g) 4th skeleton generations and (h) angled and (i) side view images of eBiobot integrated with wireless optogenetic devices in the 4th generation. The 1st design was selected from the previous study [9] and dimensions have been changed through the evolutionary computational optimizations. From the 3rd generation, wireless optogenetic devices were integrated on the top of the skeleton after attaching to the PDMS substrate, while they had been integrated inside of the beam on the stage using glue in the 1st and 2nd generations. In the 4th generation, the printed stoppers were added to prevent the optogenetic device from slipping as overserved in the 3rd generation. Red arrows indicate the locations to integrate wireless optogenetic devices. Scale bars: 2 mm.

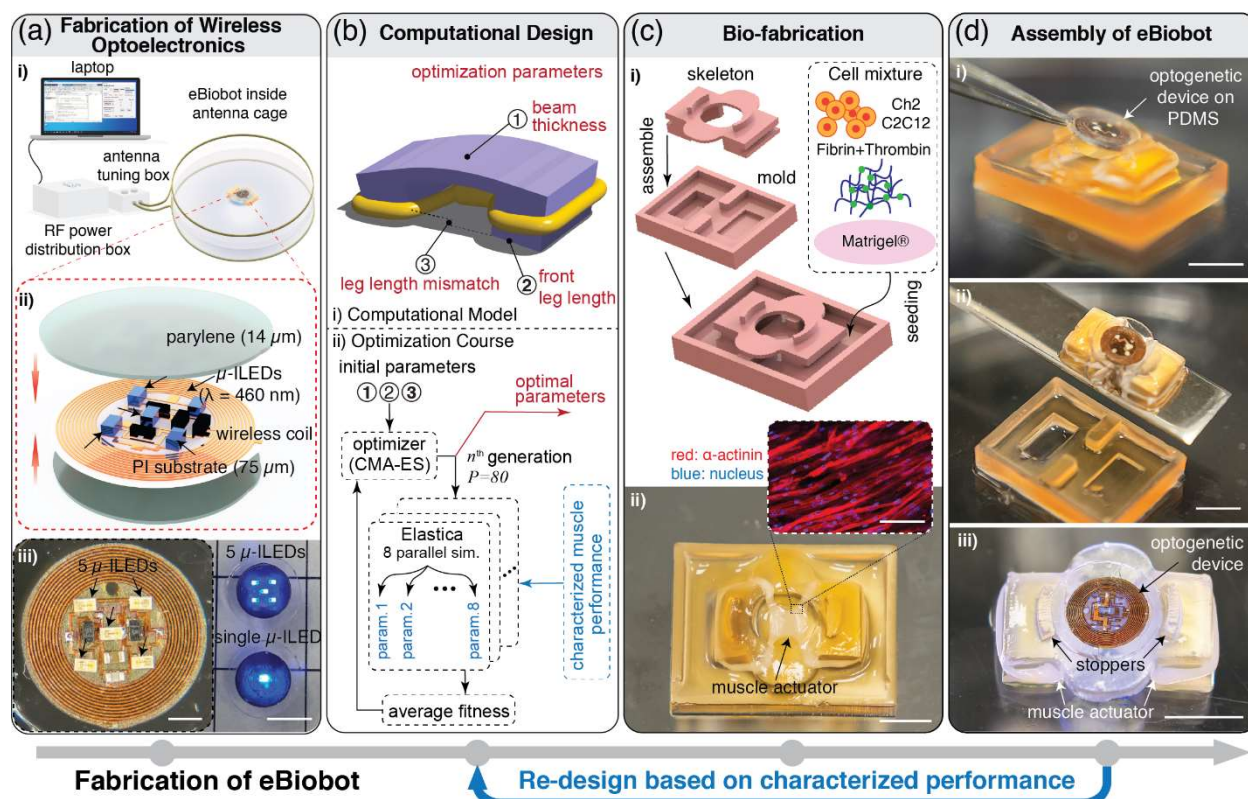


Figure 4.3. Schematic and workflow for fabrication and optimization of the single actuator eBiobot. (a) Experimental setup and fabrication of the wireless optogenetic control system. Schematics for i) experimental setup of wireless communications with the battery-free optogenetic device using μ -ILEDs and ii) layered schematics of the optogenetic device and its iii) photographic images with a single and 5 μ -ILEDs. The left inset is the expanded view of a 5 μ -ILEDs device. (b) Computational design optimization. i) Computational model using *Elastica*. The scaffold is constructed by two legs with length mismatch and a thin, connecting beam. ii) Optimization course. Three key scaffold characteristics are optimized: beam thickness, front leg length, and leg length mismatch. (c) Bio-fabrication of eBiobot. i) Schematics and steps of the fabrication and assembling of 3D printed hydrogel and engineered skeletal muscle tissue actuator. ii) Photographic image of eBiobot with muscle actuator wrapping around 3D printed skeleton inside the mold. Inset shows the fluorescence image of muscle actuator. (Red: α -actinin, Blue: DAPI) (d) Modular assembly of eBiobot. i) Assembling the wireless optogenetic device onto the skeleton using tweezers, ii) releasing the eBiobot from the mold, and iii) free-standing eBiobot. Scale bars: 5 mm for (a)-iii) (right), (c)-ii), (d)-i), ii), and iii), 1 mm for inset of (a)-iii), and $100\ \mu\text{m}$ for inset of (c)-ii).

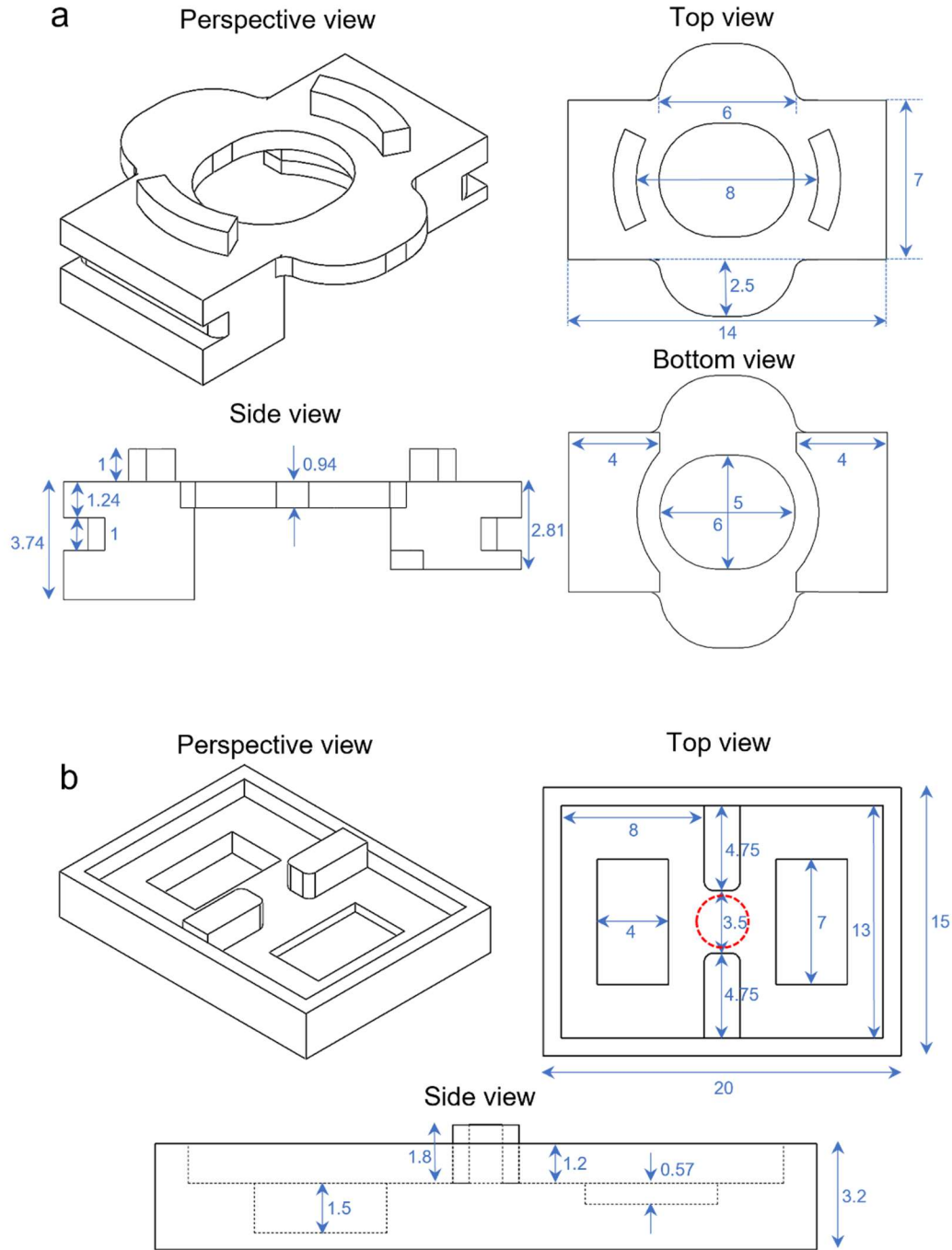


Figure 4.4. Designs of 3D printed hydrogel structures for eBiobot. CAD with dimensions of the final optimized generation (4th) of (a) eBiobot skeleton and (b) its tissue seeding mold. The red circle in (b) indicates the inner radius of the wireless optogenetics device. The width (3.5 mm) of the central part in the mold was considered to match the illuminations area of μ -ILEDs. Unit: mm.

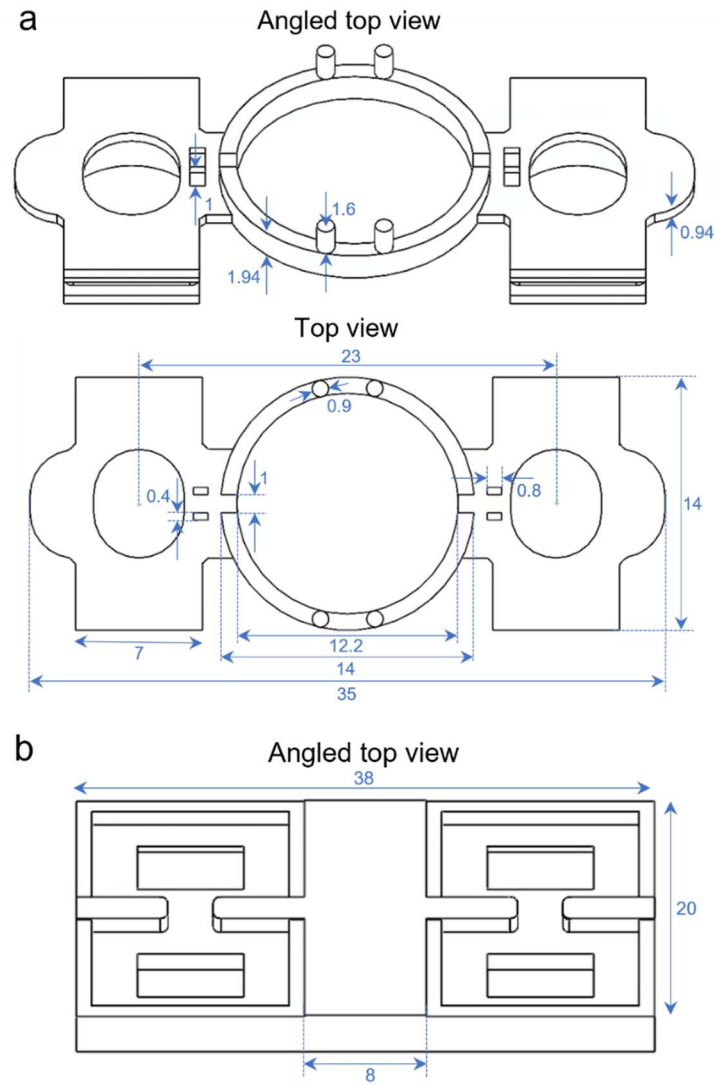


Figure 4.5. Designs of 3D printed parts for bipedal eBiobot. CAD with dimensions of (a) bipedal eBiobot skeleton and (b) its tissue seeding mold. Units: mm.

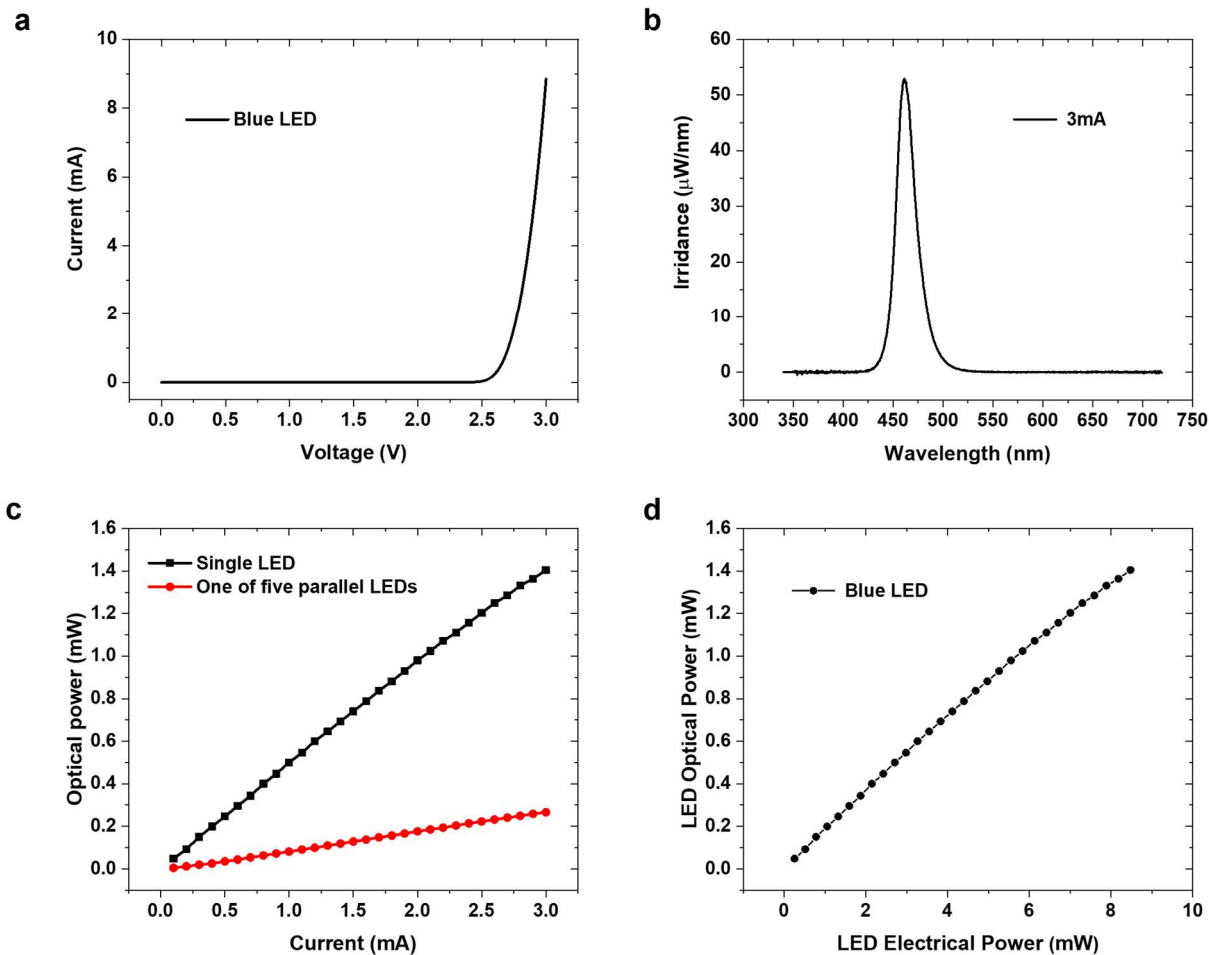


Figure 4.6. μ -ILED characteristics. (a) μ -ILED I-V curves (460 nm). (b) Emission spectra of a μ -ILED driven at 3 mA. (c) Individual μ -ILED optical power with respect to total go-through current for passive optogenetic devices with a single μ -ILED and one of five μ -ILEDs connected in parallel. (d) μ -ILED optical power vs. μ -ILED electrical power, which gives the working efficiency of 16.6%. The Figure set was produced by Zhengwei Li from the Rogers group.

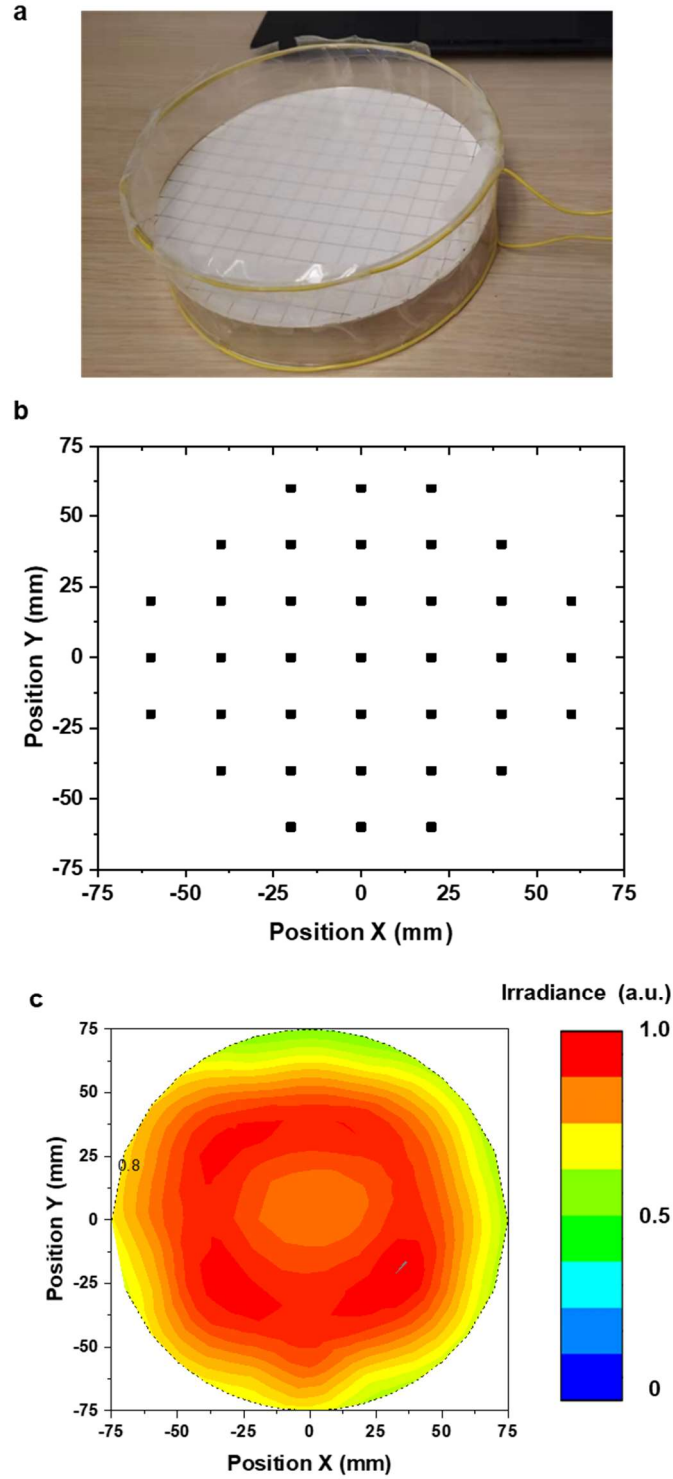


Figure 4.7. Optical irradiance distribution. (a) Optical image of the antenna box with a diameter of 150 mm. (b) The position of the measured spot in the antenna box. (c) The measured optical irradiance output intensity using a passive optogenetic device with respect to the different locations as indicated in (b). The Figure set was produced by Zhengwei Li from the Rogers group.

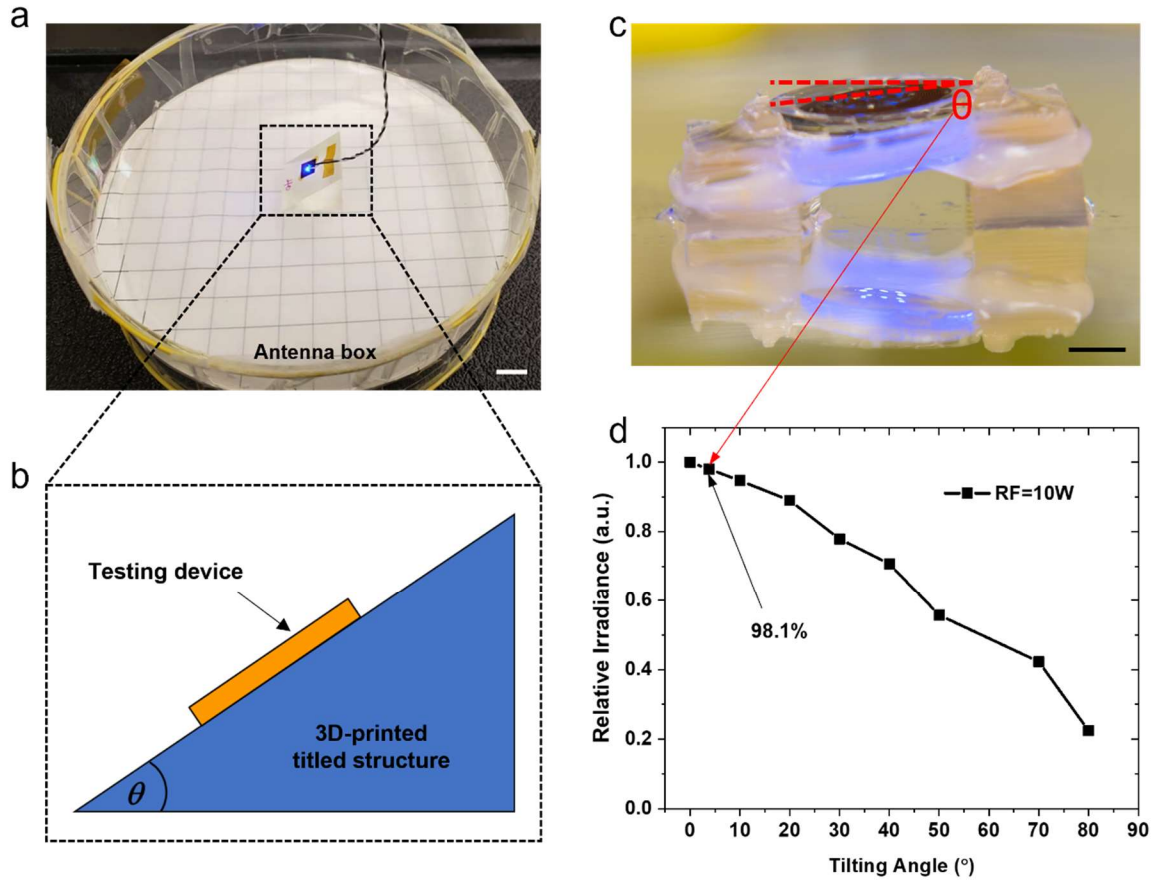


Figure 4.8. Tilting effect on the optical irradiance output. (a) Photographic image of the experimental setup to study the device tilting effect by using a passive optogenetic device. (b) Schematic of the cross-sectional view of a testing device on a 3D-printed structure with a tilted angle θ . (c) Side view image of eBiobot. The tilted angle is 3.8 degrees based on the legs difference and beam length. (d) Relative optical irradiance from a single μ -ILED with respect to tilting angle. Only 1.9% of irradiance loss occurred at 3.8 degrees. Scale bars: 1 cm for (a) and 2 mm for (c). The Figure set was produced by Zhengwei Li from the Rogers group.

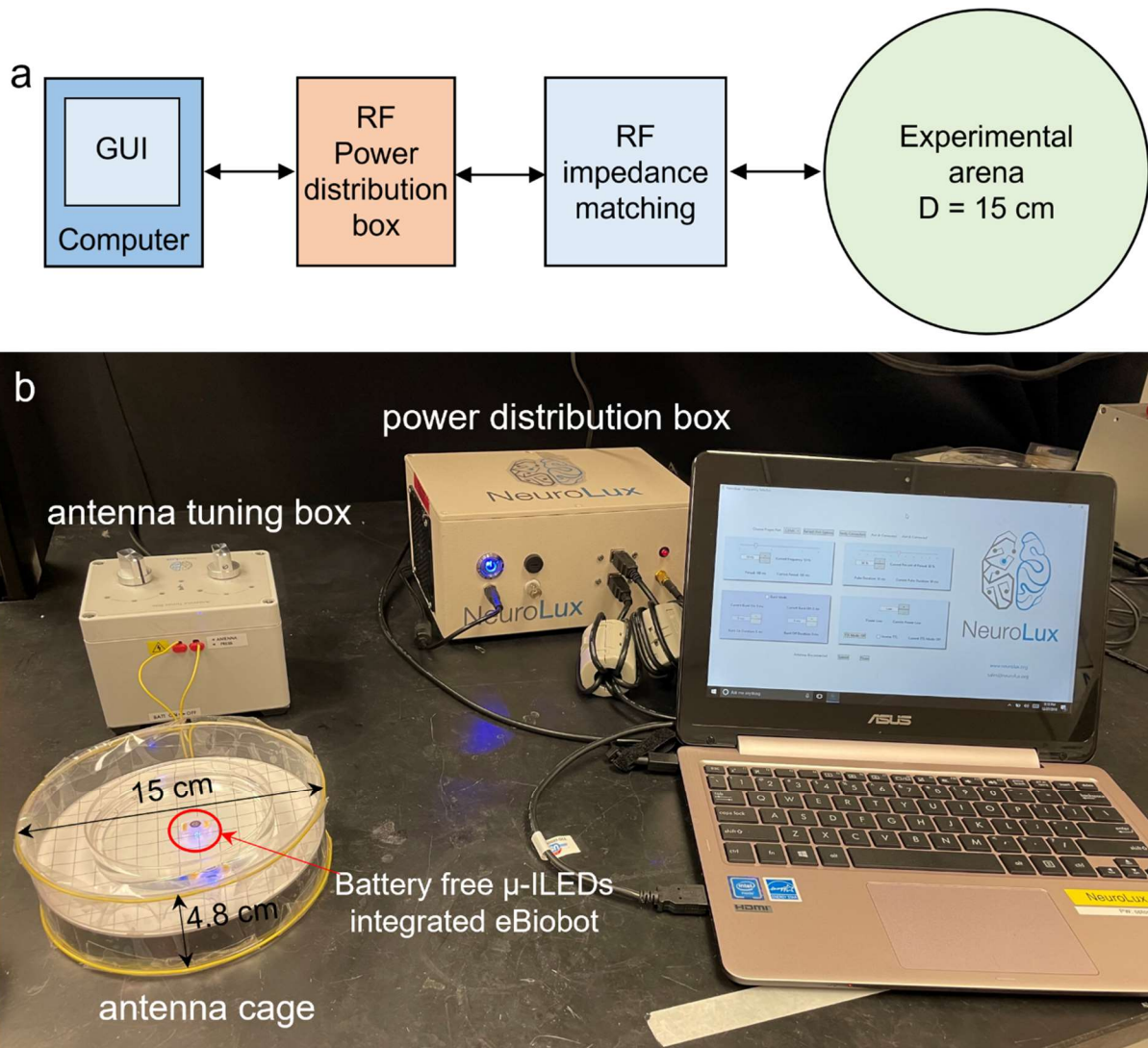


Figure 4.9. Experimental setup for wireless control of eBiobot. (a) Block diagram for the wireless control of passive optogenetic devices via resonant magnetic induction. (b) Image of experimental set up for remote control of battery-free wireless passive optogenetic device integrated eBiobot using RF power distribution box, antenna impedance matching box, the laptop with the control software, and a customized antenna with a diameter of 15 cm.

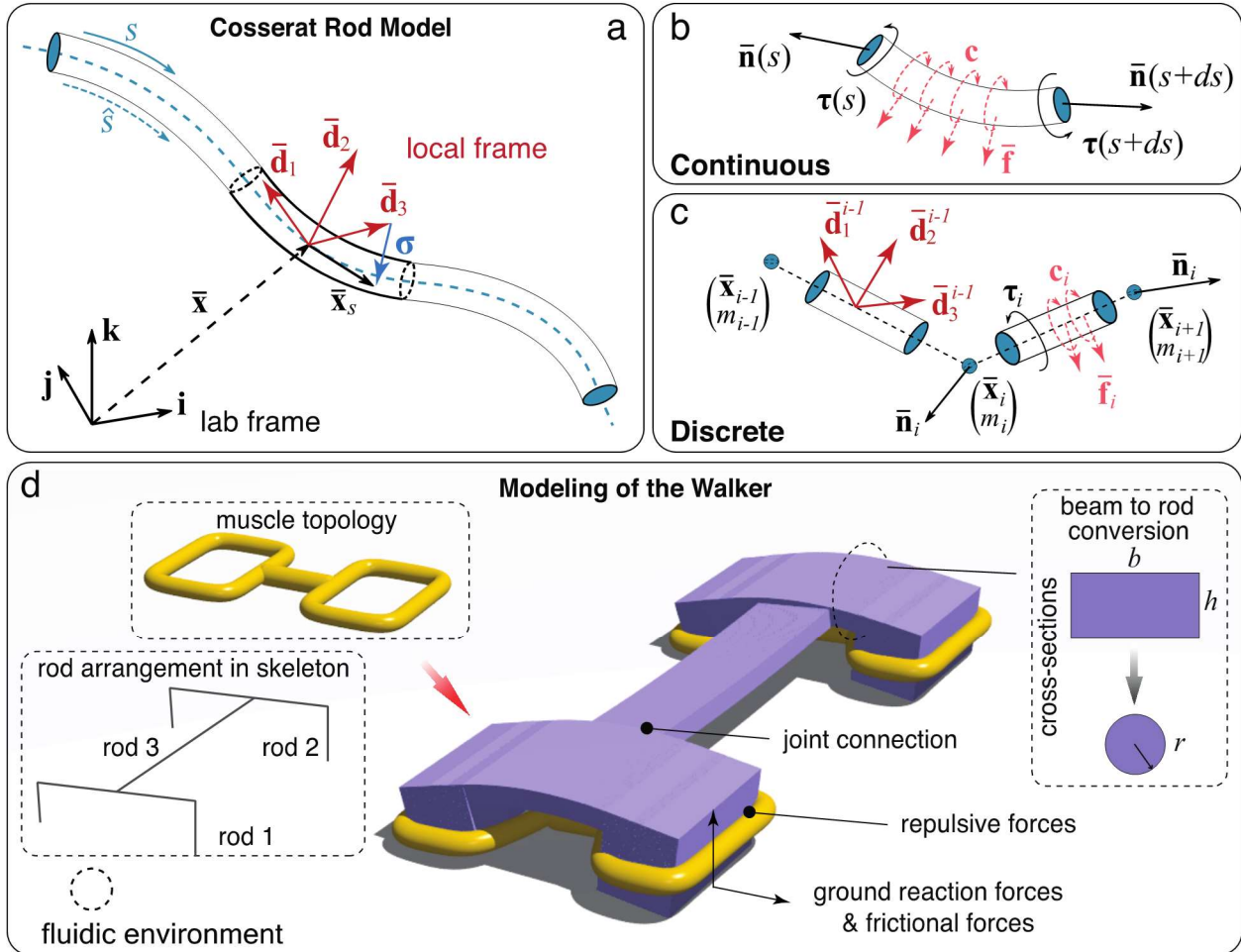


Figure 4.10. Computational model of the eBiobot using Cosserat rods. (a) Schematic for the Cosserat rod model: A soft filament described by the centerline coordinates. Continuous (b) and discretized (c) model of a rod segment. (d) Each single eBiobot is modeled as an assembly of four Cosserat rods – one representing the scaffold and three representing the muscle tissue. The bipedal eBiobot is then constructed by incorporating two same single bots and connecting them using a straight rod. Rods are assembled into the walker using joint connection schemes. The walker is fully submerged in a fluidic environment and experiences reaction and frictional forces due to the contact with the ground. The Figure set was produced by Xiaotian Zhang from the Gazzola group.

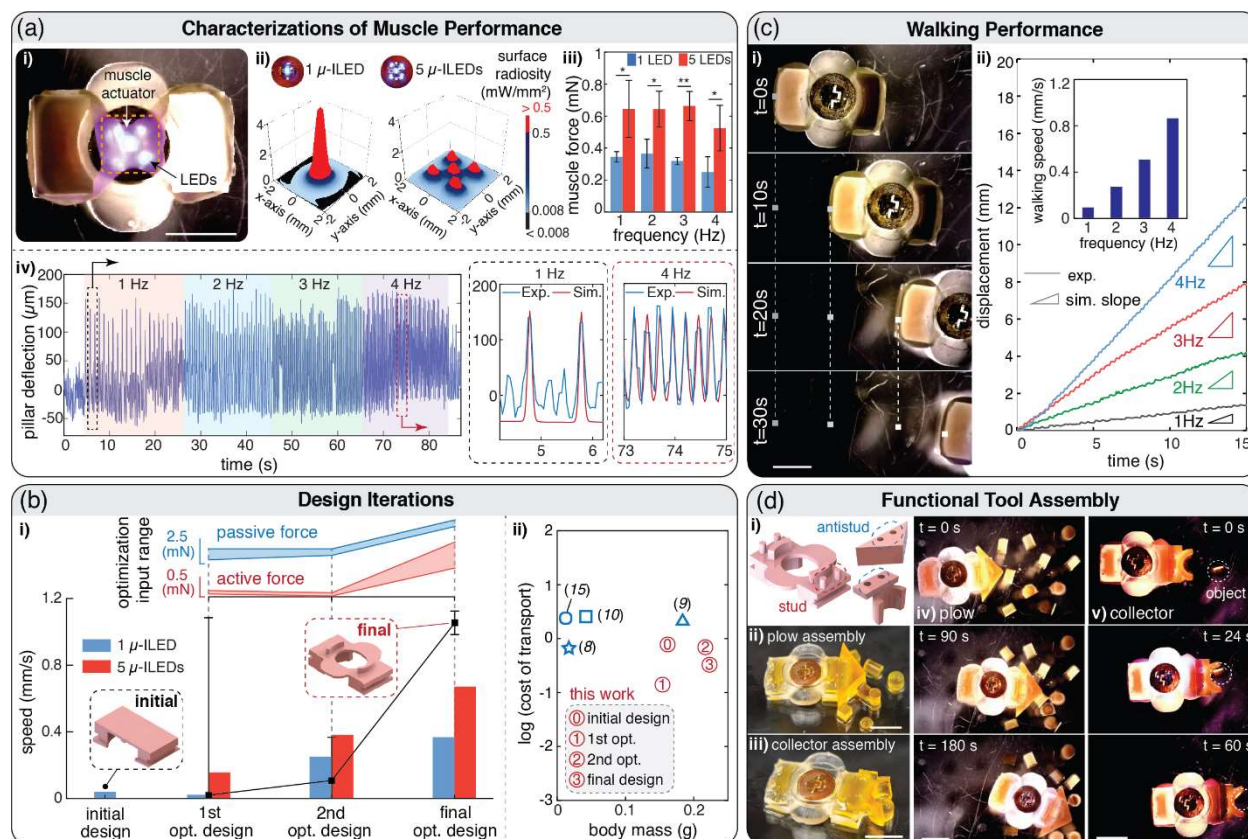


Figure 4.11. Walking and advanced functionality of single actuator eBiobot. (a) Characterizations of muscle performance in the eBiobot stimulated by μ -ILEDs wirelessly. i) Optical image of the eBiobot shining with 5 μ -ILEDs. ii) Surface optical radiosity on a muscle actuator in the region of the orange square when i) stimulated by a single and 5 μ -ILEDs. Red indicates higher light intensity for saturated force and black indicates lower light intensity for the threshold of optogenetics muscle. [3, 28] iii) Muscle active forces stimulated by a single and 5 μ -ILEDs with different frequencies. Statistical significance: * $P < 0.05$, ** $P < 0.01$. Error bars correspond to the standard deviations from three measurements. iv) Pillar deflection curves stimulated by 5 μ -ILEDs at different frequencies. Insets show the comparison with simulations. (b) Design iterations for the eBiobot. i) Each optimization incorporates muscle force sampled from the given input range, characterized by the previous iteration. Averaged simulation walking speed is compared with experimental results stimulated by 1 or 5 μ -ILEDs, while the error bar shows the normalized speed variation in each optimized design due to muscle variations. Scaffold designs are presented for initial and final generations. ii) Efficiency analysis through the cost of transport. Designs from all iterations are compared against previous biohybrid walkers. [8-10, 15] (c) Walking performances of the eBiobot equipped with 5 μ -ILEDs and driven with 10 W RF power. i) Time-lapse images of eBiobot from the last design iteration stimulated at 4 Hz. ii) Time-dependent displacement of the eBiobot at different stimulation frequencies. Inset shows the average speed. (d) Functional tool assembly. i) Schematics of Lego[®] block assembly with the skeleton. Images of ii) plow and iii) collector assemblies. Time-lapse images of iv) plow attached eBiobot displacing objects and v) collector attached eBiobot transporting an object. All scale bars: 5 mm.

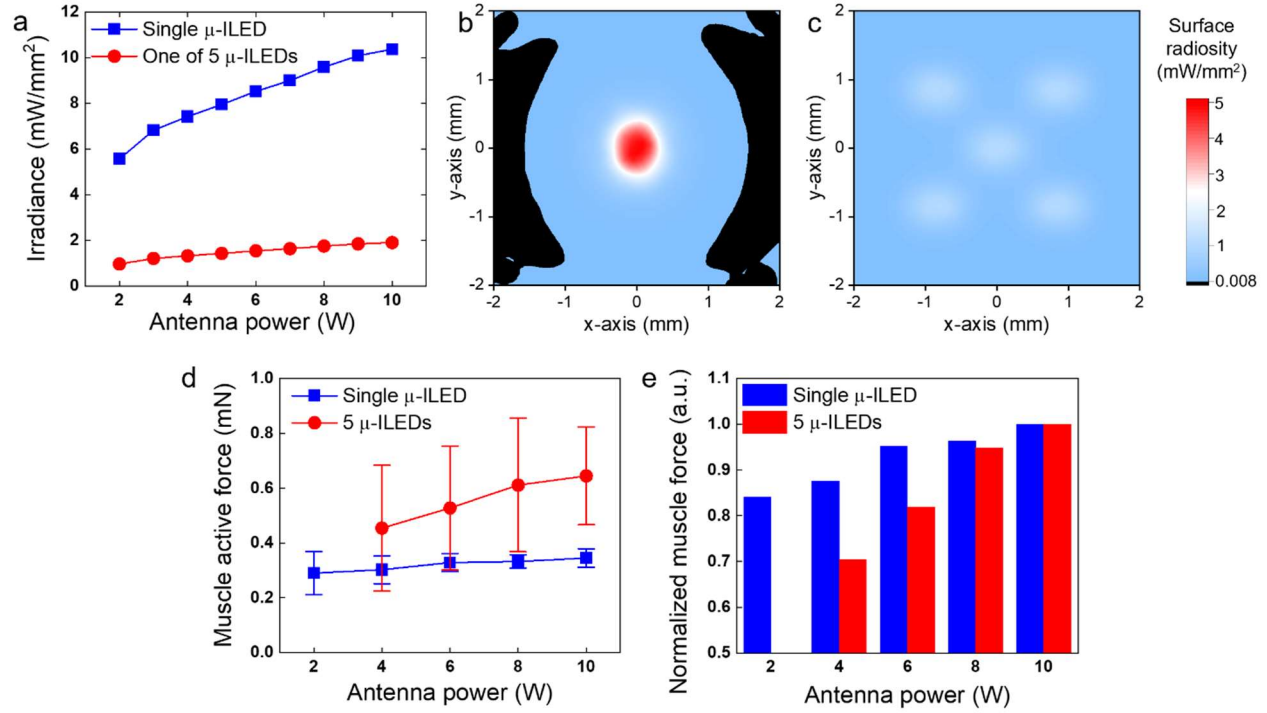


Figure 4.12. Irradiance characteristics of wireless optogenetic devices with a single μ -ILED and 5 μ -ILEDs and respective muscle forces. (a) Light irradiance with respect to applied RF antenna power of (a) a single μ -ILED and one of 5 μ -ILEDs and surface radiosity on muscle actuator from (b) a single μ -ILED and (c) 5 μ -ILEDs at 10 W RF antenna power and 50 ms of the pulse width. (d) Muscle active forces stimulated by a single μ -ILED and 5 μ -ILEDs with different RF antenna powers at 1 Hz and 50 ms pulse width and (e) their normalized muscle active forces by those stimulated by a single μ -ILED and 5 μ -ILEDs applied in 10 W and 50 ms of the pulse width. The muscle twitching in 2W stimulated by 5 μ -ILEDs was not observed.

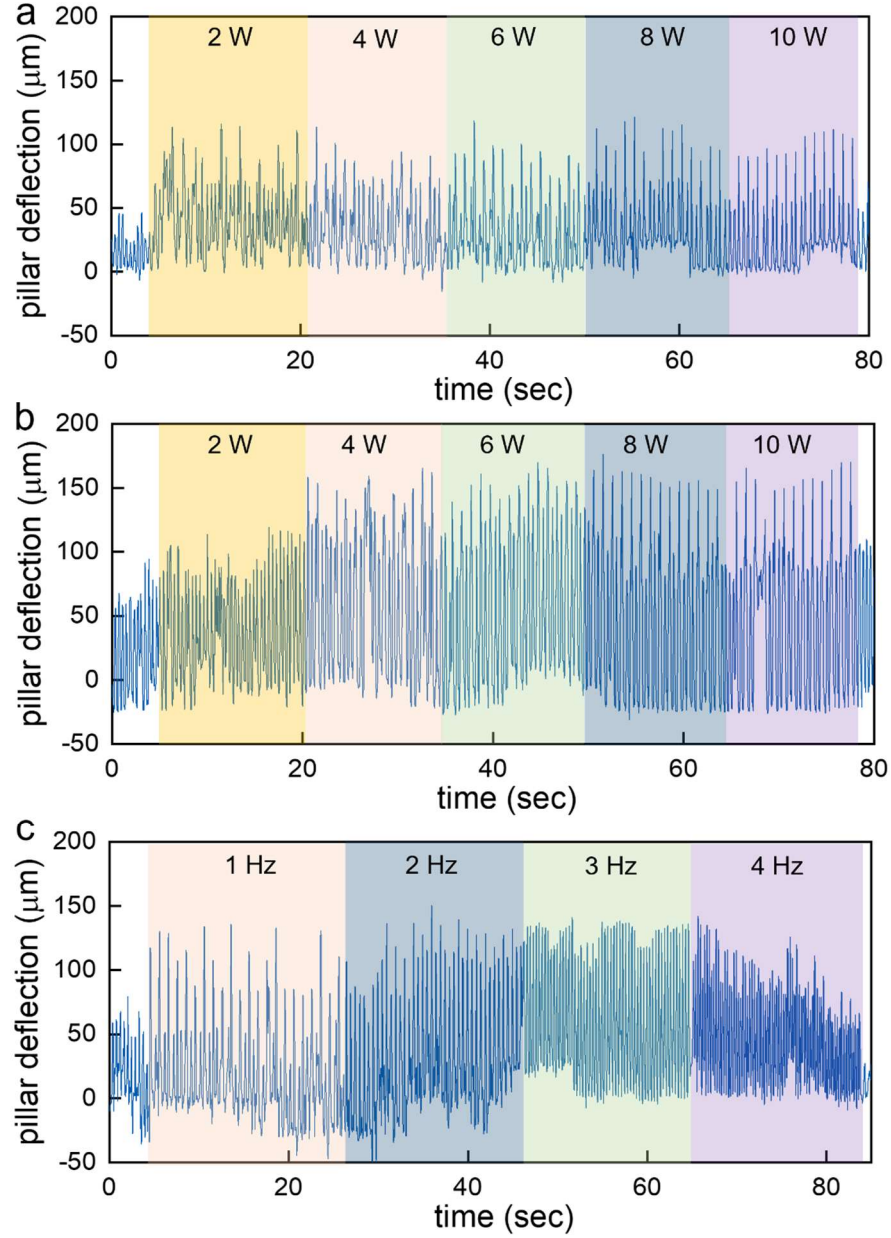


Figure 4.13. Muscle actuations stimulated by a single μ -ILED and 5 μ -ILEDs. Pillar deflection-time curves of eBiobot stimulated by (a) a single μ -ILED and (b) 5 μ -ILEDs at 1 Hz, 50 ms pulse width and with different applied RF antenna powers and by (c) a single μ -ILED at different frequencies and 10 W RF antenna power.

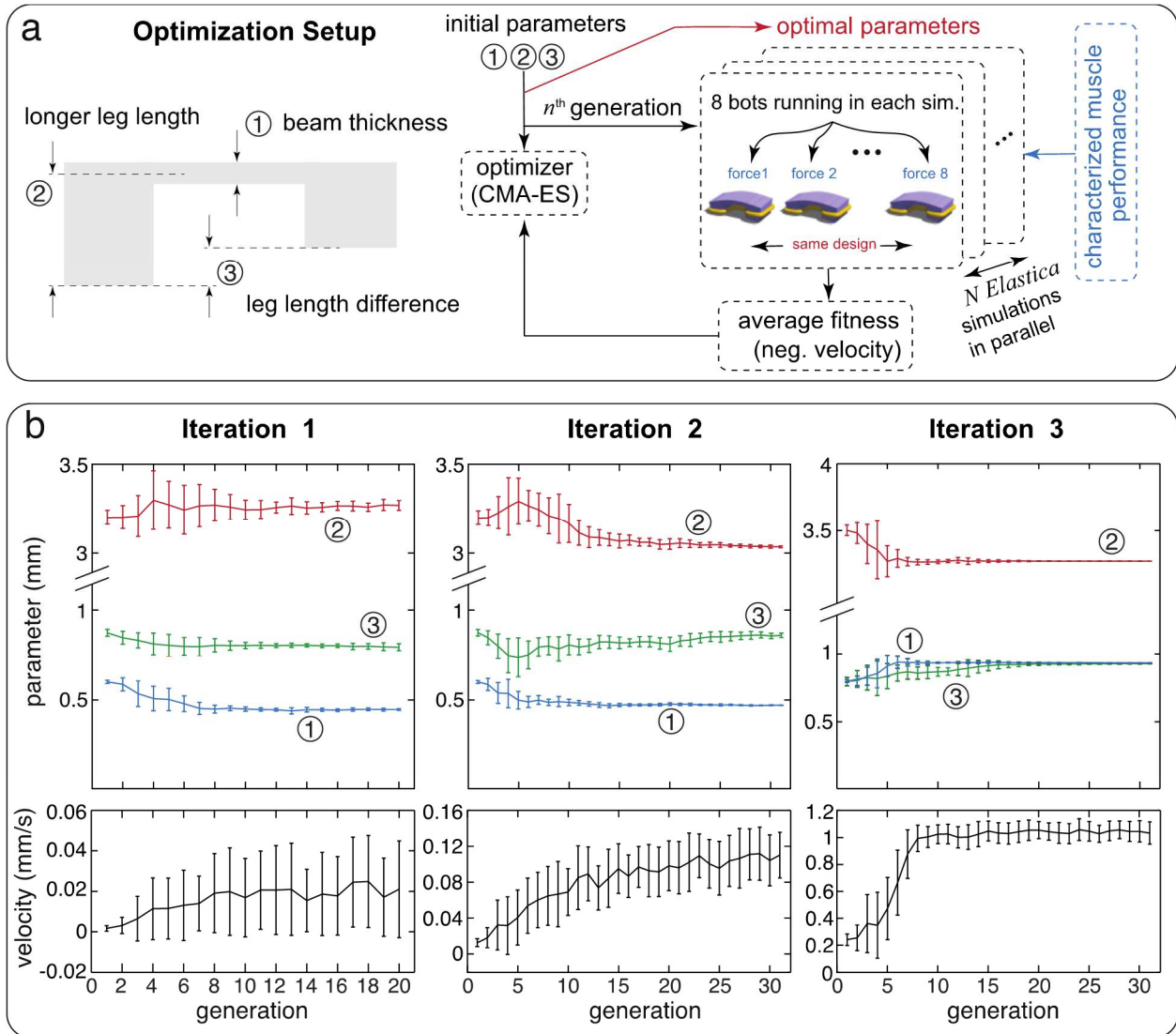


Figure 4.14. Computational design optimizations. (a) Schematics for the design parameters and optimization course. (b) Result of three optimization iterations. In each optimization course, mean values of design parameters and resulting velocities (fitness) start to converge within 20 iterations. Error bars depict the standard deviations, resulting from the randomly sampled muscle force. The Figure set was produced by Xiaotian Zhang from the Gazzola group.

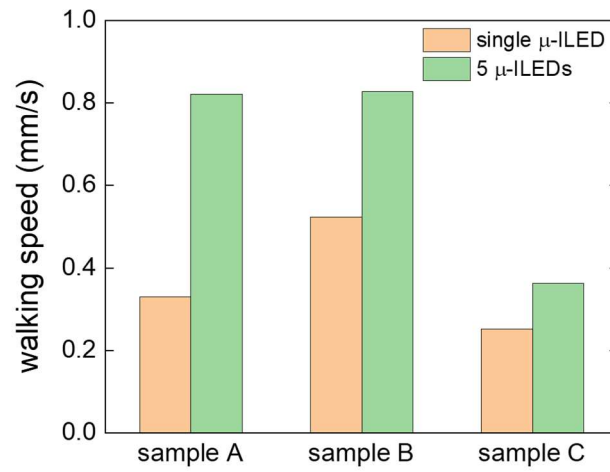


Figure 4.15. Walking speeds comparison for eBiobots stimulated by a single or 5 μ -ILEDs. Walking speeds of eBiobots stimulated at 4 Hz, 50 ms pulse width, and 10 W RF antenna power and by a single μ -ILED or 5 μ -ILEDs for three samples.

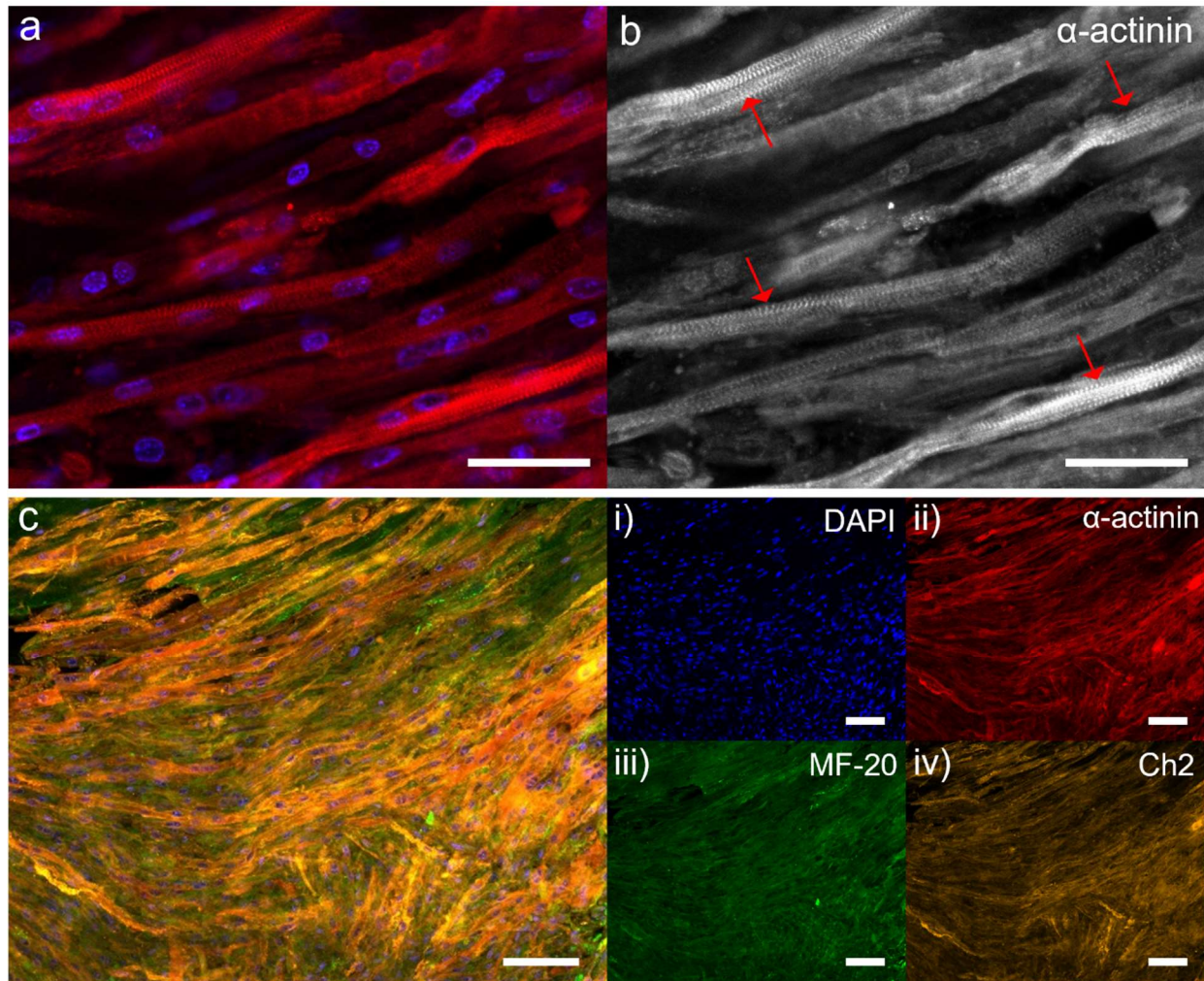


Figure 4.16. Confocal immunostaining fluorescence images of muscle actuator. (a) Immunostaining fluorescence image of muscle actuator fixed on day 21 after differentiation showing aligned myotubes with α -actinin (red) and DAPI (blue) and (b) its α -actinin image to show cross-striations in myotubes. Red arrows indicate the aligned sarcomere bands. (c) Combined fluorescence image of muscle actuator with i) DAPI (blue), ii) α -actinin (red), iii) MF-20 (green), and iv) td-tomato tagged Channelrhodopsin-2 (orange). Scale bars: 50 μm for (a) and (b), 100 μm for (c) and (c-i), ii), iii), and iv).

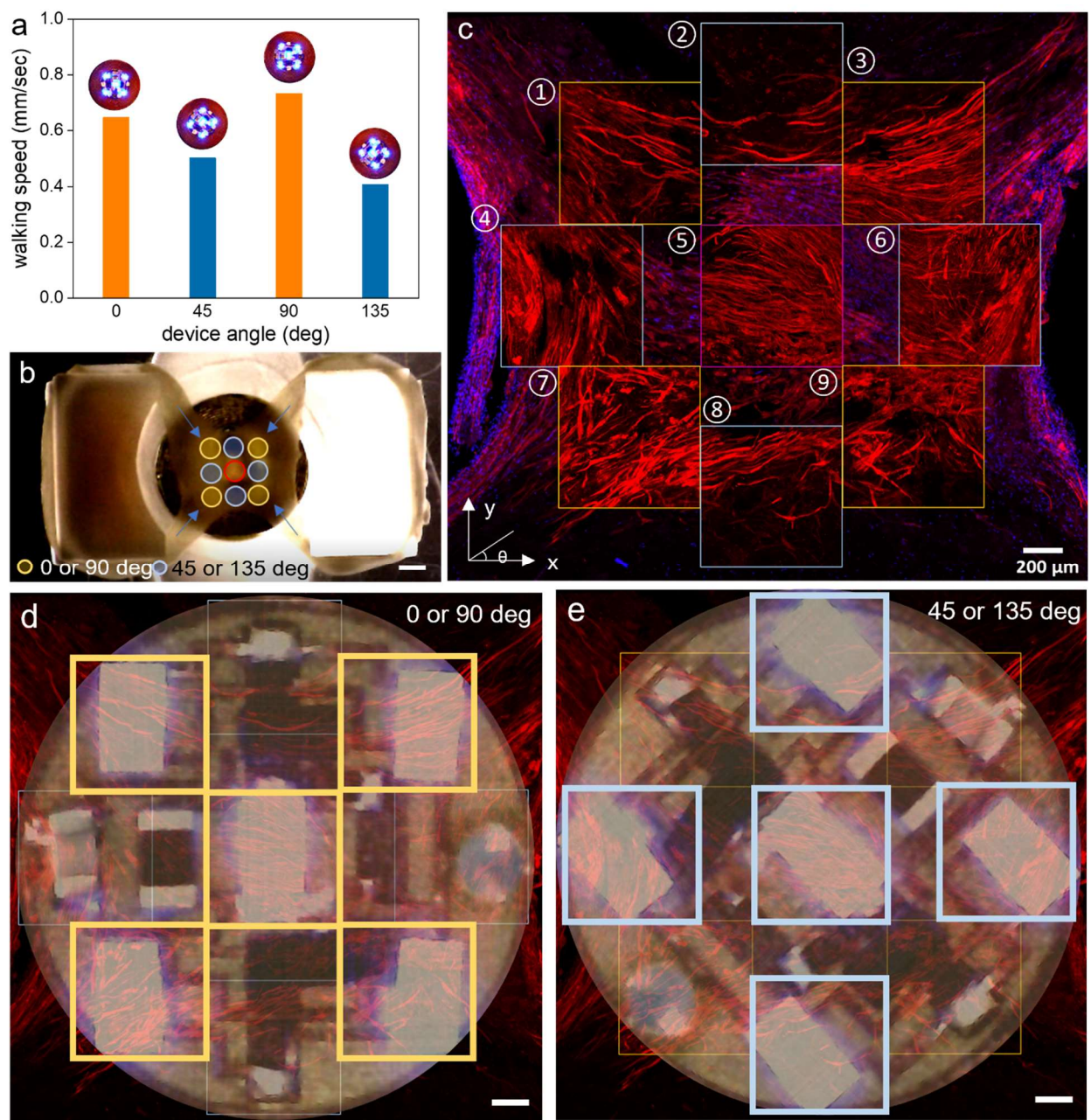


Figure 4.17. Different walking speeds depending on the orientation of 5 μ -ILEDs. (a) Walking speeds of eBiobot with different rotating angles of 5 μ -ILEDs on skeleton beam in movie S7. (b) The illumination region of 5 μ -ILEDs on the eBiobot muscle actuator. The yellow, blue, and red circles indicate the illumination areas in 0 or 90 degrees, 45 or 135 degrees, and all cases, respectively. (c) Confocal immunostaining tile image of the central part of muscle actuator stained with α -actinin as red and DAPI as blue. The axis and angle in the left bottom and its numbers for squares correspond to the myotubes alignments analysis in Figure 4.18, Figure 4.19, and Figure 4.20. Overlapped images with the 5 μ -ILEDs and fluorescence image with α -actinin of the central part of muscle actuator in wireless optogenetic device angles with (d) 90 and (e) 135 degrees. Scale bars: 1 mm for (b) and 200 μ m for (c)-(e).

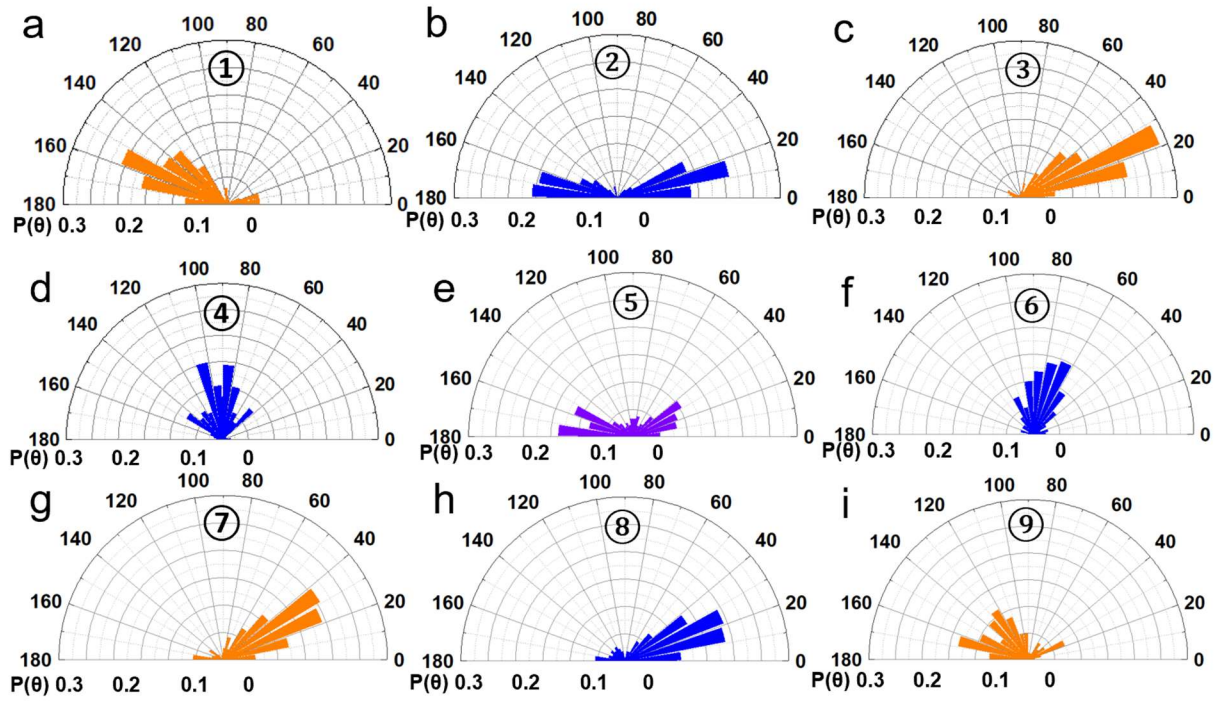


Figure 4.18. Myotubes orientations analysis of muscle actuator. Polar histograms of α -actinin-stained myotubes alignments from 3 samples in regions for (a) ①, (b) ②, (c) ③, (d) ④, (e) ⑤, (f) ⑥, (g) ⑦, (h) ⑧, and (i) ⑨ corresponding with Figure 4.17 (c).

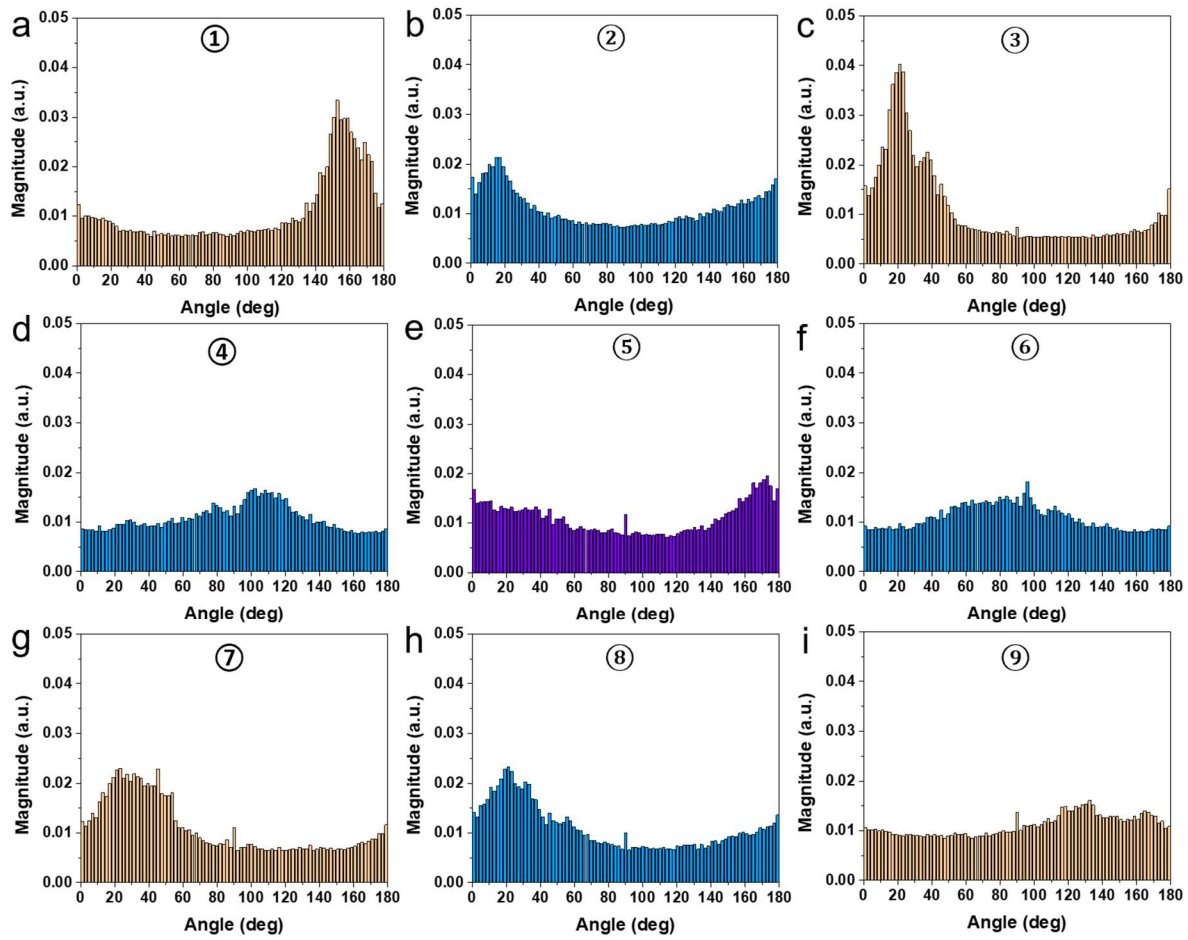


Figure 4.19. FFT analysis for myotubes alignment of muscle actuator. FFT analysis of α -actinin-stained regions for (a) ①, (b) ②, (c) ③, (d) ④, (e) ⑤, (f) ⑥, (g) ⑦, (h) ⑧, and (i) ⑨ corresponding with Figure 4.17 (c).

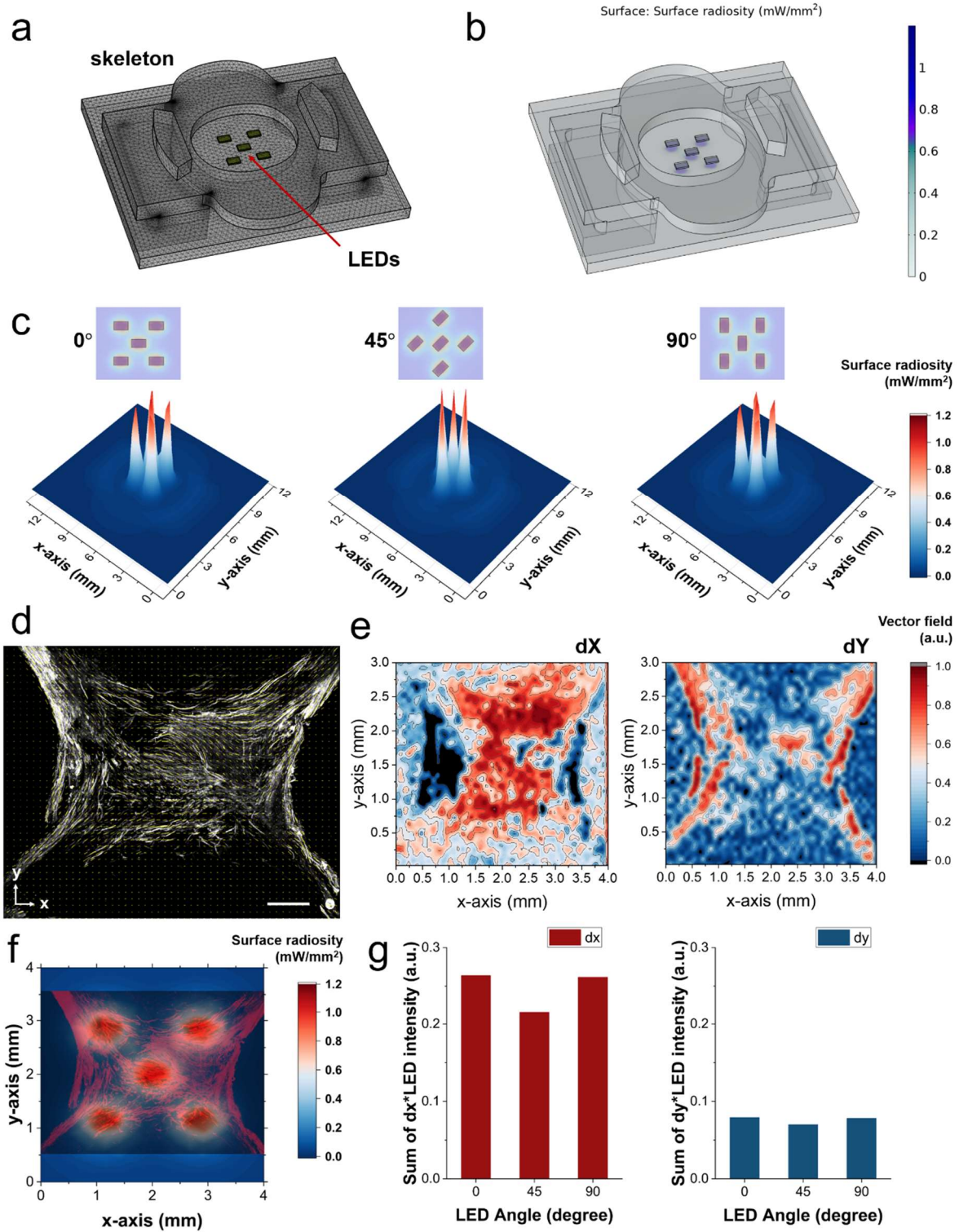


Figure 4.20. The numerical prediction of locomotion of eBibot corresponds to the different configurations of 5 μ -ILEDs. (a) Finite-element mesh used for COMSOL[®] simulation. (b) The radiation of 5 μ -ILEDs on a muscle actuator.

Figure 4.20. (Cont.) (c) Surface radiosity on a muscle actuator with different configurations of 5 μ -ILEDs (0, 45 90 degrees rotating). (d) Visual directional analysis of myotubes alignments from Figure 4.17 (c). The orientation of the vector field is evaluated according to the structure tensor computation in ImageJ software. Scale bar: 500 μ m. (e) Visualization of the vector field of myotubes alignments in x- (dX) and y-axis (dY). The vector field distribution has similar spatial trends with myotubes alignments analysis in Figure 4.18 and Figure 4.19. (f) Alignment of optical images of myotubes and the distribution of radiosity of 5 μ -ILEDs. The locomotion factor of 5 μ -ILEDs radiated eBibot is calculated by multiplying the vector field of myotubes in the x- and y-axis by the radiosity of 5 μ -ILEDs with different configurations for each pixel. (g) Bar plot of the sum of locomotion factors for every pixel in the x- and the y-axis corresponds to 0, 45, and 90 degrees of 5 μ -ILEDs. The Figure set was produced by Insu Park from the Bashir group.

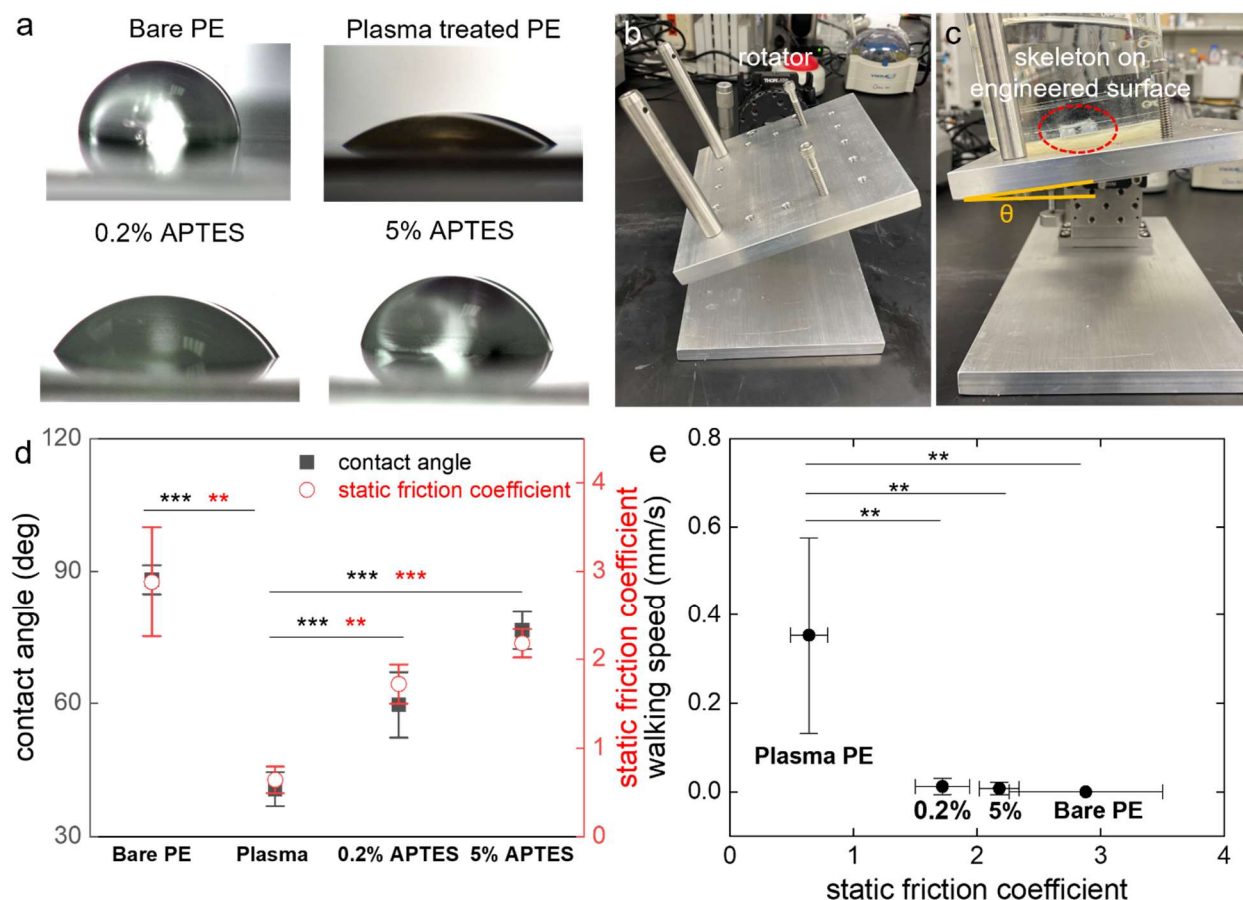


Figure 4.21. Friction study of engineered surfaces for the improved walking performance of eBiobot. (a) Side microscopic images for water drop on engineered polyethylene (PE) film. Images of (b) customized angle of the repose measurement system with the rotator and (c) how to measure the sliding angle. (d) Contact angle (black) and static friction coefficient (red) of engineered PE surfaces. (e) Walking speeds of eBiobots on engineered PE surfaces with their static friction coefficient ranges (Plasma treated, 0.2% and 5% APTES treated, bare PE surfaces from the left). Error bars correspond to the standard deviation of 5 measurements for contact angle and minimum of 3 for static friction coefficient and 6 measurements for walking speed. Statistical significance: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. The black and red asterisk in (d) corresponds to the statistical significance of contact angle and static friction coefficient, respectively.

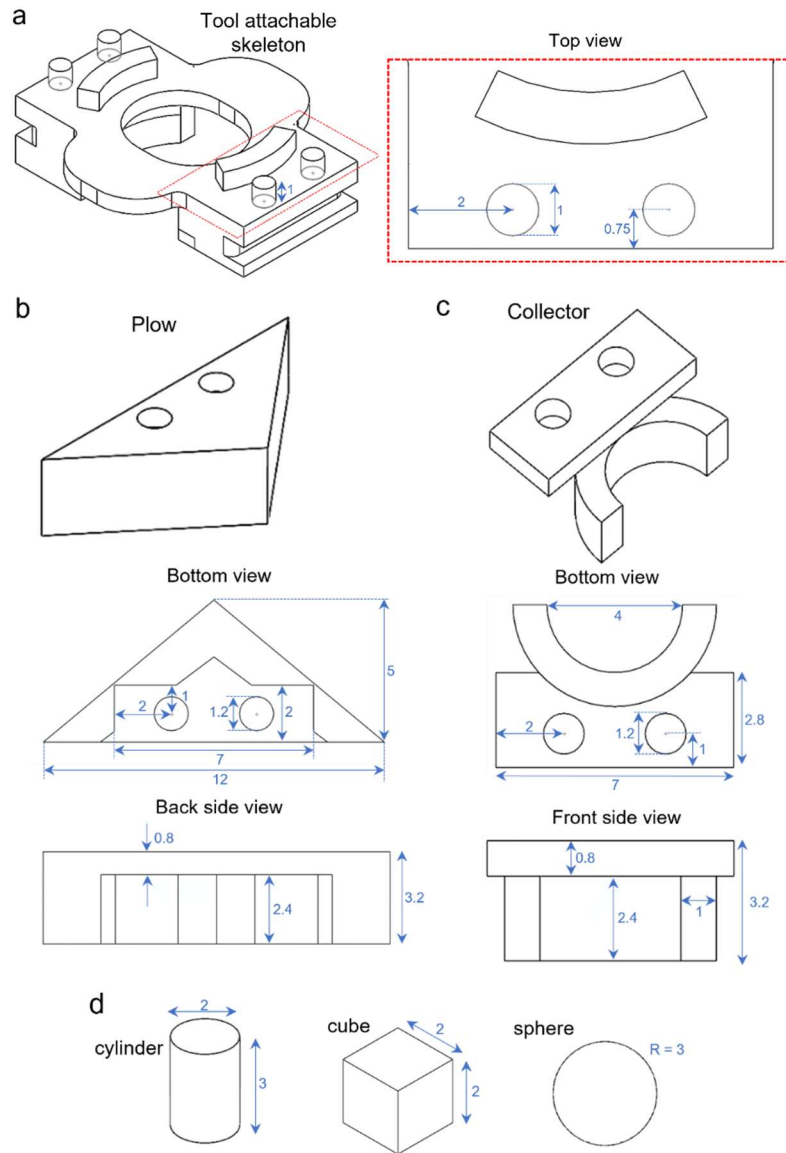


Figure 4.22. Designs of tool attachments for functional eBiobot using Lego[®]-like assembly. CAD with dimensions for (a) tool attachable skeleton for functional eBiobot, (b) plow, (c) collector, and (d) obstacles used in Figure 4.11 (d). Units: mm.

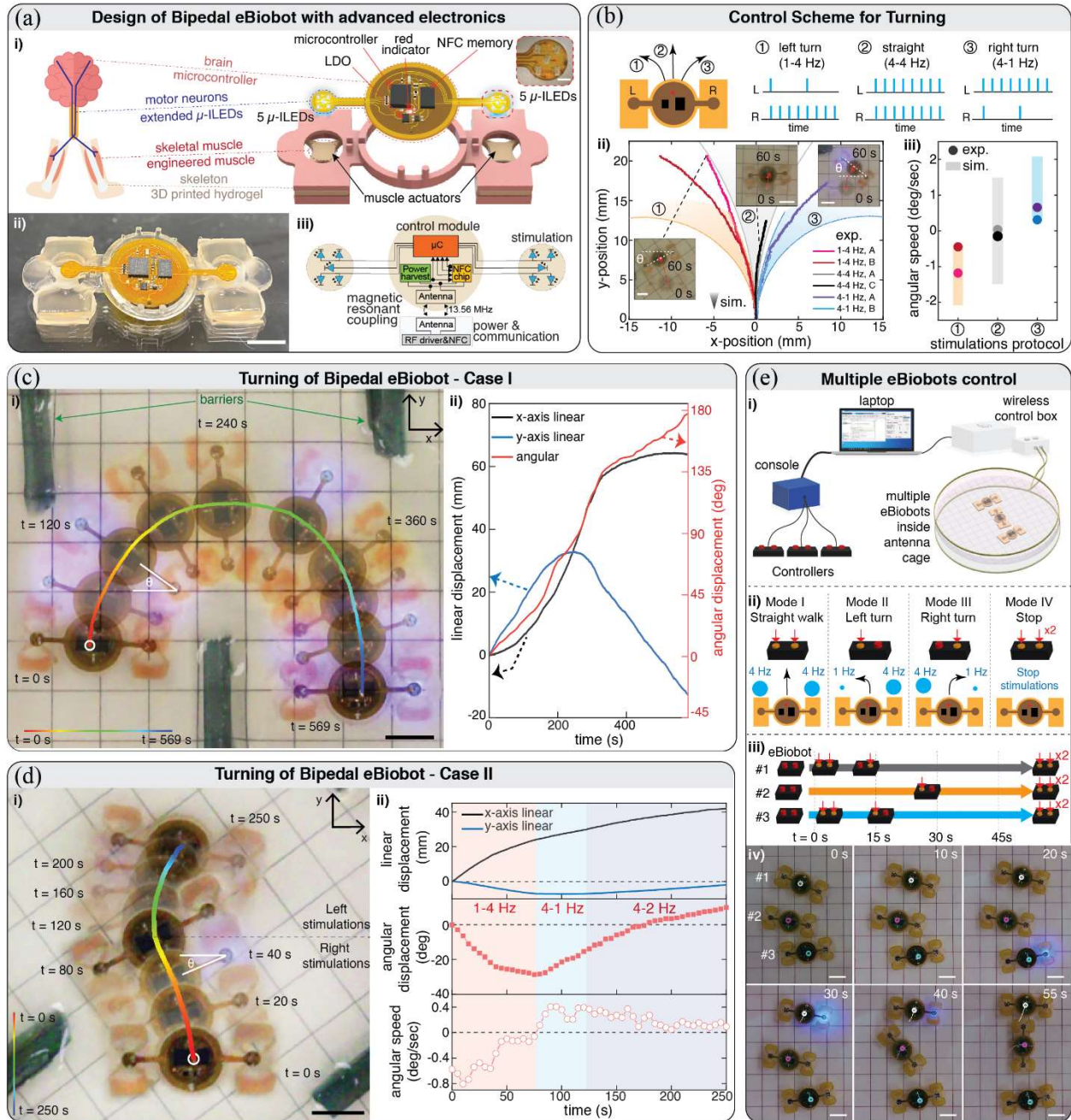


Figure 4.23. Bipedal eBiobot that can turn. (a) Bio-inspired design and advanced electronics for bipedal eBiobot. i) Schematics of biological motor units inspired design and advanced electronics with components for bipedal walking. Inset shows the image of 5 μ -ILEDs. ii) A prototype of the assembled bipedal robot. iii) Block diagrams of the advanced wireless electronic control system. The system contains a microcontroller that reads and operates the stimulation parameters updated in the NFC memory chip on an event basis in real-time. (b) The control scheme for turning. i) Schematics of bipedal eBiobot and optical stimulations scheme with its turning directions. ii) Trajectories and iii) angular speeds stimulated by dual panels μ -ILEDs with ① 1 and 4 Hz, ② 4 and 4 Hz, and ③ 4 and 1 Hz for left and right sides in 60 seconds. The computational turning scopes are obtained by considering muscle force variations.

Figure 4.23. (Cont.) (c) The first case for turning bipedal robot through barriers. i) Trajectory and time-lapse images of the turning eBiobot (in 569 seconds). The color map shows the elapsed time. ii) Linear and angular displacements with time. (d) The second case for turning bipedal eBiobot for turning both ways. i) Trajectory and time-lapse images of bidirectional turning eBiobot (in 250 seconds). The color map shows the elapsed time. ii) Linear and angular displacements and angular speed with time and stimulations scheme. (e) Controlling multiple robots using a console system. (i) Schematics of the experimental setup for individually remote-controlled multiple bipedal eBiobots using a console system connected with multiple gaming controllers. (ii) Operation modes of clickers to start, turn and stop bipedal robots corresponding with their stimulations schemes. The case for 3 robots' manipulations. (iii) Operation modes of gaming controllers for eBiobots 1, 2, and 3 with time and (iv) corresponding trajectories time-lapse images of 3 bipedal eBiobots in 55 seconds. Scale bars: 1 mm for the inset of (a)-i), 1 cm for others.

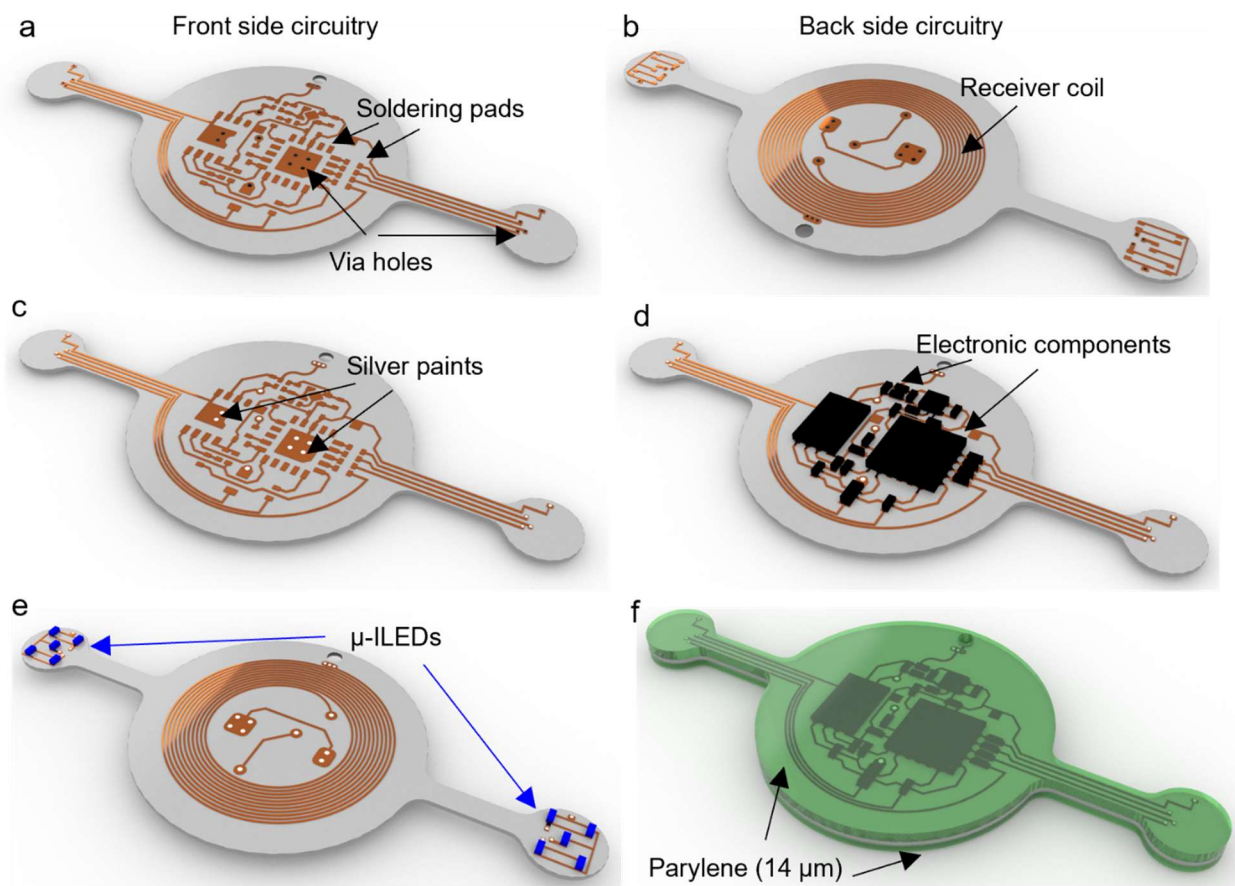


Figure 4.24. Fabrication process for advanced wireless optogenetic devices that power bipedal eBiobots. Apply aligned laser ablation process on the front (a) and back (b) surfaces of a tri-layer flexible printed circuit board (fPCB) composed of Cu (18 μm) – polyimide (PI, 75 μm) – Cu (18 μm) to define receiver coil, soldering pads, and via holes. (c) Fill via holes with silver paint to connect the top and bottom circuitry. (d)-(e) Apply hot air soldering to mount electronic components (d) and μ -ILEDs (e) on designated locations. The number of μ -ILEDs can be further adjusted based on experimental requirements. (f) Encapsulate the entire device with chemical vapor deposited parylene (14 μm). The Figure set was produced by Yiyuan Yang from the Rogers group.

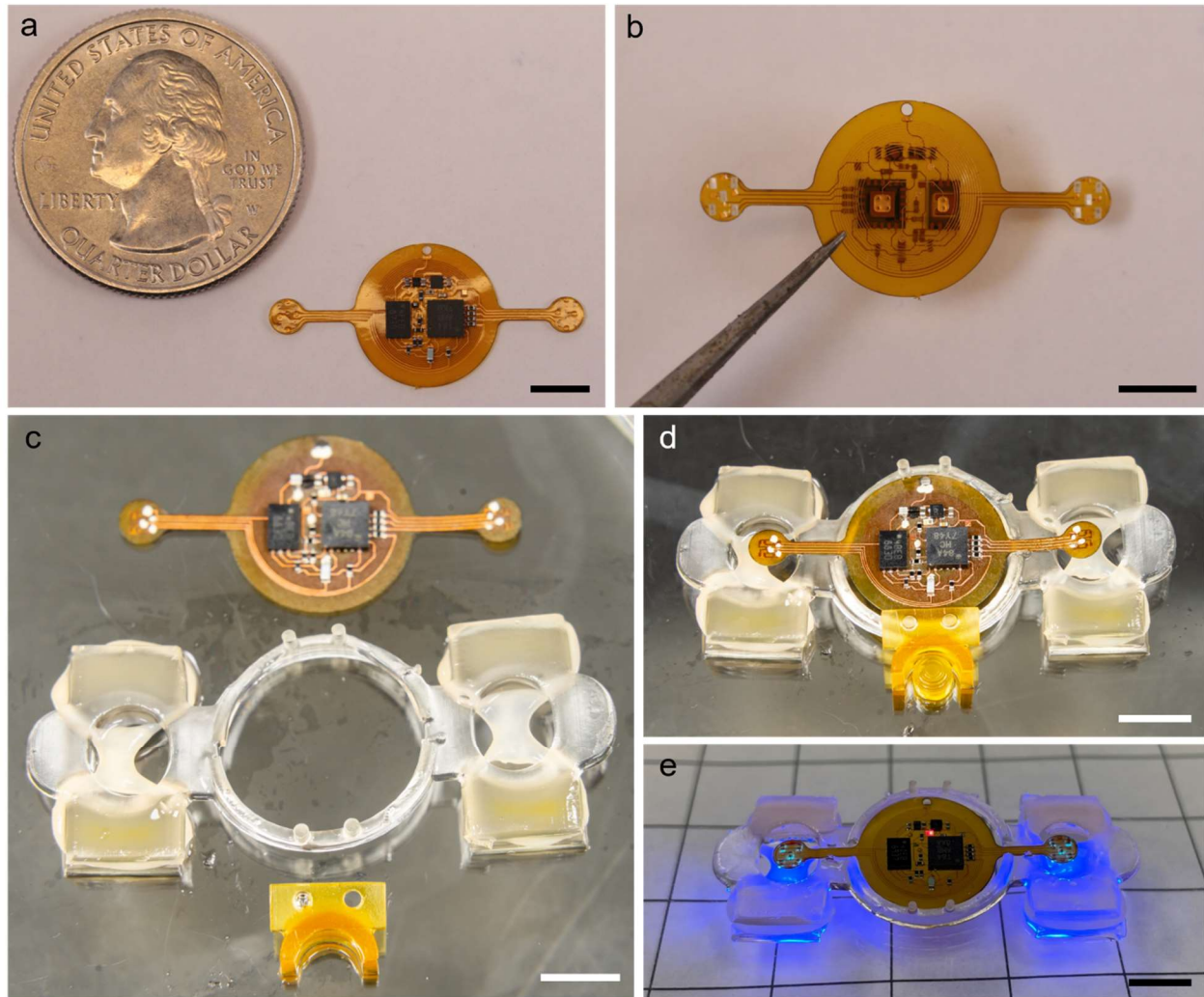


Figure 4.25. Photographic images of the advanced wireless optogenetic device and its assembly with bipedal eBiobot. Images for (a) top view and (b) bottom view of advanced wireless optogenetic device and (c) before and (d) after assembly of electronics with bipedal eBiobot and collector (e) with light illuminations from μ -ILEDs. All scale bars: 5 mm.

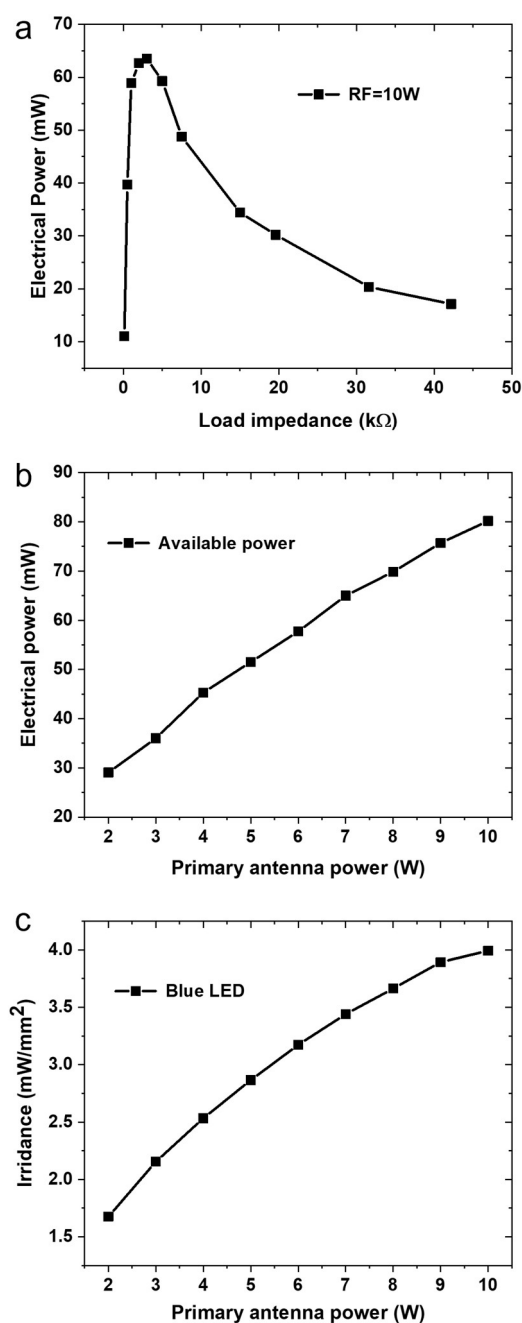


Figure 4.26. Electrical and optical characterizations of advanced wireless optogenetic devices with dual panels of μ -ILEDs. (a) Electrical power supplied to the dual panels optogenetic device as a function of loading impedance at 10 W RF antenna power. (b) Maximum total harvested power for the dual panels optogenetic device as a function of RF transmission antenna power. (c) optical irradiance output of one μ -ILED, out of ten simultaneously activated, driven by the dual panels optogenetic device as a function of transmission antenna power. The Figure set was produced by Zhegwei Li from the Rogers group.

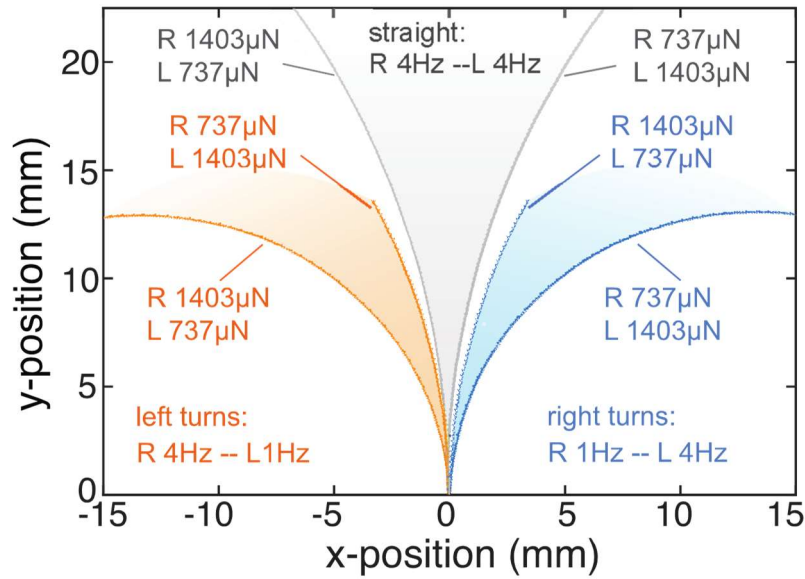


Figure 4.27. Parameters used in the simulation for predicting the turning capability and robustness of the bipedal eBiobot. R and L denote frequencies or forces set for the right or left leg, respectively. The frequency settings determine whether the bot will walk straight or turn, while the force offsets between the two legs define the trajectory envelope of each moving mode. $737\mu\text{N}$ and $1403\mu\text{N}$ forces are the lower and upper bounds of the muscle force range characterized during the last design iteration and utilized here for the prediction. The Figure set was produced by Xiaotian Zhang from the Gazzola group.

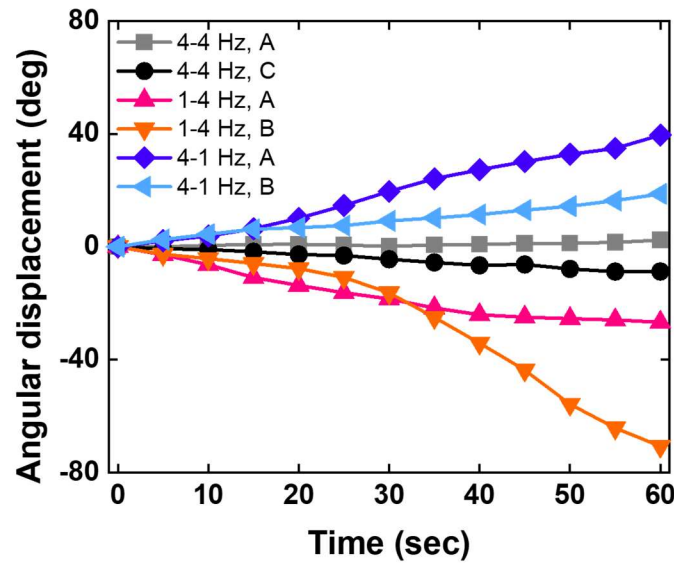


Figure 4.28. Angular displacements for turning or straight walking bipedal eBiobots. Angular displacement vs. time curves for bipedal eBiobots with different stimulation schemes from Figure 4.23 (b)-ii), iii).

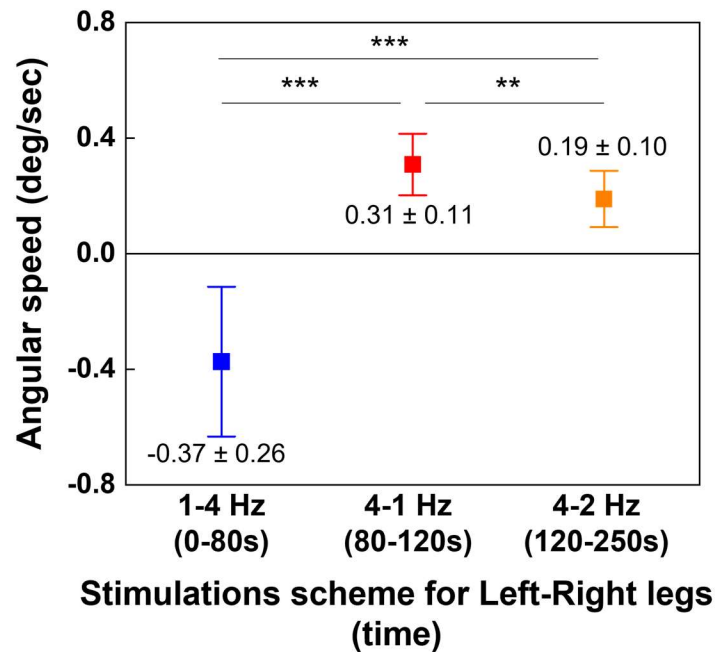


Figure 4.29. Angular speeds depending on modulation scheme. Angular speeds of turning bipedal eBiobot in Figure 4.23 (d) for every 5 seconds with different stimulations schemes. Positive angular speeds mean turning to the clockwise side, while negative means turning to the counterclockwise side. Error bars correspond to the standard deviations of calculated angular speeds for every 5 seconds in a turning bipedal eBiobot. (Mean $\pm$ standard deviation). Statistical significance: ** $P < 0.01$, *** $P < 0.001$.

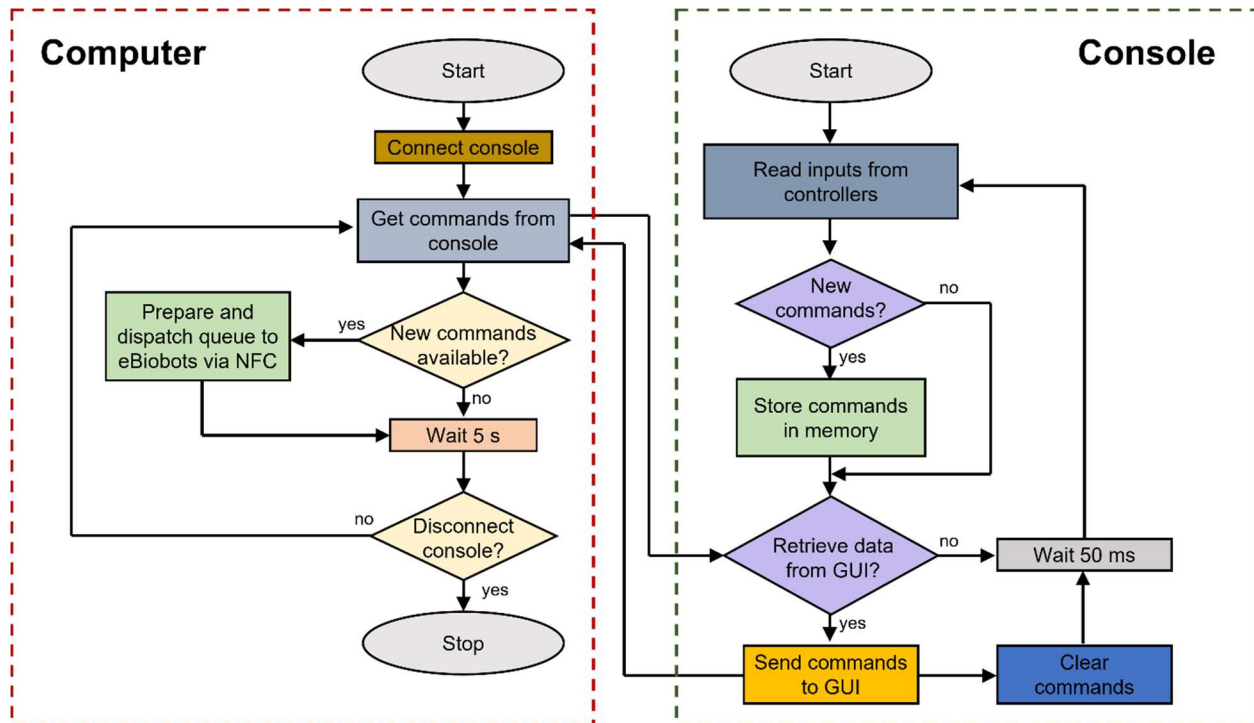


Figure 4.30. Logic diagram that depicts the operation of the console. The software running in the computer controls a temporized event to interrogate the console and retrieve the commands issued by the users on each of the 6 remote controllers. If new commands are reported, then the GUI dispatch the control commands to the corresponding eBibot. The Figure set was produced by Abraham Vazquez-Guardado from the Rogers group.

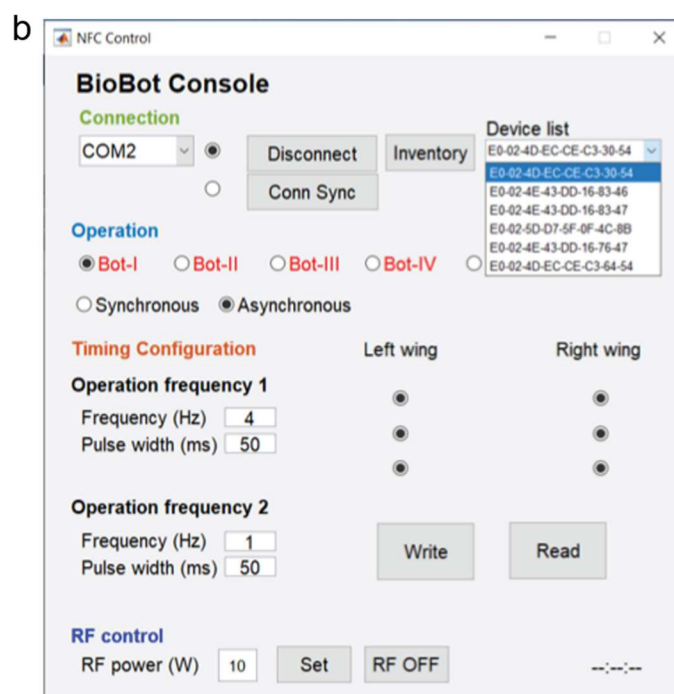
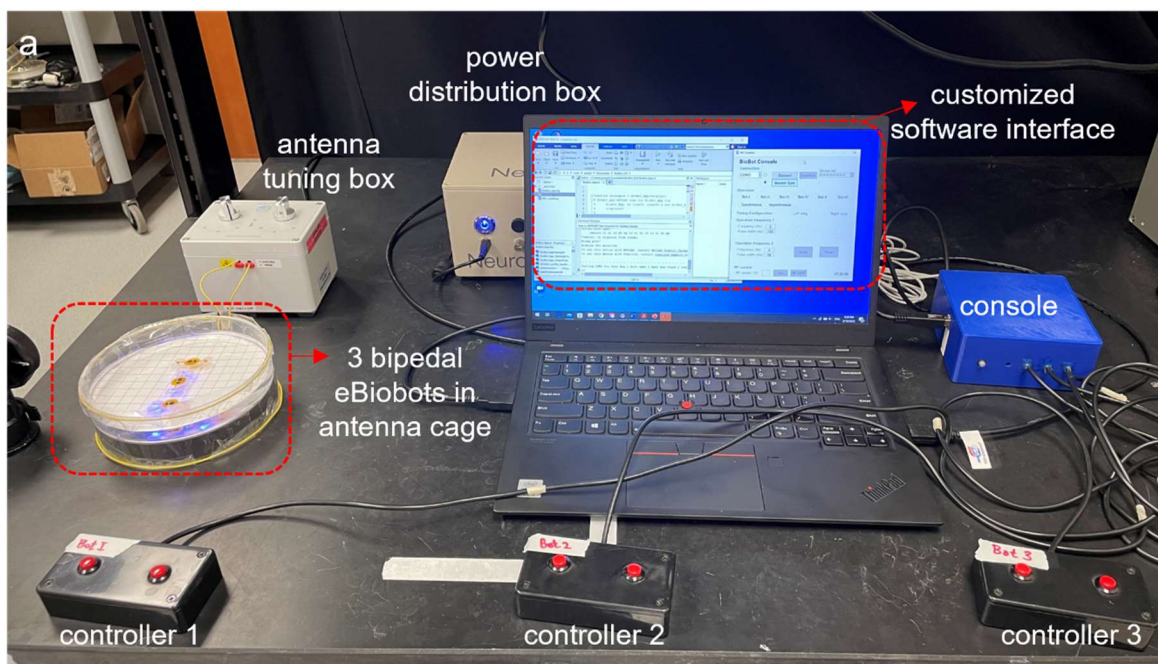


Figure 4.31. Set up for individual remote control of multiple eBiobots with gaming controllers. (a) Experimental setup image for individual remote control of multiple bipedal eBiobots using the console system connected with multiple gaming controllers, customized software, and antenna setup. (b) Software interface for console operation.

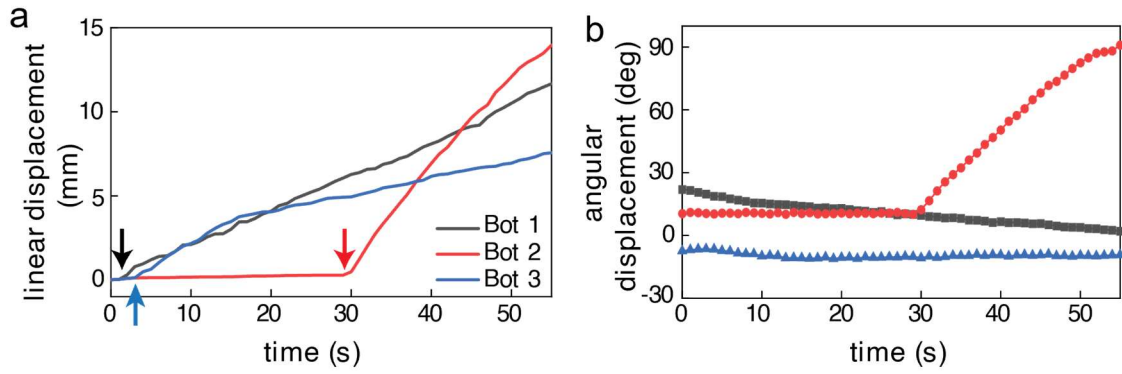


Figure 4.32. Individual remote control of three bipedal eBiobots. (a) Linear and (b) angular displacement vs. time curves for eBiobots 1, 2, and 3 from Figure 4.23 (e)-iii, iv. Arrows indicate when gaming controllers start operations.

4.6. Table

Table 4.1. Locomotion comparisons with previous biohybrid robots. Locomotion mechanisms, actuation type, body length, and maximum speeds for previous biohybrid robotics systems. eBibots in this work recorded the fastest speed among biohybrid robotic walkers or skeletal muscle-based bio-hybrid robots including swimmers. The bipedal eBibot showed 1.75 mm/sec of walking speed, another recording the fastest walking speed with the largest body scale among all biohybrid robotics systems.

Mechanism	Actuator type	Body length (mm)	Maximum speed (mm/sec)	References
Swimmer	Cardiomyocytes	6	0.4	Feinberg et al. [29]
		6	2.4	Nawroth et al. [30]
		1	0.081	Williams et al. [31]
		16.3	3.2	Park et al. [5]
		14	15	Lee et al. [6]
	NMJ-driven 3D skeletal muscle	3.2	0.0007	Aydin et al. [7]
	3D Skeletal muscle	12	0.79	Guix et al. [11]
Walker	Cardiomyocytes	0.4	0.038	Xi et al. [32]
		2	0.1	Kim et al. [33]
		7	0.24	Chan et al. [15]
	3D skeletal muscle	6	0.16	Cvetkovic et al. [22]
		14	0.5	Pagan-Diaz et al. [9]
		14	0.83	This work
		35	1.75	

4.7. References

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CHAPTER 5: CONCLUSIONS AND FUTURE DIRECTIONS

5.1. Summary and Conclusions

In summary, in this thesis work, various electronics platforms have been integrated into 3D engineered skeletal muscle tissues for electrical applications such as muscle actuation, sensing of strain, and remote optogenetic muscle controls for advanced TOC and biohybrid soft robotics applications. Chapters 2 and 3 showed TOC demonstrations using tethered skeletal muscle systems, and Chapter 4 introduced soft robotics applications using untethered skeletal muscle-driven biological machines.

Chapter 2 demonstrated 3D engineered muscle tissue actuator electrically by describing how a CVD graphene sheet was transferred using a PEGDA supportive layer with 3D printed hydrogel structures. The soft PEGDA layer enabled the 3D conformal integration of graphene sheet and millimeter-scaled 3D printed hydrogel pillars. The Raman spectroscopy analysis and electrical characterizations demonstrated the integrated graphene on 3D printed structures. Muscle tissue integrated into the platform responded to electrical pulses through the transferred 3D

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[1] Yongdeok Kim, Gelson Pagan-Diaz, Lauren Gapinske, Yerim Kim, Judy Suh, Emilia Solomon, Jennifer Foster Harris, SungWoo Nam, Rashid Bashir. *Adv. Healthcare Mater.* 9, 1901137 (2020).

[2] Hangbo Zhao⁺, Yongdeok Kim⁺, Heling Wang⁺, Xin Ning⁺, Chenkai Xu, Judy Suh, Mengdi Han, Gelson J. Pagan-Diaz, Wei Lu, Haibo Li, Wubin Bai, Onur Aydin, Yoonseok Park, Jiaojiao Wang, Yao Yao, Yishan He, M. Taher A. Saif, Yonggang Huang, Rashid Bashir, John A. Rogers, *Proc. Natl. Acad. Sci. USA*. 118, e2100077118 (2021)

[3] Yongdeok Kim⁺, Yiyuan Yang⁺, Xiaotian Zhang⁺, Zhengwei Li⁺, Abraham Vazquez-Guardado⁺, Insu Park, Jiaojiao Wang, Andrew I. Efimov, Zhi Dou, Yue Wang, Junehu Park, Haiwen Luan, Xinchun Ni, Yun Seong Kim, Janice Baek, Zhaoqian Xie, Joshua Jaeyung Park, Hangbo Zhao, Mattia Gazzola, John A. Rogers, Rashid Bashir, *Sci. Robot.* (Under review)

+These authors contributed equally to this work.

graphene electrode. The work represents a novel method for 3D graphene electrode integration as a potential platform for electrical applications for 3D biological tissue systems.

In Chapter 3, the 3D engineered optogenetic skeletal muscle tissue was integrated with the 3D compliant framework instrument capable of electrical strain sensing. The 3D frameworks utilized mechanically guided assembly processes to form a system that provides millimeter-scale mechanical supports with integrated microscale strain sensors for the measurements of tissue contractility of 3D engineered skeletal muscle tissues. These compliant 3D architectures enabled close contact and robust device-tissue coupling, for accurate electronic sensing of mechanical responses under optical and chemical stimuli. These properties of 3D frameworks are advantageous over traditional optical microscopy tracking methods for measuring and quantifying muscle force. The high sensitivity measurement of engineered muscle tissue contraction is potentially useful for muscular disease studies, drug screening systems, and medical countermeasures.

Finally, Chapter 4 presented the integration of biohybrid robots driven by optogenetic skeletal muscles with wireless optoelectronics control. The modular and bio-inspired design approaches have allowed functional biohybrid robots to be remotely steered. The design and locomotion of biohybrid robots were improved through design iterations and produced by additive manufacturing using 3D printing. The design allowed the integration of electronics and utilization, the use of muscle actuators, and Lego®-like tool attachments to the eBiobots. The bio-inspired bipedal design utilized two separated muscle actuators and a microcontroller incorporating onboard optoelectronics to actuate individual optogenetic muscles to allow turning maneuvers. The result is a suite of advanced programmable functionalities such as remote-control switching, steering, plowing, and transport, both for individual and multiple robots.

In Conclusion, various biohybrid muscle electronics systems have been demonstrated through interdisciplinary engineering approaches and active collaboration in the thesis. Integrating bioelectronics with *in-vitro* muscle systems has enabled diverse capabilities for advanced TOC and biohybrid robotics applications. It paves the way for a new class of biohybrid systems. Further future directions will be discussed in the following section.

5.2. Future directions

5.2.1. Biomedical applications using bioelectronics integrated 3D TOC platforms

Proposed TOC platforms in Chapter 2 and Chapter 3 show potential biomedical applications in drug screening systems, disease modeling, and medical countermeasures. Although these studies used mouse cell lines C2C12s, broadening cell sources to patient-derived human induced pluripotent stem cells (hi-PSCs) differentiated skeletal muscle cells or Duchenne muscular dystrophy (DMD) primary cells extracted from transgenic MDX mice can be a future direction for personalized medicine or disease model study. [1]

Not only skeletal muscle tissues but other types of tissue or multi-cellular systems can be integrated with the platforms for various biological studies. In particular, the design and fabrication scheme presented in Chapter 3 have additional potential in multifunctional sensing modalities for measurements of electrical potential, temperature, and electrochemistry with distributed collections of electronic, piezoelectric, optical, and optoelectronic components for advanced modulation of tissue functions in a wide range of applications in studies of other 3D tissue constructs using cardiomyocytes, neurons, and others. [2, 3] The ideas presented here also scale naturally to arrayed configurations for high throughput sensing in tissue engineering for drug screening and disease model studies.

5.2.2. Closed-loop system for fully automated eBiobot

Biohybrid electronics robots demonstrated in Chapter 4 also have great potential as a new class of soft robotics. Combining the electrical applications from Chapters 2 and 3 with wireless control system would enable advanced closed-loop controlled robotics applications. For example, integrating stimulation electrodes onto the inside of hydrogel legs allows the electrical actuation to non-optogenetics muscle tissues, while the optoelectronics integrated system is ideal for optogenetics cell actuation. Adopting a full Wheatstone Bridge circuit on the bending hydrogel surface could enable the electrical strain sensing of eBiobot and wireless data collection and transmission through the RF communications setup and onboard electronics. Finally, incorporating a microprocessor can establish a fully automated closed-system robotic system that operates from the advanced feedback of onboard sensors. In addition, further upgrades in the electronic control system will realize swarm robotic systems that have great potential applications in the environment, defense, and biomedicine fields. Considering that additional functionalities enabled by updated electronic systems can be added conveniently in the future, this platform paves the way for a new era of biohybrid electronics miniature robots.

5.2.3. NMJ-driven eBiobot and preliminary results

Future integration of *in-vitro* optogenetic neural tissue and innervation with skeletal muscles can be synergistic with embedded optoelectronics for neural training, realizing a new breed of the autonomous neuromuscular junction (NMJ) driven biohybrids robots equipped with cybernetic communication between biological and artificial intelligence.

In-vitro NMJ formation is a highly complex process requiring a series of orchestrated events including myogenesis, neurogenesis, the extension of neurites towards muscles, and the

establishment of a postsynaptic connection between motor neurons and myotubes. [4, 5] Recently, a few 3D *in-vitro* functional NMJ systems have been reported using an extracellular matrix (ECM) embedded muscle tissue and motor neurons integrated with a soft PDMS or 3D printed structure. [1, 4-7] Uzel et al. and Osaki et al. achieved 3D *in-vitro* functional NMJ using PDMS-based microfluidics for precise spatial control and co-culture of two different cell types with mouse embryo bodies (mEBs) differentiated motor neurons and skeletal muscles. [3, 6, 7] Aydin et al. developed an *in-vitro* NMJ-driven swimmer as a soft robotics application using an untethered PDMS structure with optical stimulations to mEBs differentiated motor neurons making muscle contractions through NMJ, which made it swim. [6] Cvetkovic et al. showed a millimeter-scale NMJ functionality using ECM embedded multi-layered tissue structures for skeletal muscle and mEBs differentiated motor neurons with 3D printed structures. [7] Based on these previous *in-vitro* NMJ studies, the design with separation between mEBs differentiated motor neurons and muscles was considered.

Figure 5.1 shows designs that integrate motor neurons for existing single and bipedal eBiobots skeletons from Chapter 4. The compartments for optogenetic mEBs differentiated motor neuron seeding (red arrows in Figure 5.1 (c) and (d)) were designed apart from the muscle part. As the proposed designs are also compatible with wireless optoelectronics used in Chapter 4, these have the potential for cybernetic communication between biological and artificial intelligence as well as neural training and control using μ -ILEDs. Figure 5.2 and Figure 5.3 present the protocols and images for seeding and co-culture of skeletal muscle and mEBs as preliminary results. The mEB compartments are separated from the central muscle part using micro-scale pillars in the skeleton (Figure 5.3 (a) and (b)). It is the platform designed to minimize the overflow to the outside of compartments when ECM-embedded mEBs are seeded inside them due to the viscous ECM/cell

mixture, while the distance between pillars is designed to allow neurites to extend from mEBs to the central part of muscle tissue between the pillars.

To improve the innervation for functional NMJ formation, stimulation training during co-culture can be a future direction. Electrical stimulations to neurons have been widely used to recover *in-vivo* nerves and NMJ regeneration after neural injuries, as electrical stimulations to electrogenic neurons are known to enhance the release of neurotrophic factors such as brain-derived neurotrophic factors and nerve growth factors. [8-11] With a similar mechanism, optical stimulations to optogenetics neurons can improve neurite outgrowth. [11, 12] Through electrical and optical stimulation training, better innervation and NMJ functionality can be achieved and NMJ driven walker is expected as a soft robotics application. This platform can also be useful for basic NMJ research, drug testing, and the establishment of clinically relevant models for diseases such as amyotrophic lateral sclerosis, also known as Lou Gehrig's disease, and muscular dystrophy. Furthermore, integrating wireless optoelectronics with mentioned platforms will be very synergic to advanced robotic functions and onboard neural computing.

5.3. Figures

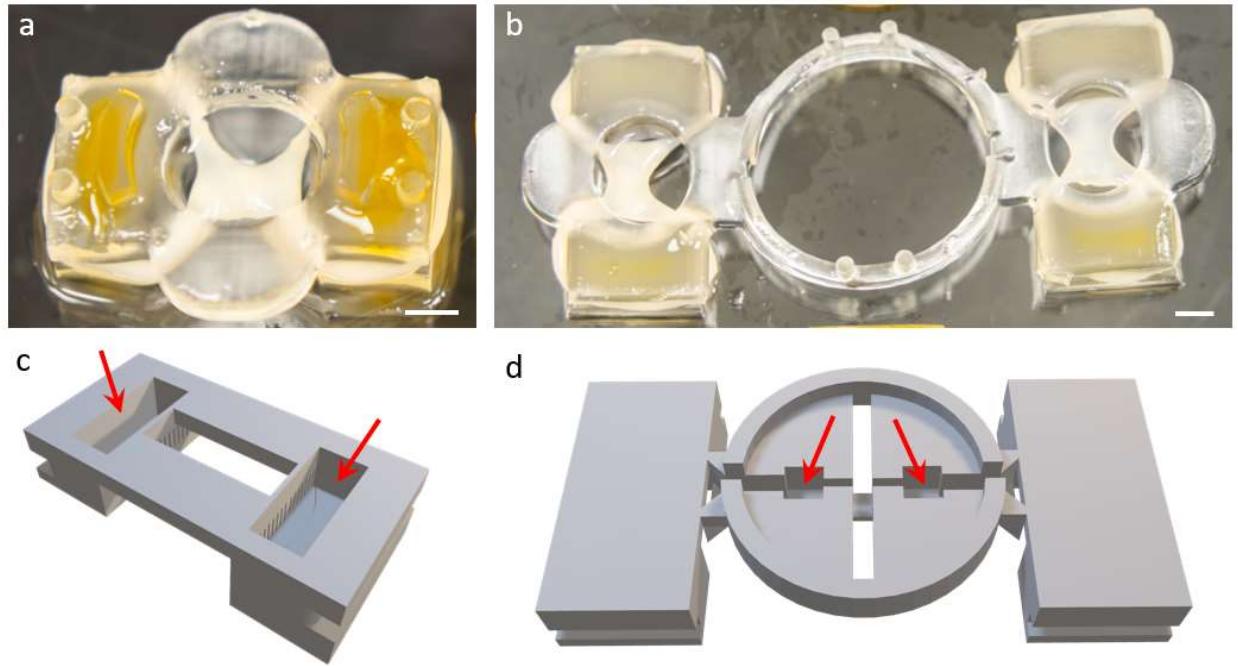


Figure 5.1. Designs for NMJ-driven robot skeleton. Optical images for muscle only driven (a) single and (b) bipedal eBiobots skeletons in Chapter 4. CAD images for NMJ-driven (c) single and (d) bipedal biobots skeletons inspired by (a) and (b). Red arrows indicate the spaces for neural seeding. Scale bars: 2 mm.

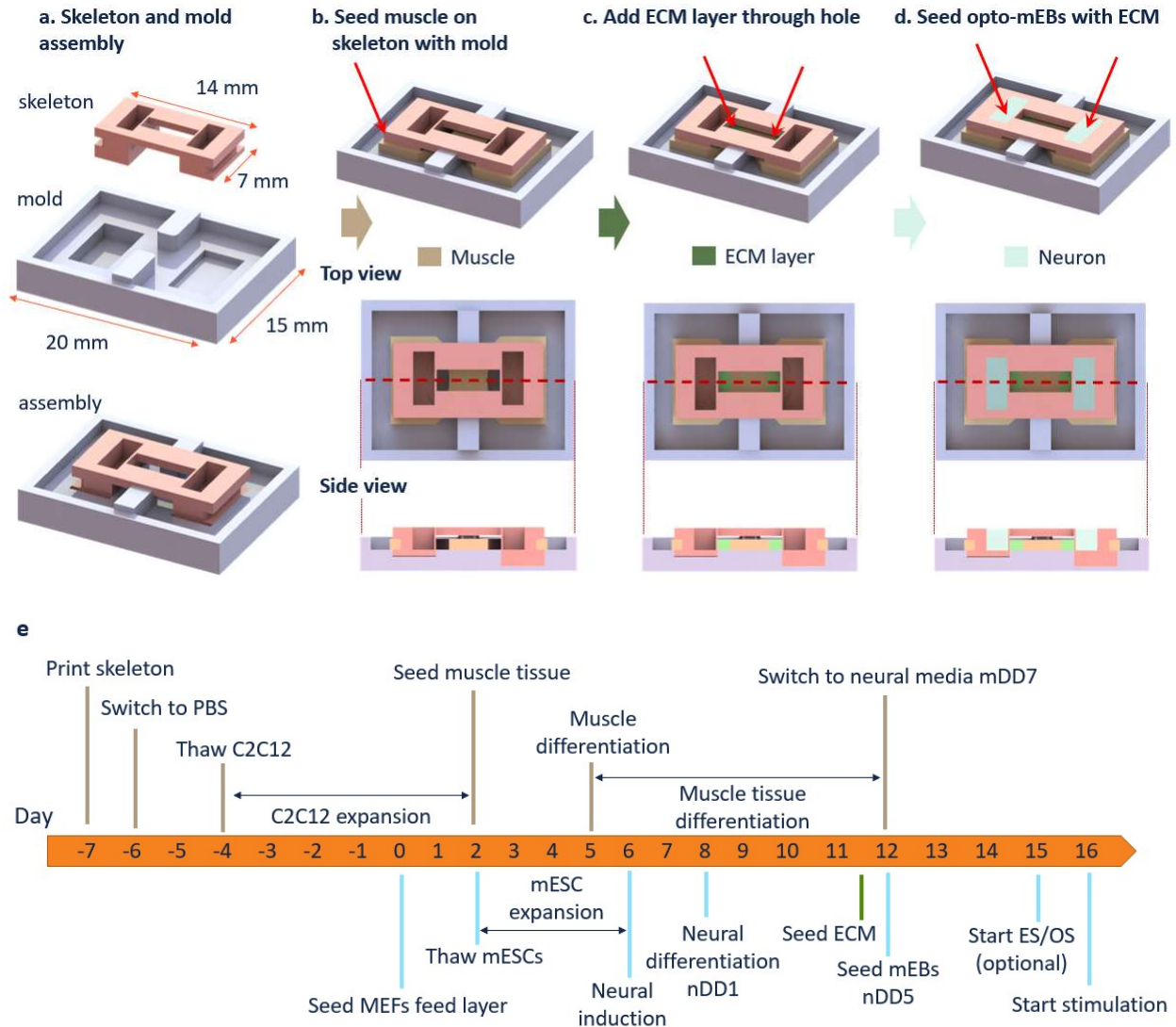


Figure 5.2. Schematics and timeline for neuron-muscle co-culture seeding protocol. (a)-(d) Schematics for seeding protocol with 3D printed skeleton and mold. (a) CAD designs for skeleton and mold for NMJ-driven biobot with dimensions. Angled, top, and cross-sectional views for (b) after seeding muscle on the skeleton with mold, (c) adding an ECM layer between the muscle and neural parts, and (d) seeding optogenetic mouse embryo bodies (opto-mEBs) with ECM inside the neural parts. (e) Seeding timeline for neuron-muscle co-culture. (MEFs: mouse embryonic fibroblast, mESC: mouse embryonic stem cells, mDD: muscle differentiation day, nDD: neuron differentiation day, ES: electrical stimulation, OS: optical stimulation)

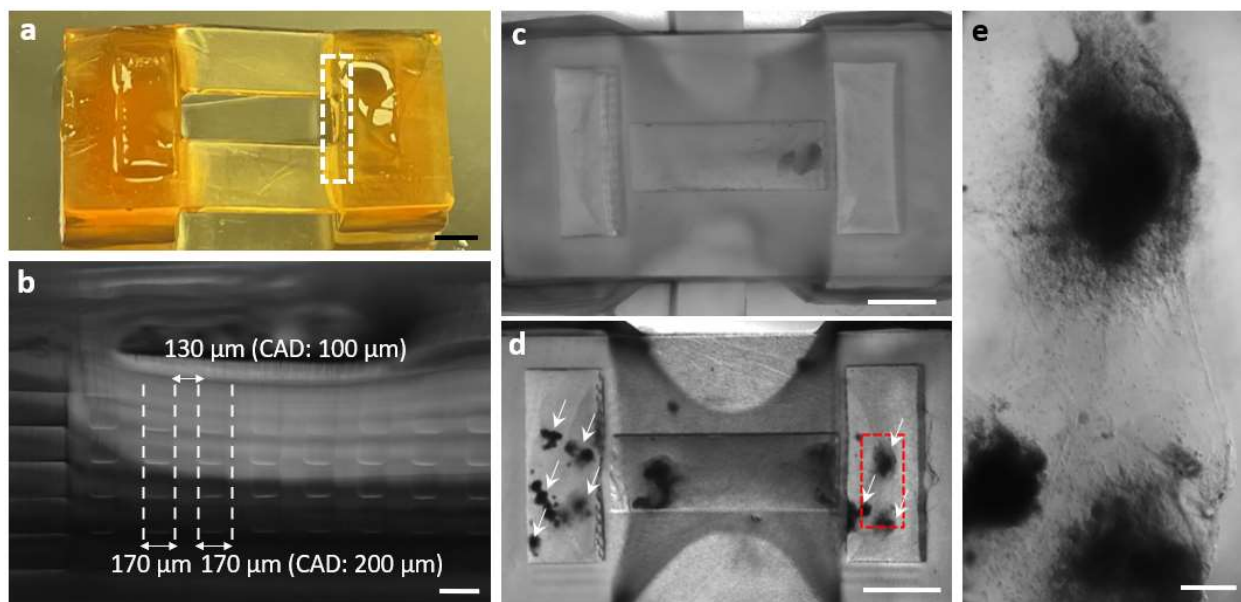


Figure 5.3. Images of neuron-muscle co-culture on NMJ skeleton. (a) Photographic image of 3D printed skeleton. Optical image of (b) pillars on the boundary of central and neural parts (side view of the white dotted square in (a)), (c) after muscle compaction and before seeding mEBs, (d) after seeding mEBs inside the neural parts, and (e) enlarged view of mEBs inside the neural part (red dotted square in (d)). White arrows in (d) indicate mEBs. Scale bars: 2 mm in (a), (c), and (d), 200 μm in (b) and (e).

5.4. References

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