Title: A fast, muscle-actuated biohybrid swimming robot

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Abstract: Integration of biological actuators and sensors with soft, synthetic scaffolds has allowed for the development of a class of microscale, emergent, and motile biohybrid robots, including small scale swimmers and walkers. Swimmers generated thrust from time irreversible dynamics of their flagella-like thin compliant tails. However, these swimmers were limited in their wider application by slow speed (0.7 micrometers per second, 0.014 body lengths/min, with *Re* ~ 10-3). Low Reynolds number (*Re*) hydrodynamic theory, appropriate for locomotion with negligible inertial effects, predicts that swimming speed can be increased by orders of magnitude by increasing angular actuation of the tails of these swimmers. This study investigates a novel design for a fast swimmer achieving speeds up to 86.8 micrometers per second, 0.58 body lengths/minute. Here, living muscle tissue applies force on the swimmer scaffold upon electrical actuation. The compliant mechanism transduces the contraction to twisting of the tails by up to 7º, resulting in high speeds. Muscle maturity was modulated through coculture with motor neurons and mechanical coupling to the compliant swimmer scaffold. Although the design was motivated by low Reynolds number theory, the swimmer achieves high speed benefiting from inertial effects with $Re \sim 10^{-1}$. Our study provides a simple design for generating high thrust using bio-actuators at small scale and a design for introducing neurons into biohybrid systems with 3D muscle tissues.

One-Sentence Summary: We present a high speed biohybrid swimmer which takes advantage of intermediate Reynolds number inertial effects.

Main Text:

INTRODUCTION

Biohybrid robots have emerged as a tool for basic science and for advancing technologies for fabrication and control (*1–5*). Several recently developed muscle actuated robots, ranging in size scale from 10^{-4} to 10^{-2} meters, have allowed for the basic study of locomotion and emergent behaviors (*6–8*). They include biomimetic swimmers resembling jellyfish (*9, 10*), manta rays (*11*), and flagellates (*12, 13*), crawling robots that use structural asymmetry to achieve unidirectional motion (*14–17*), a valveless pumping robot (*18*), and a variety of micromanipulators which function both in media (*19*) and outside of media (*20, 21*). They usually consist of cardiac or skeletal muscle

interfaced with a soft scaffold (silicone elastomers, hydrogels) and either operate autonomously or receive pacing through electrical or optogenetic stimulation.

Recent efforts focus on obtaining complex behaviors in these robots through the introduction of multiple cell types (*3*), including the introduction of motor neurons which can interface with skeletal muscle (*12, 22–25*). A flagellate-type swimmer consisting of two tails was introduced which was actuated by a skeletal muscle tissue innervated by motor neurons. Periodic muscle actuation translating the base of the tails, generating thrust through time-irreversible flagellar dynamics at Reynolds number (*Re*) on the order of 10^{-3} (swimming speeds of 0.7 µm/s or 0.04% body size/s). Their swimming was modeled using slender body elasto-hydrodynamics ignoring inertial effects (*12*). Model predictions matched closely with experimental observations.

Thrust can be generated by subjecting the base of the tails to both translation and rotation. Modelling elasto-hydrodynamics for low *Re* reveals a significant increase in thrust due to rotation at the base (*26*). The fixed end of the beam can be displaced or rotated periodically at an angular frequency ω. Nondimensionalization of the governing linear equations yields a characteristic parameter termed the Sperm number, given by $Sp = L / \left(\frac{EI}{\omega \zeta_{\perp}} \right)^{1/4}$ where L is the length of the beam, EI is its flexural rigidity, and $\zeta_{\perp}$ is the drag on the beam normal to its length per unit length (*27*). For a given set of boundary conditions, the maximum thrust occurs for an intermediate sperm number of around 2.1.

In the biohybrid flagellate-type swimmer (*12*), the tails of the swimmer undergo small amplitude displacement (~20 µm) and small amplitude rotation (~2º), subject to a muscle tissue contracting 4% of its overall length. Increasing the amplitude of displacement only increases the amplitude of the bending wave of the tail linearly, resulting in a linear increase in thrust. However, increasing the amplitude of rotation increases the amplitude of the bending wave proportional to the product of the rotation and the tail length, thereby significantly increasing the thrust. This swimmer converts the muscle contraction into tail actuation using a lever 570 µm long. With this mechanism, scaling the size of the swimmer would linearly increase the actuation amplitude of the muscle, the displacement, and the twist. Approximating the head of the swimmer as a sphere with equivalent projected forward cross-section, drag would increase linearly with size and would cancel out the effect of the increased displacement. However, increasing rotation amplitude would increase the speed overall.

The process by which muscle fibers mature, termed myofibrillogenesis, depends on a number of mechanical and chemical factors (*28*). *Mechanical factors*: During the maturation process, myoblast muscle precursor cells fuse into multinucleated nascent myotubes which stretch out and pull on the surrounding matrix. Based on in vivo study of drosophila abdominal muscles which resemble the cross-striated skeletal muscle of mammals, this pulling generates tension and is necessary for the formation of immature contractile units of actin and myosin (*29*). These immature units undergo spontaneous, calcium dependent twitching, which appears to be necessary for the organization of highly periodic cross-striated sarcomeres, the key force generating units in skeletal muscle. This implies that the mechanical stiffness of the swimmer scaffold that serves as an anchor for the muscle needs to be sufficient to allow the muscle tension to develop, and yet compliant enough to allow the muscle to twitch. *Chemical factors*: In vitro studies with the immortalized cell line of mouse myoblasts, C2C12, reveals that this maturation process requires a reduction in growth factors, which is achieved by reducing the amount of serum in the media (*30, 31*). A remarkable finding from in vitro studies of neuromuscular junction (NMJ) formation is that the spontaneous

twitching of C2C12 derived skeletal muscle increases in the presence of motor neurons (*22*, *32*). The presence of neurons in the vicinity of muscle culture enhances muscle maturation, contraction and force, although the underlying mechanism of their cross talk remains unclear. We consider both mechanical and chemical factors in the development of our fast, biohybrid flagellate swimmer.

RESULTS

Biohybrid swimmer design*:*

Design overview

Our novel swimmer is designed to convert muscle contraction into large amplitude rotation of a pair of thin tails to generate high amplitude bending waves and propulsion (Fig. 1). It is also designed to support muscle maturation through mechanical and chemical cues. The swimmer consists of a soft, polydimethylsiloxane (PDMS) scaffold, a ring of skeletal muscle made from C2C12 mouse myoblasts, and a cluster of mouse embryonic stem cell (mESC) derived motor neuron. The muscle tissue functions as the onboard actuator. The scaffold consists of (*1*) a head with a reservoir for neuron spheroids, (*2*) a compliant mechanism with anchors for muscle ring, and (*3*) two slender tails. An immature muscle ring is mounted on the anchors. The ring matures with time while it receives mechanical cues from the compliant mechanism and chemical cues from the nearby neurons on the head. Upon actuation, the muscle contracts, pulling the anchors towards each other, resulting in translation and rotation of the base of the tails. The dynamics of the tails generate net time-averaged thrust ($\langle F_{prop} \rangle$) for the swimmer. For high thrust, we need large amplitude muscle contraction which requires a strong, mature muscle. The compliant mechanism offers appropriate mechanical stiffness cue for optimal muscle maturation and yet sufficient compliance to allow for maximal angular rotation of the tails. The wide head of the swimmer increases drag against swimming but also supports a long muscle strip and minimizes the hydrodynamic interactions between the tails by increasing their separation distance. At low Reynolds number, the time averaged velocity of the swimmer, denoted by ($\langle v \rangle$, is the time average of the thrust divided by the total drag on the untethered system γ, given by $\langle v \rangle = \langle F_{prop} \rangle / \gamma$. We approximate the drag on the head (γ_{head}) to be that of a sphere at Stoke's flow with the same projected area in the direction of swimming.

At low *Re*, thrust generated by a thin filament comes from the asymmetry between the drag per unit length tangent to the filament ($\zeta_{\parallel}$) and perpendicular to the filament ($\zeta_{\perp}$), such that $F_{prop} \propto (\zeta_{\perp} - \zeta_{\parallel})$ (*27*). In the limit of the length of the filament to the radius of the filament $L/r \to \infty$, $\zeta_{\perp} = 2\zeta_{\parallel}$ (*33*). In our design, where $L/r \approx 90$, $\zeta_{\perp} = 1.6\zeta_{\parallel}$ (*26*). When the tails are oriented along the direction of swimming, they contribute to the drag of the overall system $\gamma_{tails} = 2L\zeta_{\parallel}$. The overall drag of the system is $\gamma = \gamma_{head} + \gamma_{tails}$.

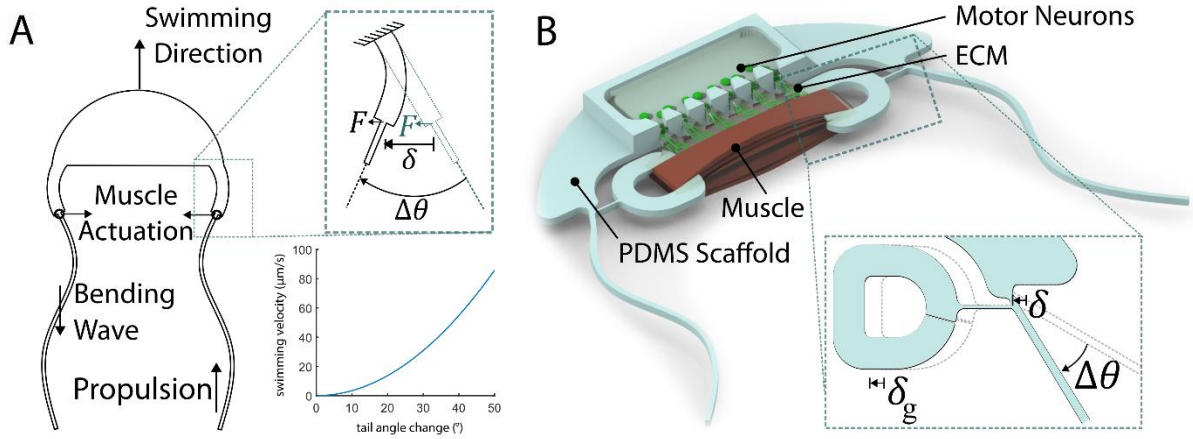

Fig. 1. Design for the a fast biohybrid swimmer. (**A**) Conceptual design highlighting the role of large angular rotation of the tails for generating large amplitude bending waves and propulsion. (**B**) Rendering of the biohybrid implementation of the conceptual design.

Muscle tissue design

To accommodate manual mounting of muscle ring on the swimmer grips, the inner diameter of the ring is set at 3 mm for grips 4 mm apart and a scaffold thickness of 150 µm. This allows for some slack between the ring and the grip. After mounting, the muscle ring continues to compact, pulling the grips towards one another as it matures.

For increasing muscle contractility, we recall a previous study that found optimal contractility in muscles anchored between two pillars with an overall stiffness of 2 µN/µm (*22*). The tissues measured around 500 µm in length with a cross-sectional area around 300 µm^2. We hypothesize that this optimal stiffness might scale with the geometry of the tissue. One way to do this is to keep the ratio of the tensile stress σ and tensile strain ϵ constant between tissue culture platforms. Let L_0 be the free distance between the pillars, and that during maturation the muscle contracts by ΔL. Also suppose that the pillars have a linear stiffness k so that the passive tension in the muscle is $k\Delta L$. The ratio of the tensile stress $\sigma = k\Delta L / A_c$ to the tensile strain $\epsilon = \Delta L / L_0$ is then kL_0 / A_c where A_c is the cross-sectional area of the tissue. Keeping this constant with respect to the previous study gives a stiffness of 14 µN/µm per grip for the current swimmer scaffold.

Swimmer tails

The swimmer tails attach to the head of the swimmer via a compliant mechanism which translates the muscle contraction into rotation. The two tails cancel out each other's thrust normal to the direction of swimming. Like the scaffold head, the tails contribute drag to the system, which at low *Re* can be estimated with resistive force theory (*33*). In the limit $Re \to 0$, and for an arbitrarily slender tail, there exists a difference between the drag per unit length along the tail $\zeta_{\parallel}$ and perpendicular to the tail $\zeta_{\perp}$. This difference is referred to as drag asymmetry, and it is necessary for the generation of net propulsion at low *Re* (*27*). Because $\zeta_{\parallel}$ is less than $\zeta_{\perp}$, the tails contribute the least drag to the overall system when their resting configuration orients along the direction of swimming. During development, the muscle generates tonic tension, pulling the tails towards the middle of the swimmer. To compensate for this, the tails are initially oriented 60º away from the muscle. For a 4 mm long muscle which shortens by *4*% of its length during electrical stimulation (*12*), the absolute displacement for each grip (anchoring the muscle) would be roughly 80 µm. This displacement of the grips δ_g is converted by the compliant mechanism into displacement δ and

rotation $\Delta\theta$ of the tails (Fig. 1B). For fixed actuation amplitudes and tail cross section, the propulsion generated by the tails is fully determined by their sperm number, *Sp*, which varies with length, actuation frequency, and flexural rigidity. For low Re swimmers, propulsion is negligible for $Sp < 1$ and is at a maximum around $Sp = 2.1$ (*27*). The present swimmer is designed with tails with $Sp = 1.4$ during 5 Hz actuation.

Motor neurons contribution to muscle performance

Evidence has been emerging that in vitro muscle matures better in coculture with motor neurons, potentially due to interaction mediated by mutual chemical cues. Neurons extend neurites towards the muscle, some of which may form neuro-muscular junctions. Muscle maturation is facilitated by spontaneous twitching and tension for the formation of cross-striated sarcomeres. Muscle development from myoblasts requires fusion of myoblast into multinucleated nascent myotubes, followed by the development of those myotubes into immature myofibrils with the formation of linear, loosely organized actin-myosin structures. A study of the abdominal muscles of drosophila, which resemble the cross-striated skeletal muscle in mammals, found that tension within nascent myotubes precedes and is required for their development into immature myofibrils. These immature myofibrils exhibit calcium dependent twitching, and this twitching appears necessary for the self-assembly of organized, cross-striated sarcomeres (*29*). Studies have observed increased spontaneous twitching in skeletal muscle in the presence of motor neurons. Aydin et al. (2020) developed a platform for the fabrication of C2C12 derived muscle tissues in the presence of mESC derived motor neurons. They observed preferential outgrowth of neurites towards the muscle, higher spontaneous contractility and a 60% higher ratio of cross-striated myofibers to immature myotubes in co-cultured samples. Andersen et al. (2020), developed a system for culturing cortical, spinal, and muscle organoids together. Several times more spontaneous twitching events were observed in the muscle in the presence of the spinal organoid, although the variability was high sample to sample. Transmission electron microscopy images of the multi-organoid samples show highly organized, cross-striated sarcomeres, which is evidence of maturity.

To improve maturity of the swimmer muscle through neuronal signals, we dispense mouse stem cell derived motor neurons on a PDMS holder affixed to the head of the swimmer. The holder also increases the rigidity of the head against bending and buckling out of plane. We fill the holder with mouse embryonic stem cell (mESC) derived motor neuron spheroids.

The holder keeps the motor neurons around 0.5 - 1 mm away from the muscle tissue to avoid any contact between neuron and muscle cell bodies. Studies have shown that spheroids of motor neurons in contact with skeletal muscle tissue form localized, functional neuromuscular junctions (*23, 34*), resulting in weak, localized muscle activity. In contrast, neurons positioned a minimum of 200 µm from the muscle formed NMJs and exhibited robust tissue-wide contraction (*22, 35*). In our design, extracellular matrix is used to bridge the gap between the motor neuron spheroids and the muscle. Neurites extend through the ECM bridges towards the muscle while the holder prevents the neuron spheroids from slipping onto the muscle during or after placement (Fig. 1B). When ECM is deposited around a muscle tissue, we observe that the muscle tissue pulls the ECM towards itself over several days. In addition, when motor neurons innervate muscle, they build up tension in their axons (*36*). Without mechanical anchorage, this force might pull the neurons into the muscle should innervation occur. Note that the goal here is not to rely on NMJ formation and neural stimulation of the muscle tissue. Rather, we intend to enhance muscle maturation with biochemical cues from nearby neurons.

Implementation and characterization:

Scaffold fabrication and calibration

The scaffold is molded in a PTFE coated microfabricated silicon mold. The neuron spheroid holder is cast in a separate SLA printed resin mold and is attached to the scaffold with liquid PDMS. For the purpose of analyzing its deformation response, the swimmer scaffold has been sub-divided into four components: the grip, the coupler, the beam, and the head (Fig. 2A). Stiffness of the anchors (mechanical cue to the muscle) is measured by pulling the grip of the swimmer with a tungsten wire with known spring constant (0.43 µm/s, Fig. 2B). Displacement of the grip during this pull (i.e., compliance of the grip) is contributed by the deformations of the head (x_1), bending of the small segment of the tail connecting the head and the coupler (x_2), the stretch of the coupler (x_3), and deformation of the grip itself (x_4). Note that the bending of the tail near the base gives rise to the desired rotation which generates thrust (Fig. 2C). Initially, a sacrificial support attaches the grips to the head of the swimmer, resulting in an overall stiffness of 1.83 µN/um per side, behaving linearly up to 400 µN. When the sacrificial support is removed, the muscle exhibits nonlinear softening, with a stiffness of 1.75 µN/µm per side at small deformations, reducing to 0.97 µN/µm at 400 µN (Fig. 2D). This means that the muscle is subjected to higher mechanical stiffness during maturation, but lower stiffness after maturation when it is stimulated to induce swimming. The effect of such change in stiffness during muscle maturation has never been explored before. We discuss this in detail in a following section. The rate at which the tail rotates with respect to the grip exhibits slight nonlinearity, beginning at around 0.09º/µm and decreasing to 0.05º/µm at an x_4 displacement of 400 µm (Fig. 2E). This drop in the rotation rate works against the expected swimmer performance.

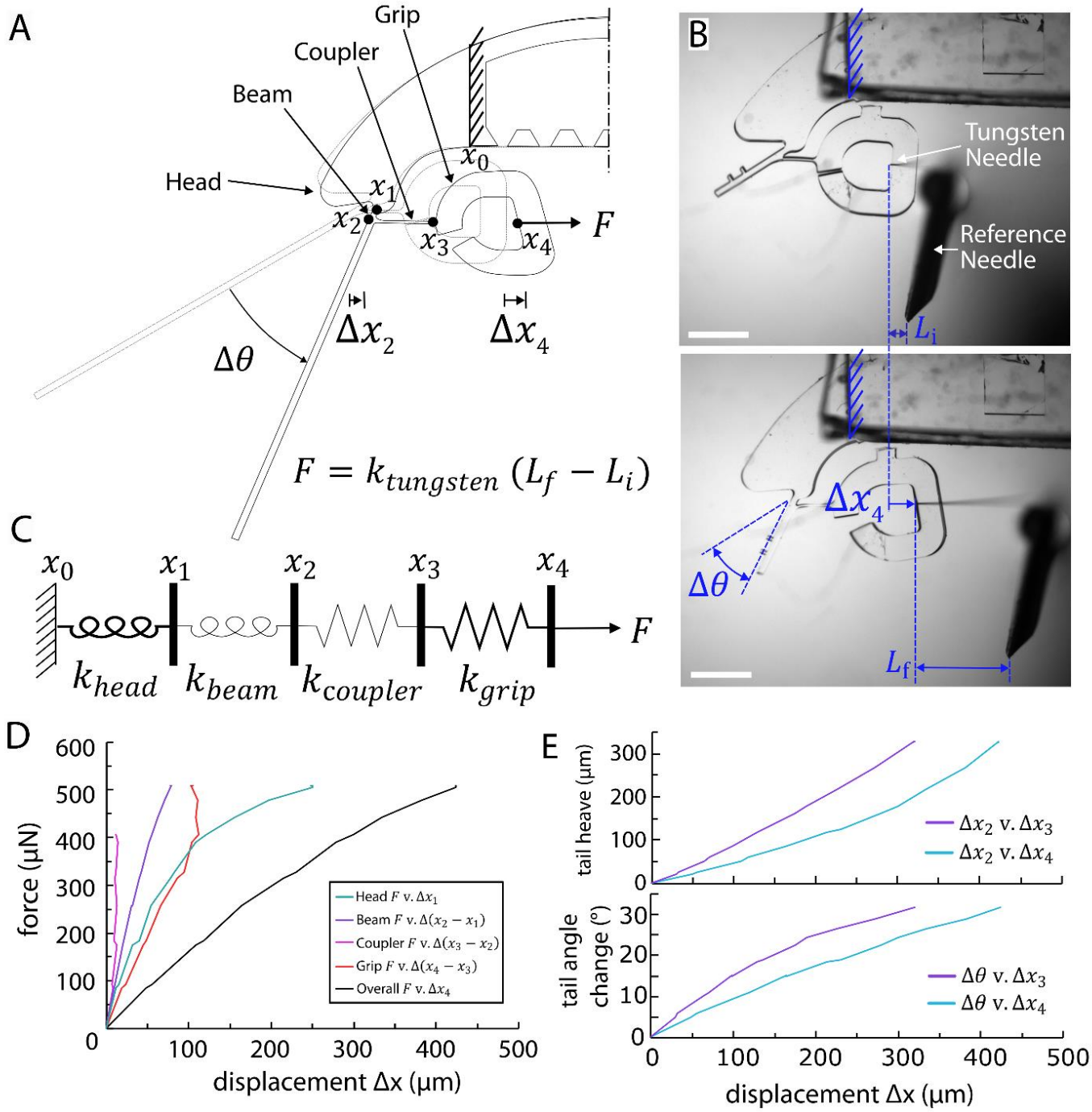

Fig. 2. Calibration of the PDMS swimmer scaffold. (**A**) Schematic of the swimmer scaffold decomposing the overall system into four compliant components. (**B**) Diagram of the setup for calibrating the PDMS scaffold with respect to the stiffness of a tungsten needle (0.43 μN/μm) with a hypodermic needle acting as a reference. (**C**) Diagram of the compliant components of the scaffold. The head and the beam contribute to the rotation of the tail. (**D**) Force-displacement relationships for the components of the scaffold. (E) Tail angle-displacement relationship which applies to contraction of the muscle.

Timeline for biological component assembly

The timeline of the fabrication and assembly of the biological components of the swimmer appears in Fig. 3. The muscle ring fabrication and mounting process (Fig. 3C) are shown in Supplementary Video 1. During mounting, sacrificial supports attach the grips to the head of the swimmer. They offer rigidity against grip displacement and facilitate mounting of the muscle. In order to initiate

C2C12 myoblast fusion into nascent myotubes, the concentration of serum in the media is reduced, following the protocol in Olson (1992).

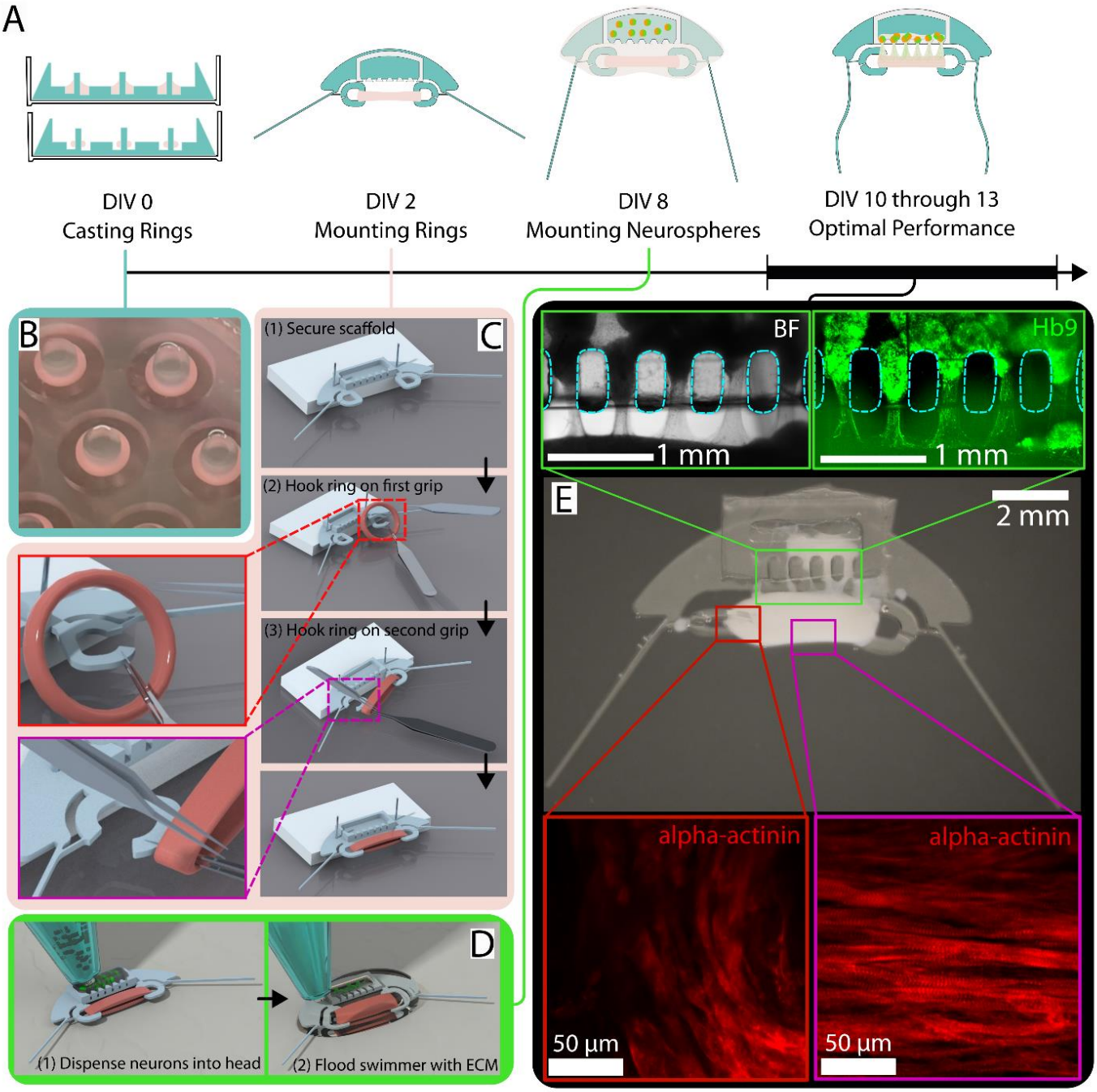

Fig. 3. Timeline for the fabrication and assembly of the biological components of the swimmer. (**A**) Overall timeline for the biological components. Some preparation steps occur beforehand, including culturing of C2C12 myoblasts and differentiation of mouse embryonic stem cells into motor neuron spheroids (see Materials and Methods). (**B**) Ring molds used for casting the muscle tissue rings. (**C**) Renderings of the steps involved in mounting the muscle rings on the scaffold. Callouts show examples for precise tweezer placement necessary to put the muscle into the grips without causing damage. (**D**) Renderings of the steps involved in mounting the motor neuron spheroids in the head of the swimmer. A hydrophobic parafilm substrate keeps the ECM from flowing away. (**E**) Photograph of the swimmer with

callouts showing the live imaging of the growth of neurites towards the muscle and confocal images of α-actinin assembled into sarcomeres localized in the middle of the muscle tissue taken after fixing. The photograph of the swimmer was post-processed by darkening the background around the end of the PDMS scaffold.

Role of temporal variation of stiffness on muscle development

Although the circumference of the muscle is long enough to wrap around the anchors without deforming the scaffold, in practice, the tissue possesses some rigidity and resists being stretched out. It exerts around 100 µN of force on the anchors on immediately after mounting. On DIV 2, the swimmers are placed in low serum (2% HS) differentiation media. Over the following two weeks, the muscles continue to compact, developing tonic tension against the anchors. In the presence of the sacrificial supports (Fig. 5A) when the compliance of the head, the tail and the coupler are turned off, the muscle is subjected to a stiffness of 1.9 µN/µm per grip (Fig. 4A, 4B). After the supports are cut, the stiffness drops. The amount it drops depends on the force in the muscle. On DIV 3, cutting the supports drops the stiffness to 1.5 µN/um per grip. On DIV 6 or 9, the stiffness drops to 0.8 µN/um per grip. We wanted to know if the time history of the stiffness felt by the muscle would have a long-term effect on the rate and extent of the build-up in tension in the muscles. To test this, three groups of swimmers were prepared with muscle rings (no neurons). The sacrificial supports were cut 1, 4, and 7 days after starting differentiation media (DIV 3, 6, 9). The length of the muscle is measured with respect to the points joining the grips and the couplers (x_3 to x_3) (Fig. 4C).

When the sacrificial supports are cut, the muscles shorten and their tonic force drops (Fig. 4D,E). The muscles rebuild this tension within a day (Fig. 4Ei,ii). Throughout the development process, the lengths and tonic force of the muscles in the different groups follow a similar course. Before DIV 5, the overall stiffness felt by the groups varies by less than 25%, which may contribute to this similarly. However, even on DIV 8, when the stiffness varies by 90% between the samples with the without the sacrificial supports, the tonic force in all the samples is insignificantly different. Except on DIV 4 and on DIV 9 after cutting the supports in the DIV 9 release group, the tonic force in all the samples is insignificantly different. The different anchor stiffnesses do not appear to affect the tonic force development, and the variation in the time history of the stiffness felt by each of the muscles does not appear to have left a lasting effect on tonic force or length of the muscles. This might be the result of a homeostatic state of tension that the muscle arrives at during maturation, independent of the support stiffness and corresponding force history. On DIV 11, when the muscles would be used for swimming, the average tension in all of the muscles was 498 ± 8.5 µN (n = 10, mean $\pm$ SEM).

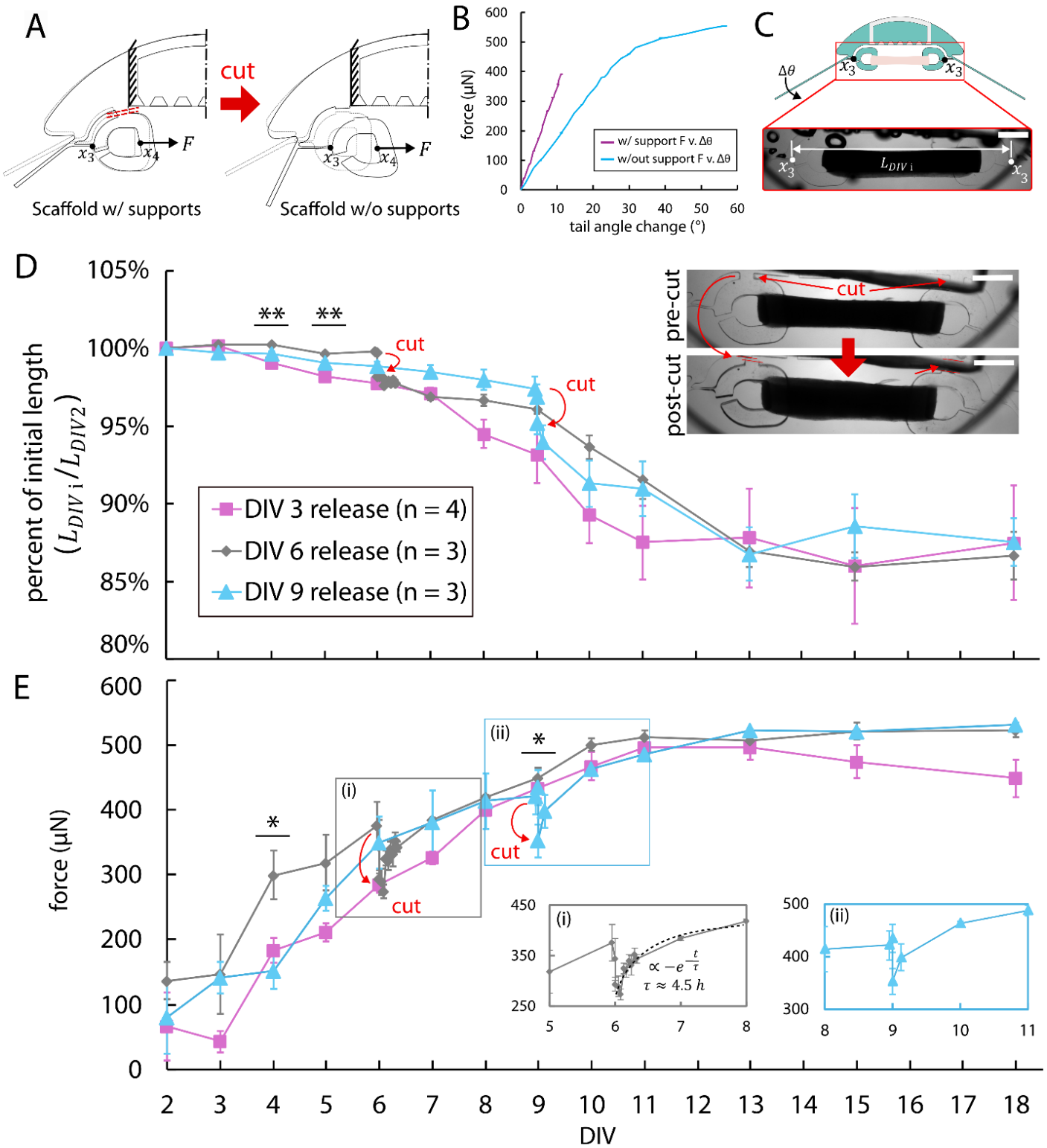

Fig. 4. Contraction and tonic force increase of the muscle during development. (A) Schematic showing the change in scaffold geometry following removal of the sacrificial supports. (B) Force-displacement curves showing the stiffness measured from x_3, the junction between the grip and the coupler before and after cutting the sacrificial supports. (C) Diagram defining how the contraction of the muscle is extracted from bright field images. (D,E) Tracking the variation in the contraction and tonic force of the muscles when their sacrificial supports are removed at DIV 3, 6, and 9. Statistics indicate the results of one-way ANOVA statistical tests comparing the percent of initial lengths of the muscle of the three groups (** = p-

value < 0.01, * = p-value < 0.05, n.s. = p-value > 0.05). For DIV 6 and 9, statistics reflect length measurements taken one hour after the removal of the sacrificial supports. By DIV 9, after cutting the sacrificial supports on the last group of samples, large variation among the muscle lengths contributes to the groups being indistinct from one another (p-value > 0.05 by one-way ANOVA). (Ei,ii) insets for change in force following release on DIV 6 and 9. (Ei) exponential curve fit gives an approximate time constant of 4.5 hours for recovery of the force after release.

Starting between DIV 6 and 8, the muscle rings tend to exhibit small amplitude, spontaneous twitching. This indicates that immature myofibrils or matured cross-striated myofibers have developed. To increase the amount of spontaneous twitching, motor neuron spheroids are added on DIV 8. To do this, the swimmers are placed on wax paper, excess media is aspirated, and the motor neuron spheroids are added to the holder on the swimmer's head (Fig. 3D). Next, the neurons and muscle are covered with ECM. This protects the muscle and neurons from drying out while the ECM polymerizes. When the swimmer is lifted away from the wax paper, some ECM stays attached to the narrow gaps between the muscle and the holder, while excess ECM sticks to the wax paper and tears off. Over the next two days, loose ECM gets pulled into the muscles by the C2C12 cells, and the ECM between the holder and the muscle forms a series of tense bridges. Neurites are seen to grow along these bridges towards the muscle (Fig. 3E). By DIV 8, swimmers with neurons tend to exhibit strong, spontaneous twitching on the order of $1.11 \pm 0.27\%$ (n = 9, mean $\pm$ SEM) the length of the muscle. This twitching rotates the tails of the swimmer enough to see with the naked eye (Supplementary Video 2). As a note, the serum concentration is the same for all samples after DIV 2 (2% HS), but the basal media varies between aneural and cocultured samples (see Methods and Materials).

Motor neurons increase spontaneous and evoked muscle contraction

By DIV 10, swimmers with and without neurons tend to reach their peak dynamic contractility in response to electrical stimulation. Muscle contractility is measured as a percent change in the muscle's resting length (Fig. 5A). The amplitude of spontaneous and evoked twitching is compared between swimmers with and without neurons (Fig. 5B). On DIV 10, only 4 of 11 swimmers without neurons exhibit spontaneous twitching, as compared to all 9 of the swimmers with neurons. During electrical stimulation, the swimmers with neurons contract 4.2 times more than the swimmers without neurons on average. Contractility is observed to increase with increasing voltage up to 5 V/cm (Fig. 5C). However, voltages above 3V/cm are sometimes observed to excite prolonged, spontaneous twitching, which hampers our ability to turn off the movement of the swimmer. Contractility at low frequency is around 2.8% of the muscle length (Fig. 5D). At stimulation frequencies above 4 Hz, the muscle is unable to fully relax before the next pulse, resulting in tonic contraction and decreased actuation amplitude.

We used motor neurons expressing ChR2, a light sensitive ion channel which opens in the presence of blue light (465 nm) and causes the neurons to depolarize (*37, 38*). At DIV 10, the swimmer samples with neurons were subjected to blue light using the setup from Aydin et al. (2019). In previous studies, stimulation of optogenetic motor neurons has been able to cause global depolarization of an associated muscle tissue (*12*). In those cases, this may have been due to widespread innervation of the muscle by the neurons or due to gap junction (found in immature skeletal muscle) between the myofibers capable to transmitting the wave of depolarization (*39, 40*). In our samples, there were no significant changes in the spontaneous twitching pattern of the muscles during or after optical stimulation of the neurons nor in the amplitude of contraction of the muscle. We take this to suggest that any NMJs which might exist in the system are too few in number or too immature to cause the global depolarization of the tissue observed in other studies. It is worth noting that the neurons only contacted a small area on one side of the 0.8 mm^2 thick

muscle tissues. The total volume of neurons measures on the order of 1 mm^3 as compared to the volume of the muscle which is around 4 mm^3. It is unknown whether the neurites would have been able to penetrate into the interior of these muscle tissues.

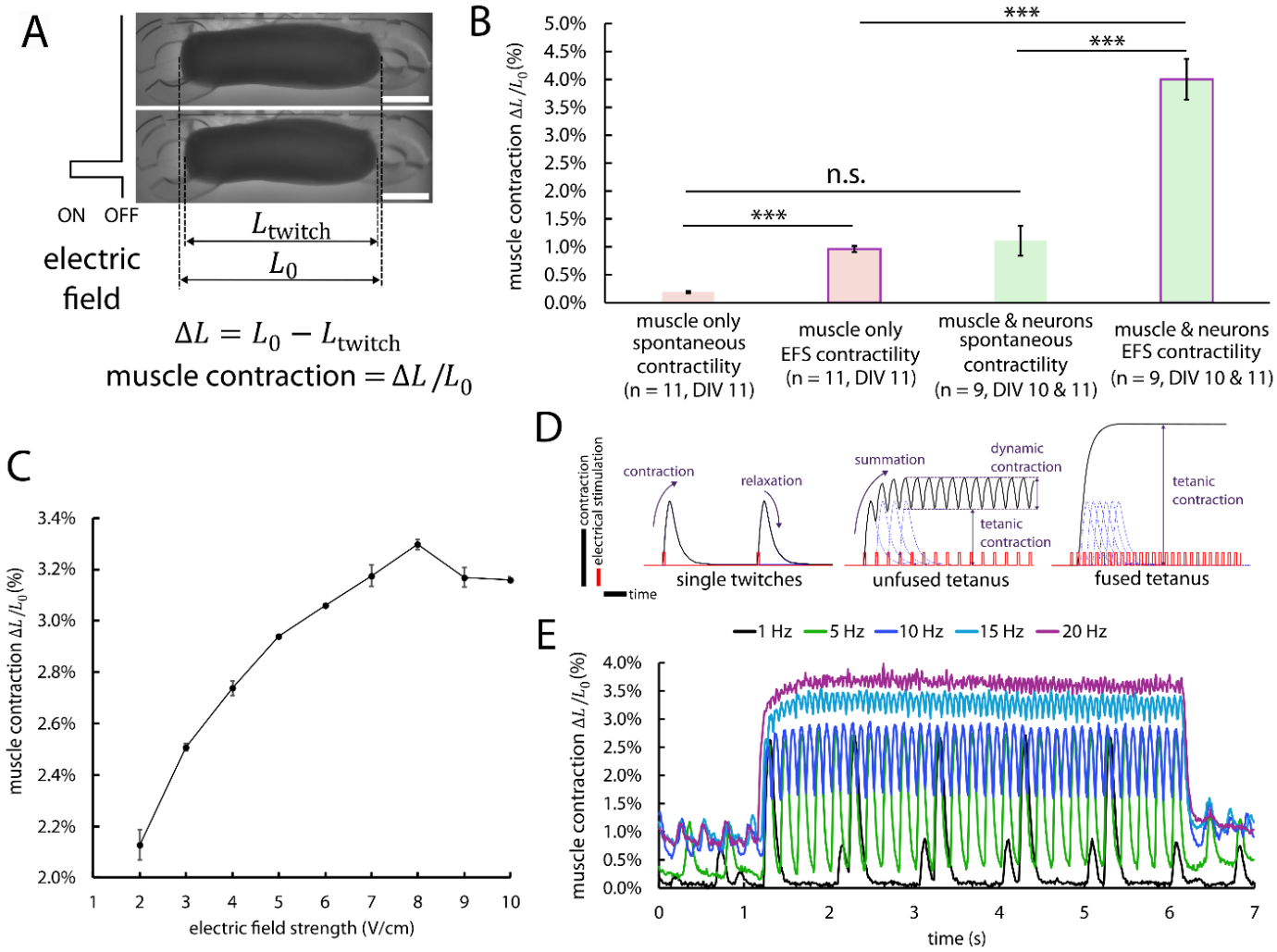

Fig. 5. Characterization of the spontaneous and evoked contractility of the muscle tissues. (A) Diagram for how the muscle contractility is measured with respect to the resting length of the muscle. (B) Comparison of the spontaneous and evoked contractility of swimmers with and without the inclusion of motor neurons. Statistics refer to t-tests assuming independent samples (*** = p-value < 0.001, n.s. = p-value > 0.05, error-bars indicate SEM). (C) Variation in contractility with increasing electrical field strength for a typical swimmer. Values refer to the mean of the contraction peaks observed when the muscle is stimulated for 4 pulses at 1 Hz for each voltage (error-bars indicate SEM of the peak values). (D) Diagram of nomenclature used to describe muscle contraction. Muscle contraction follows membrane depolarization and calcium influx. And because the refractory period for the depolarization of muscle is 2-3 ms (*41*), the contractions of a muscle in response to membrane depolarizations can overlap. At low frequency (≤ 5 Hz), a muscle can relax between depolarizations, leading to single isolated twitches. At higher frequencies, the muscle cannot fully relax between depolarizations, leading to a constant shortening referred to as tetanic contraction. The muscle is said to exhibit unfused tetanus. At very high stimulation frequencies (> 20 Hz), the muscle contraction appears smooth and sustained, and the muscle is said to exhibit fused tetanus. (E) Variation in the muscle dynamics with increasing frequency for a typical swimmer. Muscle is subjected to electrical stimulation at each frequency in ascending order, at a field strength of 3V/cm, and with 10 second

rest between stimulations. Low amplitude spontaneous twitches (~1%) can be seen interspersed with the evoked spikes. At higher frequencies, the muscle is unable to fully relax between stimulation frequencies and exhibits spontaneous twitching at increased frequency with comparable maximum contraction amplitude (~1%). High frequency and high field strength both tend to excite spontaneous twitching, which may indicate the dysregulation of the calcium cycling in the muscle fibers.

Swimming experiments:

Analyzing swimming velocities

Swimmers exhibiting strong contractility (>2%) to stimulation at 3 V/cm are placed in a dish containing a mixture of coculture media and Percoll (P1644, Sigma Aldritch). The Percoll induces a density gradient, allowing the swimmer, slightly denser than water (1.06 g/mL), to float at a desired height in the media (above the floor and below the air-media interface) due to buoyancy (*42*). Of twelve swimmers prepared following the timeline in Figure 3, eleven exhibit a response to electrical stimulation, of which six exhibit forward swimming. Swimmer displacement is extracted from videos using an ImageJ template matching plugin (*43*) (Fig. 6A). Points tracked on the body of the swimmer tend to oscillate with the muscle contractions, with the overall path of the swimmer moving along a straight trajectory (Fig 6B). The projection of the swimmer trajectory in the direction of travel is used to extract the velocity of the swimmer before and during electrical stimulation (Fig. 6C). Contraction of the muscle is calculated by tracking a point on the grip with respect to the head of the swimmer and then adding the displacement of the grip due to the tonic contraction of the muscle. At rest, the swimmers are observed to drift at speeds around 1-2 µm/s. This is attributed to convective currents in the media. In the example (Fig. 6B,C), the swimmer moves with a time averaged velocity of 63.6 µm/s, which corresponds to a *Re* of 0.33 (Supplementary Video 3). During each muscle twitch, the tails rotate an average of $4.8 \pm 0.04°$ with a peak rotational rate of $176 \pm 1.6° / s$ (n = 39, mean ± SEM).

At higher frequencies, the muscle undergoes tetanus as it is unable to relax between stimulation pulses. The onset of tetanus brings the tails together, giving the swimmer a sudden high velocity kick. Similarly, when stimulation ends, the release of tetanus causes allows the tails to move apart, causing a high velocity kick backwards. These transients are excluded from the calculation of time averaged swimming velocity.

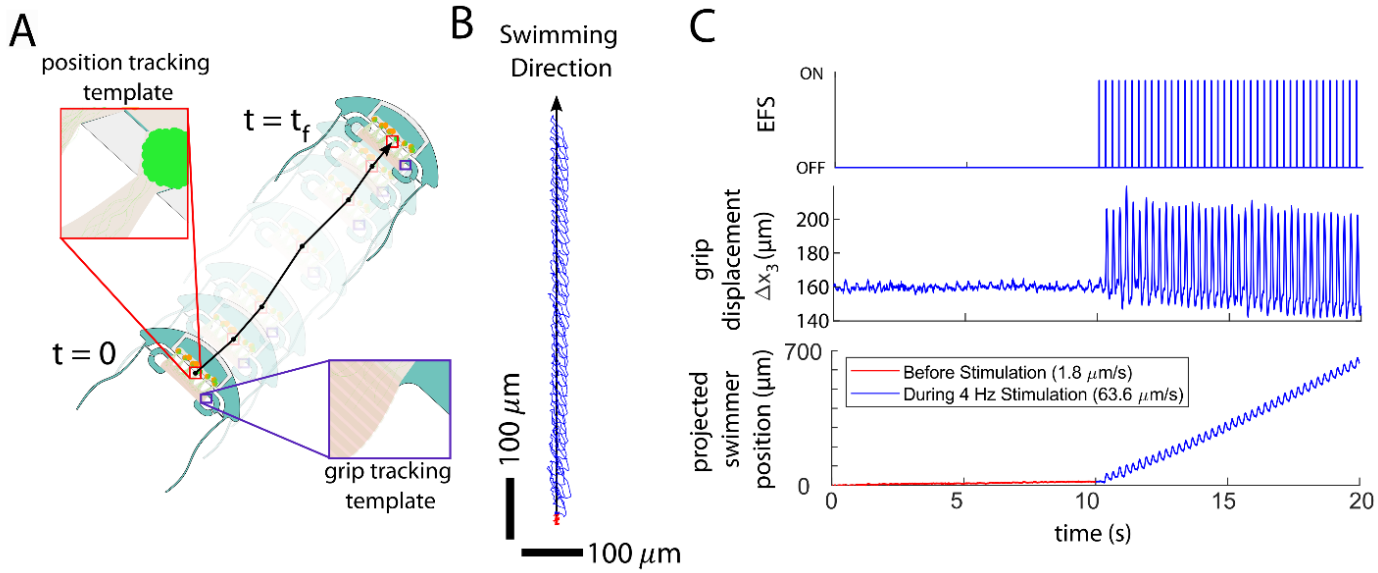

Fig. 6. Swimming position tracking and analysis. (A) Diagram of the template tracking method employed to extract the swimmers position and muscle contraction waveforms. (B) 2D projection of the path of a point on the head of the swimmer before (red) and during (blue) 4Hz electrical stimulation. (C) The corresponding stimulation, grip displacement, and position of the swimmer during the 4 Hz electrical stimulation. The position is taken by projecting the swimmer's path onto the direction of travel. Before stimulation, the swimmer is drifting with a small velocity. This is attributed to convective currents in the dish.

Persistent motion of the swimmer

We varied actuation frequency (0.5 to 20 Hz) and measured swimmer dynamics (Fig. 7A-D) (Supplementary Video 4). At frequencies 5 Hz and below, where the muscle experiences minimal tetanic contraction, the swimmer appears to reach a steady time-average velocity within a few cycles. And once the stimulation ends, the swimmer appears to continue moving forward while decelerating (Fig. 7E*i*). At frequencies ≥ 8 Hz, the initial onset of unfused tetanus causes the swimmer to move forward rapidly at a speed of 178 ± 16 µm/s (n = 30, mean $\pm$ SEM). Over 1-2 seconds, the swimmer appears to decelerate towards a steady, non-zero time averaged velocity, which we attribute to the small amplitude dynamic contractions of the muscle (Fig. 7E*ii*). Once stimulation stops, the muscle relaxes back to its baseline causing the swimmer to move backwards at a speed of 159 ± 13 µm/s (n = 30, mean $\pm$ SEM) before decelerating (Fig.7E*iii*). Across all samples during 0.5 Hz stimulation, the muscle length during contractions changes at a peak rate of 1.54 ± 0.10 mm/s and relaxes slower, with a peak rate of 1.31 ± 0.14 mm/s (n = 18, mean $\pm$ SEM). This may account for some of the difference between the forward and backward velocities of the swimmer due to tetanus.

We investigated the source of the swimmer's deceleration with a model of ballistic motion which only accounts for the drag force. Suppose the swimmer has mass m, and experiences drag proportional to its velocity with a constant drag coefficient γ. And suppose that the swimmer has some initial velocity v_0 and position x_0 at time t_0. Let $x(t)$ be the position of the swimmer over time in the lab reference where the fluid is drifting with some small velocity v_{drift}. From the force balance, $m\ddot{x} = -\gamma(\dot{x} - v_{drift})$. Solving the initial value problem gives $x(t) = v_0 \frac{m}{\gamma} e^{-\frac{t-t_0}{m/\gamma}} + v_{drift}(t - t_0) + x_0$. The characteristic time constant τ for the deceleration is mass divided drag m/γ. We fit experimentally measured $x(t)$ of the swimmer to the above equation for the following 3 cases: (i) soon (1 s) after low frequency (0.5 – 5 Hz) stimulation is stopped, (ii) during high frequency (8 – 20 Hz) stimulation when muscle reaches tetanus state with low amplitude contraction, and (iii) soon (1 s) after high frequency stimulation is turned off. The delay of 1 s is to avoid any transient dynamics soon after applying or removing stimulation. All the data in Fig. 7 comes from a single swimmer with a wet mass of approximately 27 mg. Based on the speed of the swimmer at the start of the experiment, the drift velocity v_{drift} is approximately 1.76 µm/s. Each curve fit for each case yields a slightly different value for the time constant τ, with an average value of 2.8 seconds. Using τ = 2.8s, we repeat the fitting to calculate values for v_0 and x_0. Using these values for v_0 and x_0, each of the curves can be nondimensionalized to isolate the first-order dynamics (Fig. 7E).

The velocities seen for this example swimmer range from 10 µm/s to 80 µm/s, which corresponds with $Re \sim O(10^{-1})$. The value of *Re* can be thought of in terms of a ratio of the contribution of inertial forces and viscous forces in the hydrodynamics of a body. At these intermediate values (*Re*

~ 1), we would expect both the inertial and viscous forces to be present in the propulsion generating mechanism of the swimmer. Whereas viscous forces contribute to propulsion through drag asymmetry of the tails, inertial forces would contribute by imparting momentum to the surrounding fluid. This regime of *Re* is not considered for flagellate swimmers (*12, 27*).

For a wet mass of 27 mg, the average time constant of 2.8 s corresponds to a linear drag coefficient of 9.6 mg/s. This is lower than the predicted drag for the two tails, γ_{tails} = 21.6 mg/s. This is significantly lower than our full prediction of the drag of the swimmer that includes the head drag, γ = 42.6 mg/s (for a projected forward cross-sectional area of 3 mm^2). In the following section, a low *Re* model of the swimmer dynamics is used to produce a digital twin of our experiments, taking the experimental muscle waveforms as the inputs and producing predictions for the swimming velocity. This calculation assumes that the swimmer comes to rest immediately once the propulsion force F_{prop} from the tails is removed, such that the velocity $v(t) = F_{prop} / \gamma$.

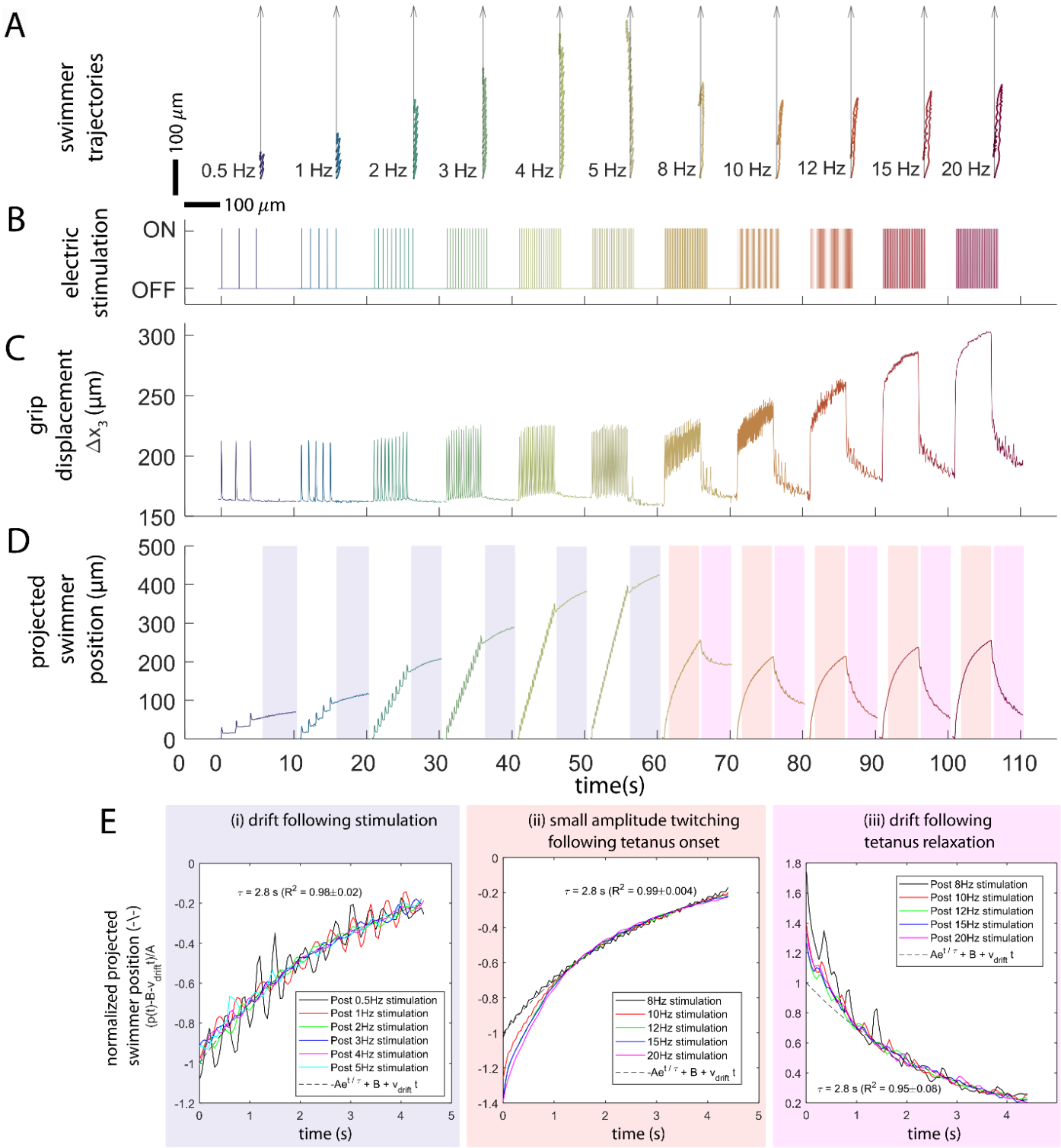

Fig. 7. Swimming waveforms for a high performing swimmer. (**A**) x-y coordinate plots for the swimming trajectory of the swimmer at multiple frequencies. (**B**) Electrical stimulation waveforms (3V/cm, 10 ms pulse width). (**C**) Muscle contraction measured from motion of x_3, the junction between the grip and the coupler. At frequencies above 5 Hz, the muscle experiences significant tetanic contraction. (**D**) swimmer position calculated by projecting the trajectory in the direction of swimming. At frequencies ≥ 8 Hz, the onset and relaxation of tetanus gives the position waveform a distinct sawtooth shape. (**E**) Nondimensionalization of the swimmer dynamics with time constant τ = 2.8s for each of the cases

considered. Background colors refer to time windows in (D). During these time windows, we hypothesize that the dominant force on the swimmer is drag. They include (i) when the muscle is almost motionless, so that the only force exerted on the swimmer is drag, (ii) when the initial propulsion from the onset of tetanus is large compared to the propulsion from subsequent dynamic contractions, and (iii) when the propulsion from tetanus relaxation is large compared to the subsequent, slower muscle relaxation.

Comparison between experimental swimming and low Re simulations

We plot the velocities of our six swimmers as a function of tail angle amplitude. Swimming velocities increase with increasing tail angle across aggregated data from all six swimmers (Fig. 8A). In the case of frequencies which induced tetanic contraction, the tail angle change corresponds with the dynamic contraction and excludes the tetanic contraction (Fig 5D). In some cases, the swimmer was observed to move backwards during stimulation. Across the frequencies tested, peak swimming velocity was observed at 5 Hz. In order to assess agreement with our low *Re* elasto-hydrodynamic model (*26*), swimmer velocities were calculated with drag coefficients $\gamma = 9.6$ mg/s and 42.6 mg/s, and experimental muscle contraction waveforms for frequencies in the range of 0.5 to 5 Hz (Fig. 7C) and for $\Delta\theta \in (0°, 8°]$ (see Methods and Materials). Neither drag coefficient yields velocity predictions above 40 µm/s. When considering a single swimmers performance at different velocities, we find model predictions are several times slower than experimental values (Fig. 8B). This suggests more thrust is being produced than predicted by low *Re* dynamics. This increased thrust and speed is likely contributed by inertial effects. The evidence for this comes from the deceleration of the swimmer from any arbitrary initial velocity with a single time constant in the absence of any actuation or external force.

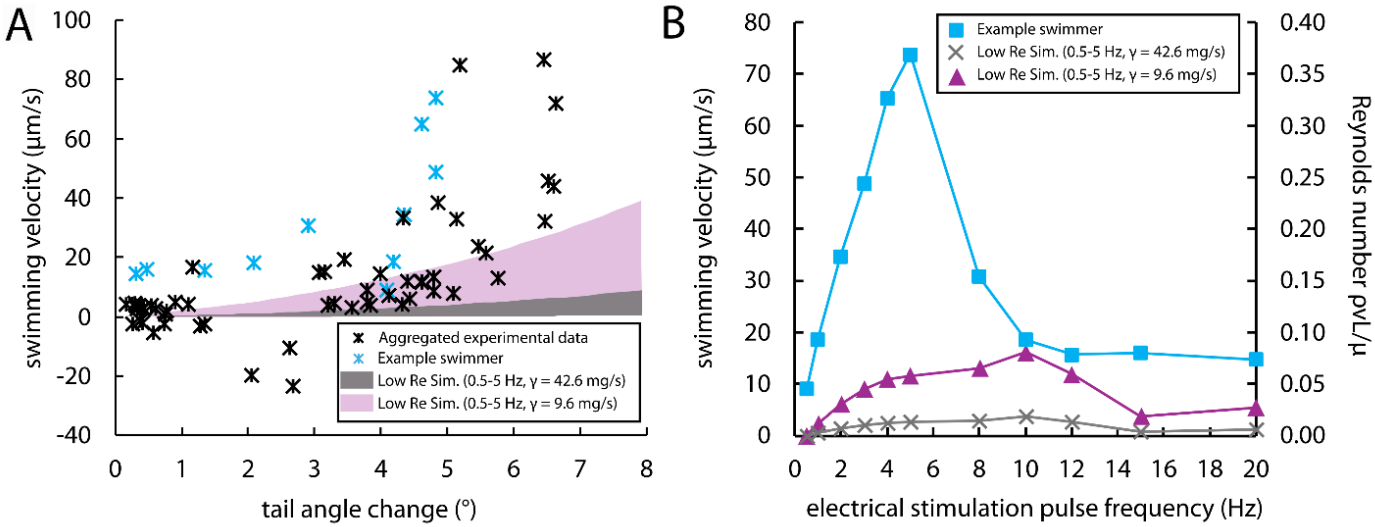

Fig. 8. Comparison between experimental swimmer velocities and predictions from our low *Re* model. (**A**) Aggregated swimming velocities and tail angle changes across all samples (n = 6) against regions corresponding to model predictions generated with the indicated frequencies, tail angle changes, and drag coefficients. Tail angle changes correspond with the dynamic contraction (Fig. 5D) of the input waveform, excluding tetanus contraction. Simulations are run using experimental muscle contractions waveforms as the model inputs. Inputs are scaled to achieve the associated tail angle change. (**B**) Comparison of experimental and predicted swimmer velocities at multiple stimulation frequencies for a single swimmer. The predicted swimming velocities are generated using the experimental muscle contraction for this swimmer as the model input.

DISCUSSION

In this study, we fabricate and test a muscle-powered biohybrid swimmer designed to swim at low *Re* using the non-reciprocal motion of a pair of tails to generate net-thrust. Our design focuses on supporting the maturation of a muscle through mechanical and chemical cues to provide a large displacement to a compliant mechanism which rotates the tails. When electrically stimulated, swimmers reach velocities two orders of magnitude higher than previous designs. When stimulation stops, the swimmers are observed to drift forward under their own inertia. Low *Re* elasto-hydrodynamic simulations made to mimic our experiments predict speed around four times slower than are observed. Taken together, our results suggest that inertial forces, expected at intermediate $Re \sim O(1)$, are playing a role in the dynamics generating thrust by our swimmer.

Adaptability of the muscle to attachment stiffness.

The tonic tension in the developing muscles grows at a similar pace irrespective of when the sacrificial supports are removed, with the only significant difference between the three groups being observed on DIVs 4 and 9 following the removal of the sacrificial support in the last group (Fig. 4E). We observed the tonic tension build-up in three groups of muscles over a 2-week period The muscle builds up tonic tension against a constant or softening stiffness. When the stiffness drops due to the removal of the sacrificial support, the muscle quickly contracts to reach the same level of tonic tension it had before the drop. We interpret this as the muscle adapting to its changing environment to reach a homeostatic tension. This tension appears to increase up to DIV 15 before leveling off. A comparable result was observed in Powell et al. (2002) with tissue-engineered human skeletal muscle. On the days between DIV 9 and 14, researchers applied a constant stretch to the muscle, increasing it by 5% every two days. And despite the perturbation, the tonic tension in the muscle stayed in the range of 400 to 600 μN. This is similar to the tonic tension found in our muscles, and by coincidence, our muscle rings and their tissues have similar cross-sectional areas of roughly 1 mm^2. In their case, applying a constant stretch has the effect of increasing the tonic force in the muscle, and the muscle is able to relax and re-establish its homeostatic tension. In our case, the muscles are able to rebuild their tonic tension after drops in tension and changes in stiffness. The drop itself is only 25-90% depending on the day of release, and in all cases, the stiffness less than 14% of the hypothesized optimal stiffness of 14 μN/um. We suspect that for a range of "soft" stiffnesses, the increase in tonic force of the muscle will follow a similar time-course which is robust to changes in the stiffness to other values within this range.

Robustness to changes in attachment stiffness within a range would allow for increased flexibility in the design of biohybrid machines. With the ability to adjust to perturbations in stiffness, muscle-powered machine can be fabricated with sacrificial supports that allow for the system to gradually reach a final configuration. Already, supporting structures are a common element in biohybrid system (*8, 18, 45*). Our results do not indicate how different stiffnesses will impact other aspects of the muscle aside from tonic force. A comparison between muscle development on soft and stiff pillars has been attempted before in the context of training in vitro tissue constructs with electrical stimulation (*46*). It was found that the benefits of training with electrical stimulation depended on the anchor stiffness. On a soft pillar, the training leads to increased contractility, but on a stiff pillar, training over multiple days had no improvement over stimulating the muscle for 1 hour on the last day of the test. So, although tonic forces may be comparable for different stiffnesses, the performance of the muscle and its response to other cues may vary with stiffness. In addition, it has been found that muscles cocultured with motor neurons exhibits hypertrophy from chronic electrical stimulation while muscle tissues by themselves do not (*47*). We would therefore suspect that the performance of skeletal muscle and its response to training will depend on anchor stiffness and whether it is cocultured with motor neurons. Such an investigation would benefit from granular

tunability of the spring stiffness and traditional measures of muscle maturity including mRNA markers (*47*), sarcomere numbers, and ECM composition (*48*).

Effects of motor neurons on muscle performance

Muscles grown in coculture with motor neurons demonstrate several-fold higher contractility compared to those grown by themselves (Fig. 5B). Although neurites are observed to grow to the muscle (Fig. 3E), the improvement to contractility occurs within two days of adding the neurons. This may be too soon for the formation of mature NMJs, which takes three to five days in vitro (*12, 49, 50*). Considering the asymmetry between the volume of neurons and the volume of the muscle (1:4), as well as restrictions on where the collagen bridge connects the neurons to the muscle in our swimmer design, we suspect that the improvement in muscle contractility is due to chemical cues from crosstalk between the cell types. In our samples, the neurites were able to sense and grow towards the muscles, which may have been due to their proximity and the presence of a physical bridge of ECM between them. Although functional improvements in muscle due to coculture with neurons has been observed many times, the coculture parameters necessary for this improvement are unclear, and could be studied with this platform by adjusting the proximity and intermediary between the muscle and neurons.

Inertia in swimmer propulsion

We observed persistent motion of the swimmer following stimulation, following the onset of tetanus, and following the release of tetanus. This is evidence of momentum which is not expected at ultra-low Reynolds numbers. Experimental velocities are 3-4 folds higher compared to predictions based on Stoke's flow (Fig. 8). This is not surprising since $Re \sim 10^{-1}$ found in our experiments is much higher than that usually considered in the Stoke's flow regime, for example, $Re \sim 10^{-3}$ for sperm dynamics (*51–53*). If inertial forces are contributing to the propulsion, this would mean that the tails of the swimmer are imparting momentum to the fluid. Because the density of the swimmer is almost identical to the density of the fluid, evidence of inertial drift of the swimmer (Fig. 7E) suggests inertial drift of the fluid.

These intermediate Reynolds numbers have been examined theoretically in the literature for some special cases (*54–57*). Their authors looked at the speed of a swimmer with respect to its flapping Reynolds number $Re_f = \rho_{body} A\omega L / \mu$, calculated from the frequency ω and amplitude A of actuation, and the body density ρ_{body}. Theoretical studies predict swimming speed proportional to Re_f^{α} for dense swimmers (body density $\rho_{body} >>$ fluid density $\rho_{fluid}, \alpha = 1$) and swimmers near asymmetric boundaries ($\alpha \in \{1/2, 1\}$). In both cases, the introduction of inertia to the propulsion is continuous with increasing *Re*. The applicable case to our swimmer is one with swimmer density similar to the surrounding fluid ($\rho_{body}\ \rho_{fluid}$), which has not been investigated theoretically for the general case (*56*). However, an analysis was performed for the specific case of a dimer swimmer by linearizing the Navier-Stoke's equations around $Re \sim 1$ (*58*)

A few biological swimmers have a similar size scale to that of our swimmer, including newborn tadpoles, however they operate at a higher *Re*, on the order of 10^2 to 10^4 (*59, 60*). A species of sea slug called *Clione antarctica* exhibits both non-reciprocal low *Re* and reciprocal high *Re* locomotion. Studies looking at the transition between the two swimming modes have found that inertial propulsion would arise discontinuously when the amplitude and frequency of the flapping of the slugs "wings" exceeded a threshold (*61*). They attributed it to overcoming a hydrodynamic instability. They computed this threshold with respect to the unsteady Reynolds number $Re_{\omega} = \rho_{body} \omega L^2 / \mu$, which is calculated based on the frequency of flapping ω and the length scale

of the body L. It is a measure of how quickly the sea slug flaps its wings. They found that the threshold lies between $Re_{\omega} \in [5, 20]$, which may be specific to their organism. A more appropriate parameter for our swimmer might be the velocity of the tails during stimulation, which is around 15.5 mm/s at the tip and depends mostly on the muscle contraction speed and less on the stimulation frequency. Future work could seek this threshold by using rigid swimmer tails and progressively shortening them between experiments.

Although our reasons for increasing the tail rotation come from low *Re* theory, there are benefits to intermediate *Re* swimming (Fig. 8A). Newborn tadpoles are able to reach velocities in the mm/s to cm/s range by undulating their bodies. With further improvements in our ability to generate large amplitude rotations using muscle tissues, it may be possible to design a system which achieves these speeds without increasing the size.

In summary, we demonstrate a novel flagellate-like biohybrid swimmer capable of reaching velocities two orders of magnitude greater than previous designs of similar size. The velocities also exceed predictions based on Stokesian flow (low $Re \sim 10^{-6}$). This is attributed to inertial forces contributing to thrust in our swimmers at $Re \sim 10^{-1}$. Higher thrust was achieved by (i) the novel design of the swimmer scaffold which transduces muscle contraction to the twisting and translation of the swimmer tails at their base. Time averaged swimming velocity increases rapidly with change in the tail angle during muscle contraction; and (ii) several-fold increase in muscle contractility in the presence of motor neurons which show growth towards the muscle. We found that C2C12-derived muscle adjusts its contractility or the tonic tension towards a homeostatic value, irrespective to the history of stiffness cues it experienced during maturation. This is a critical finding from the study that deserves further investigation to reveal how changing stiffness effects the response of tissue-engineered muscle to other supportive cues, such as electrical, chemical, and mechanical stimulation. Future iterations of the swimmer design may turn towards optimization algorithms involving Finite Element analysis to maximize tail angle change with respect to muscle contractions.

MATERIALS AND METHODS

Swimmer scaffold fabrication and calibration

The swimmer scaffold is molded from polydimethylsiloxane (PDMS, Dow Corning Sylgard 184, 10:1 base to crosslinker ratio) at 60ºC for two days using a silicon wafer mold for the head and tails (⟨100⟩ orientation, 500 µm thickness, undoped) and a resin mold for the neuron holder. The wafer is patterns with SPR220 and etched to a depth of 155 µm using deep reactive ion etching (DRIE) in an STS Pegasus. It is then coated with PTFE to allow for the demolding of PDMS. The resin mold is printed in from Clear V4 resin in a Form3 SLA 3D printer with a minimum feature size of around 200 µm. The two components are manually assembled with liquid PDMS and cured at 60ºC for two days. The assembled swimmer scaffold is autoclaved in distilled water to drive out monomers and then in air to sterilize. The force-displacement relationship at the grips of the scaffold is calibrated in water using a tungsten needle (Living Systems Instrumentation, Young's modulus 411 GPa, 0.43 µN/µm stiffness). During calibration, the scaffold is adhered to a dish with double sided tape at the edge of where the neuron holder sits.

Skeletal muscle ring fabrication and mounting

Rings of C2C12 mouse myoblasts are fabricated and matured following standard protocols (Z. Li et al., 2019; Mancini et al., 2011). Briefly, P4 C2C12 mouse myoblasts are thawed from cryogenic storage and resuspended in growth media (Gibco's DMEM, 1X GultaMAX, 1X Penicillin/Streptomycin, 10% FBS) and plated at a density of 20k cells/cm2 in a treated culture

flask. The growth media is changed every other day, and the cells are passaged once reaching 70% confluency.

Ring molds with inner diameter 3 mm are cast from PDMS using from a SLA printed resin negative. To prevent cell adhesion, the ring molds are incubated in a 1% Pluronic solution (Sigma-Aldrich Pluronic F-127, reconstituted from powder and filtered through 0.22 µm pores) at 20ºC overnight. Afterwards, the solution is removed, and the ring molds are rinsed 3 times with PBS.

A C2C12-ECM mixture is prepared containing 2.5M cells/mL, 2 mg/mL Type I rat tail collagen, and 2mg/mL Matrigel. The collagen is neutralized with a 7% sodium bicarbonate solution and 10X MEM. Rings are cast from 70µL of the mixture and cured at 37ºC for 30 minutes. The mold is then inundated in growth media. The rings are left to compact until DIV 2. The compaction ratio is around 10:1, resulting in rings with cross section 1 mm by 0.5 mm and an inner diameter of 3 mm.

On DIV 2, the rings are mounted on the biobot scaffold as shown in Figure 2. Briefly, the biobot scaffold is fixed to a block of PDMS using insect pins, and places under a stereoscope. The scaffold is inundated with growth media, and the ring is added to the dish. Using two pairs of tweezers, the grips of the scaffold are opened, and the ring is placed inside. Then, the media is changed to reduce the chance of infection. The rings are maintained in low serum differentiation media until DIV 18 or until motor neurons are added (Gibco's DMEM, 1X GultaMAX, 1X Penicillin/Streptomycin, 2% HS, 1X ITS). The muscles tend to start responding to electric stimulation on DIV 6 (monophasic, 10 ms pulsewidth, 3V/cm field strength).

The sacrificial supports connecting the grips to the head of the swimmer are removed using surgical scissors under a stereomicroscope. The scissors cutting into the PDMS causes the grips to twist. The muscle rings tend to survive this step because they can flex along with the grips. Attempts are making tissues with finger-like grips, like those found in (Emon et al., 2021), fail because the adhesion of ECM to PDMS is insufficient to tolerate this torsion.

Preparation and seeding of the motor neuron spheroids

The procedure for preparation of motor neuron spheroids from optogenetic mouse embryonic stem cells line ChR2H134R-HBG3 Hb9-GFP is described elsewhere (Aydin et al., 2019; Uzel et al., 2016b). The procedure for adding these spheroids to the swimmer is shown in Figure 2. The swimmers are removed from their mounting pedestals and placed on a parafilm sheet with a 100 µL droplet of media. Spheroids are mixed with the same reconstituted mixture of collagen and Matrigel as the muscle rings. The droplet of media is entirely removed from around the swimmer, and the spheroid-ECM mixture is injected into the neuron holder on the head of the swimmer. An additional 20 uL of ECM is dispensed around the head of the swimmer and the muscle to protect it from dehydration and to form a continuous bridge of ECM from the muscle to the neurospheres. The ECM is cured at 37ºC and 100% humidity for 30 minutes. Next, the swimmers are pinned back onto their mounting pedestals and inundated with coculture media (see recipe in Supplementary Information). Loose ECM dislodges, but the ECM surrounding the neurospheres and which has anchored itself in the narrow space between the muscle and the head of the swimmer tends to remain intact, forming a bridge. This ECM bridge frequently survives reinundation when the sacrificial supports are still intact.

Electric field stimulation and recordings

Typically, for voltages exceeding 1.5V, electrolysis will cause bubbles to form at the electrodes and the pH of the media to shift. However, even at 20V, a 10 ms pulsewidth is short enough that

bubbles do not form. This is important because bubble formation can induce currents in the media which push the swimmer.

The performance of the muscle is recorded (Olympus IX81, 2X lens, brightfield, 100fps) subject to electrical field stimulation (monophasic, 10 ms pulsewidth) delivered by a pair of graphite plates (20 mm by 20 mm by 3 mm) positioned on either side of the swimmer. A short pulsewidth is used to prevent electrolysis which changes the pH of the media and induces flow through the production of bubbles. To evaluate swimming performance, a swimmer is placed in a 100x15 mm polystyrene dish (Thermo Scientific, BioLite) containing 30 mL of coculture media and 10 mL of Percoll (Sigma-Aldritch). Using tweezers to break the surface tension, the swimmer is submerged. It is seen to float in the Percoll gradient around 5 mm above the bottom of the dish. The graphite electrodes are placed 4 cm apart to provide clearance for the swimmer. Recordings are taken at 20 or 100 fps, depending on the length of the experiment due to RAM limitations. Additional recordings are taken with a Canon Rebel Ti camera (29.97 fps), which allows for tracking the swimmer over a larger field of view.

Image processing and analysis

Tracking of the head of the swimmer is performed using the template matching plugin in ImageJ. Swimming displacement is quantified by projecting the motion of the swimmer onto its time-averaged swimming trajectory. For a given test, the trajectory may vary with time to account for rotation of the swimmer during the recordings. Once the overall motion of the swimmer has been subtracted, the displacements of the grips are tracked from a point on the grip near the coupler.

Low Re elasto-hydrodynamic model simulations

The full details of the elasto-hydrodynamic model simulated in this paper appear in Aydin et al., (2024), and the relevant MATLAB codes are available (https://github.com/wdrennan/Biohybrid-Swimmer-Digital-Assets). A second order accurate, implicit finite difference method is used for simulating the filament dynamics. The simulation takes the heave (x_2) and twist ($\Delta\theta$) of the tails as boundary conditions. These are g are calculated based on the grip movement at x_3 for the test shown in Fig. 7 using the calibrations shown in Fig. 2E. When simulating the swimming speed for a wider range of angles in Fig. 8A, the $x_3(t)$ waveform for a given frequency is scaled so that the amplitude corresponds with the appropriate twist angle change using these same calibrations. The simulations are run using the experimentally derived drag coefficient (γ = 9.6 mg/s) as well as the predicted drag coefficient (γ = 42.6 mg/s). As a note, in the low *Re* model, the thrust depends on the drag asymmetry of the tails, $\zeta_\perp - \zeta_\parallel$. The experimentally derived drag coefficient (γ = 9.6 mg/s) is smaller than that calculated for the tails (γ = 21.6 mg/s). This means that the experimental drag coefficient contradicts our calculated value of $\zeta_\perp$ and $\zeta_\parallel$. In the low *Re* model, we use the predicted values of $\zeta_\perp$ and $\zeta_\parallel$ to compute the thrust, even when this constitutes a contradiction with the experimental drag coefficient.

Immunostaining of the muscle rings

For immunofluorescence imaging, samples were fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) overnight at 4°C. The samples were then permeabilized using 0.2% Triton X-100 in PBS and blocked with a solution of 3% bovine serum albumin (BSA) and 5% normal goat serum (NGS) in PBS. Following this, the samples were incubated overnight at 4°C with a rabbit anti alpha-actinin primary antibody (1:250, Abcam #ab68167). After washing the samples five times with PBS (5 minutes each wash), they were incubated with the goat anti rabbit Alexa fluor 568 secondary antibody overnight at 4°C. The next day, samples were washed three

times with PBS and incubated with 4′,6-diamidino-2-phenylindole (DAPI) (1:1000, Invitrogen) for 10 minutes, followed by another three PBS wash. Images were acquired using a Zeiss LSM 700 confocal microscope equipped with an EC Plan-Neofluar 20X/0.5 N.A. objective (Carl Zeiss AG).

Statistical methods

A series of 3-independent sample, weighted, one-way ANOVA tests were used to compare the force and length of the aneural swimmer in each of the three sample populations on each day (Fig. 4D,E). On days where the sacrificial supports were cut, the force and length values measured after cutting were used. P-values are used to reject the null hypothesis that all the swimmer samples come from the sample population. A series of Students two-tailed t-tests assuming independent samples were used to compare the spontaneous and evoked contractility of aneural and cocultured swimmer samples (Fig. 5B). The behavior of a swimmer during inertial drift was characterized by least-square curve fitting with the given exponential function with affine offset (Fig. 7E). The value for v_{drift} is taken by fitting a linear function to the projected swimmer position over time before stimulation began. R^2-values are presented as means $\pm$ SEM for the given groupings.

Supplementary Materials

Supplementary Video 1 – Seeding and mounting C2C12 muscle rings (*3*)

Supplementary Video 2 – The cocultured swimmer samples twitching spontaneously in their petri dishes

Supplementary Video 3 – 4Hz electrical stimulation test of the swimmer sample shown in Fig. 6

Supplementary Video 4 – Frequency sweep test of the swimmer sample shown in Fig. 7 and 8

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