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TITLE:

Assessing Functional Metrics of Skeletal Muscle Health in Human Skeletal Muscle Microtissues

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human skeletal muscle, electrical stimulation, calcium handling, force measurement, immortalized myogenic progenitor cells, minimally invasive, tissue engineering, high content, three-dimensional, cell culture, polydimethylsiloxane.

SUMMARY:

This manuscript describes a detailed protocol to produce arrays of 3D human skeletal muscle microtissues and minimally invasive downstream in situ assays of function, including contractile force and calcium handling analyses.

ABSTRACT:

Three-dimensional (3D) in vitro models of skeletal muscle are a valuable advancement in biomedical research as they afford the opportunity to study skeletal muscle reformation and function in a scalable format that is amenable to experimental manipulations. 3D muscle culture systems are desirable as they enable scientists to study skeletal muscle ex vivo in the context of human cells. 3D in vitro models closely mimic aspects of the native tissue structure of adult skeletal muscle. However, their universal application is limited by the availability of platforms that are simple to fabricate, cost and user-friendly, and yield relatively high quantities of human skeletal muscle tissues. Additionally, since skeletal muscle plays an important functional role that is impaired over time in many disease states, an experimental platform for microtissue studies is most practical when minimally invasive calcium transient and contractile force measurements can be conducted directly within the platform itself. In this protocol, the fabrication of a 96-well platform known as 'MyoTACTIC', and en masse production of 3D human skeletal muscle microtissues (hMMTs) is described. In addition, the methods for a minimally invasive application

of electrical stimulation that enables repeated measurements of skeletal muscle force and calcium handling of each microtissue over time are reported.

INTRODUCTION:

Skeletal muscle is one of the most abundant tissues in the human body and supports key body functions such as locomotion, heat homeostasis and metabolism[1]. Historically, animal models and two-dimensional (2D) cell culture systems have been used to study biological processes and disease pathogenesis, as well as for testing pharmacological compounds in the treatment of skeletal muscle diseases[2,3]. While animal models have greatly improved our knowledge of skeletal muscle in health and in disease, their translational impact has been hampered by high costs, ethical considerations and interspecies differences[2,4]. In turning to human cell-based systems to study skeletal muscle, 2D cell culture systems are favorable due to their simplicity. However, there is a limitation. This format often fails to recapitulate the cell–cell and cell–extracellular matrix interactions that occur naturally within the body[5,6]. Over the last several years, three dimensional (3D) skeletal muscle models have emerged as a powerful alternative to whole animal models and conventional 2D culture systems by allowing the modeling of physiologically and pathologically relevant processes ex vivo[7,8]. Indeed, a plethora of studies have reported strategies to model human skeletal muscle in a bioartificial 3D culture format[1]. One limitation for many of these studies is that active force is quantified following the removal of the muscle tissues from the culture platforms and attachment to a force transducer, which is destructive and hence, limited to serving as an endpoint assay[9–21]. Others have designed culture systems that allow for non-invasive methods of measuring active force, but not all are amenable to high content molecule testing applications[7–10,14,18,22–29].

This protocol describes a detailed method to fabricate human muscle microtissues (hMMTs) in the skeletal muscle (Myo) microTissue Array deviCe To Investigate forCe (MyoTACTIC) platform; a 96 well plate device that supports the bulk production of 3D skeletal muscle microtissues[30]. The MyoTACTIC plate fabrication method enables generation of a 96 well polydimethylsiloxane (PDMS) culture plate and all corresponding well features in a single casting step, whereby each well requires a relatively small number of cells for microtissue formation. Microtissues formed within MyoTACTIC contain aligned, striated, and multinucleated myotubes that are reproducible from well to well of the device, and upon maturation, can respond to chemical and electrical stimuli in situ[30]. Herein, the technique to manufacture a PDMS MyoTACTIC culture plate device from a polyurethane (PU) replica, an optimized method to implement immortalized human myogenic progenitor cells to fabricate hMMTs, and the functional assessment of engineered hMMT force generation and calcium handling properties are outlined and discussed.

PROTOCOL:

1. PDMS MyoTACTIC plate fabrication

NOTE**:** PDMS MyoTACTIC plate fabrication requires a polyurethane (PU) negative mold, which can be manufactured as previously described[30]. The computer-aided design (CAD) SolidWorks file for the MyoTACTIC plate design has been made available on GitHub

(https://github.com/gilbertlabcode/MyoTACTIC-SolidWork-CAD-file).

1.1. Prepare ~ 110 g of PDMS polymer solution in a disposable plastic cup at a 1:15 ratio of monomer to curing agent using the components in the silicone elastomer kit. Stir the polymer solution for 2–3 min using a 5 mL disposable serological pipette until completely mixed and homogenous.

CAUTION: Avoid PDMS polymer solutions contact with skin and eyes; and avoid inhalation. Always wear a lab coat and disposable gloves when handling the liquid PDMS mixture and refer to Safety Data Sheet (SDS) for specific safety protocols.

NOTE**:** Protect equipment surfaces (e.g., scale, bench top, etc.) with a disposable covering in case of PDMS polymer solution spill.

1.2. Degas the mixture at room temperature by placing the cup within a benchtop vacuum chamber connected to a standard duty dry vacuum pump for ~30 min, or until all bubbles are removed. Break the vacuum every 5–10 min to aid in degassing. Use an empty 50 mL syringe barrel to plunge air and blow out any remaining bubbles on the surface of the polymer mixture as needed.

NOTE**:** A piece of 6.35 mm ID tubing can be used for connecting a P1250 pipette tip to the barrel to improve the velocity and accuracy of the air stream.

1.3. While the PDMS polymer solution is degassing, remove any leftover pieces of PDMS adhered to the PU negative mold by gently wiping down the edges and surface with a dry paper towel. Use facility compressed air, set to 70–100 kPag to remove remaining fine particles.

NOTE: Protect the PU negative mold from damage and from particulate accumulation by placing it in a sealable plastic bag and storing it within a drawer that is protected from laboratory traffic.

1.4. Place the PU mold in a chemical hood that has been protected with a disposable covering. Stand the mold horizontally at ~75° and spray the active surface with an even layer of release agent. Spray the mold from top to bottom, then left to right, while holding the can 15–20 cm from the mold and using a fluid back and forth sweeping motion. Rotate the mold 180° and repeat spray motions from step 1.5, then let the PU mold sit in the chemical hood for 10–15 min to dry.

CAUTION: Ensure release agent is used within a chemical fume hood, avoid contact with skin and eyes, and refer to Safety Data Sheet (SDS) for specific safety protocols

NOTE: Remove or cover other items in the fume hood and avoid over-spraying the mold with release agent. A thin film needs to fully cover the active surface, but excessive coating can be transferred to the PDMS in the next step, which can in turn negatively impact hMMT seeding. The surface should feel slick to the touch but not wet.

1.5. Pour 100 g of PDMS evenly into the PU mold and place within a vacuum chamber connected to a rotary vane vacuum pump. Degas the PDMS-filled mold for ~45 min, breaking the vacuum seal every 5–10 min for the first 20 min to accelerate the process. Leave within the vacuum chamber until the PDMS mixture is completely void of all bubbles.

NOTE: A rotary vane vacuum pump is used to achieve a lower vacuum and reduce the time required for this step. Degassing of the PDMS-filled mold can be performed using the benchtop vacuum chamber and the standard duty dry vacuum pump, however, the time to void the mixture of all bubbles will be longer. What is essential is the removal of all bubbles from the PDMS polymer solution, especially in those regions destined to become hMMT anchoring posts. Bubbles in this region will result in anchor post breakage, the loss of culture wells, and in turn result in a small piece of PDMS remaining wedged in the mold.

1.6. Transfer the degassed PDMS-filled PU mold to a 65 °C oven, incubating overnight to cure the liquid rubber.

1.7. Remove the cured PDMS positive plate from the oven and cool the plate at room temperature for at least 30 min.

1.8. With a bladeless scalpel, gently detach cured PDMS from the PU mold by running the back of the handle between the PDMS and walls of the mold. Start along the upper edge and separate all 4 sides, before pushing down the base of the mold and detaching this portion. This step is complete when the back of the handle runs smoothly between the PDMS and all 4 walls of the PU mold.

NOTE: Work slowly and carefully with the bladeless scalpel to prevent cracking the PU mold or tearing the PDMS. This step should take 15–20 min.

1.9. Starting from one end, use the bladeless scalpel to lift-up the edge of the PDMS and work fingers between the cured PDMS culture plate and PU mold. Then, using both hands, push fingers further underneath the plate, slowly peeling up and out of the PU mold.

NOTE: Work slowly and use both hands to evenly peel the plate. Minimize bending to reduce the likelihood of anchor post breakage. This step should take 5–10 min.

1.10. Use a single edge razor blade to cut the plate into groups of 6 ± 2 MyoTACTIC wells (**Figure 1**) for tissue seeding purposes. Place full MyoTACTIC plates, or plate portions, into an instrument sterilization bag and autoclave for a 25 min dry cycle (20 min of sterilization time, and 5 min of dry time) at 120 °C and 100–140 kPag.

2. Culture of immortalized human myoblast progenitor cells

NOTE: The immortalized myoblasts used in this protocol were obtained from the Institut de Myologie (Paris, France)[31].

2.1. Obtain one vial of frozen cells from the liquid nitrogen dewar and quick thaw the vial in a 37 °C water bath (in less than 1 min). Cells are frozen at a density of 7.5 x 10^5 cells per 1 mL of freezing media consisting of 90% fetal bovine serum (FBS) and 10% dimethyl sulfoxide (DMSO).

2.2. Gently transfer the contents of the vial to a 15 mL conical tube containing 9 mL of pre-warmed wash medium consisting of 90% DMEM 1x, 10% FBS and 1% Pen/Strep to dilute out the DMSO. Spin the 15 mL conical tube at 400 x *g* for 10 min and then aspirate away the media taking care to avoid the cell pellet.

2.3. Resuspend the pellet in 1 mL of growth media consisting of 84% Skeletal Muscle Cell Basal Medium with Skeletal Muscle Cell Growth Medium Supplement Mix, 15% FBS and 1% Pen/Strep and then transfer to a 50 mL conical tube with 29 mL of growth media.

2.4. Transfer 10 mL of media containing approximately 2.5 x 10^5 cells into a 100 mm x 20 mm cell culture dish. Repeat this step with the remaining 20 mL of the cell solution. Then transfer cell culture dishes to a humidified cell culture incubator set to 37 °C and 5% CO_2.

2.5. Refresh the culture media every other day. Culture the cells until they achieve ~ 70%–80% confluency (typically 4–5 days), at which point the cells are prepared for seeding. 1.5 x 10^5 cells are required to generate each hMMT. Therefore, passage the cells as needed to achieve the requisite number of cells.

NOTE: The immortalized myoblast progenitor cell lines should never exceed 80% confluency before seeding cells into MyoTACTIC wells, passaging the cells, or preparing freezer stocks. hMMTs fabricated from cells that have exceeded 80% confluency often fail to reach contractile maturity. Passaging procedures are identical to methods described below in step 3.

3. Seeding engineered hMMTs with MyoTACTIC

3.1. Preparation of MyoTACTIC culture wells and reagents for hMMT seeding.

NOTE: This protocol provides specific details to produce 6 hMMTs.

3.1.1. 2–3 h before cell seeding, place a 6 well MyoTACTIC plate portion into a 10 cm cell culture dish. Prepare each individual MyoTACTIC culture well by adding 100 µL of a 5% Pluronic F-127 solution into each well. Put the lid on the 10 cm culture dish, apply paraffin to seal the space between the 10 cm dish and lid, and the place the 10 cm plate containing the MyoTACTIC portion into a centrifuge outfitted with a plate spinner adaptor.

NOTE: If a centrifuge plate spinner adaptor is not available, a p20 pipette tip can be used to carefully remove bubbles from behind the posts, thereby ensuring that the entire culture surface is evenly coated.

3.1.2. Centrifuge at 1,550 x *g* for 1 min to remove all bubbles within culture wells, especially behind the posts. Store the 10 cm cell culture dish containing the MyoTACTIC plate portion containing Pluronic F-127 solution at 4 °C until the cells are prepared for seeding.

NOTE: Pluronic F-127 coating can be applied for as little as 2 h to as long as 24 h. For example, wells can be filled with Pluronic F-127 solution at the end of the day for use the next day. Do not exceed 24 h as this will negatively impact hMMT remodeling and fail to form healthy tissues that reach contractile maturity.

3.1.3. Slowly thaw one 50 µL basement membrane extract aliquot and one 10 µL thrombin aliquot (100 U/mL stock solution) on ice within the culture hood.

NOTE: Do not refreeze basement membrane extract aliquots. They are single use. Thrombin aliquots are reusable, therefore, re-freeze up to 5 times after use.

3.1.4. Weigh ~7 mg of powdered fibrinogen in a 1.5 mL microcentrifuge tube and then transfer to the cell culture hood. Add 700 µL of 0.9% (wt/vol) NaCl solution in water (or saline solution) to arrive at a 10 mg/mL final concentration solution. Do not vortex fibrinogen to dissolve, instead place the tube in a 37 °C cell culture incubator for 3–5 min.

3.1.5. Remove and flick the tube gently, then pulse spin the dissolved solution in a benchtop mini centrifuge (microfuge) and return to culture hood. Filter the fibrinogen solution using a 1 mL syringe outfitted with a 0.22 µm syringe filter. Transfer the dissolved fibrinogen solution to ice alongside the basement membrane extract and thrombin aliquots.

3.1.6. Lastly, prepare the hMMT seeding media that will be introduced to the culture wells after seeding of the tissues. This media contains Skeletal Muscle Cell Basal Medium supplemented with 20% FBS, 1% P/S and 3% 6-aminocaproic acid (ACA; 1.5 mg/mL final concentration is achieved by diluting from a 50 mg/mL stock solution; % is expressed as v/v). Pre-warm the media at 37 °C before use.

3.2. Preparation of cells for hMMT seeding.

3.2.1. Collect the cell culture plates from the culture incubator. Aspirate the media from each plate, then wash cells once with D-PBS by adding 5 mL of D-PBS into each culture plate. Next aspirate the D-PBS and detach the cells by adding 1 mL of 0.25% Trypsin-EDTA into each culture dish. Place in the cell culture incubator for 3 min.

3.2.2. Halt the trypsin by adding 3 mL wash medium (89% of 1x DMEM + 10% FBS + 1% Pen/Strep) to the culture dish. Transfer the cell solution to an appropriately sized conical tube, and then pellet the cells by centrifuging at 400 x *g* for 10 min. Aspirate the media taking care to avoid the cell pellet. Then resuspend the pellet cell slurry in 1 mL of the wash medium.

3.2.3. Count the cells using a hemocytometer and trypan blue dye under brightfield microscopy.

If using multiple plates of cells, this cell suspension will be very concentrated. Dilute cell suspensions prior to counting as needed.

3.2.4. Since each tissue requires 150,000 cells resuspended in 15 µL of the extracellular matrix (ECM) mixture, therefore, to seed 6 tissues, prepare a total of 900,000 cells in 90 µL of ECM mixture. To account for cell–ECM solution loss that occurs during the preparation process due to bubble formation or pipette loss, prepare extra cell–ECM mixture (i.e., 8 tissues, or 1,200,000 cells in 120 µL of ECM). Transfer the volume of cell suspension containing 1,200,000 cells into a new conical tube. Increase the volume to 10 mL with wash medium and spin at 400 x *g* for 10 min.

3.2.5. While cells are spinning down, prepare 150 µL ECM mixture in a 1.5 mL microcentrifuge tube using the following recipe. First add 60 µL of DMEM, then add 60 µL of Fibrinogen 10 mg/mL solution (40% volume) and lastly add 30 µL of basement membrane extract (20% volume). Store the ECM mixture on ice until use.

NOTE: Always use tips pre-chilled at -20 °C when working with solutions containing basement membrane extract. ECM mixture contains 4 mg/mL of fibrinogen.

3.3. Seeding hMMTs

3.3.1. Collect the conical tube containing the spun down cells and aspirate the media taking care to avoid the cell pellet. Vigorously flick the end of the tube with a gloved finger to dislodge the pellet and continue flicking until the pellet appears as a cell slurry.

NOTE: It is important to aspirate as much wash media as possible to ensure the cell–ECM suspension is at the desired dilution. Use a pipette to remove remaining wash media if needed.

3.3.2. Transfer 120 µL of the ECM solution to the tube containing the pellet cell slurry. Pipette up and down to thoroughly resuspend the cells within the ECM to generate a single cell suspension. Pipette slowly and carefully to avoid introducing bubbles and thereby reducing the working volume. Then, place the cell–ECM solution on ice until use.

3.3.3. Retrieve MyoTACTIC plate portions containing Pluronic F-127 coated MyoTACTIC wells from the 4 °C refrigerator and place the 10 cm dish containing the MyoTACTIC wells on top of an ice pack inside of the cell culture hood.

3.3.4. Aspirate the Pluronic F-127 solution from each well. The PDMS is porous and will soak up the Pluronic F-127 solution, especially if plates were spun down with a centrifuge. Allow residual Pluronic F-127 solution to release and settle to the bottom of the well by letting wells sit for 5 min, then aspirate again.

NOTE: Use a Pasteur pipette with a p1250 tip attached at the end, and a p200 tip over the p1250 tip when aspirating the Pluronic F-127 solution. This will improve precision to prevent accidental

aspiration of the post structures. Avoid scraping the oval pool shaped cell seeding area at the bottom of the well with the pipette as this disrupts the Pluronic F-127 coating and interferes with the hMMT self-organization process. The proper technique is to hover the pipette tip just over the oval pool while aspirating the Pluronic F-127 solution.

3.3.5. Pipette the cell–ECM suspension carefully to resuspend any cells that may have sunk to the bottom of the tube. Then transfer 105 µL of cell–ECM suspension into a fresh, pre-chilled 1.5 mL tube. Take care to grasp the tube close to the top to prevent the solution from warming.

3.3.6. Add 0.84 µL of the 100 U/mL thrombin stock solution to the 105 µL cell–ECM suspension to arrive at a final concentration of 0.2 U/mg of fibrinogen. Pipette rapidly, carefully, and thoroughly to mix, while avoiding introduction of bubbles.

NOTE: Thrombin initiates the rapid conversion of fibrinogen into a fibrin clot. As such, there is limited time to seed the tissues upon thrombin addition. To avoid premature clotting before cell–ECM mixture is transferred into the culture wells, set a p20 pipette to 15 µL before adding the thrombin. Use pre-chilled tips or dip the pipette tip into ice cold DMEM for a few seconds before collecting and transferring 15 µL of the cell–ECM mixture into each well. It is a best practice to prepare cell–ECM mixture aliquots such that no more than 6 hMMTs are seeded at a time.

3.3.7. To seed the tissues, add 15 µL of cell–ECM mixture (i.e., 150,000 cells) to each individual well. Carefully add cell-ECM mixture to the center of the oval pool and avoid pressing the pipette tip into the bottom of the well. Then, with two light motions, spread the cell suspension behind each post in the well. Once the surface is evenly coated in the cell-ECM suspension, move on to the next well.

NOTE: Work efficiently to avoid premature gelation of the cell–ECM mixture in the tube before all tissues are seeded. When seeding the wells, it is extremely important to avoid transferring bubbles as the bubble will interfere with the remodeling of the hMMT, rendering the hMMT unusable.

3.3.8. Place the lid on the 10 cm culture plate containing the seeded wells and transfer to a 37 °C tissue culture incubator for approximately 5 min.

NOTE: Place the microcentrifuge tube with residual cell–ECM mixture into the incubator as a confirmation of the gel polymerization process.

3.3.9. After the cell–ECM mixture has polymerized, add 200 µL of prewarmed hMMT seeding media to each MyoTACTIC well. Replace the lid on the 10 cm dish and return MyoTACTIC plate portions within the 10 cm dish to the incubator. This time point is referred to as Day -2. Do not disturb tissues until the next step.

3.4. Differentiating hMMTs

3.4.1. After 2 days of incubation, remove the hMMT seeding media carefully using a pipette and replace with 200 µL of prewarmed differentiation media containing 2% horse serum, 1% Pen/Strep, 4% ACA (i.e., 2 mg/mL final concentration from a stock concentration of 50 mg/mL; % is expressed as v/v) and 10 µg/mL human recombinant insulin in DMEM. This time point is referred to as Day 0 of differentiation.

3.4.2. Every other day thereafter, exchange half of the media with fresh differentiation media until day 12 of differentiation, the last day of culture (**Figure 2a**).

NOTE: Protocol details to fabricate hMMTs using primary human myoblasts have been published elsewhere[30]. If there are concerns for cell viability post seeding, incubate hMMTs with calcein and propidium iodide to quantify viability.

4. Electrical stimulation and analysis of hMMT-induced post deflection

4.1. Set up a clear bottom glass or plastic stage mount on an inverted microscope and attach a smartphone camera to the microscope eyepiece using a microscope–camera mount. Phase contrast microscopy at 10x magnification will be used (**Figure 3a**).

NOTE: Any inverted phase contrast microscope outfitted with a 10x magnification objective will be appropriate. The PDMS well constructs will be removed from the 10 cm dish and placed directly onto the clear bottom stage mount, and hence, open to the air. Sterilize all surfaces and equipment with 70% ethanol and minimize traffic in the area during experimentation.

4.2. To prepare the electrical stimulation electrodes, cut ~30 cm of tin-coated copper wire. Then, starting at the hub of a 25 G regular bevel needle, wrap ~10 cm of the wire tightly around the top half, leaving ~20 cm of excess wire. Repeat for a second needle and then cut the hub off each needle.

NOTE: The wire must be tight around needle to ensure it does not move and the wire wrapped portion of the electrode must not touch the PDMS as this can impede electric field generation.

4.3. Attach BNC to alligator clip connector cable to an output channel on the waveform generator and set the channel for square pulses with 20% duty cycle, 5 V amplitude (electrical field strength of 10 V/cm). The frequency will alter between 0.5 Hz and 20 Hz for twitch and tetanus contractions, respectively.

NOTE: Waveform generator settings may require experiment-specific optimization

4.4. Place a MyoTACTIC plate portion containing hMMTs on the microscope stage and gently insert each electrode bevel end into the PDMS directly behind each post in the oval pool. Carefully tape down the 20 cm sections of excess wire to the microscope stage so that the needles remain vertical and ~10 cm of each wire remains free for connection (**Figure 3a**).

CAUTION: Never place a bare finger over either end of the electrode. Ensure that a thimble is worn on the index finger to apply light pressure to the electrode on insertion into the PDMS.

4.5. Focus the microscope field of view on one of two posts, such that focus is sharp on the post edge closest to the electrode. Then, Lock the smartphone camera focus to the clearest area of the post. This is important for the downstream analysis as a change in focus while recording will interfere with analysis.

4.6. Connect the free ends of the taped wires to the waveform generator via alligator clamps (**Figure 3a**).

4.7. Start video recording on the smartphone camera, and then turn on the channel output to initiate stimulation. Induce the hMMT to undergo 3 twitch and 3 tetanus contractions, with 2 min of rest between the twitch and tetanus stimulation series.

4.8. Turn off channel output, detach alligator clips from the tin-coated copper wires, remove the tape from the wires, and wipe each electrode with 70% ethanol before inserting into subsequent hMMT well. Repeat procedure from step 4.5 for all hMMTs.

NOTE: Limit the time that hMMTs spend outside of incubator by stimulating no more than 3 tissues at a time. If additional hMMTs within the MyoTACTIC plate portion remain to be analyzed, return the plate to the incubator for 10 min to allow hMMTs to return to 37 °C.

4.9. To analyze post deflection so as to calculate hMMT force generation, use the custom script, which has been made available on GitHub (https://github.com/gilbertlabcode/myoTACTIC). Follow the instructions in README.md to set up and launch the script, then open each post tracking video to conduct the force analysis.

4.10. Select a region of interest (ROI) along the post edge to be tracked for post displacement. Press **Enter** to confirm the ROI and press **Enter** again to run the script (**Supplemental Video 1**).

NOTE: Large deflections cause the tracker to fail. If the tracker fails during contraction, the ROI size can be increased to make the tracking less sensitive but permit tracking of large deflections. Three sizes are provided in the code and can be tuned by adjusting the script comments.

4.11. The script ends with two input requirements. First, enter **y** to confirm the video contained multiple contractions. Second, enter **y** (yes) or **n** (no) for exporting results to a .CSV file (**Supplemental Video 1**).

4.12. Ensure post deflection results are reported as displacement in pixels for each contraction. Convert pixels to μm values for the microscope 10x magnification setting. Then, convert post displacement numbers to absolute contractile forces (μN) by multiplying values (μm) by the force-displacement conversion factor of 2.36 μN / μm, which corresponds to the 1:15 curing agent to monomer ratio of the PDMS used in MyoTACTIC fabrication[30].

5. Calcium transient analysis using electrical stimulation

NOTE: For calcium handling experiments, immortalized myoblasts were stably transduced with the MHCK7-GCAMP6 reporter as previously described[11,12]. Transduced cells were FACS sorted for GFP to obtain the positive population, and then used to fabricate hMMTs. Alternative methods for calcium imaging such as using ratio-metric dyes like Fura-2 AM and Indo-1 or fluorescence lifetime imaging of calcium indicators (e.g., Fluo-4 or Oregon Green BAPTA1) may be amenable to our system.

5.1. Set up the microscope stage and stimulation equipment (electrodes, waveform generator, etc.) as previously described in step 4. For this experiment, use a 4x magnification.

NOTE: A dark room and an epifluorescence microscope equipped with a CCD camera will be needed.

5.2. Launch microscope imaging software, select the **FITC filter channel** (blue light), and select **movie recording function**. Set at an exposure of 500 ms and a resolution of 680 x 510 (Binning 2x2).

NOTE: Imaging software may vary and exposure is manually set by the user. Take care to avoid hMMT over exposure prior to stimulation. At rest, a dark tissue outline/shadow with spontaneous fluorescence is normal while a clear tissue image is over exposure (**Supplemental Video 2**). Ensure the exposure is consistent for all hMMTs within an experiment.

5.3. Close the microscope shutter and keep the FITC channel off until ready to record calcium handling.

5.4. When all equipment and software is set up, retrieve the MyoTACTIC plate portion containing the hMMTs to be analyzed and set it directly on the microscope stage. Then, insert and connect electrodes as previously described in step 4.

5.5. Use brightfield to focus the field of view on the center of the selected hMMT, then, turn off the lamp.

5.6. Open the microscope FITC channel shutter, confirm blue light is on, and then select **Movie Record** in the software.

5.7. Turn on the output on the waveform generator to initiate the electrical stimulation. Induce the hMMT to undergo 8 twitch and 8 tetanus contractions. Allow for 2 min of rest between the twitch and tetanus stimulation series, during which time the FITC shutter is in the closed position.

NOTE: Allow for 10 s of spontaneous activity before and after electrical stimulation to record minimum fluorescence for calculation and data analysis purposes.

5.8. Turn off the channel output, detach alligator clips from the tin-coated copper wires, remove the tape from the wires, and wipe each electrode with 70% ethanol before inserting into subsequent hMMT well. Repeat stimulation and recording procedure for all hMMTs.

5.9. Save movies in TIFF file format for analysis in ImageJ, or alternative imaging software.

NOTE: Limit the time that hMMTs spend outside of incubator by stimulating no more than 3 tissues at a time. If additional hMMT within the MyoTACTIC plate portion remain to be analyzed, return the device to the incubator for 10 min to allow hMMTs to return to 37 °C.

5.10. To analyze calcium transient data, first open ImageJ. Select **Analyze**, then **Set Measurements**, then select the **Mean Grey Value** and deselect all other options. When completed, open a calcium (.tiff) video (**Supplemental Video 2**).

5.11. Outline the border of the microtissue using a polygon selection tool and save this area as the ROI (**Supplemental Video 2**). Under **More** in the ROI Manager window, select **Multi Measure** and measure fluorescent intensity for all file slices at one row per slice (**Supplemental Video 2**).

5.12. Copy all measurements, including slice number (frame), to a spreadsheet and compare the fluorescent intensity of each slice to the minimum spontaneous fluorescent intensity from the file using $\Delta F/F_0 = (F_{immediate} - F_{minimum})/F_{minimum}$ (**Supplemental Video 2**).

5.13. Calculate the time for each frame by multiplying the movie recording frame speed by slice number (frame), and plot $\Delta F/F_0$ against time for the hMMT calcium transient response to stimulation (**Supplemental Video 2**).

5.14. Select the peak calcium transient signal for each of 6 consecutive contractions and average values to calculate the relative mean peak fluorescent intensity change of each hMMT (**Supplemental Video 2**).

NOTE: Always exclude data arising from the first twitch contraction from the overall analysis. A spreadsheet entitled “Calcium Handling Template.xlsx” has been provided on GitHub (https://github.com/gilbertlabcode/Calcium-Handling-Template-) to facilitate the hMMT calcium transient analysis. Fillable cells where values and inputs are to be entered are highlighted in grey. Make sure to adjust peak selection numbers as this is only a guide to aid in peak selection (**Supplemental Video 2**).

REPRESENTATIVE RESULTS:

Described herein are methods to cast a 96-well PDMS-based MyoTACTIC culture platform from a PU mold, to fabricate arrays of hMMT replica tissues, and to analyze two aspects of hMMT function within the culture device–force generation and calcium handling. **Figure 1** offers a schematic overview of the preparation of MyoTACTIC culture wells before hMMT seeding. PDMS is a widely used silicone-based polymer, that can be easily molded to create complex devices[32].

A PDMS-based protocol was designed to cast an unlimited number of 96-well culture devices from a manufactured negative PU mold[30]. The final cured PDMS positive plate is flexible and can be easily cut into smaller-sized functional units with the aid of a single edge razor blade (**Figure 1**). This allows the user to scale the device to meet the hMMT replica needs specific to their experimental design. These smaller functional units are also beneficial for overcoming a limitation of working with an ECM scaffold that polymerizes rapidly, such as fibrin hydrogels, by easily tuning the number of wells to match the cell seeding speed of the user. Moreover, PDMS is readily autoclavable and it can be stored for an indefinite period. Finally, from a logistics standpoint, a full MyoTACTIC plate can be covered by any standard 96-well plate lid, and the dimension and profile of MyoTACTIC plate portions are amenable to incubation within a 10 cm culture plate, to ensure hMMT culture sterility. Pluronic F-127 solution coating ahead of cell seeding serves a dual role of providing an additional measure of culture well sterilization, and as a non-ionic surfactant, preventing cell adhesion in favor of uniform cell – ECM remodeling (**Figure 1**).

When the immortalized myogenic progenitor cell population is ready for experimentation, the cells are then encapsulated in a hydrogel comprised of fibrinogen and basement membrane extract. The cell–ECM mixture is then evenly deposited in the oval pool at the bottom of each MyoTACTIC well, and then over a 14-day culture period, the cells spatially self-organize across these two vertical posts to form a 3D microtissue populated with a bed of aligned, multinucleated, striated myotubes that resemble aspects of native tissue organization (**Figure 2a,g,h**). Over the remodeling process, uniaxial tension is generated between the two anchorage points, and this guides the tissue self-organization process to in turn drive the formation of a compact tissue. During the differentiation period, the width of the hMMTs constructed from myoblasts derived from healthy patients remains fairly consistent. However, in events of errors that interfere with hMMT remodeling, this uniformity is lost (**Figure 2a-f**), making this simple metric useful in hMMT quality control assessment. For example, overzealous mixing of the cell – ECM suspension can result in the formation of bubbles. Bubbles carried over to the MyoTACTIC well impede hMMT remodeling. In such instances, bubbles displace some or all of the myoblasts from the area occupied by the bubble, eventually forming a crevice in the final hMMT (**Figure 2b**; see red arrow). Another example relates to the integrity of the Pluronic F-127 coating in the wells. Pluronic F-127 is a non-ionic surfactant that prevents cells from adhering to the MyoTACTIC well. If the coating is scraped off during aspiration, myoblasts will adhere to the surface of the MyoTACTIC wells, forming new anchorage points and resulting in myotubes that are not aligned across the two posts (**Figure 2c**). Additional errors can also result in aberrant hMMT remodeling, as seen in **Figure 2d–f**. Cell vitality post seeding can also be evaluated using calcein / propidium iodide-based staining assays. When these technical errors are avoided, hMMTs are populated by a bed of aligned and multinucleated myotubes that extend across the length of each hMMT (**Figure 2g**). The myotubes are fairly uniform in size across their length and between different hMMTs (**Figure 2h–i**). Myotubes are characterized by the presence of sarcomere striations which are visualized by immunostaining for sarcomeric α – actinin (SAA; **Figure 2h**).

Functional properties can also be examined in hMMTs beginning around 7 days of differentiation. The experimental set up to complete these functional studies is illustrated in **Figure 3a**. The

microscopy setup is pictured on top of a vibraplane table; however, this is not required to complete functional investigations. In functional studies, electrodes generated as described in the electrical stimulation section of the protocol are inserted into the PDMS, such that electrodes reside behind the posts. It is important to have good visualization of the post region before inserting the electrode as the need to reinsert may result in displacement of the tissue from the post. Proper insertion of the electrodes is also important to obtain sharp focus of the post so that post-deflection can subsequently be tracked with the python script. **Figure 3b** shows a representative displacement of the MyoTACTIC well plate post in response to low (twitch, 0.5 Hz) and high (tetanus, 20Hz) frequency hMMT electrical stimulation. Quantification of post displacement can be used to calculate the induced contractile force of hMMTs (**Figure 3c**). When this information is combined with hMMT cross-sectional area, specific force can be determined[30]. Moreover, calcium transients play an important role in muscle contraction. Stable transduction of skeletal muscle myoblasts with a genetically encoded calcium indicator such as GCaMP6[12,30,33,34], enables live surveillance of calcium transients[12,30,34]. Calcium transients were analyzed using the afore described electrical stimulation setup up to stimulate day 12 hMMTs generated with immortalized myoblasts expressing the MHCK7-GCAMP6 reporter (**Figure 4a**), and quantified as mean peak fluorescent intensity during low (twitch, 0.5 Hz) and high (tetanus, 20Hz) frequency electrical stimulation (**Figure 4b**). It is observed that hMMT calcium handling properties measured across different experiments are relatively consistent (**Figure 4b**). Quantification methods for post deflection and calcium handling are outlined in **Supplementary Video 1** and **Supplementary Video 2**.

FIGURE AND TABLE LEGENDS:

Figure 1: Preparation of MyoTACTIC PDMS plate prior to hMMT seeding. The 96-well platform referred to as MyoTACTIC is fabricated by first curing polydimethylsiloxane (PDMS) within a negative polyurethane (PU) mold. The PDMS-based culture platform is then carefully peeled from the negative PU mold. In an academic setting, the PDMS device is then cut into smaller units containing 6 ± 2 functional wells. These wells are then placed in a sterilization pouch, autoclaved and stored until use. Up to one day before use, the desired number of device portions are placed within a 10 cm culture plate and Pluronic F-127 is added to each well. The 10 cm dish lid is added, the edge is sealed with parafilm before incubating the plate at 4˚ for 2–24 h.

Figure 2: MyoTACTIC supports hMMT self-organization and formation of aligned myotubes. (**a**) (top) Schematic timeline of hMMT self-organization and subsequent myotube maturation over a 14-day culture period. (bottom) Representative 4x phase-contrast images to illustrate the kinetics of immortalized myoblasts self-organization across the two vertical posts that result in the formation of a 3D hMMT. Scale bar, 500 µm. (**b–f**) Representative 4x phase-contrast images of hMMTs on day 12 of differentiation showing hMMT outcomes caused by (**b**) a bubble within the cell – ECM at time of seeding (red arrow points to bubble induce hMMT damage), (**c**) incorrect aspiration of the Pluronic F-127 solution coating, and (**d–f**) hMMT over-remodeling that can signal improper ECM gelation, lack of ACA activity in culture media, improper care of myoblast parental line, etc. Scale bar, 500 µm. (**g**) Representative tiled and flattened confocal image of a Day 12 hMMT taken at 10x magnification. hMMTs are fixed directly in the PDMS mold, then

carefully removed and placed in a 96 well culture plate for staining. Sarcomeric α-actinin (SAA) shown in magenta, and Hoechst 33342 nuclear counterstain shown in cyan**.** Scale bar, 500 µm. (**h**) Representative confocal image of a Day 12 hMMT at 40x magnification. hMMT myotubes and nuclei are visualized by immunostaining for SAA (magenta) and counterstaining with Hoechst 33342 (cyan). Scale bar, 50 µm. (**i**) Dot plot graph of mean hMMT myotube diameter at day 12 of differentiation. Values are reported as mean ± SEM. n = 8 hMMTs from 3 biological replicates, distinguished by symbol shapes, whereby a minimum of 30 myotubes per hMMT are analyzed.

Figure 3: In situ measurement of contractile force within the MyoTACTIC platform. (**a**) Representation of the electrical stimulation arrangement (left). Although pictured in this setup, a turntable is not required. A smartphone camera and mount was outfitted to the eyepiece of an inverted microscope equipped with a fluorescence lamp for the video recordings of hMMTs electrical stimulation post displacements (left). 25 G needles were wrapped with tin-coated copper wires (bottom right), connected to a BNC to alligator clip connector cable (top left) and were affixed up to an arbitrary waveform generator. Placement of electrodes during electrical stimulation is shown in bottom right. (**b**) Representative snapshots taken from post deflection videos acquired from hMMTs on Day 12 of differentiation. Videos were captured at 10x magnification. Solid white vertical lines show post placement when hMMTs are relaxed, while dotted white vertical lines show post displacement following high (tetanus 20 Hz) or low (0.5 Hz) frequency stimulation. Scale bar, 100 µm. (**c**) Dot plot graph of mean hMMT twitch (0.5 Hz)- and tetanus (20 Hz)-induced contractile force on Day 12 of differentiation. Values are reported as mean ± SEM. n = 9 hMMTs from 3 biological replicates, distinguished by symbol shapes.

Figure 4: In situ measurement of calcium handling within the MyoTACTIC platform. (**a**) Representative images from a 4x magnification video recording of spontaneous GCaMP6^{+} calcium transients and calcium transients in response to low (twitch contraction, 0.5 Hz) and high (tetanus contraction, 20 Hz) frequency electrical stimulation. hMMTs are outlined by a dotted yellow line. Scale bar, 200 µm. (**b**) Dot plot graph showing quantification of mean peak fluorescent intensity per hMMT following low (twitch contraction, 0.5 Hz) and high (tetanus contraction, 20 Hz) frequency electrical stimulation. Values are reported as mean ± SEM. n = 9 hMMTs from 3 biological replicates, distinguished by symbol shapes.

Supplemental Video 1: Demonstration of methods to analyze post-deflection data. A representative video of post deflection analysis at day 12 of differentiation being tracked by the custom script during tetanus stimulation. First the ROI size is selected directly within the post tracking script. Next the video is opened by selecting the play button to run the script. A sharp focused region of the post is selected as the ROI and the blue post tracking box is placed. **Enter** is pressed to lock ROI and pressed again to run the script. “y” is entered as response to the prompt “Multiple contraction video? [Y/N]”, then ‘n’ is entered as a response to the prompt “Export post locations as .csv file? [Y/N]”. Post deflection as relative displacement in pixels is the final output.

Supplemental Video 2: Demonstration of methods to analyze GCaMP6 calcium transient data. A representative video of epifluorescence calcium handling analysis at day 12 of differentiation during tetanus stimulation. First, a video of calcium transients (saved as a series of tiff images)

has been opened in ImageJ and the user has navigated through frames until the hMMT is visible. Then, the required analysis measurement "mean grey value" is confirmed and the hMMT is outlined using the polygonal drawing tool. This outline is saved as the region of interest and the fluorescent intensity of each video slice is analyzed using multi-measure. These measurements are copied to the spreadsheet "Calcium Handling Template" with frame data and calcium transient peak values filled in and confirmed.

DISCUSSION:

This manuscript describes methods to fabricate and analyze a 3D hMMT culture model that can be applied to studies of basic muscle biology, disease modeling, or for candidate molecule testing. The MyoTACTIC platform is cost-friendly, easy to manufacture, and requires a relatively small number of cells to produce skeletal muscle microtissues. hMMTs formed within the MyoTACTIC culture platform are comprised of aligned, multinucleated, and striated myotubes, and respond to electrical stimuli by initiating calcium transients that trigger contraction (**Figure 2, Figure 3, Figure 4**). Prior studies showed that hMMTs offer a similar response to biochemical stimuli and can reach maturation levels matching those reported in larger format 3D skeletal muscle models[12,30].

A critical theme throughout the MyoTACTIC plate fabrication and hMMT generation is ensuring that bubbles are prevented, and if formed have been removed. To facilitate single step casting of an operable PDMS mold, a PU mold had been generated downstream of the initial 3D-printed plastic mold previously described by Afshar et al.[30]. The PU mold that has been generated allows for users to produce a 96 well footprint of PDMS mold whereby a maximum of 96 wells are functional (contain two posts), as dictated by the PU mold fabrication step. During fabrication of the PDMS MyoTACTIC plate, new users often miss smaller bubbles that remain behind in the liquid PDMS, even after degassing. Bubbles that localize to regions of the PU mold corresponding to the anchoring flexible post structures will result in post breakage upon separation of the PU mold from the PDMS culture plate, and in the process, leaves behind a small piece of cured PDMS in the PU mold. Compressed air can be used to remove these cured PDMS remnants, and a failure to do so will effectively reduce the number of functional wells available for future plate castings. Therefore, it is critical to ensure all bubbles have been removed before the PDMS plate is cured as an advantage of this platform is that is it a stand-alone device, whereby all of the features of the plate are cast in a single step rather than requiring each individual microtissue anchoring point to be introduced to the culture wells manually[35–37]. Most academic laboratory studies do not require an entire MyoTACTIC culture plate. To maximize use of each PMDS plate, it is manually cut into groups of 6 ± 2 MyoTACTIC wells. To improve this process a PU mold that lets users peel functional units of 6 ± 2 MyoTACTIC wells can entirely eliminate this plate cutting step. Moreover, large bubbles introduced into the cell–ECM solution while mixing or during the tissue seeding procedure will impair proper tissue formation. These large bubbles displace cells from the region the bubble occupies, often resulting in hMMTs with regions with few myotubes that will succumb to contractile stress and snap during electrical stimulation.

A notable advantage of MyoTACTIC is the ability to quantify active force generation and calcium handling properties in situ. This is a challenge faced by many other 3D culture systems, where

investigating 3D tissue contractile force is implemented as an endpoint assay following removal of the tissue from the culture device[12,37]. Therefore, MyoTACTIC is well-suited to supporting longitudinal studies, for example, understanding the temporal effects of drug treatment on skeletal muscle. One limitation of the method described herein to quantify active force generation and calcium handling properties in situ is the manual placement of electrodes, which limits the use of this system for high content molecule testing applications. A possible solution would be to engineer a clear lid of electrodes set up as a parallel circuit that can be directly inserted into a standardized set of functional wells enabling electrical stimulation of multiple tissues at once. Alternatively, use of a myogenic progenitor cell line stably expressing a channelrhodopsin construct would allow muscle cell membrane depolarization, and hence, tissue contraction induced by blue light exposure. Moreover, active force is captured in videos as post deflection and quantification of active contractile force can be conducted in an unbiased manner by using a custom semi-automated python script to track post deflection in short videos. Therefore, unbiased longitudinal assessment of stimulated contractile force is enabled by MyoTACTIC[30]. To improve the efficiency of data analysis, smartphone applications that are designed to measure force while simultaneously capturing videos of post deflection, are ideal to increase throughput.

Finally, the value of this platform has also been previously described in its ability to accurately predict drug response. Similar to clinical outcomes, we have previously reported that treatment of hMMTs (made with primary myoblasts) with myotoxic compounds (dexamethasone, cerivastatin) induced myotube atrophy and decreased active contractile force[30]. Moreover, the predictive value of MyoTACTIC generated hMMTs was validated by showing that a clinically relevant dosage of a chemotherapeutic used to treat pancreatic cancer, a disease that is often associated with cachexia[2], significantly reduced hMMT quality and contractile force[30]. Therefore, the simple fabrication of MyoTACTIC and its ease of use in the generation of 3D hMMTs shows many advantages for high-content data capture as demonstrated by its reliability with regards to structural and functional outputs. A general limitation of PDMS-based cell culture devices is that PDMS adsorbs proteins. The method described herein makes use of serum-containing culture media, which overcomes this limitation by serving to 'block' the device and allow the effects of molecule and drug treatments to be observed. However, owing to this limitation, definitive conclusions about molecule doses are to be avoided, and dose response curves are encouraged. Conversely, this platform is not suitable for serum-free microtissue culture as the additives will adsorb to the PDMS, thereby hindering tissue health and development. In the future, integrating techniques such as 3D bioprinting and/or automated liquid handling will improve throughput in manufacturing hMMT cultures.

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DISCLOSURES:

The authors have no conflicts of interest to declare.

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Figure 1

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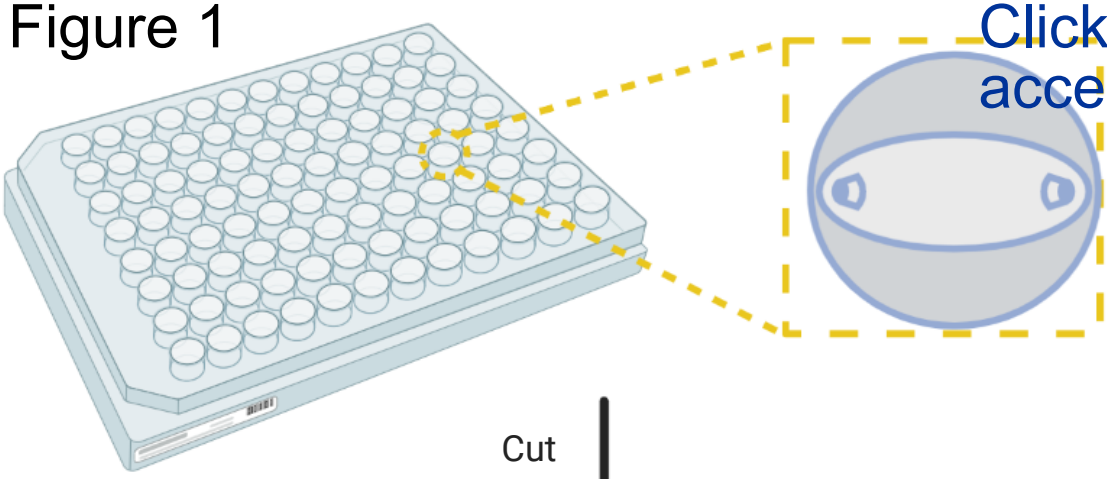
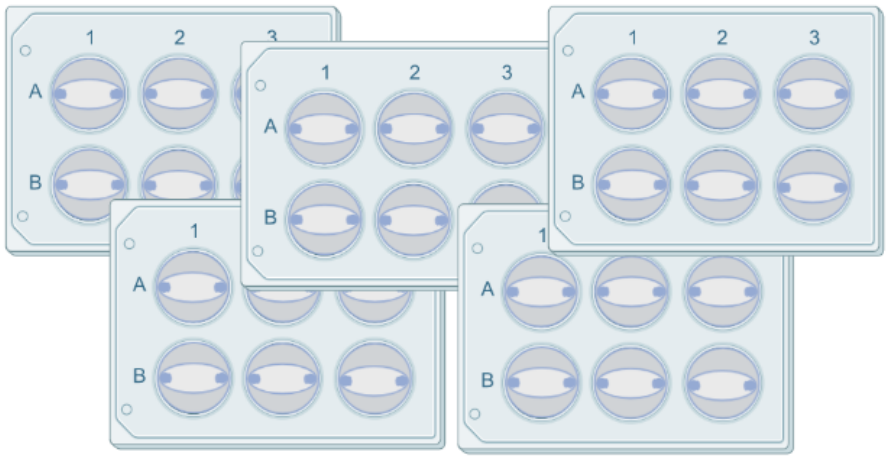


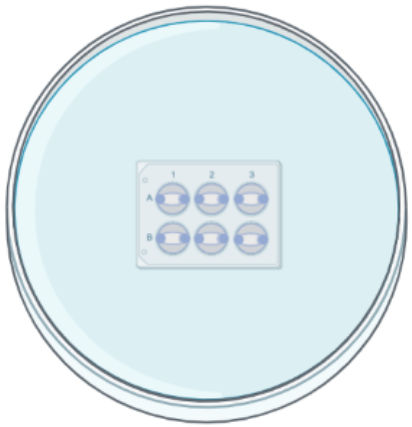
Final PDMS positive plate generated from negative PU mold

Cut



Cured PDMS plate is cut in 6 ± 2 wells

Autoclave



Cut wells are placed in culture dish and coated before seeding

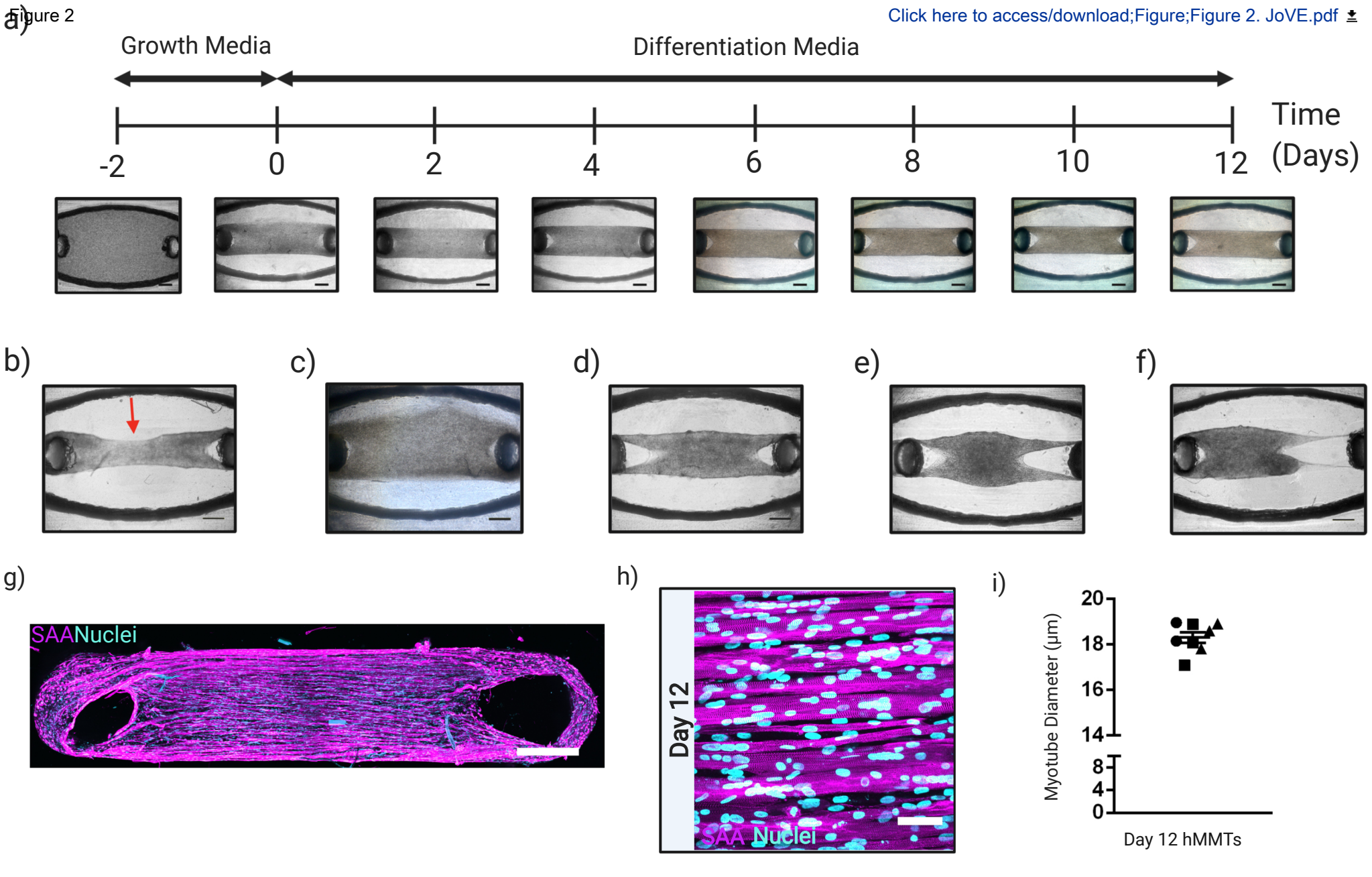


Figure 3

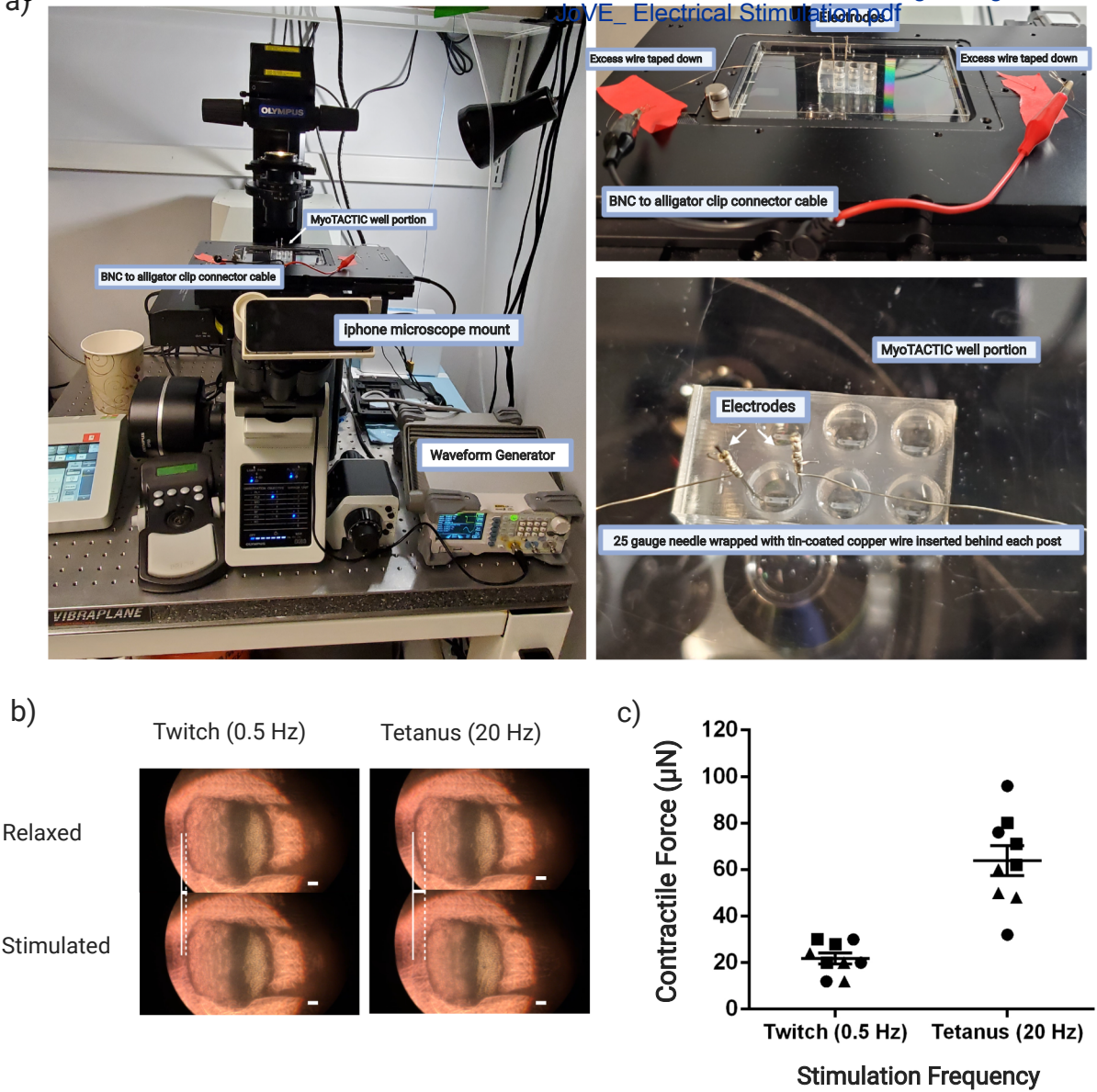
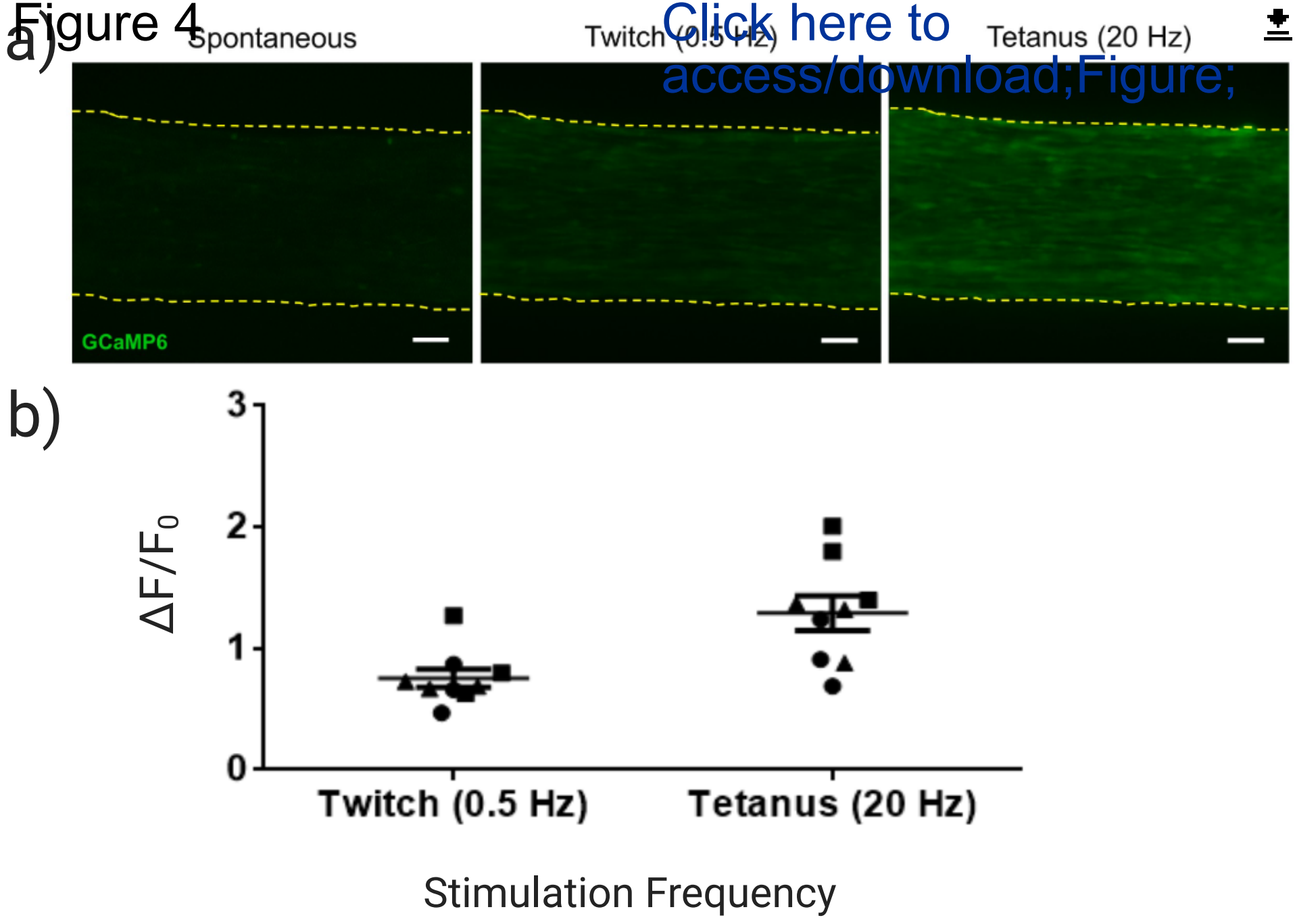


Figure 4



Supplemental Video Figure 1

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Video or Animated Figure

Supplemental Figure 1. Post_Deflection_Analysis.mp4

Click here to access/download

Video or Animated Figure

Supplemental Figure 2. Calcium_Handling_Analysis.mp4

Table of Materials

Click here to access/download;Table of Materials;JOVE_Materials List (1).xlsx

Name of Material/ Equipment	Company
0.9% Saline Solution, Sterile	House Brand
25G Needle	BD, Medstore, University of Toronto
6-Aminocaproic Acid, ≥99% (titration), Powder	Sigma - Aldrich
6.35 mm ID Tubing	VWR
AB1167 Myoblast Cell Line	Institut de Myologie (Paris, France)
Arbitrary Waveform Generator	Rigol
Basement Membrane Extract (Geltrex)	Thermo Fisher Scientific
Benchtop Vacuum Chamber	Sigma - Aldrich
BNC to Aligator Clip Cable	
Culture Plastics	Sarstedt
Dimethyl Sulfoxide	Sigma - Aldrich
DPBS, Powder, No Calcium, No Magnesium	Thermo Fisher Scientific
Dulbecco's Modified Eagle Medium (DMEM) (1X)	Gibco
Fetal Bovine Serum	Fisher Scientific
Fibrinogen from Bovine Plasma	Sigma - Aldrich
Filtropur Syringe Filter, 0.22um Pore Size	Sarstedt
Horse Serum	Gibco
Human Recombinant Insulin	Sigma - Aldrich
Image Acquisition Software	Olympus
Image Processing Software	National Institutes of Health
Isotemp Oven	Thermo Fisher Scientific
Microscope	Olympus
Microscope - Camera Mount	Labcam
Penicillin-Streptomycin (10,000 U/mL)	Gibco
Plastic Disposable Syringes, 1cc	BD
Plastic Disposable Syringes, 50cc	BD
Pluronic F-127, Powder, BioReagent	Sigma - Aldrich
Polydimethylsiloxane (Sylgard 184 Silicone Elastomer Kit)	Dow
Polyurethane Negative Mold	In House
Release Agent	Mann Release Technologies
Rotary Vane Vacuum Pump	Edwards
Scalpel	Almedic, Medstore, University of Toronto
Single Edge Razor Blade	VWR
Skeletal Muscle Cell Basal Medium	Promocell
Skeletal Muscle Cell Growth Medium (Ready-to-use)	Promocell
Smartphone (iPhone)	Apple

Standard Duty Dry Vacuum Pump	Welch
Sterilization Bag	Alliance
Thimble	Igege
Thrombin from human plasma	Sigma - Aldrich
Tin coated copper wire	Arco
Trypan Blue Solution, 0.4%	Thermo Scientific
Trypsin-EDTA, 0.25%	Thermo FIsher Scientific
Vacuum Chamber 2	SP Bel-Art

Catalog Number	Comments/Description
1010	10 mL aliquots of the solution are made and stored at 4°C
2548-CABD305127	
A2504-100G	A 50 mg / mL stock solution is generated by dissolving 5 mg of 6-aminocaproic acid powder in 100 mL of autoclaved, distilled water. The solution is vaccum filtered and 10 mL aliquots are stored at 4°C
60985-528	
DG1022Z	
A14132-02	Stored as aliquots of 50 µL or 100 µL at -80°C
D2672	
	Ordered from Amazon
	Includes culture plates, serological pipettes, etc
D8418-250ML	
21600069	
11995-065	This is a high glucose DMEM with L-glutamine and sodium pyruvate
10437028	
F8630-5G	Aliquots ranging from 7 - 10 mg of fibrinogen powder are made and stored at -20°C
83.1826.001	
16050-122	
91077C	Stock solution is 100X and made by dissolving 1 mg of human recombinant insulin in 1 mL of DMEM and 1 µL of NaOH 10N. Solution is filtered and stored as 1 mL aliquots at 4°C
cellSens Dimension	
ImageJ	
201	
IX83	
Labcam for iPhone	Ordered from Amazon
15140-122	
2606-309659	
2612-309653	
P2443-250G	A 5% stock solution of pluronic acid is made by dissolving 5 g of pluronic acid powder in 100 mL of chilled, autoclaved, distilled water. The solution is vaccum filtered and 10 mL aliquots are stored at 4°C
4019862	Kits are also available at Thermo Fisher Scientific, Sigma - Aldrich, etc.
200	
A65401906	
2586-M36-0100	
55411-050	
C-23260	30 mL aliquotes are generated and at stored at 4°C.
C-23060	42 mL aliquots are generated and stored at 4°C.
SE	

2546B-01	
211-SCM2	
	Ordered from Amazon
T6884-250UN	100 units of thrombin is dissolved in 1 mL of a 0.1% BSA solution. 10 µL aliquots are prepared and stored at - 20°C.
B8871K48	Ordered from Amazon
15250061	
25200072	
F42027-0000	

Response to Reviewers

Manuscript ID: JoVE62307
Title: Assessing functional metrics of skeletal muscle health in human skeletal muscle microtissues
Author(s): Heta L., Brennen M., Majid E., Penney M.G*.
Corresponding Authors: Penney M.G

Date of Initial Submission:

We thank the reviewers for their insightful comments and questions. We have addressed these comments in the revised version of the manuscript with tracked changes and have provided a specific set of responses (in *italicized* font) below. We believe these changes have improved the quality of the paper.

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Each author has conducted a thorough proofread of the manuscript and made edits as needed.

2. Please revise the following lines to avoid previously published work: 539-540, 1056-1058, 1096-1100)

We have revised the listed lines.

3. Please provide an institutional email address for each author.

We have included institutional emails for each author.

4. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

We have revised the manuscript to exclude personal pronouns.

5. JoVE cannot publish manuscripts containing commercial language. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials: e.g., MyoTACTIC, Apple, iPhone, LabCam, Rigol DG 1022Z, SolidWorks, Geltrex, etc. We must maintain our scientific integrity and prevent the subsequent video from becoming a commercial advertisement.

Thank you for your feedback. We have removed all instances of commercial language.

6. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

We have revised to ensure actions are described in the imperative tense.

7. For time units, please use abbreviated forms for durations of less than one day when the unit is preceded by a numeral, throughout the protocol. Do not abbreviate day, week, month, and year. Examples: 5 h, 10 min, 100 s, 8 days, 10 weeks

We have abbreviated all forms of duration that are less than one day.

8. Line 674-699: The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step.

The protocol has been simplified to include 2-3 actions per step.

9. Line 591/905: Please consider removing the links. Please cite the reference if required.

We have opted to keep the links in the manuscripts as it leads to a repository that contains valuable files for the scientist to complete this protocol.

10. Please include a one line space between each protocol step and highlight up to 3 pages of protocol text for inclusion in the protocol section of the video. This will clarify what needs to be filmed.

We have included a one line space between each protocol step and highlighted 3 pages of protocol text for inclusion in the video.

11. Please include any limitation of the presented protocol.

We now include limitations of the protocol in the discussion.

12. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or

.docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

All figures provided are original and have not been reused from previous publications.

13. Figure 3b/ 4A: Please include scale bars in all images of the panel.

Figure 4A and 3B have been updated to include scale bars in all images.

14. Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials.

We have removed these symbols from the Table of Equipment and Materials.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
This article propose a protocol for the creation of a 3D model of muscle with electrical stimulation.
The protocol is well described, clear, with a time window of the entire process. This reviewer does not have any major concerns.

Major Concerns:
None

Minor Concerns:
Please better specify how many cells are seeded inside each mold, in 1 single well. I suggest to add a note where authors evaluate cell vitality after seeding in the platform.
Please check for typos.

We have now specified in several places in the protocol how many cells are seeded inside a single well of a mold, and how one can test for cell viability post-seeding.

Reviewer #2:

I wish to congratulate the authors for a well written manuscript, clearly describing the methods and outlining steps that require attention. Only a few very minor details could be further clarified.
Page 3: step 3: define the desired vacuum (in bar)
Page 3: step 8: define the desired vacuum (in bar). Since another pump is applied than in step 3, is this a lower vacuum ?

We have edited the description by removing statements of vacuum pump power from both steps and instead define each compressor type. In addition, we have added notes to clarify the

goal of these vacuum steps and thereby make clear what one can expect if using lower vs higher vacuum pressures.

Page 4: step 11: with a new PU mold, what fraction of the 96 wells in the PDMS is fully functional (2 posts present) ? Typically, how often do PDMS post breaks off while removing the PDMS plate from the PU mold ? Or put differently, on average, how many wells become dysfunctional upon each new cycle of reusing the PU mold ? A solution for removing broken posts is suggested in the discussion (page 16, use of compressed air), it would be better to move this suggestion to the corresponding step on page 4.

Thank you for your feedback, we have addressed these questions in both the protocol and the discussion. Briefly, a PU mold will have up to 96 functional wells and this is dictated by a) the quality of the 3D printed device that is used to cast the PU mold, and the users' experience in casting. We have cast 3 PU molds in our lab, and have been using just a single one of these molds over the past 4 years to cast all of the PDMS devices we have used over the years (100s of plates). In our experience, the number of functional wells in the PDMS plates is very close to the number of defect-less wells in the PU mold. On occasion 1-2 wells of the PU mold will be plugged with PDMS, but in all cases, the rubber remnant can be removed, thereby restoring use of that well for subsequent PDMS casting steps.

Page 5 step 1: centrifugal force should be defined. If RPM is given, then also the diameter of the rotor must be given to define the force. Alternatively, force can be expressed as RCF.

We apologize for any confusion. We have updated the manuscript to express the force as RCF.

Page 6: step6: for ACA it would be more clear to indicate that % is v/v. (3% w/v would give a different concentration)

Thank you for raising this point, which we have now clarified within the seeding portion of the protocol.

Page 13: lines 1041-1044: references to figure 1 are probably figure 2?

We have edited this section to reference figure 2, which is indeed the correct image. Thank you for catching our error.

Page 14: legend figure 2g: it is not clear how the confocal image was acquired. Is this possible when the tissue is still present in the mold ? Or was the tissue removed from the mold ?

This is an excellent point and we now clarify this point within the figure legend.

Figure 3a: the microscopy setup is on top of a vibraplane table. Are any data available that would indicate additional noise when such table is not used ? If yes, it would be helpful to comment on this.

A vibraplane table is not required for this investigation and has been clarified in the text as thus.

Reviewer #3:

Manuscript Summary:

This article describes the steps for engineering 3-D muscle tissues in a multi-well plate format and quantifying contractile stresses and calcium transients. The fabrication techniques are simpler compared to other approaches, an advantage that makes this system relatively easy to adopt and thus of interest to the field. The authors also provide links to design files and analysis code, which will further help others adopt these techniques. The protocol is overall clear and detailed, although some parts need clarification or further discussion (see below).

Major Concerns:

The assembly and use of this is not clear: (Line 608) NOTE: Connect a p1250 μL pipette tip to a 50 mL syringe outfitted with a small piece of connector tubing and use to remove any remaining bubbles on the surface of the polymer mix as needed.

We agree that our description was confusing and have altered the note to make this clearer.

It would be helpful to briefly describe passaging procedures for the cells, or simply note if they are the same as the procedure describe in Line 749-752.

We have updated the text to include a note about passaging the cells.

At Line 857, it is not clear what the "clear bottom stage mount" is - is it simply a piece of glass or plastic?

The stage mount has now been specified in the protocol.

At Line 877, it is not clear how the thimble is used.

We have now updated the text to make it clear how and why the thimble is used for this protocol.

It would be helpful to note and reference other methods for calcium imaging (Fluo-4, etc.) because transduction with GCAMP is not always accessible for different users or cell types.

Thank you for your feedback, alternative methods for calcium imaging have now been noted and referenced in the revised manuscript.

The described electrodes require manual placement, which limits the use of this system for high content molecule testing applications, as mentioned in the Introduction. This limitation should be acknowledged in the Discussion, ideally with potential solutions.

Thank you pointing out this oversight on our part. The limitation you raised, as well as others have now been acknowledged in the Discussion together with possible solutions.

Minor Concerns:
Lines 1041-1044 should refer to Figure 2 not Figure 1.
Lines 1051 should refer to Figure 3b and 1054 to Figure 3c.
In the figures, I assume the circles, squares, and triangles refer to biological replicates, but this should be explicitly stated in the captions.

Thank you for catching our errors, this is greatly appreciated. All minor concerns have been addressed in the revised submission.