Title: Automated, high temporal resolution mechanics measurements during incubation of contractile tissues.

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Keywords: Muscle Mechanics, Tissue Incubation, Equipment development, Smooth Muscle, Mechatronics

Wordcount: 3155

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Summary

Muscle tissue mechanics and contractility measurements have a great advantage over cultured cell level experiments as their mechanical and contractile properties are much closer to *in vivo* tissue properties. However, tissue level experiments cannot be combined with incubation with the same time resolution and consistency as cell culture studies. Here we present a system in which contractile tissues can be incubated for days while intermittently being tested for their mechanical and contractile properties. A two-chamber system was developed with control of temperature in the outer chamber and CO_2 and humidity control in the inner, sterile chamber. Incubation medium, to which biologically active components may be added, is reused after each mechanics test to preserve both added and released components. Mechanics and contractility are measured in a different medium to which, through a high accuracy syringe pump, up to 6 different agonists in a 100-fold dose range can be added. The whole system can be operated through fully automated protocols from a personal computer. Testing data shows accurate maintenance of temperature, CO_2 and relative humidity at pre-set levels. Equine trachealis smooth muscle tissues tested in the system showed no signs of infection after 72 hours with incubation medium replacement every 24 hours. Methacholine dosing and electrical field stimulation every 4 hours showed consistent responses. In conclusion, the developed system is a great improvement on the manual incubation techniques being used thus far, improving on time resolution, repeatability and robustness, while reducing contamination risk and tissue damage from repeated handling.

Introduction

In muscle physiology, changes in contractile mechanics can occur in response to biological interactions with cytokines, immune cells and other exposures, and these effects can appear and disappear at varying timescales (Matusovsky et al. 2014; Mitchell et al. 1994; Farghaly et al. 2008). Recent advancements in imaging technology and automation have allowed for high temporal resolution live cell measurements of anything that can be fluorescently labelled or imaged in brightfield (see (Nketia et al. 2017) for review). These measurements have revealed that the traditional 24h interval measurements of incubated cells often missed important dynamics in the cell responses. Gel contraction assays (Ngo et al. 2006) and traction force microscopy (Style et al. 2014) have also added the ability to measure contractile force in these studies. However, in contractile tissues, in particular in smooth muscle, cell culture studies show contractile stresses that are 2 (3D culture, ~2kPa (West et al. 2013)) to 3 (2D culture, ~100Pa (Park et al. 2015)) orders of magnitude smaller than in vitro tissue contractile stresses (30-200kPa, (Borsdorf et al. 2019; Ijpma et al. 2015; Matusovsky et al. 2015)). This change in phenotype in cell culture is likely caused by a dramatic loss of contractile proteins in culture, as shown in (Chamley-Campbell, Campbell, and Ross 1979; Arafat et al. 2001). This indicates that cultured smooth muscle cells are not an ideal model of in vivo mechanical and contractile behaviour. Thus, when studying smooth muscle contractile mechanics, intact tissue preparations provide the best in vitro approximation of in vivo contractile behaviour.

Tissue mechanics studies are performed with inherently lower throughput than cell studies, but recent advancements in cryostorage (Ijpma et al. 2018) now allow testing the effects of incubation with numerous biological mediators on tissues from the same source. This has also enabled the use of human tissues for coincubation studies with immune cells or serum from the same subject, even when isolation protocols for these take longer than the tissues can otherwise be kept alive. Current methods for combining measurements of contractile force and incubation require tissues to be physically moved from an incubator to a mechanics set-up and back (Matusovsky et al. 2014; Matusovsky et al. 2019), which may reduce tissue viability, risks introduction of contaminants and limits the temporal resolution that can be reasonably achieved. Thus, ideally, such measurements would be performed in a single system that combines incubation and mechanics assessment, without external interference.

In this paper we present such a system in which multiple contractile tissues can be tested in parallel for their contractile properties, at regular intervals, in a stable and sterile incubator environment, and in a fully automated manner, allowing for prolonged incubation with biological mediators. We tested the viability of the system with cryostored tissues to demonstrate the utility of this methodology for repeated analysis of tissues from the same source/subject under varied conditions.

Equipment

Environment Separation

[insert figure 1]

When combining incubation with measuring contractile properties of tissues, one must merge a sterile, high humidity, high CO_2 environment for the tissue, with sensitive electronics to measure force and control tissue length. The humidity must be carefully controlled to minimize evaporation of incubation medium while preventing condensation and subsequent corrosion or shorting electrical components. To minimize exposure of sensitive electronics to the high humidity environment of the tissue incubator, environmental separation was implemented, separating the temperature control from the humidity and CO_2 control by creating two nested chambers (Fig.1A). The outer enclosure is temperature regulated using positive temperature coefficient (PTC) heating elements (Walfront 110V-250W PTC thermistor heater element) and a temperature signal from a thermistor element in a proportional-integral-derivative (PID) loop (Auber Instruments WS-1510EBPM). Incubation media and liquid pumps as well as their control electronics reside in this temperature-controlled enclosure. The inner chamber (Fig. 2 item 1) is heated primarily by heat transfer through its stainless steel main structural element, an L bracket (Fig. 1D), stabilizing the incubator temperature and avoiding noise and contamination risks associated with the high air flow rates required for accurate temperature control. This inner chamber is attached with hinges to a stainless-steel base plate containing the fluid manifolds, which rests on passive noise dampening feet (Newport VIB100-0512 5-12) to isolate the tissues from further noise generated by the environment, pumps and fans. The inner chamber, which contains the tissue bath fluid manifolds, force transducers and length actuators (Fig. 2, items 4, 5 and 6 respectively), receives low air flow circulation for humidification and CO_2 control. This air flow contains ~90% relative humidity, providing a balance between allowable evaporation of medium and risk of condensation on the electronics. CO_2 is controlled using CO_2 readings from inside the incubator (Gas Sensing Solutions, ExplorIR-WH-20) and a solenoid valve connected to an external CO_2 tank. The chamber is sealed by a glass door on rubber seals.

[insert figure 2]

Tissue Bath and Fluid Control

The tissue is held between a force transducer (Aurora Scientific 400A) and a length actuator (Aurora Scientific 322C) to which hooks are attached (Fig. 1B). Small tissue strips can be clamped in aluminum or stainless-steel foil clips which have a prefabricated hole for mounting on the hooks. Larger tissues could be placed directly on the hooks and vascular rings or small airways could slide right over the hooks. Platinum electrodes are attached to the tissue bath walls on either side of the tissue. A second set of platinum electrodes are mounted just above the bath on either side (Fig 1B, #4) to detect floods, as a safety measure in case of malfunction of fluid level control, through changes in resistance between the two electrodes.

Tissue contraction testing requires regular refreshing of the bathing medium as nutrients are consumed during muscle activation. Tissue contraction set-ups therefore either use small constant flow-through baths or large baths where the entire medium is replaced at regular intervals. In contrast, incubation of muscle tissues does not usually require regular refreshing of the incubation medium beyond a once-a-day replacement for nutrient replenishment. Depending on the experiment, serum as well as whichever biological mediators are being tested, might be added to the incubation medium. These ingredients can be expensive and thus the medium is preferably reused between contractility measurements. Furthermore, the tissues or co-incubated cells might release cytokines, vesicles etc. that might modulate the tissue response and need to remain in the medium for optimal effect. To recycle the incubation medium, tissue bath manifolds were created with 3 fluid streams: incubation medium, mechanics measurement medium, and waste flow (Fig. 1B). The incubation medium is stored in syringes on individual custom syringe pumps (Progressive Automations, PA-07-2-5) inside the outer enclosure to allow two-way flowing of incubation medium in and out of the tissue baths (Fig. 1C). Mechanics medium (for the mechanics medium used, see “Experiments”) is flown into the bath continuously during contractility measurements at the same rate as the waste is flown out. Both waste and mechanics medium are pumped with stepper motor driven peristaltic pumps (Welco, WPM-P1EA-WP) to avoid liquid contact with pump parts while maintaining continuous flow ability. To assure a constant fluid level in the bath for the mechanics medium, ultrasonic distance transducers (Microsonic, zws-15) with focus tubes are placed above each bath to measure the fluid level constantly, providing a feedback signal for the waste pump (Fig. 1B, Fig. 2 item 2). The fluid level sensing is used for the incubation medium as well to assure the bath is fully filled when needed, and compensate for potential evaporation. To minimize the number of required pumps, one mechanics medium pump and one waste pump provide flow for all 4 baths, with the baths selected by an in- and an out flow-valve. Preliminary testing showed that solenoid valves introduce electromagnetic fields at the moment of switching that affect the sensitive electronics of the force transducers and length actuators. Shape memory valves (Takasago Fluidics, SMV-2R-BNF1) eliminate this problem as these valves operate more gradually by passing a small current through a shape memory wire, resulting in rapid shrinking of the wire which opens or closes the valve, depending on the type used.

Fluid overflow protection in case of failures in the fluid level maintenance electronics or control is provided by two flood sensor electrodes. When fluid starts cresting above the tissue bath, the two flood sensor electrodes will short and signal to a microcontroller to stop inflow to this bath and start outflow until the short is cleared.

The fluid manifolds are 3D printed using a biocompatible resin (Formlabs Biomed Amber v1), connected to the fluid lines with Idex flangeless fittings. A 1mm undercut ridge was designed at the base of the

bath (Fig. 1B) to act as a channel to assure no fluid remains in the edges of the bath as a result of surface tension when the bath is emptied between switching solutions. An elevated entry for the mechanics medium is used to enhance solution mixing in the bath. Earlier iterations of the bath design showed layering of the solutions in the bath with in and outflow in plane at bottom of the bath, visualized by adding food colouring to the solutions. .

To reduce the risk of contamination of tissues the mechanics medium is flown through quartz tubes inside a UV sterilizing chamber.

Dose control

To stimulate the tissue chemically a stepper motor-controlled syringe pump coupled with a rotational 9 port selector valve is used (Fig. 1C,(Cavro Centris syringe pump, 250µL glass syringe, 9 port ceramic selector valve)). 6 lines of the valve can be connected to different chemical stimulants, while one line connects to the waste flow, one to the mechanics medium flow and one to a reservoir with deionized (DI) water for cleaning. A 250µL glass syringe allows for precise control of 1 to 1000 µL per minute. When switching from one chemical stimulant to another, the syringe and the PolyEther Ether Ketone (PEEK) fluid line connecting to the mechanics medium flow need to be rinsed with DI water repeatedly to avoid mixing of the current with the previous stimulant.

Software and Control

The system is controlled by a central personal computer that connects to 3 control units: an Aurora 850A system, an internal and an external Printed Circuit Board (PCB). The Aurora 850A system controls length, provides electrical field stimulation (EFS) and performs signal processing of force and length signals. The internal PCB uses a Teensy 4.1 (PJRC) to control the peristaltic pumps (Fig. 1C, In and Out) and bath lighting for visibility during tissue mounting, switches the shape memory valves and reads the ultrasonic distance transducers, the flood sensors and the relative humidity and temperature signals. The external PCB uses a Teensy 4.0 to control the medium syringe pumps, the CO_2 solenoid valve and reads the CO_2 sensor. The PC runs an inhouse developed visual C# windows application to communicate with the two Teensies, the Aurora 850A system and the dosing syringe pump.

The custom C# software reads all the signals, displays force and length data and allows direct manual as well as fully automated control of all parameters. A protocol creator allows for the generation of protocols on a 24h continuous repeat that can control parameters for individual baths allowing different protocols to be performed automatically on each of the baths. Because of the use of a single mechanics medium pump with switching valves to each bath, only one bath can receive fluid control at any one time. However, to protect sensitive electronics, if a flood is detected in any one bath, flow is stopped immediately and the flooded bath is emptied, regardless of whether that bath was under fluid control at the time.

Experiments

Experiments were performed on equine trachealis smooth muscle tissues procured from the Faculty of Veterinary Medicine, University of Montreal (St.-Hyacinthe, PQ, Canada). All procedures were approved by the Animal Care Committee of the University of Montreal (Protocol Rech-1324) and complied with the guidelines of the Canadian Council on Animal Care. Smooth muscle tissues with epithelium and connective tissue were dissected in Hanks Balanced Salt Solution (composition in mM: 5.3 KCl, 0.44 KH_2PO_4, 137.9 NaCl, 0.336 Na_2PO_4, 2.33 $CaCl_2$, 0.79 $MgSO_4$, 10 glucose, 10 HEPES buffer, pH adjusted to

7.4 with NaOH) on ice and cryostored according to (Ijpma et al. 2018). After thawing, the tissues were placed in ice cold calcium free Krebs-Henseleit (KH) solution (composition in mM: 110 NaCl, 0.82 $MgSO_4$, 1.2 KH_2PO_4, 3.4 KCl, 25.7 $NaHCO_3$, and 5.6 glucose, pH at 7.4, aerated with 95/5% O_2/CO_2 gas mixture) and the epithelium and connective tissue were removed. Small strips (~5x1x0.5mm) were attached to stainless steel foil clips (Aurora Scientific 810A-1), pinned at a fixed length and placed overnight in Dulbecco's Modified Eagle's Medium(DMEM) with 2% Fetal Bovine Serum (FBS) and 1% Penicillin-Streptomycin-Amphotericin (PSA) in an incubator to facilitate tissue recovery from dissection and cryostorage and to reduce the risk of contamination in the system (as in (Ijpma et al. 2018)). The next day the tissues were mounted at the same length in the four tissue baths. During mechanics measurements, 1ml/min of mechanics medium (KH solution,as above + 2.4mM $CaCl_2$) was flown into the tissue baths, while during incubation the tissues were submerged in DMEM with 2% FBS and 1% PSA.

Cleaning

Tissue baths and fluid lines are cleaned by, sequentially, flushing 10% acetic acid to dissolve salts, DI water, 3% hydrogen peroxide to sterilize and again DI water. The dosing syringe pump and PEEK fluid lines are cleaned by flushing with a 10% bleach solution to sterilize, followed by DI water. Internal surfaces are cleaned with a 3% hydrogen peroxide solution. Electronics inside the incubation chamber can be removed to be sterilized with ethylene oxide and sterilizing UV light strips inside the incubation chamber are used to decontaminate surfaces during cleaning protocols.

Results

Several experiments were run to test the stability and control of the system. To test the environmental control, the system was run without tissues. Temperature, relative humidity and CO_2 levels (Fig. 3A-C) were recorded. Temperature inside the incubation chamber showed a slight overshoot prior to equilibration of the entire system at the set point of 37°C, with stabilization at around 1h after turning on the temperature control. As the tissues should not be placed inside the system until after the temperature has equilibrated, this overshoot is not an issue. Relative humidity showed a two-phase response: an initial rapid rise to an initial plateau humidity level, followed by a slower increase towards a second plateau. The second plateau is delayed due to the water in the humidity reservoir starting at room temperature, taking approximately 45 min to increase to the incubator temperature. CO_2 equilibrated rapidly, but with a sawtooth pattern caused by excess pressure in the supply line. A lower supply pressure will decrease the amount of CO_2 injected once CO_2 levels drop below a set value and reduce the sawtooth pattern amplitude.

[insert figure 3]

To test the performance of the tissue incubator, equine horse trachealis tissues were placed in the system after overnight incubation. All experiments were performed isometrically. Once mounted, the chambers were sealed, and CO_2 control and humidity supply were turned on. To stabilize the response, the tissues were first kept in mechanics medium at 1 ml/min and contracted, repeatedly at 55-minute intervals using 10^{-5}M methacholine (MCh). After each tissue was contracted three times the system was set to a fixed protocol in which every 12 hours each tissue was tested for its EFS response (10s, 50Hz, 2ms pulse width, 15 V cm^{-1}) followed by MCh dose response, exposed to doses between 10^{-7}M to 10^{-4}M with half log intervals, followed by a single dose of 10^{-6}M isoproterenol (Iso) to measure relaxation response to a broad bronchodilator. The same protocol was repeated without the Iso dose with a 6h offset, every 12h to compare the Iso response to relaxation from washing out the MCh. Every 3 hours a

single EFS contraction and a single MCh contraction (10^{-5} M) was applied for a finer time resolution of contractile response without risking saturation or fatigue issues from repeated Isoproterenol exposures. Dose response traces from one representative bath are shown in figure 3D. Forces are shown in mN. With the estimated tissue cross-section and previously established approximate smooth muscle content of these tissues at 60% (Matusovsky et al. 2015) the maximum contractile stress in the tested tissues varied from 32-60 kPa, similar to values reported for these tissues in (Matusovsky et al. 2015). This particular protocol resulted in gradual decrease of the maximal force response over the 64 hours of the experiment, which indicates the need for further optimization of protocols for specific experimental needs (FBS concentration, frequency of contraction repetitions, other exposures, medium changes). Analyzed dose response curves (Fig. 3E) further show this gradual decrease in force. EFS force response drops more rapidly over time (Fig. 3F), which is to be expected as the nerve cells responsible for these contractions degrade fast and the medium used is not optimized for their survival (Paynter 2008). Additionally, the 10s of stimulation at below maximal response levels might also be affected more by a change in sensitivity of the muscle. As the peak force is below the half maximum force for MCh at 0h, the effects of a shift in sensitivity and reactivity are compounded for those experiments. The response to Iso (Fig. 3G) remained stable with the protocol used.

Discussion

Here we have shown the feasibility of a 4-bath tissue incubation and mechanics system for the measurement of the evolution of mechanical properties during prolonged incubation of contractile tissues. The proposed equipment is capable of accurately controlling temperature, humidity and CO_2 while minimizing exposure of sensitive electronics. We've shown accurate control of dosing, allowing up to 6 chemical stimulants in a 100-fold dose range each. Tissues were shown to remain free of signs of contamination (cloudy or discolored medium, no visible growth under microscope inspection) for at least 5 days, with daily incubation medium changes.

The primary reason tissue mechanics measurements systems are not usually placed in incubators is the risk of damage to sensitive electronics caused by the high humidity environment of the incubator. Medium evaporation and incubation chamber humidity tread a tricky balance between the risk of reduced tissue contractility from over-concentration of salts in the medium and damage to electrical components from high humidity and condensation. The sensitive force transducers used can drift considerably with variations in relative humidity, particularly for relative humidity values close to saturation. Additionally, opening the incubation chamber after or during an experiment lets in cold air that may result in cooling of the force transducer housing at a rate faster than the humid air inside the transducer can escape, resulting in condensation on the inside of the transducer. This may be mitigated by bypassing the humidifier circuit prior to opening the chamber and injecting the dryer air from the enveloping chamber. However, higher humidity values also increase chances of contamination as more favorable conditions for bacterial growth will exist on all surfaces in the incubator. An alternative approach is to accept a degree of evaporation or to compensate for evaporation by injecting DI water into the bath to dilute the medium back to its original level. This could be achieved using the dosing syringe pump.

Preliminary experiments showed a risk of cross contamination stimulants used in previous protocols when there were insufficient washing cycles of the syringe and tubing. Filling the syringe with DI water

after the washing cycles and keeping it filled until the next dose response measurement reduced this issue substantially. This indicates that some of the high dose stimulants get trapped either in the tubing connectors or the syringe pump and selection valve, which is then diluted by a prolonged soaking in DI water.

While not demonstrated here, the dosing syringe pump can also be used to sample the incubation medium throughout the experiment to measure components released by the tissue or any co-incubated cells. Repeated filling of the dose syringe pump from line 9, the mechanics solution line, and emptying to line 1, the waste line will bring incubation medium into the syringe. The syringe contents can subsequently be emptied through an unused line of the selector valve into a reservoir for analysis, all without breaking the seals on any of the chambers.

Conclusions and future directions

The muscle mechanics measurement and incubation system developed here is a great improvement on the manual incubation techniques being used thus far, improving on time resolution, repeatability and robustness, while reducing contamination risk and tissue damage from repeated handling. This opens up the field of muscle mechanics to a much wider array of incubation and co-incubation studies.

Acknowledgements:

The authors thank Dr. J.-P. Lavoie (U. de Montreal) for procurement of equine trachealis tissue. This work was supported by the Natural Sciences and Engineering Research Council of Canada and Aurora Scientific Inc. through the Collaborative Research and Development grant CRDPJ-519929-17 for the Lauzon Lab.

Disclosure:

McGill University has signed a revenue sharing agreement with Aurora Scientific Inc. for the commercial development of a system derived from the one presented here, with Gijs Ijpma and Anne-Marie Lauzon as primary beneficiaries.

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Figure 1:

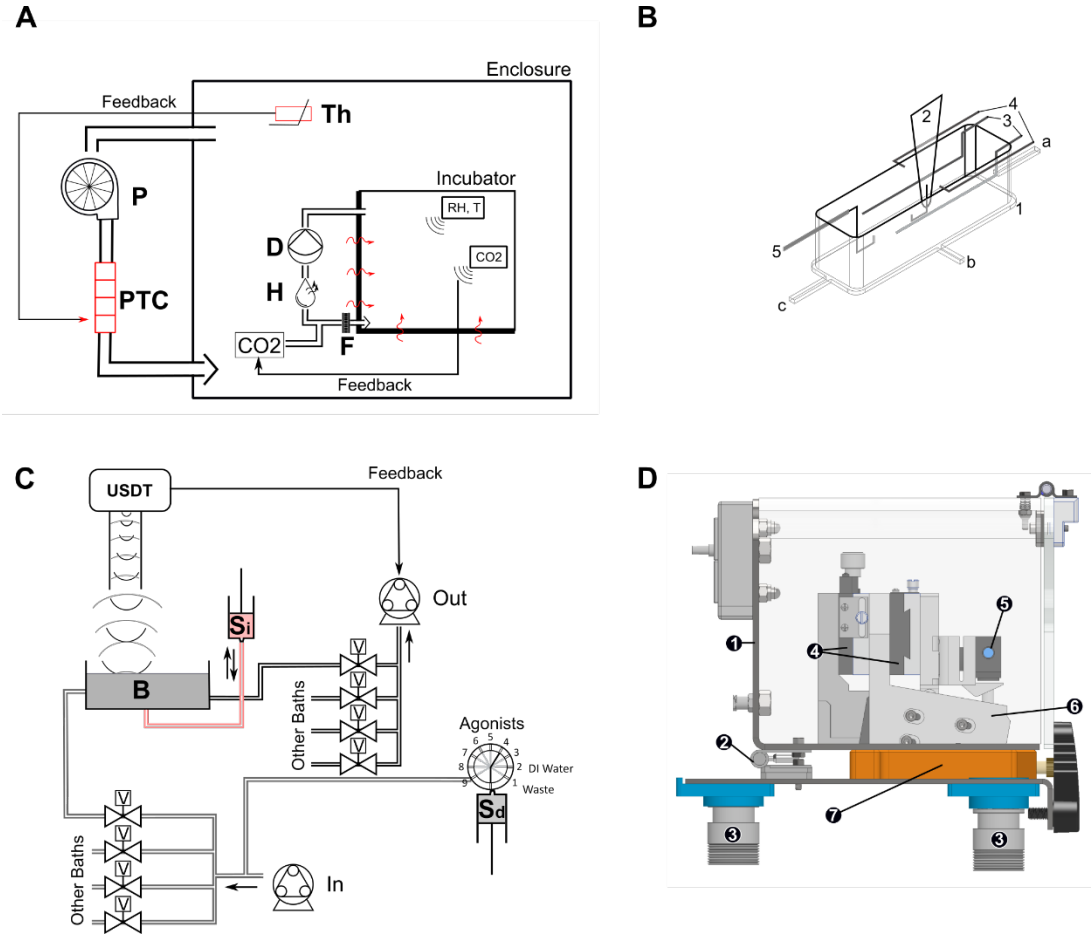

Figure 1: A) *Environmental control separation. Incubator is placed inside a temperature-controlled enclosure. Air is pumped out of the enclosure by blower pump P, passed through Positive Temperature Coefficient (PTC) heating element regulated by a thermistor (Th) signal and fed back into the enclosure. A separate air circuit pumps air with a diaphragm pump (D) from the incubator through a humidification stage (H). Subsequently CO_2 is injected based on CO_2 level measurement inside the incubator and the air is filtered (F) before returning to the incubator chamber. Air inside the incubator is heated indirectly through conduction of the stainless-steel L bracket.* ***B)*** *Tissue bath overview. 1: undercut ridge to enable complete draining of bath. 2: length actuator lever. 3: electric field stimulation electrodes. 4: overflow sensor electrodes. 5: force transducer hook. a, b, c, inflow, medium flow and outflow ports respectively.* ***C)*** *Schematic of fluid control. Peristaltic inflow (In) and outflow (Out) pumps control solution level in bath (B), using a feedback fluid level signal from the ultrasonic distance transducer (USDT). Flow to and from individual baths is controlled by valves (V). Agonists can be injected with Dose Syringe pump (Sd) connected to a 9-port rotary selector valve. Each bath is also connected to a syringe pump for incubation medium (Si) supply and storage.* ***D)*** *Side view of incubator subsystem. 1: stainless steel L bracket. 2: hinge allowing access to tissue baths. 3: passive noise dampers. 4: vertical and horizontal translators for tissue positioning. 5: ultrasonic distance transducer. 6: force transducer holder. 7: fluid manifold.*

Figure 2:

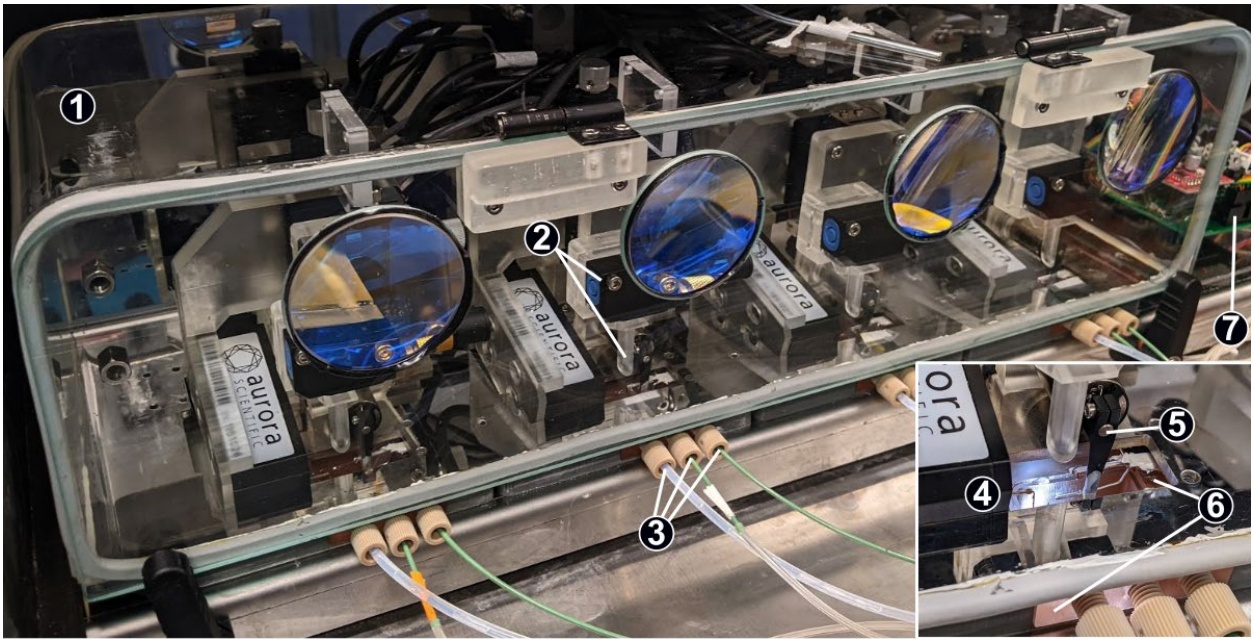

Figure 2: System overview. Inset: muscle bath. 1: Incubation chamber; 2: Ultrasonic distance transducer and focus tube; 3: Fluid connectors (left-to-right: waste, incubation medium and mechanics medium; 4: Force transducer; 5: Length actuator; 6: Fluid manifold; 7: Controller board.

Figure 3:

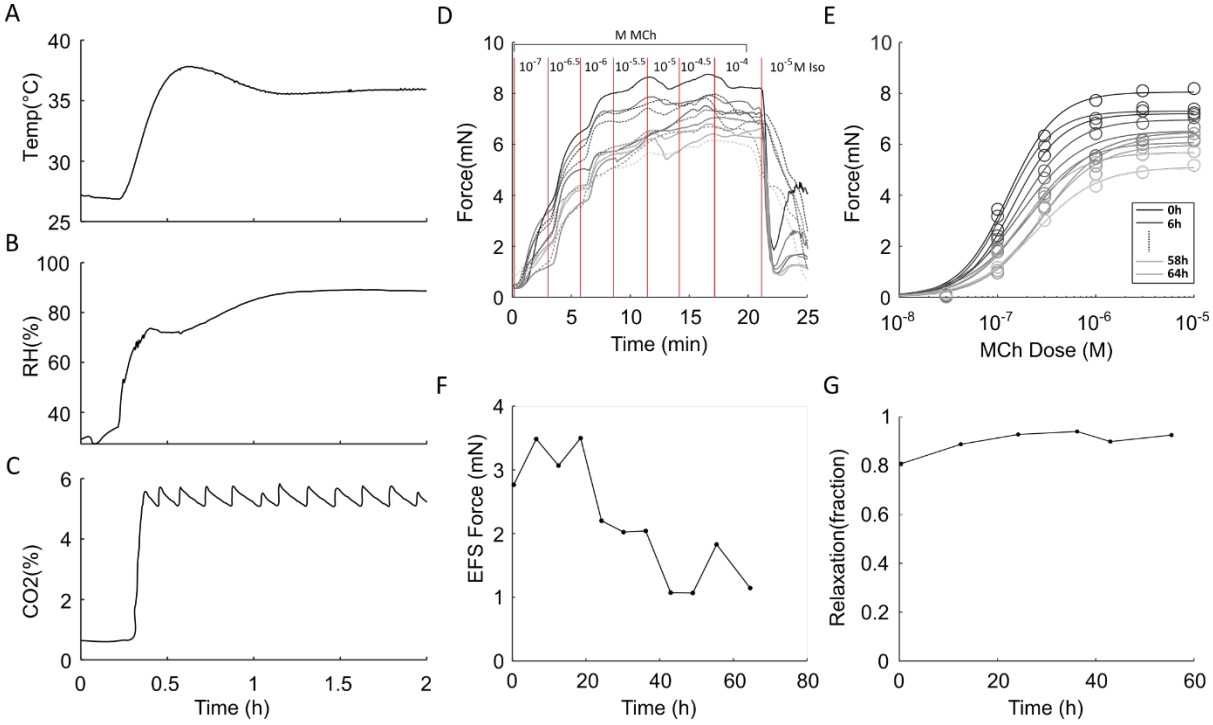

Figure 3: Representative sample data. A-C: environmental data to illustrate system response time. A) Temperature data: temperature controller turned on at 0.25h. B) Relative humidity data: water added to humidity reservoir at 0.25h. C) CO_2 data: CO_2 control turned on at 0.3h. D-G 64 hour run with methacholine (MCh) dose response and Electrical Field Stimulation (EFS) every 6 hours and Isoproterenol (Iso) relaxation response every 12h. D) MCh Dose response traces. Solid lines were exposed to Isoproterenol at 21 min, dashed lines did not see Iso exposure. E) Analyzed MCh dose response data. F) EFS force over time. G) Relaxation in response to Iso as a fraction of contractile force.