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Engineering 3D cellularized collagen gels for vascular tissue regeneration

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Corresponding Author:	Diego Mantovani, Ph.D. Laval University Quebec city, Quebec CANADA
Corresponding Author Secondary Information:	
Corresponding Author E-Mail:	diego.mantovani@gmn.ulaval.ca
Corresponding Author's Institution:	Laval University
Corresponding Author's Secondary Institution:	
First Author:	Sebastien Meghezi
First Author Secondary Information:	
Other Authors:	Sebastien Meghezi
	Dawit Seifu
	Nina Bono
	Larry Unsworth
	Kibret Mequanint
Order of Authors Secondary Information:	
Abstract:	Synthetic materials are known to initiate clinical complications such as inflammation, stenosis, and infections when implanted as vascular substitutes. Collagen has been extensively used for a wide range of biomedical applications and is considered a valid alternative to synthetic materials due to its inherent biocompatibility (ie. low antigenicity, inflammation, and cytotoxic responses). However, the limited mechanical properties and the related low hand-ability of collagen gels have hampered their use as scaffold materials for vascular tissue engineering. Therefore, the rationale behind this work was first to engineer cellularized collagen gels into a tubular-shaped geometry and second to enhance SMCs-driven reorganization of collagen matrix to obtain tissues stiff enough to be handled. The strategy described here is based on the direct assembling of collagen and smooth muscle cells (construct) in a 3D cylindrical geometry with the use of a molding technique. This process requires a maturation period, during which the constructs are cultured in a bioreactor under static conditions (without applied external dynamic mechanical constraints) for 1 or 2 weeks. The "static bioreactor" provides a monitored and controlled sterile environment (pH, temperature, gas exchange, nutrient supply and waste removal) to the constructs. During culture period, thickness measurements were performed to evaluate the cell-driven remodeling of the collagen matrix, and glucose consumption and lactate production rates were measured to monitor the cells metabolic activity. Finally, mechanical and viscoelastic properties were assessed for the resulting tubular constructs. To this end, specific protocols and a focused know-how (manipulation, gripping, working in hydrated environment, and so on) were

	developed to characterize the engineered tissues.
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Cover Letter

UNIVERSITÉ LAVAL

Canada Research Chair in Biomaterials and Bioengineering for the Innovation in Surgery
Laboratory for Biomaterials and Bioengineering
Faculty of Science and Engineering

Quebec City, Oct 4th, 2014

Eric Veien, Ph.D.
Science Editor - Bioengineering
JoVE
1 Alewife Center, Suite 200, Cambridge, MA 02140

Subject: Submission/Invited JoVE manuscript

Dear Dr Veien:

Following our correspondence and phone exchanges, please find enclosed the manuscript I am submitting today on behalf of co-authors.

The manuscript is absolutely original, it has never been submitted to any other journal, and actually was prepared only for this submission.

I suggested 6 reviewers, unbiased and without any conflict of interest. Concerning the videos, they are available online, and we provided a blind link (not available to general public).

I am looking forward to receive news from your side.

Please feel free to contact me for any information you might require.

Thanks for this opportunity to submit a visually-oriented manuscript, which I found very suitable in the field of Bioengineering & Regenerative Medicine.

With my highest regards,

Diego Mantovani, PhD, FBSE
Director, Lab Biomaterials and Bioengineering
Holder, Canada Research Chair I
Adjunct Director, Regenerative Medicine Division

Pavillon Adrien-Pouliot, 1745E
1065, avenue de la Médecine
Québec (Québec) G1V 0A6
CANADA

+1 418 656-2131 poste 6270
Télécopieur : + 1 418 656-5343
Diego.Mantovani@gmn.ulaval.ca
www.lbb.ulaval.ca ; www.iBiomat.ulaval.ca

TITLE:
Engineering 3D cellularized collagen gels for vascular tissue regeneration

AUTHORS:
Sébastien Meghezi
Laboratory for Biomaterials and Bioengineering
Department Min-Met-Materials Eng & *CHU de Québec* Research Center
Canada Research Chair I for the Innovation in Surgery
Laval University
Quebec City, Canada
sebastien.meghezi.1@ulaval.ca

Dawit G. Seifu
Laboratory for Biomaterials and Bioengineering
Department Min-Met-Materials Eng & *CHU de Québec* Research Center
Canada Research Chair I for the Innovation in Surgery
Laval University
Quebec City, Canada
dawit-gezahegn.seifu.1@ulaval.ca

Nina Bono
Laboratory for Biomaterials and Bioengineering
Department Min-Met-Materials Eng & *CHU de Québec* Research Center
Canada Research Chair I for the Innovation in Surgery

Department Electronics, Information and Bioengineering
Politecnico di Milano
Milan, Italy
nina.bono@polimi.it

Larry Unsworth
Department of Chemical and Materials Engineering
University of Alberta
Edmonton, Alberta, Canada

National Institute for Nanotechnology
National Research Council (Canada)
Edmonton, Alberta, Canada
lunswort@ualberta.ca

Kibret Mequanint
Department of Chemical and Biochemical Engineering
University of Western Ontario
London, Ontario, Canada

kmequani@uwo.ca

Diego Mantovani
Laboratory for Biomaterials and Bioengineering
Department Min-Met-Materials Eng & *CHU de Québec* Research Center
Canada Research Chair I for the Innovation in Surgery
Laval University
Quebec City, Canada
Diego.Mantovani@gmn.ulaval.ca

CORRESPONDING AUTHOR:
Diego Mantovani, Ph.D., FBSE.

KEYWORDS:
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SHORT ABSTRACT:
In this work, we present a technique for the rapid fabrication of living vascular tissues by direct culturing of collagen, smooth muscle cells and endothelial cells. In addition, a new protocol for the mechanical characterization of engineered vascular tissues is described.

LONG ABSTRACT:
Synthetic materials are known to initiate clinical complications such as inflammation, stenosis, and infections when implanted as vascular substitutes. Collagen has been extensively used for a wide range of biomedical applications and is considered a valid alternative to synthetic materials due to its inherent biocompatibility (i.e., low antigenicity, inflammation, and cytotoxic responses). However, the limited mechanical properties and the related low hand-ability of collagen gels have hampered their use as scaffold materials for vascular tissue engineering. Therefore, the rationale behind this work was first to engineer cellularized collagen gels into a tubular-shaped geometry and second to enhance SMCs-driven reorganization of collagen matrix to obtain tissues stiff enough to be handled.

The strategy described here is based on the direct assembling of collagen and smooth muscle cells (construct) in a 3D cylindrical geometry with the use of a molding technique. This process requires a maturation period, during which the constructs are cultured in a bioreactor under static conditions (without applied external dynamic mechanical constraints) for 1 or 2 weeks. The “static bioreactor” provides a monitored and controlled sterile environment (pH, temperature, gas exchange, nutrient supply and waste removal) to the constructs. During culture period, thickness measurements were performed to evaluate the cell-driven remodeling of the collagen matrix, and glucose consumption and lactate production rates were measured to monitor the cells metabolic activity. Finally, mechanical and viscoelastic properties were assessed for the resulting tubular constructs. To this end, specific protocols and a focused know-how (manipulation, gripping, working in hydrated environment, and so on) were

developed to characterize the engineered tissues.

INTRODUCTION:

Vascular tissue engineering envisions different strategies aimed at the fabrication of engineered vessels, including grafts based on synthetic scaffolds, cell sheet-based tissue-engineered blood vessels (TEBVs), and extracellular matrix (ECM) components-based TEBVs. Among these approaches, synthetic polymers exhibit good mechanical properties, but share a common drawback as they lack bioactivity[1]. The cell sheet-based method allows the production of engineered vascular substitutes with high mechanical properties, but the time required to produce such grafts is approximately 28 weeks[2]. Natural biopolymers of the ECM, such as collagen, elastin, fibrin[3] or a combination thereof, remain the gold standard materials for tissue engineering scaffolds. This is primarily for the reason that these materials possess a generally good biocompatibility while being able to induce functional cellular responses[4-5]. Among these biopolymers, type I collagen is one of the most abundant and predominant load-bearing protein of the ECM in many tissues such as skin, blood vessels and tendons. Extensive work has been conducted on the mechanical properties of collagen[6–8], but there have been only a few studies on cellular remodeling of collagen gels during static maturation. Cellular remodeling refers to the structural modifications of the collagen matrix induced by cells that could affect the stability of the collagen fibrils network[9]. As a natural scaffold, relatively large quantities of type I collagen can be isolated, sterilized and stored from different sources such as rat-tail tendons[10]. Understanding cellular interactions with collagen and the related overall mechanical behaviors of the cellularized collagen scaffolds (constructs) is an essential step for the construction of tissues. Collagen-based TEBVs can be processed by directly mixing cells with collagen during gel preparation and further molded into specific shapes such as tubular and planar[11]. Vascular cells inside the gels proliferate and remodel type I collagen[12]. Thus, this method bypasses the need for specific macroporosity that represents one of the significant issues in the development of scaffolds for tissue engineering applications. However, the major drawbacks of collagen gels are their low mechanical properties compared to synthetic materials[13].

In this study, a viable tissue with homogenous distribution of cells was engineered by direct mixing of collagen with cells in a one-step process. “Static bioreactors” were used for the 1- or 2-weeks of static maturation of the cellularized collagen gels (without applied external dynamic mechanical constraints). During the culture, collagen matrix remodeling occurred, thus providing structural reinforcement to the constructs. Furthermore, these constructs were ready to be transferred to a rotating-wall bioreactor and a homogenous endothelium was achieved. In addition, in this work a specific mechanical testing protocol is also proposed to provide an appropriate novel approach in characterizing the mechanical properties of tubular soft tissues.

In summary, this work presents a method for the *in vitro* rapid fabrication and maturation of vascular tissues that are strong enough to be handled not only for biological and mechanical characterizations, but also for further mechanical conditioning in a dynamic bioreactor, which is considered a crucial step in the regeneration of tissues.

PROTOCOL:

1. Fabrication and assembly of the static bioreactor

1.1 Fabrication of the reservoir

1.1.1 Prepare 50 mL centrifuge tubes as a medium culture reservoir for the bioreactor.

1.1.2 Make two ports by drilling two 5 mm diameters holes at 20 mm from the bottom and the top of the reservoir, respectively. Then insert two luer fittings in 5 mm length silicone tubes. Press-fit these luer fittings through the holes, and seal all the connections with medical grade silicone glue.

1.1.3 Insert a 0.22 μm filter into the upper port of the reservoir (**Figure 1, a**).

1.1.4 Insert a luer septum into the lower port of the reservoir (**Figure 1, a**).

1.2 Mandrel-cap assembly

1.2.1 Drill a 4.5 mm diameter hole at the center of the ventilated cap of the reservoir tube without damaging the filter membrane that covers the aerating holes.

1.2.2 Prepare a stir bar (diameter = 4.5 mm, length = 100 mm) as a mandrel for the construct.

1.2.3 Prepare two silicone conical stoppers (length = 10 mm, middle hole diameter = 4.5 mm).

1.2.4 Assemble the mandrel and the cap (mandrel-cap complex) as described in **Figure 1, b**.

1.2.4.1. Press-fit the mandrel into the hole. Insert the 2 stoppers over the mandrel so that the cap is fitted between them. Adjust the position of the mandrel so that its useful length is 78 mm.

1.2.4.2. Apply a primer and then medical grade silicone glue to the surfaces that will be in contact before joining the cap and the silicone conical stoppers together. Remove the excess glue on the cap.

1.2.5 Let it dry at room temperature for 1-3 days.

1.3 Fabrication of the gauze-grips

1.3.1 Prepare 3 silicone tubes (tube1: inner diameter = 6.4 mm, length = 5 mm; tube2: diameter = 6.4 mm, length = 10 mm, and tube3: diameter = 3.1 mm, length = 12 mm).

1.3.2 Assemble the gauze-grips as described in **Figure 1, c**.

1.3.2.1. Cut tube1 longitudinally, and open it over tube2. Stick them together with the silicone glue.

1.3.2.2. Cut sterile surgical gauze to 5 cm x 7 cm sheet, and then roll tightly the gauze over the tube 3 along the longest side of the gauze. Insert the tube 1-tube 2 complex over the gauze.

1.3.2.3. Add silicone glue to stick together the gauze, the tube 1-tube 2 complex and the tube 3. Cut the gauze at a length of 8 mm.

1.4 Assembly and Sterilization

1.4.1 Assemble the mandrel-cap complex and the gauze-grips as described in **Figure 2**.

1.4.1.1. Coat the mandrel with medical grade grease (**Figure 2, a**). Place the gauze-grips over the mandrel (**Figure 2, b**). Distance the grips at fixed value of 35 mm from each other.

1.4.1.2. Prepare a tubular mold by removing the bottom part of a 10 mL syringe using a table saw (final length = 8 mm) (**Figure 2, b**).

1.4.2 Insert the mold over the gauze grips-equipped mandrel-cap assembly (housing-mold complex), snap-fitting the mold on the silicone stopper (**Figure 2, c**).

1.4.3 Autoclave the reservoir and the housing-mold complex.

Note: Be careful to hold on the silicone stopper tightly when inserting the mold to avoid its detachment.

2 Engineering SMCs-collagen gel-based constructs and static maturation

2.1 Engineering constructs

2.1.1. Expand porcine aortic smooth muscle cells (pSMCs) in 175 cm^2 culture flasks filled with 20 mL of complete culture medium consisting of Dulbecco's Modified Eagle Medium supplemented with 10% (v/v) porcine serum (PS), 10% (v/v) fetal bovine serum (FBS), 1% (v/v) penicillin-streptomycin (pen-strep).

2.1.2. At ≈ 90% confluence, detach pSMCs (passage 2-4) by removing the culture medium from the flask of pSMCs, adding 5 mL of trypsin solution (1X in phosphate-buffered saline solution, PBS), and incubating for 10 min (T = 37 °C, 5% CO_2, 100% humidity).

2.1.3. Resuspend the pSMCs at a concentration of $4x10^6$ cells/mL in complete culture medium.

2.1.4. Prepare collagen solution as previously described[10].

2.1.4.1. Extract and collect collagen bundles from rat-tail tendons in a PBS solution.

2.1.4.2. Transfer the collagen fibers subsequently into acetone (5 min), isopropanol 70% (v/v) (5 min) and acetic acid (0.02 N, 48 h, 4 °C) solutions.

2.1.4.3. Blend the viscous solution and freeze at -20 °C for 3 days.

2.1.4.4. Lyophilize the frozen solution to obtain collagen sponges.

2.1.4.5. Solubilize the collagen sponges into acetic acid solution (0.02 N) at a concentration of 4 g/L and centrifuge at 29581 g force for 45 min.

2.1.4.6. Sterilize the collagen solution through dialysis process against subsequent solutions of acetic acid (0.02 N, 1 h), chloroform 1% (v/v, 1 h) and acetic acid (0.02 N, sterile solution changed every 2 days for 1 week).

2.1.4.7. Collect the sterile collagen solution (4 g/L) in a sterile cell culture hood.

2.1.5. Prepare cellularized collagen gels as shown in **Figure 3**.

2.1.5.1. Prepare 50 mL of sterile buffer solution by mixing 35 mL of DMEM (5X), 4 mL of HEPES (1 N), 3 mL of NaOH (1 N) in 8 mL of sterile deionized water.

2.1.5.2. Prepare cells and collagen gel mixture by mixing 50% (v/v) of sterile collagen solution (4 g/L of acetic acid 0.02 N) with 25% (v/v) of buffer solution and 25% (v/v) of the suspension of pSMCs in complete culture medium.

2.1.6. Measure the pH of the mixture and ensure that it is between 7.0 and 7.4.

2.1.7. Pour gently 9 mL of cells-and-collagen mixture into the above mentioned housing/mold complex (step 1.4.3, **Figure 3, a-b**).

2.1.8. Let it gel at room temperature for 1 h under the cell culture hood (**Figure 3, b**).

2.2 Maturation in static bioreactor

2.2.1. Remove the mold (**Figure 3, c**) and transfer carefully the construct into the reservoir, containing 35 mL of culture medium (**Figure 3, d**).

2.2.2. Incubate the construct (T = 37 °C, 5% CO_2, 100% humidity) in vertical position for 1 or 2 weeks of static maturation.

2.2.3. Install a web camera (sealed in order to ensure insulation) inside the incubator in front of the construct.

2.2.4. Change the culture medium every 2 days by aspirating the old medium from the luer septum port and re-filling the reservoir with an equivalent amount of fresh culture medium.

2.3 Measurement of thickness and metabolic activity of SMCs-collagen gel-based constructs during static culture

2.3.1. Place the scanning laser interferometer into the cell culture hood and flip it from the vertical to the horizontal position using a spirit level.

2.3.2. Transfer the bioreactor into the cell culture hood and remove the construct from the reservoir.

2.3.3. Transfer the construct (still mounted on the mandrel) into the pathway of the laser beam, and place it strictly orthogonally with respect to the beam axis (as shown in **Figure 4**).

2.3.4. Read the value displayed on the screen of the scanning laser interferometer, corresponding to the external diameter of the construct.

2.3.5. Calculate the wall thickness of the construct based on its external and internal diameter (*i.e.* the mandrel diameter).

Note: Repeat the steps 2.3.1 to 2.3.5 every hour for the first 12 hours and then every 24 hours.

2.3.6. Use 1 mL of the old culture medium (sampled when changing the medium culture, step 2.2.4) for measuring the lactate and glucose concentrations with the blood gas analyzer.

2.3.7. Use 1 mL of the fresh culture medium as a baseline level for the glucose and lactate concentrations measurements[14].

Note: Repeat the steps 2.3.6 and 2.3.7 every 2 days after culture medium changing.

2.4 Construct harvesting for further mechanical and biological characterizations

2.4.1. After 1 or 2 weeks of static maturation period, transfer the static bioreactor into the cell culture hood.

2.4.2 Transfer gently the mature construct from its mandrel (**Supplemental Video 1**) to a 100 mm diameter Petri-dish containing 40 mL of fresh culture medium (**Figure 5 and Figure 7, a**).

3 Mechanical characterization of the constructs in the longitudinal and circumferential directions

3.1. Install the experimental set-up consisting of the micromechanical tester equipped with a 5 or 10 N load cell and a bath containing PBS at 37 °C to keep the samples at pseudo-physiological conditions (**Figure 6**).

3.2. Balance the load cell and the extensometer.

Note: Balancing is a function integrated into the micromechanical tester consisting in resetting the displayed extension value and the displayed load value while no sample is mounted onto the machine. This function allows defining the reference for both measurements.

3.3. Mounting the tubular constructs onto the mechanical apparatus: longitudinal direction

Note: Perform longitudinal fatigue tests directly on the entire tubular constructs. Use in-house-built gripping devices to connect the gauze grips of the constructs to the load cell and to the base of the PBS bath.

3.3.1. Mount the tubular construct onto the gripping devices (**Figure 7, b**), following the harvesting procedure (section 2.4).

3.3.2. Wrap the gripping devices and the gauze grips together with Teflon tape to prevent any slipping of the gauze grips during the test. Mount the sample onto the micromechanical tester (**Figure 7, c**).

3.4. Mounting the ring-shaped constructs onto the mechanical apparatus: circumferential direction

Note: Perform circumferential fatigue tests on ring-shaped specimens sectioned from the tubular constructs. Use two stainless steel bars as grips to hold the specimens.

3.4.1. Mount the tubular construct onto a plastic pipe as a support marked with 5 mm gaps (**Figure 7, b)**, following the harvesting (section 2.4).

3.4.2. Cut 10 mm rings from the tubular construct.

3.4.3. Measure the length of the specimen using a vanier caliper for the further analyses.

3.4.4. Mount the ring-shaped specimen onto the stainless steel bars of the micromechanical tester (**Figure 7, c**). Make sure to place the specimen at the center of the bars.

Note: The plastic pipe in the step 3.4.1 and a cutting system as shown in **Figure 7, b** are used to avoid any damage to the construct during cutting.

3.5. Fatigue test on constructs in the longitudinal or circumferential direction

3.5.1. Stretch the construct to its initial gauge length.

3.5.2. Maintain the construct in this position for 10 minutes in pseudo-physiological environment.

3.5.3. Apply 10% cyclic strain of the initial gauge length (30 cycles) to the construct at 5%/s strain rate.

3.5.4. Repeat step 3.5.3 at incremental steps of 10% cyclic strain until failure of the sample.

Note: The use of the pseudo-physiological environment requires taking into account the buoyancy and the inertia of the gripping system that affect the measurement of the applied load.

3.5.5. Record the background as follows:

3.5.5.1. Move the load frame to the initial gauge length.

3.5.5.2. Repeat the steps 3.5.3 and 3.5.4 without any sample mounted, and keeping the gripping devices connected to the load cell (only 1 cycle is required).

4. Luminal endothelialization of constructs

Note: After following the harvesting protocol (section 2.4), the constructs withstand handling to be mounted in the rotating-wall bioreactor for the further endothelialization.

4.1. Rotating-wall bioreactor design

4.1.1. Drill a 4.5 mm diameter hole at the center of the ventilated cap of the reservoir tube without damaging the filter membrane that covers the aerating holes.

4.1.2. Press-fit a mandrel (diameter = 4.5 mm, length = 40 mm) into the hole and fix the mandrel as described in step 1.1.2.

4.1.3. Prepare two C-shaped silicone support for the construct external diameter = 14 mm; internal diameter = 8 mm).

4.1.4. Position a rotating motor in one end of the rotating-wall bioreactor and a bearing on other end (**Figure 8, b**).

4.2. Lumen endothelialization

4.2.1. Expand human umbilical vein endothelial cells (HUVECs) in 25 cm^2 culture flasks with 5 mL of M199 culture medium supplemented with 10% (v/v) PS, 10% (v/v) FBS, 1% (v/v) pen-strep in Petri dish inside an incubator (T = 37 °C, 5% CO_2, 100% humidity) till 90% confluence.

4.2.2. Prepare 1.5 mL of the protein coating solution per construct required for optimal cell adhesion by diluting the concentrate protein mixture to 10.5 ng/mL in serum-free endothelial cell culture medium.

4.2.3. Measure the length of the construct using a vernier caliper.

4.2.4. Calculate the luminal volume V and the luminal area A of the construct as: $V=\pi D_{in}^2 L/4$ and $A=\pi D_{in} L$ respectively (where D_{in} is the inner diameter corresponding to the mandrel diameter, and L is the length of the construct).

4.2.5. Position the construct at the center of the reservoir following the harvesting procedure (section 2.4). Use C-shaped silicone support to fix the construct at both ends to the reservoir (**Figure 8, a**).

4.2.6. Fill the reservoir with 35 mL of culture medium.

4.2.7. Fill 75% of the calculated luminal volume of the construct (V) with the protein coating solution prepared in step 4.2.2. Close both of the extremities of the construct to avoid any leakage of the protein coating solution (Figure 8, a).

4.2.8. Assemble the rotating-wall bioreactor system inside the cell culture hood.

4.2.9. Start the rotation of the bioreactor at 4.02×10^{-5} g force for 1 h to allow the luminal coating as shown in **Figure 8, b**.

4.2.10. Open the upper extremity of the construct and aspirate the protein coating solution from the lumen.

4.2.11. Detach HUVECs (passage 2-3) by removing the culture medium from the flask of HUVECs and adding 3 mL of the trypsin solution (1X in PBS). Incubate for 5 min (T = 37 °C, 5% CO_2, 100% humidity).

4.2.12. Resuspend the HUVECs at a concentration of 4×10^6 cells/mL in supplemented M199 culture medium.

4.2.13. Inside the cell culture hood, seed HUVECs into the lumen of the construct with a density of 1000 cells/cm^2 [15]. Close the upper extremities of the construct to avoid any leakage of the HUVECs solution.

4.2.14. Incubate the constructs (T = 37 °C, 5% CO_2, 100% humidity) hosted into the rotating-wall bioreactor (**Figure 8, b**) and culture for 2 days at a constant rotation of $4.02x10^{-5}$ g force.

4.2.15. Harvest the construct after 2 days of culture in sterile conditions and prepare it for further biological characterization as described in section 2.4.

REPRESENTATIVE RESULTS:

This work describes the fabrication of engineered tubular collagen-based constructs containing vascular cells. Already after 1 h of early gelation, cells-and-collagen mixture was directly assembled in a 3D tubular geometry, with the external diameter equal to the diameter of the corresponding mold (around 14 mm). All along static maturation, measurements revealed the rapid reduction of the external diameter of the tubular cellularized structures, as shown in **Table 1**. The diameter of the cellularized collagen gels shrunk of about 60% of its initial value after 1 day of static culture, and of almost 85% within 7 days (**Supplemental Video 2**). SMCs within the constructs are responsible for the observed shrinking and the related mechanical reinforcement, as this phenomenon does not occur in non-cellularized collagen scaffolds. Note that no gradient of any type (thermal, biochemical, mechanical, or others) was applied. The cells-driven contraction resulted in a material with greater collagen density that could be handled and subdued to mechanical solicitations (**Supplemental Video 3** and **4**).

To relate the cells-driven remodeling to the overall mechanical and viscoelastic properties, fatigue tests were performed on the constructs (**Supplemental Video 5** and **6**). These tests consisted in cycling the constructs (30 times) at different constant strains (10, 20 and 30% of initial gauge length) and to record the stress as the response of the constructs to the mechanical solicitation over time. The representative results for one construct are shown in **Figure 9**. The construct withstood higher stresses in the longitudinal direction (75 kPa) than in the circumferential direction (16 kPa) when subjected to the same strain range (30% strain). Meanwhile, at each cycle, the stress peak value reached for the targeted maximum strain decreased over time. This behavior is typical of the high viscoelastic properties exhibited by these collagen-based constructs.

The biological activity of the cellularized constructs was assessed during static maturation. Hence, metabolic activity of SMCs was evaluated by measuring the glucose consumption and lactate production during static culture. Culture medium was sampled every 2 days and glucose and lactate concentrations were measured using a blood gas analyzer. The constant increase in glucose consumption and lactate production combined to the important shrinking of the constructs, attest the SMCs activity all along static culture (**Figure 10**).

The increased mechanical stability due to the cell-driven remodeling allowed the manipulation of the constructs and the subsequent endothelialization process. Masson's trichrome staining performed on the endothelialized constructs showed a highly homogenous endothelium. SMCs exhibited a spindle-like shaped morphology and appeared homogenously dispersed through the wall, while HUVECs appeared well spread in the luminal side (**Figure 11**).

Figure 1: Components of the static bioreactor. The static bioreactor consisted of a modified 50 mL centrifuge tube (A) and a mandrel-equipped cap (B). The tube served as medium reservoir, and was equipped with a port for a 0.22 µm filter, for the gas exchange, and a septum, for the medium sampling and changing. A mandrel present in the ventilated cap allowed the fabrication of constructs in tubular shape. The gauze-grips (C) were designed and fabricated to support the gelation of the constructs over the mandrel. Moreover, these grips allowed the constructs to be handled after the static maturation and to be fixed to the mechanical apparatus. The external diameter of the mandrel was 4.7 mm.

Figure 2: Assembling of the static bioreactor. Assembling phases of the bioreactor before the sterilization. The gauze-grips were mounted on the mandrel (A) at a fixed distance. A mold was inserted (B) and tightly fixed to the silicone stopper (C). The external diameter of the mandrel was 4.7 mm.

Figure 3: Fabrication of the constructs in sterile conditions. The cells and collagen mixture was poured into the housing-mold complex (A), and let gel for 1 h at room temperature (B). Afterwards, the mold was removed (C), the static bioreactor was assembled (D) and transferred inside a reservoir for the static maturation of the construct in incubator (T = 37 °C, 5% CO_2, 100% humidity). The external diameter of the mandrel was 4.7 mm.

Figure 4: Measurement of the thickness/external diameter of the constructs. A laser scanning interferometer was used to perform the measurement of the external diameters of the constructs. The construct was placed into the pathway of the laser beam and generated a shadow. The width of the shadow, corresponding to the external diameter of the construct, was then measured and displayed on the screen.

Figure 5: Morphological appearance of the harvested construct. (A) Right after gelation and (B) after cells-driven remodeling during static maturation for 2 weeks.

Figure 6: Experimental set-up for mechanical characterizations. It consisted of the micromechanical tester equipped with a 5 or 10 N load cell and a bath containing PBS at 37 °C to keep the samples in pseudo-physiological conditions.

Figure 7: Sample preparation for mechanical characterizations. Sample harvesting (A) and preparation (B) for fatigue tests performed in the longitudinal and the circumferential directions (C). The external diameter of the mandrel was 4.7 mm.

Figure 8: Rotating-wall bioreactor. (A) The tubular constructs were assembled in the center of the reservoir with the help of c-shaped silicone support. Both of the extremities of the construct were closed to avoid any leakage of the HUVECs solution. (B) The constructs were cultured in incubator (T = 37 °C, 5% CO_2, 100% humidity) in rotation at 4.02×10^{-5} g force for 2 days.

Figure 9: Mechanical characterizations. Results of fatigue tests performed on constructs in longitudinal (A) and circumferential (B) directions after cell-driven remodeling.

Figure 10: Metabolic activity of SMCs within the collagen gels. Measurements of glucose consumption rate and lactate production rate were performed with the blood gas analyzer every 2 days, after the culture medium changing. Fresh culture medium was used as a baseline level for the glucose and lactate concentrations measurements.

Figure 11: Lumen endothelialization. Histological images of the radial cross-sections of tubular constructs. Masson's Trichome staining of tubular constructs cultured statically for 1 week (A) and 2 weeks (B). H & E staining of a tubular construct (C).

Table 1: Rapid contraction of construct diameter during the static maturation. Wall thickness of the constructs and the contraction rate as a function of time of static culture. Contraction was measured by determining the external diameter of the tubular constructs with a scanning laser interferometer (Series 183B, LaserMike 136). After 24 h, the constructs contracted to 57±2% of their molded dimensions. Data are expressed as mean ± SD (n = 3). The presence and the activity of living smooth muscle cells was the only responsible for such major changes.

Supplemental Video 1: Harvesting of the non-remodeled tubular collagen gels.

Supplemental Video 2: Cells-driven contraction of tubular collagen gels.

Supplemental Video 3: Manipulation of the non-remodeled tubular collagen gels.

Supplemental Video 4: Manipulation of the cells-remodeled tubular collagen gels.

Supplemental Video 5: Longitudinal fatigue test (at 30%) on cells-remodeled tubular collagen gels.

Supplemental Video 6: Circumferential fatigue test (at 30%) on cells-remodeled tubular collagen gels.

DISCUSSION:

Among the community of vascular tissue engineers, tremendous efforts have been done to reproduce the tunica media layer responsible for the mechanical stability of blood vessels[16]. Since the pioneering work of Weinberg and Bell[17], collagen has been widely used as a scaffold for vascular tissue engineering because of its biocompatibility, non-immunogenic properties and availability. However, the use of collagen represents a big challenge for researchers, as this material is not easy to handle, due to the intrinsic lack of mechanical stiffness. Manipulations during scaffold preparation may damage the scaffolds, compromising them for further use.

The technique described in this work allows: *i)* to engineer cellularized collagen gels into a tubular-shaped geometry; *ii)* to engineer biological tissues strong enough to be handled after a

short static maturation period (1 or 2 weeks); iii) to assess mechanical and viscoelastic properties of such tubular-shaped biological tissues in 2 directions. Cells in the gel play a key role in the collagen matrix remodeling. During the maturation period, contractile SMCs led to the compaction of the gels yielding a construct with higher mechanical stability that could be assessed in the longitudinal and circumferential directions. Afterwards, HUVECs seeded in the luminal side of the constructs generated a homogenous and viable endothelium, thus demonstrating the suitability of the collagen gels for vascular tissue engineering applications.

The bioreactor described in this work for the fabrication of the static maturation of the construct was specifically designed to provide an optimal environment for cells maturation. In addition, the devices developed for the characterization of the mechanical and viscoelastic properties of the constructs were designed with the aim to reduce any potential damage inherent to the manipulation of such delicate materials. Hence, the static bioreactor was equipped of a 0.22 µm filter and a filter membrane on the cap (step 1.1.2, **Figure 1, a** and **b**) that allowed gas exchange between culture medium inside the reservoir and the incubator, while keeping a sterile culture environment. The luer septum at the bottom was used as a port for culture medium sampling and changing during static culture. Some critical steps have to be considered during construct fabrication and characterizations. All the manipulations (performed in the step 2.1.1 and in the subsequent steps) that might alter the sterility of the system were performed in a sterile biological hood. Cells and collagen gel mixture preparation was handled on ice in order to delay the gelation process (steps 2.1.4 to 2.1.7). At step 2.1.7, any air-bubbles entrapped in the mixture prior to gelation are potential stress concentration areas that can compromise the stability of the constructs. Therefore, removal of such air-bubbles requires slightly shaking the assembly or using medical vacuum for 3 min for degasing in sterile conditions. Finally, the grips were specifically designed for maintaining the axis of the mandrel central in the tubular mold during gelation and for allowing delicate manipulation of the constructs during harvesting (removal of the mandrel, section 2.4), for endothelialization, and for facilitating the mounting onto the mechanical system (longitudinal tests).

The present protocol proposes an original easy-to-process alternative approach of reinforcement of collagen gels constructs based on the natural inherent contractile potential of SMCs. Common techniques of collagen matrices reinforcement involve the use of physical and chemical crosslinking agents that can have deleterious effects on cells-matrix interactions[18–20] The fabrication technique presented in this work allows directing this cells-driven remodeling process to yield a tissue-engineered construct with targeted mechanical properties without any physical or chemical treatment.

Characterization of mechanical and viscoelastic properties of hydrated collagen gels is a great challenge. In this perspective, the present protocol describes an original simple and efficient method for assessing the mechanical properties of tubular soft tissues. This characterization can be performed not only in the circumferential direction, but also in the longitudinal direction, directly on the whole tubular structure. During mechanical characterization, temperature, aqueous environment, pH and ionic strength are some of the environmental factors that are known to drastically affect the mechanical behavior of biological tissues[21].

Hence, the present work suggests an original set-up and protocol for the mechanical characterization of biological tissues in a highly reproducible pseudo-physiological environment (saline solution at 37 °C and pH 7.4). To the best of our knowledge, this kind of characterization has never been reported elsewhere.

In conclusion, the technique proposed in this work demonstrates the high potential of direct mixing of cells with collagen for vascular tissue engineering applications. This method together with the mechanical characterization and endothelialization process constitute high polyvalent protocols. Hence, through slight modifications of the set-ups and protocols while keeping the same rationale, main requirements for engineering vascular tissue equivalents can be addressed such as rapid and uncomplicated processing, including endothelialization, and the possibility to be transposed to a wide range of soft tissues with various lengths and diameters. Furthermore, different adherent cell types, ECM proteins and molded geometries can be investigated for a number of targeted applications, such as engineering tendons, skin grafts, cardiac patches, nerves, among others. Although the mechanical properties of the constructs are encouraging, they are still lower than those of native tissues. In this context, we strongly believe that a very short static maturation period is a crucial step toward the dynamic stimulation into a bioreactor, thus leading to a higher structural integrity and mechanical stability. However, the possibility to rapidly produce tissue-engineered cellularized collagen-based constructs suitable for mechanical and histological analyses makes the static bioreactor described herein a useful and promising tool to provide insight into the interplay between cells and ECM during growth and remodeling, or even to be used as a model for therapies and drug delivery systems.

DISCLOSURES:
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Figure 1

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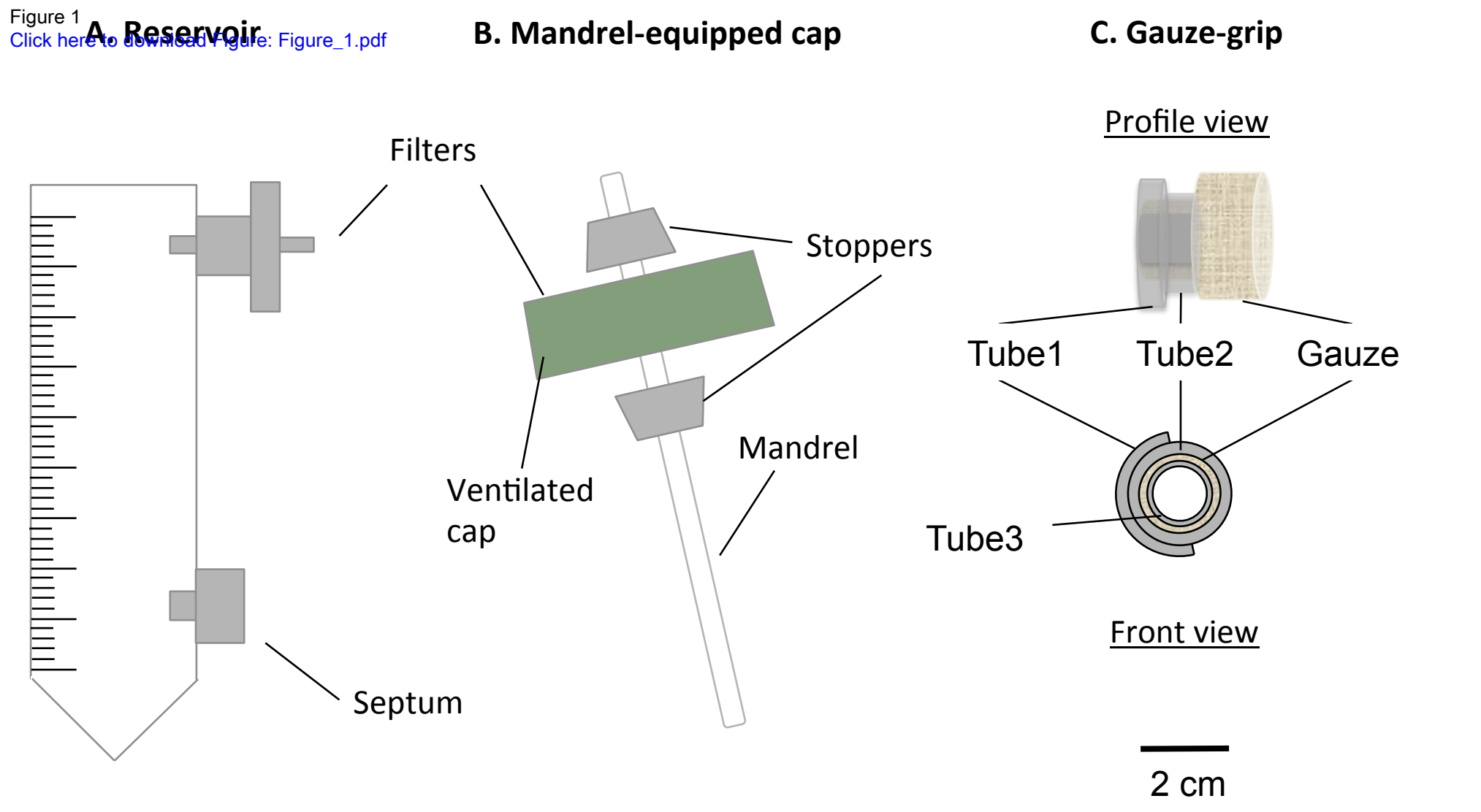

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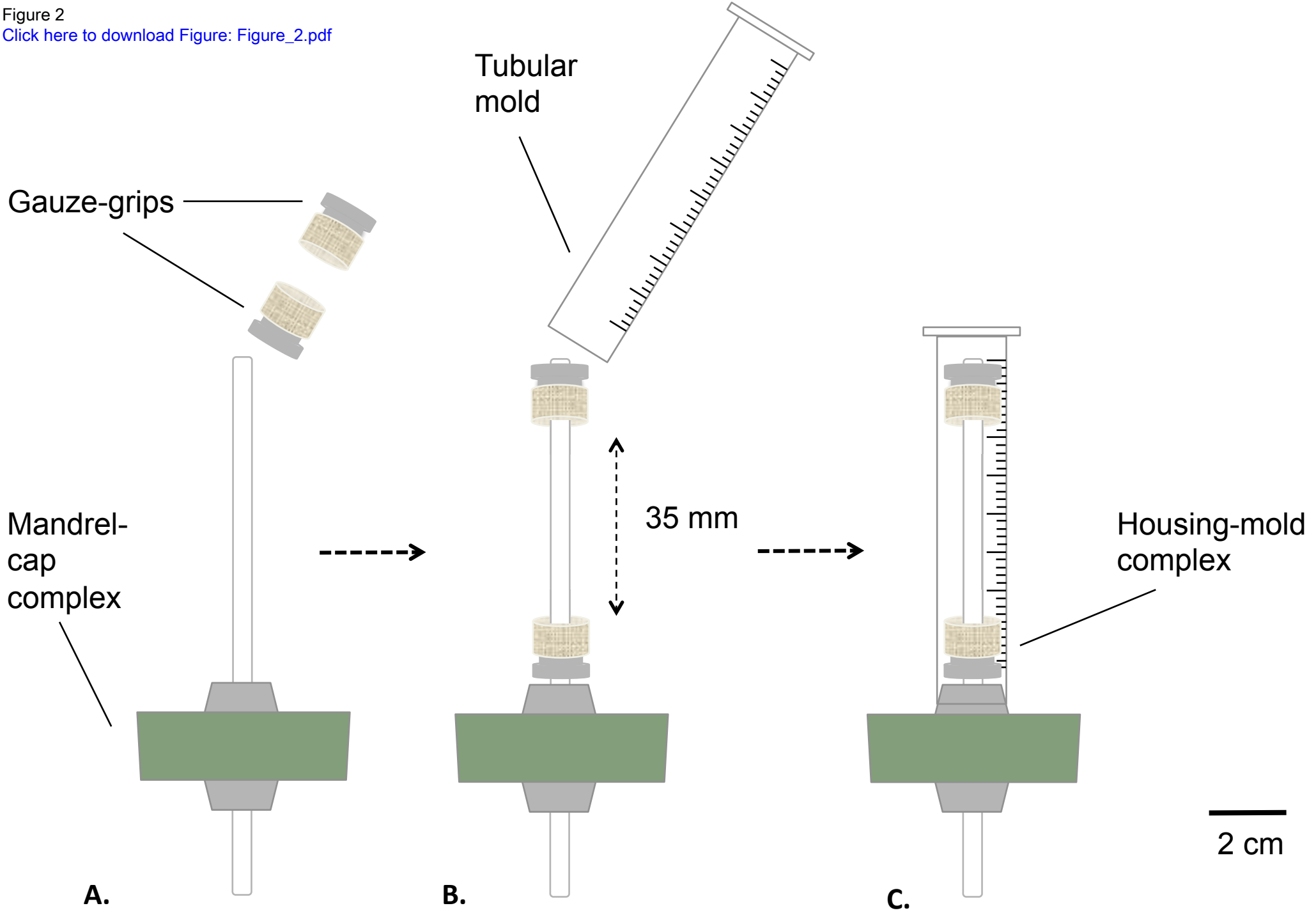

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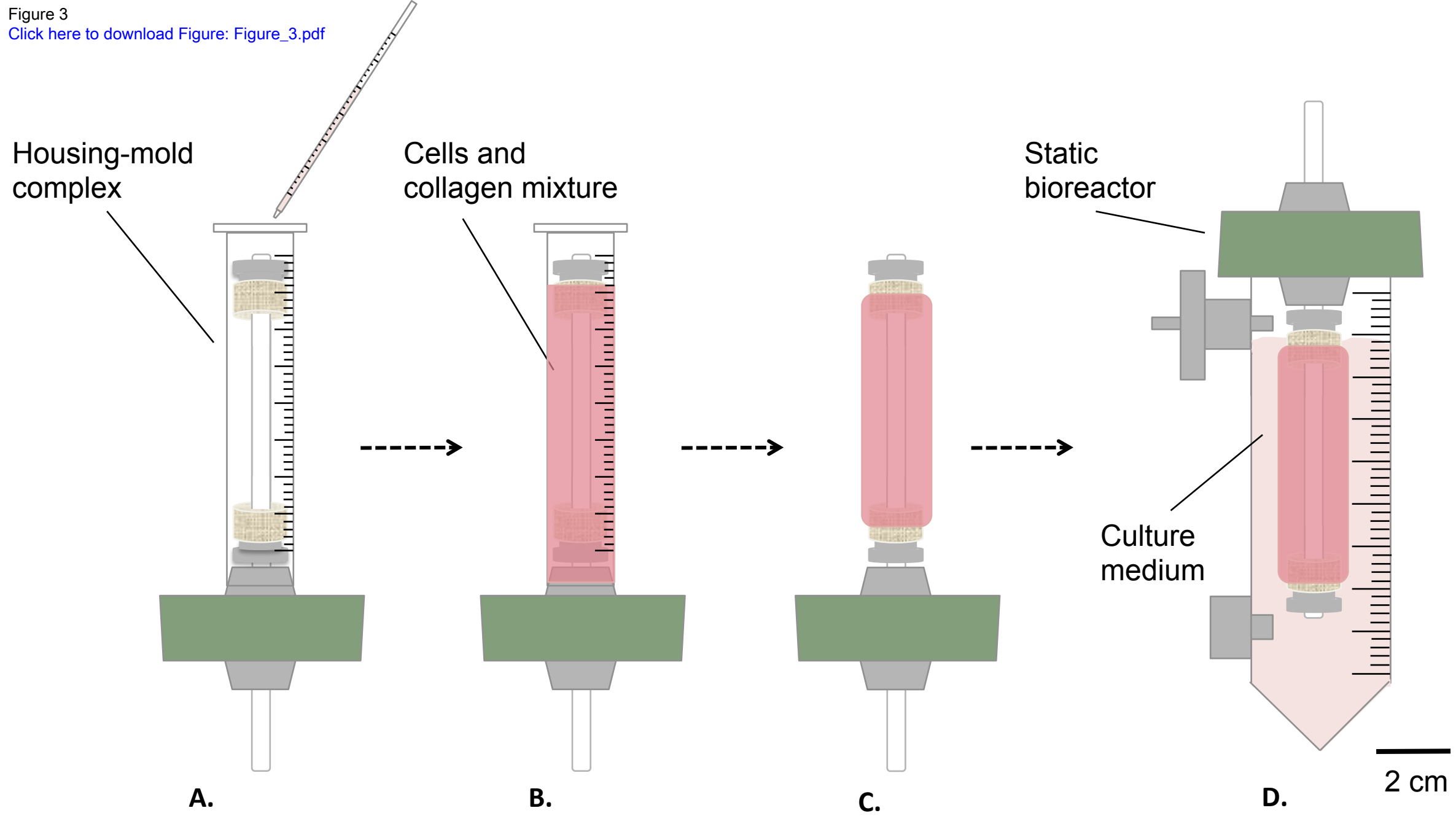

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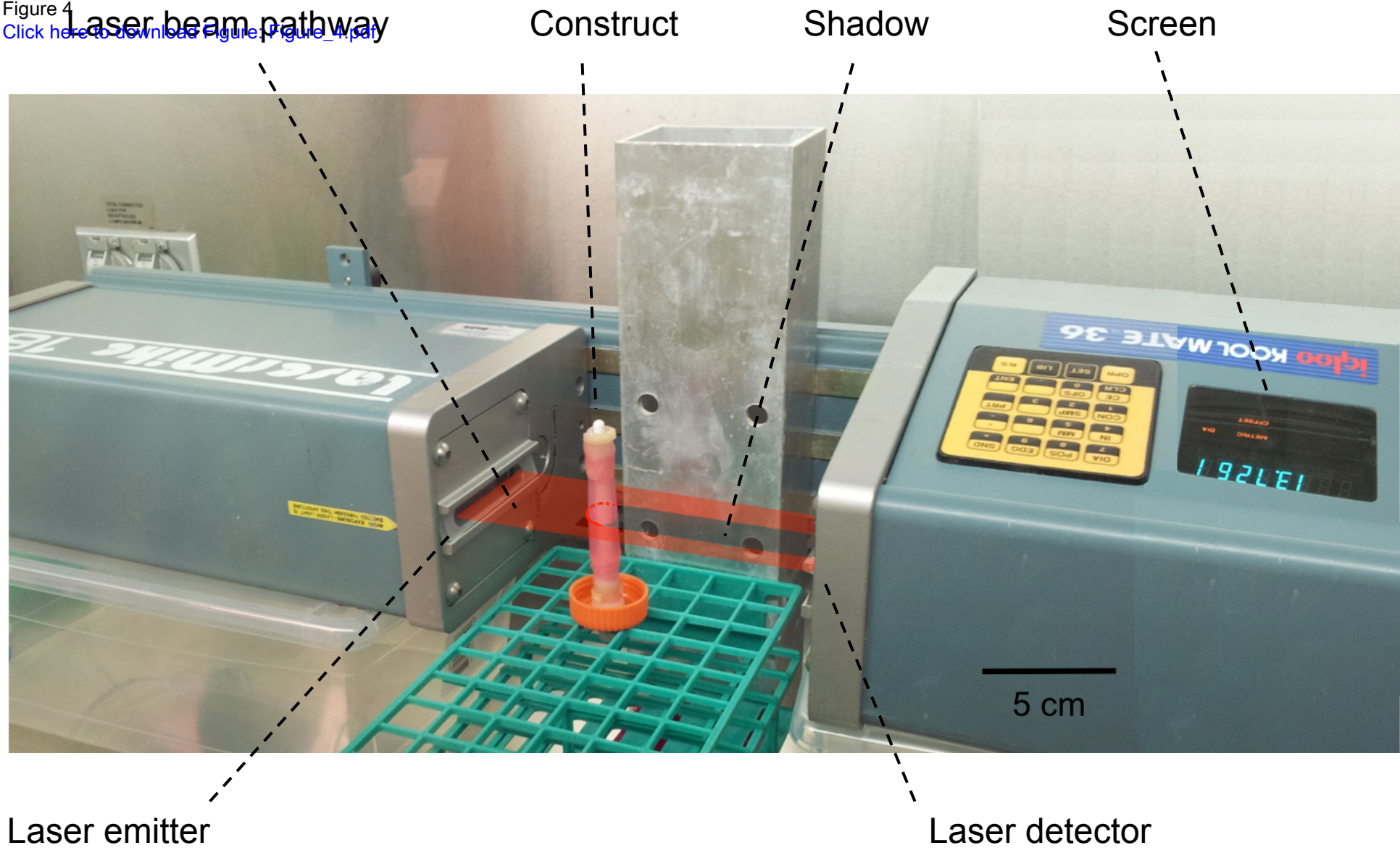

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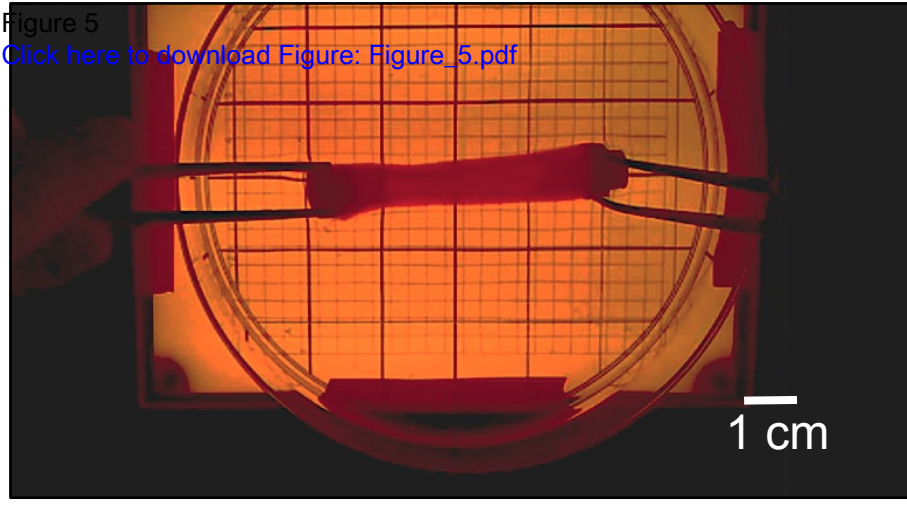

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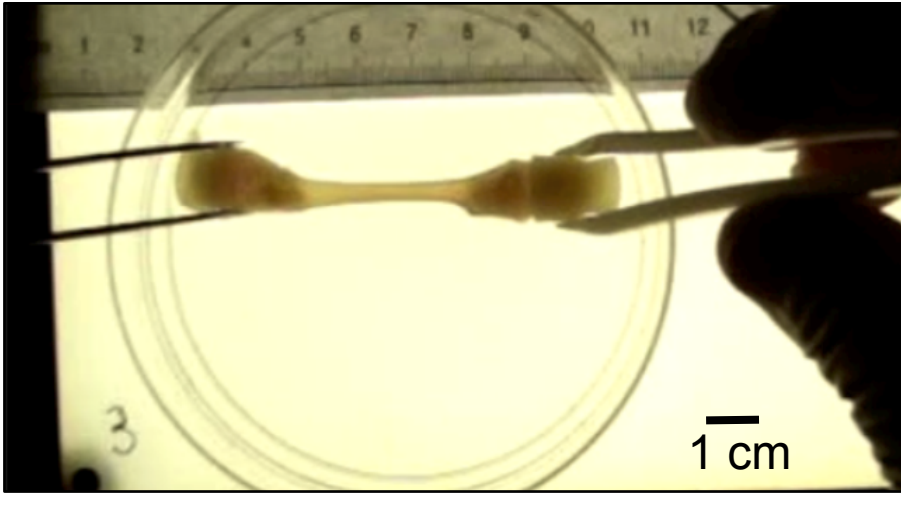

B.

Figure 6

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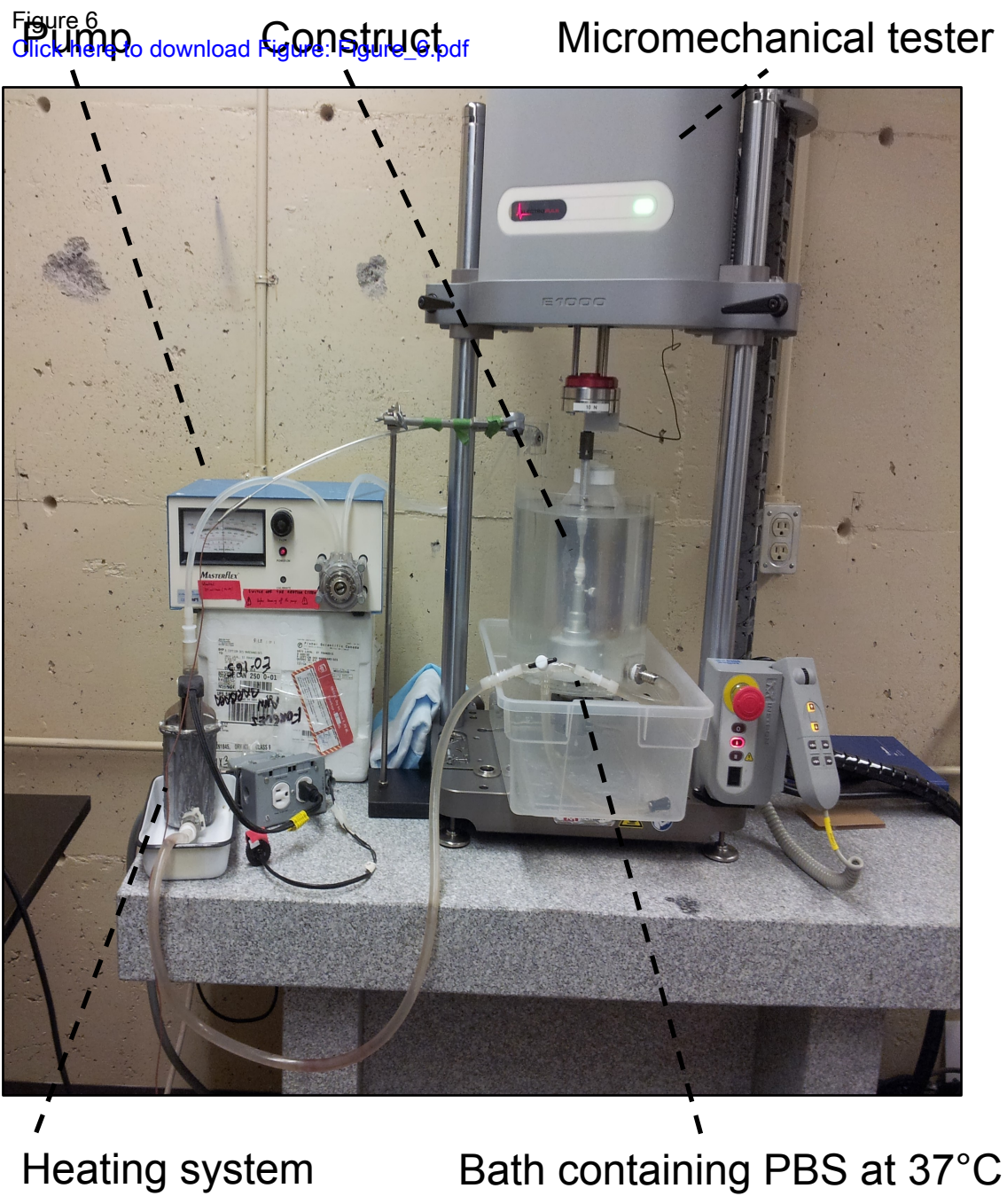

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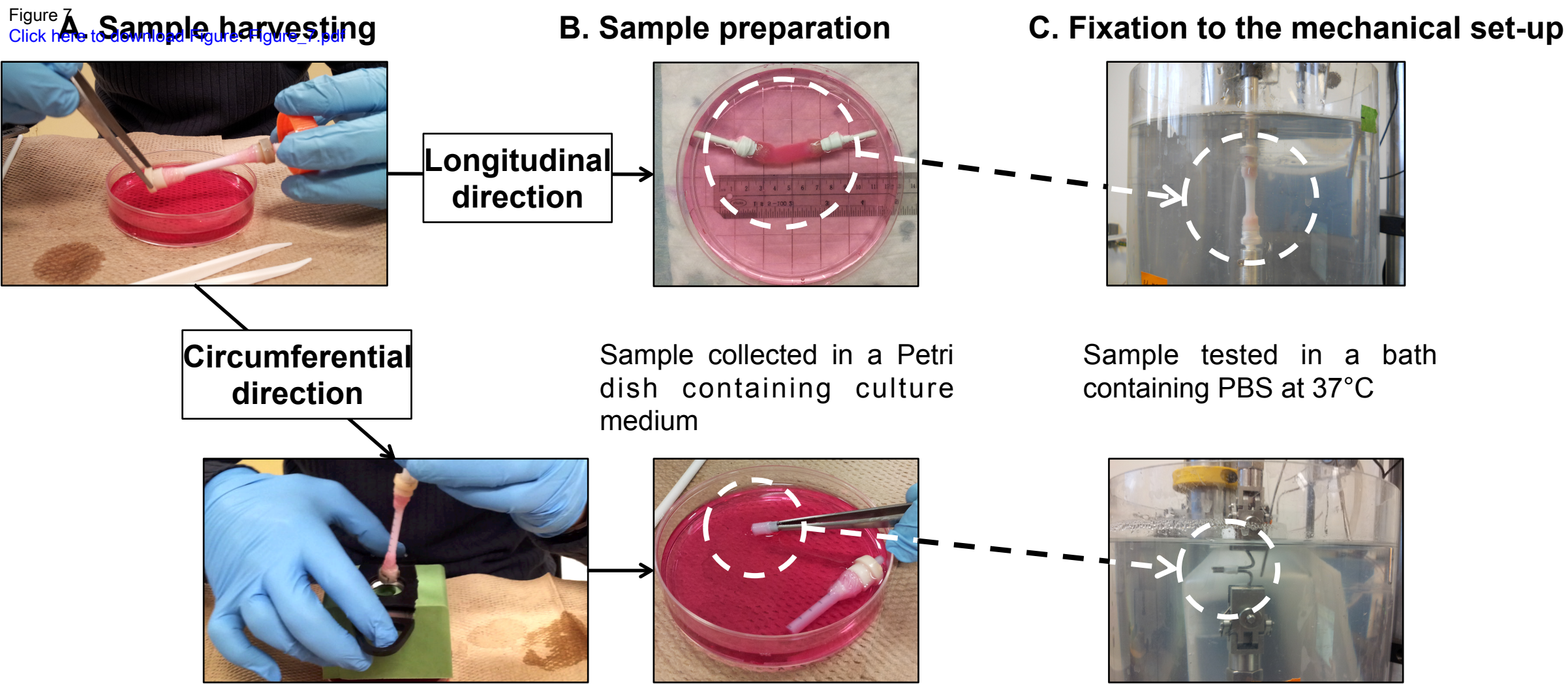

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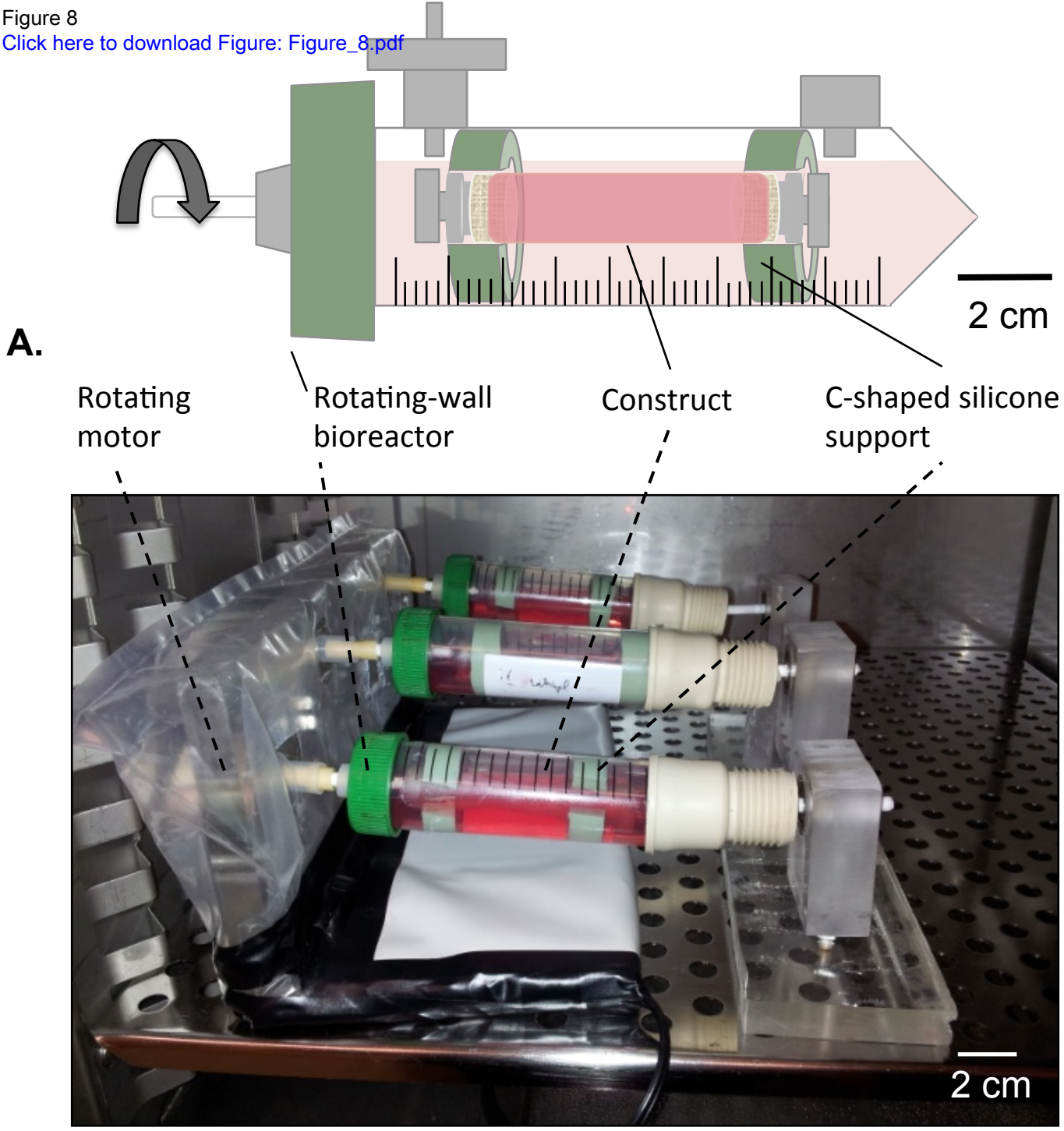

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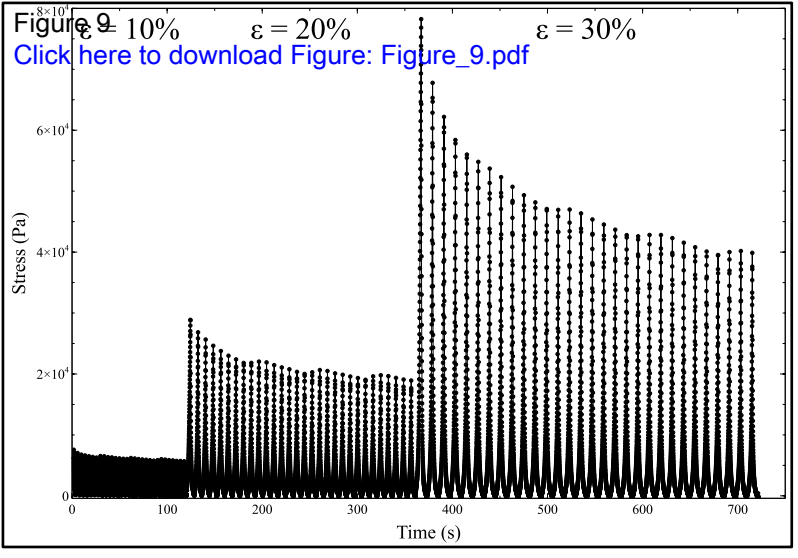
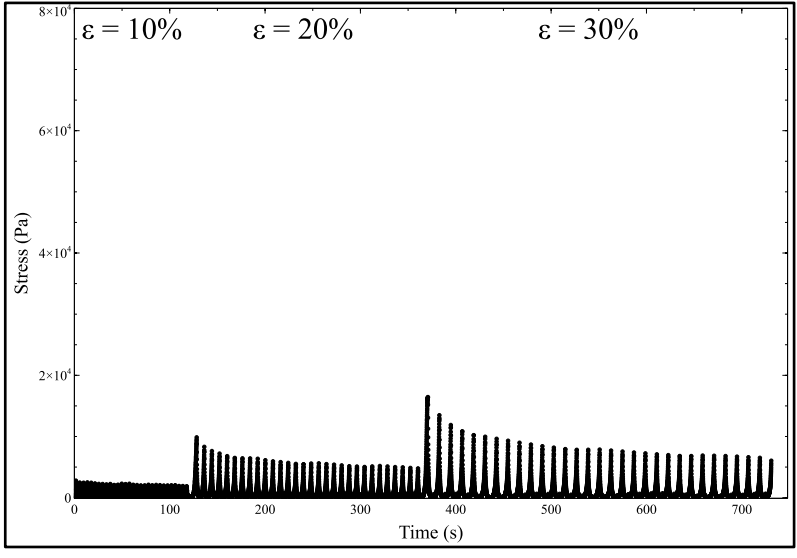


A.



B.

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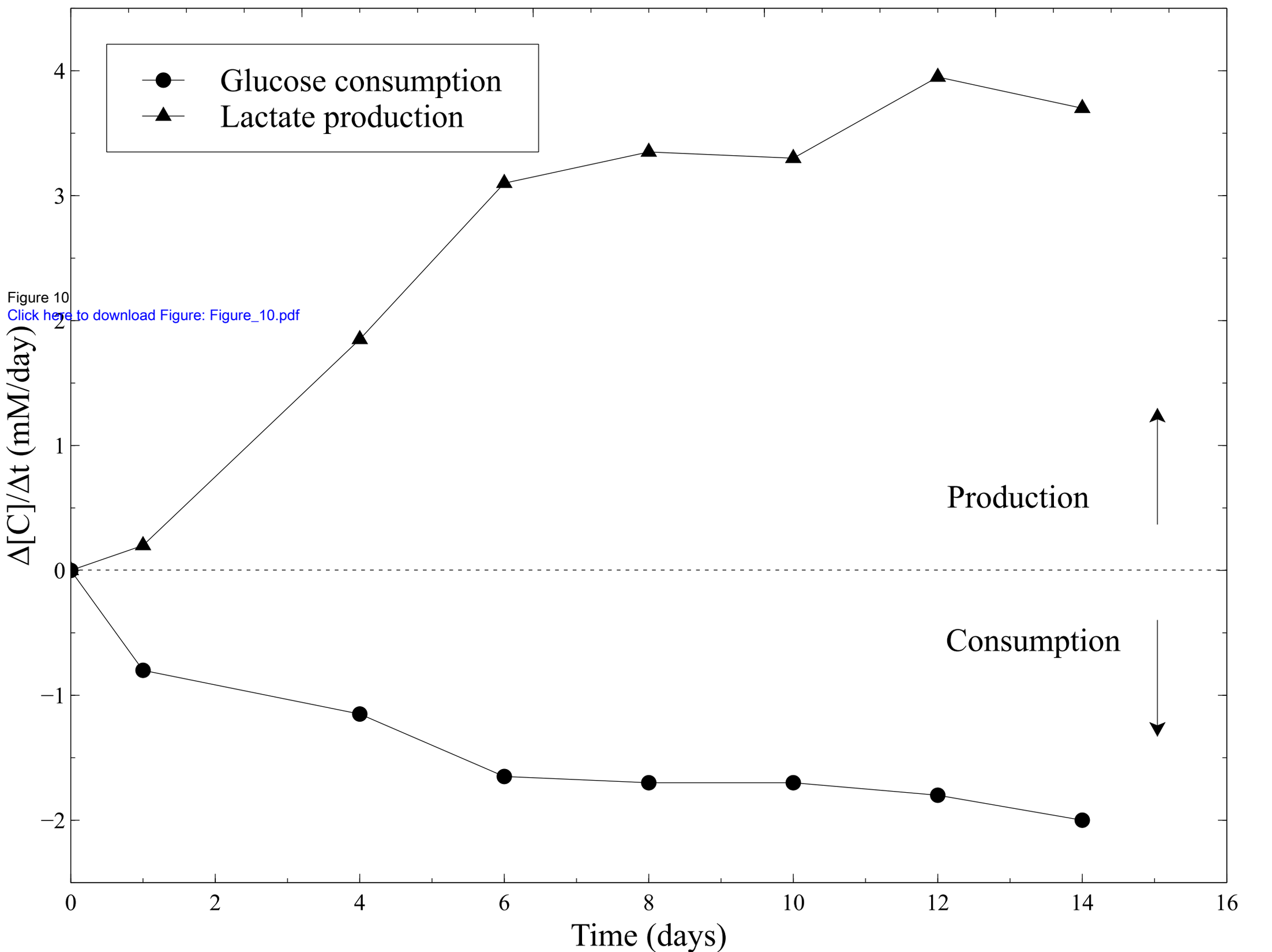

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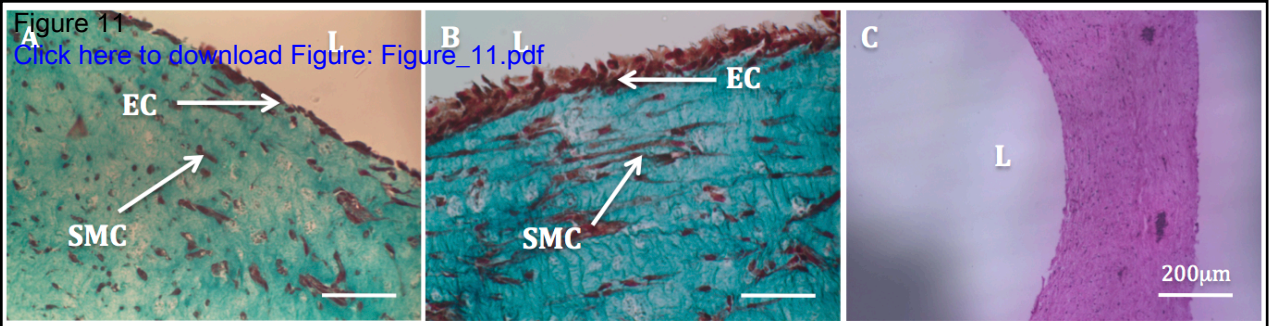

Table 1

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Time	Thickness (mm)	Contraction
0h	4.83±0.02	0±0%
2h	4.26±0.02	12±0%
4h	4.21±0.03	13±1%
6h	4.06±0.10	16±2%
12h	3.16±0.07	35±1%
1 day	2.08±0.11	57±2%
1 week	0.68±0.07	86±1%
2 weeks	0.36±0.00	93±0%

Excel Spreadsheet- Table of Materials/Equipment

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Name of Material / Equipment	Company / Sources	Catalog Number
CellTreat 50 mL Bio-Reaction tubes	CELLTREAT Scientific Products	229-475
Male luer with lock ring x 1/8" hose barb. PP. 25/pk	Cole Parmer	RK-45503-04
Female luer x 1/8" hose barb adapter. PP. 25/pk	Cole Parmer	RK-45500-04
Masterflex platinum-cured silicone tubing L/S 17. 25 ft.	Cole Parmer	RK-96410-17
Masterflex platinum-cured silicone tubing L/S 16. 25 ft.	Cole Parmer	RK-96410-16
Silastic Medical adhesive silicone, type A	Dow Corning	-
Polyvent 4 Vessel venting filters	Whatman	6713-0425
Rod. PP. stirring. 8''	Scienceware	377660008
Stopper silicone rubber 00 PK12	VWR	59590-084
Krytox PFPE/PTFE Greases	Dupont	GPL 202
Trypsin-EDTA (0.5%)	Gibco	15400-054
Xiameter RTV-4130-J base and curing agent	Dow Corning	-
Dulbecco's modified Eagle medium, DMEM, high glucose, pyruvate	Gibco (Life Technology)	11995-065
Pure acetone (99%)	Laboratoire Mat Inc.	AP0102
Isopropyl alcohol (HPLC grade, 99.9%)	Fisher Scientific	AC610080040
0.02 N acetic acid (glacial acetic acid, HPLC grade, 99%; Fisher Scientific,	Fisher Scientific	FL070494
Chloroform solution (99%)	Laboratoire Mat Inc.	CR 0179
Hepes	Sigma-Aldrich	163716
NaOH	Laboratoire Mat Inc.	SR-0169
LaserMike 136	LaserMike	Series 183B
ElectroPulse MicroTester	Instron Corporation	-
HyClone Media M199/EBSS, 500 mL	GE Healthcare Life Sciences	SH30253.01
Fetal bovine serum HI - 500mL	Gibco	SH 30396.03
Porcine serum (PS)	Sigma-Aldrich	P9783
Penicillin-Streptomicin	Gibco	15140-122
Phosphate buffered saline (PBS)	Fisher Scientific	BP661-50

Tissue culture flask T17CN Vent Cap Red	Sarstedt Inc.	83.1812.002
ColorpHast- pH-indicator strips (pH=6.5-10.0)	EMD	9583
Matrigel Basement Membrane Matrix Growth Factor Reduced, 5 mL vial	BD Biosciences - Discovery Labware	356230
LifeCam VX-3000	Microsoft	-
Biochemical analyzer, DxC600	Beckman Coulter Unicell Synchron	-
Collagen fibers	Rat tails	-
Porcine smooth muscle cells (pSMCs)	Porcine aortas	-
Human umbilical vein endothelial cells (HUVECs)	Human umbilical veins	-

Comments / Description
Centrifuge tube
Luer fittings for "gas-exchange port"
Luer fittings for the "medium sampling port"
Tube 1 and 2 for the gauze grippers
Tube 3 for the gauze grippers
Silicon glue for the fabrication of the static bioreactor
Filter for "gas exchange port"
Mandrel
Stopper for the insertion of the mandrel to the vented cap of the centrifuge tube
Medical grade grease for covering the mandrel
Cell culture
C-shaped silicone support for endothelialization
Cell culture
Chemical for collagen extraction
Chemical for collagen extraction
Chemical for collagen extraction
Chemical for collagen extraction
Chemical for construct preparation
Chemical for construct preparation
Scanning laser interferometer
Micromechanical Tester
Component of cell culture medium
Component of cell culture medium
Component of cell culture medium
Component of cell culture medium
Saline solution

Cell culture

pH measurements

Concentrate protein mixture for endothelialization process

Thickness measurement

Glucose and lactate concentrations measurements

Collagen was extracted in the laboratory
pSMCs were isolated in the laboratory
HUVECs were isolated in the laboratory

Video 1

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Video 2

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CORRESPONDING AUTHOR:

Name:	Diego Mantovani
Department:	Lab Biomaterials and Bioengineering
Institution:	Laval University
Article Title:	Engineering 3D cellularized collagen gels for vascular tissue fabrication
Signature:	Diego Mantovani (Diego Mantovani)
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Engineering 3D cellularized collagen gels for vascular tissue regeneration
(Identification number for JoVE: 52812_R2_RE_Revised_Manuscript)

Authors:
Sébastien Meghezi, Dawit G. Seifu, Nina Bono, Larry Unsworth, Kibret Mequanint, Diego Mantovani

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General comments
-The authors would like to take this opportunity to thank the science editor, the Reviewer #1 and the Reviewer #2 for valuable work in improving the overall quality of the manuscript. Authors really appreciated the pertinence of the comments, and the well thought out of the suggestions.

Editorial comments:
The manuscript has been modified by the Science Editor to comply with the JoVE formatting standard. Please maintain the current formatting throughout the manuscript. The updated manuscript (52812_R2_RE.docx) is located in your Editorial Manager account. Please download the .docx file and use this updated version for any future revisions.
- Accordingly to the comment, the authors agreed the formatting of the text proposed by the Editor. Moreover, the authors uploaded all the Supplemental Videos to the Editorial Manager with revised captions. Please, note that a new video has been added to the protocol in section 2.4.2, which is related to the harvesting process of the construct. Numbering of the subsequent Supplemental Videos has been changed as consequence.

Changes made by the Science Editor:

1. There have been edits made to the manuscript. Please accept (or address) all tracked changes.
- The authors found all the comments and the edits made to the manuscript by the Science Editor very pertinent. Accordingly, the authors addressed all tracked changes.

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.
- Accordingly to the comment 1, the authors proofread the manuscript spelling or grammar issues were corrected in red. While reviewing the text, the authors agreed to revise the title too, mainly because the word “fabrication” does not reflect the status of the work. Moreover, the word “regeneration” will allow the manuscript to be referenced and accessible by keyword search much easier then the word “fabrication”.

2. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. The highlighted steps should form a cohesive

narrative with a logical flow from one highlighted step to the next. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.
Currently, there are 3.5 pages of highlighted protocol text. This limit is to ensure that the videography can occur in one day.
- Accordingly to the comment 2, the authors highlighted (2.75 pages, in yellow) all the steps of the protocol that identify the essential steps of the protocol for the video.

3. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.
- Accordingly to the comment 3, the authors included all relevant details required to perform the step in the highlighting.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
This article is absolutely relevant to similar tasks undergone on vascular tissue engineering labs around the world. The description is adequate and the authors provide some detail on potential problems.

Major Concerns:
None

Minor Concerns:
None

Additional Comments to Authors:
N/A

Reviewer #2:
Manuscript Summary:
The manuscript focus a direct strategy to assemble collagen and smooth muscle cells in a 3D cylindrical geometry useful for the production of vascular tissue and also a way to characterize its mechanical properties in an environment similar to the physiological.

Major Concerns:
No major concerns were detected. The manuscript was well organized and well written, and the results attainable by the use of both strategies proposed have very good potential use in the production and characterization of biomaterials.

Minor Concerns:
Please find below a list of recommendations to improve the overall quality of the work:

1) In Figure 4, the text showing the brands/models and the screen of the equipment is upside down.
To ensure the validity of the measurement, the longitudinal axis of the construct and the plan of the laser beam have to be perfectly orthogonal to each other. The construct or the laser has to be flipped from the vertical position to the horizontal position. Flipping the construct would require

additional equipment in the cell culture hood, which could compromise the sterility of the environment. Therefore, the scanning laser interferometer is flipped and its horizontal position is ensured with a tubular spirit level.
The authors added additional information in step 2.3.1 accordingly to the previous explanation.

2) Data on glutamine consumption and ammonium formation in addition to glucose consumption and lactate formation would be an excellent addition, given that glutamine is not only an important source of nitrogen to animal cells in culture, but also of carbon and energy. Its consumption results in formation of ammonium, a compound that can be deleterious to cells if accumulated in high proportions. Also, since glutamine metabolism is strongly related to glucose metabolism and that animal cells frequently shown unbalanced (overflow) metabolism in vitro, results of the evolution of concentration of the four compounds mentioned would be more complete regarding the analysis of the metabolic activity of the cells.
The authors would like to thank the Reviewer #2 for pointing out this really important point. The results presented in this work are only representative. Data on glutamine consumption and ammonium formation would certainly improve the overall cell metabolism for further investigation.

3) Still regarding glucose and lactate data, the authors in fact do not report values of rates of consumption and production of these compounds in Figure 10. Variations (deltas) are reported as a function of time, but not the derivatives of concentrations with time (dC/dt), which would indeed represent the rates. Also, are the data shown cumulative? It seems to be the case, given that the culture medium was changed frequently and so, similar values would be expected in equal analysis periods if the metabolism of the cells did not vary greatly in the evaluated intervals. Finally, the caption of Figure 10 describes two figures0 (A and B), but both graphs are shown in the same figure.
Please then correct the discussion and the caption regarding Figure 10.
-According to the comment 3 of Reviewer #2, the authors replaced the graph in figure 10. The new graph now reports the derivatives of concentrations with time (dC/dt), which is more pertinent. In addition, the authors modified the caption as suggested.

4) Please check/correct the following sentences:
- Item # 1.2.1, lines 150-151: Drill a 4.5 mm diameter hole at the center of the ventilated cap of the reservoir tube without damaging filter membrane that covers the aerating holes.
Recommendation: insert a THE between "filter" and "membrane

-Item # 2.4.2, lines 283-284: Transfer gently the mature construct from its mandrel to a 100 mm diameter Petri-dish containing 40 ml of fresh medium culture (Figure 5, b and Figure 7, a).
Recommendation: change "medium culture" to "culture medium"

- Item Disclosure: No funding was received organizations or agencies with potential conflict of interests.
Recommendation: change to "No funding was received from organizations or agencies with potential conflict of interests".

- Change "posphate" to "phosphate" in the List of Material/Equipment.

The authors addressed the recommendations suggested by the Reviewer #2 in the comment 4.

5) Please provide the description of the sources/Companies/Catalog Numbers for the following items:
- collagen (despite the description is available in another publication, this compound is too important in the body of the present paper and should be ideally described again)
- pSMCs cells
- HUVECs cells

The authors detailed the extraction process in step 2.1.4 as suggested by the Reviewer #2 in the comment 5. In addition, the authors provided information about collagen and cell sources in the material list (file: 52812_R1_102714_Materials_Reply.xls).

Additional Comments to Authors:
None