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Fibroblast-derived human engineered connective tissue for screening applications

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TITLE:

Fibroblast Derived Human Engineered Connective Tissue for Screening Applications

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tissue engineering, cardiac fibroblast, collagen matrix, tissue stiffness, screening

SUMMARY:

Presented here is a protocol to generate engineered connective tissues for a parallel culture of 48 tissues in a multi-well plate with double poles, suitable for mechanistic studies, disease modeling, and screening applications. The protocol is compatible with fibroblasts from different organs and species and is exemplified here with human primary cardiac fibroblasts.

ABSTRACT:

Fibroblasts are phenotypically highly dynamic cells, which quickly transdifferentiate into myofibroblasts in response to biochemical and biomechanical stimuli. The current understanding of fibrotic processes, including cardiac fibrosis, remains poor, which hampers the development of new anti-fibrotic therapies. Controllable and reliable human model systems are crucial for a better understanding of fibrosis pathology. This is a highly reproducible and scalable protocol to generate engineered connective tissues (ECT) in a 48-well casting plate to facilitate studies of fibroblasts and the pathophysiology of fibrotic tissue in a 3-dimensional (3D) environment. ECT are generated around the poles with tunable stiffness, allowing for studies under a defined biomechanical load. Under the defined loading conditions, phenotypic adaptations controlled by

cell-matrix interactions can be studied. Parallel testing is feasible in the 48-well format with the opportunity for the time-course analysis of multiple parameters, such as tissue compaction and contraction against the load. From these parameters, biomechanical properties such as tissue stiffness and elasticity can be studied.

INTRODUCTION:

A major obstacle in the study of fibrotic diseases is the lack of representative human 3D tissue models that provide insight into the behavior of fibroblasts and their pathological derivatives. To study fibrotic processes, standard 2D culture systems are sub-optimal since isolated fibroblasts transdifferentiate rapidly into α-smooth muscle actin (SMA)-expressing myofibroblasts when cultured on non-compliant 2D substrates[1-3]. Thus, fibroblasts in the standard 2D culture do not reflect a regular "healthy" tissue phenotype[3-6]. Cultures on pliable substrates have been introduced to simulate non-fibrotic (10 kPa) and fibrotic (35 kPa) tissue environments[7], but these lack the third dimension, which is very important with respect to pathophysiology. Tissue engineering provides the opportunity to overcome this limitation by allowing fibroblast culture in a defined and experimentally tunable extracellular matrix (ECM)-context, for example, by alterations in the cellularity, ECM composition, and ECM concentration, all of which can determine the tissue biomechanics.

Various 3D models have been generated using fibroblasts. Floating discs and microspheres were among the first and demonstrate that collagen is remodeled and compacted in a time-dependent manner. Fibroblasts exert traction forces on collagen fibrils, a process which can be facilitated by the addition of pro-fibrotic agents such as transforming growth factor-beta 1 (TGF-β1)[8-16]. However, freely floating cultures do not allow for the controlled external loading and, therefore, constitute continuously shrinking or compacting models. Sheet-like engineered tissues opened the possibility of studying homeostatic regulation of biomechanical properties of tissues, namely through uni-, bi-, multi-axial, or cyclic strain testing[17-20]. These models have been used, e.g., to demonstrate the influence of the cell number on the tissue stiffness, which was found to correlate positively with cytoskeleton integrity and actomyosin cytoskeleton contractility[18,19]. However, it is important to note that force-to-strain conversions are complicated by the non-uniform tissue deformation around clamp points of force transducers and anchor points. This inherent limitation can be bypassed by dog-bone or ring-shaped tissues, offering some tissue enforcement at anchor-points[21-23]. Ring-shaped tissues can be prepared by distributing a cell-collagen hydrogel into ring-shaped molds. As the hydrogel compacts, a tissue forms around the uncompressible inner rod of the mold, which offers resistance for further tissue contraction[24-27]. After initial and typically maximal compaction, tissues may also be transferred to adjustable spacers to further restrain circular ECT at a defined tissue length[3,24-30]. Biophysical properties can be assessed in standard horizontal or vertical strain-stress devices with appropriate load cells under unidirectional or dynamic strain[3]. As the tissues have a largely uniform circular structure and can be held on bars/hooks (anchorage points and/or force transducers), although these may still enclose compression areas around the loading bars, this format allows a more uniform strain variation as compared to clamping[3]. Furthermore, anchored tissues elicit a bipolar cell shape, and cells adapt to the tissue forces by elongation along force lines promoting anisotropic traction[31-36]. We have previously applied ring-shaped ECT from rat and human cardiac fibroblasts (CF)

around a single stiff pole in functional stress-strain experiments and performed gain and loss of function studies by using virally transduced fibroblasts[24-26] and pharmacological studies[37]. Further, we could identify sex differences in CF-mediated fibrosis in the ECT model[27].

The following protocol for the generation of human ECT, exemplified with primary human CF obtained as cryopreserved CF from commercial vendors (see **Table of Materials**), combines the advantages of ring-shaped tissues with an easy and fast way of producing macroscopic tissues for a 48-well platform designed for parallel high-content testing.

Importantly, the ECT model is not restricted to a specific fibroblast type, with the documented use in the investigation of other fibroblasts, e.g., skin fibroblasts[38,39]. Moreover, fibroblasts from patient's biopsies work equally well, and the choice of fibroblasts ultimately depends on the scientific question to be addressed.

The platform used for the generation of ECT described in this protocol is a commercially available 48-well 3D cell/tissue culture plate (**Figure 1A**). The methods for the preparation, culturing, and monitoring ECT formation and function under a defined geometry and mechanical load with the help of the 48-well plate are described. The formed ECTs are held by integrated flexible poles and the mechanical load can be fine-tuned according to the final purpose by using poles with different hardness (Shore A value 36-89), influencing their bending stiffnesses. Therefore, poles with a shore A value of 46 are recommended. The protocol is, in addition, compatible with a previously described custom circular mold, where the ECT is held around a single stiff rod[37]. The dimensions of this mold are given in **Figure 1B**.

PROTOCOL:

All steps must be undertaken in a Class II biosafety hoods installed in laboratories under containment level 1. Depending on local regulations and type of manipulations to be performed, such as viral-mediated gene transfer, the containment level must be increased to the biosafety level 2 or 3. All cultures are maintained at 37 °C in a cell culture incubator with a humidified atmosphere of 5% CO_2 in the air. Note that the volumes (Steps 1 and 2) are provided for a T75 cell culture flask. Adjust the volumes to different culture formats according to the standard cell culture recommendations.

1. Thawing and pre-plating primary cardiac fibroblast (CF) for monolayer culture (5-12 days)

NOTE: As an alternative, HFF-1 cells can be used following the standard sub-culture protocol as advised by the supplier.

1.1. Prepare the fibroblast growth medium (FGM) according to the manufacturer's instructions. Optionally, add antibiotics such as 100 U/mL penicillin and 100 mg/mL streptomycin. Allow for the complete mixing of all the components before use. Store at 4 °C for up to 14 days.

1.2. Warm FGM to 20-25 °C.

1.3. Thaw the cryopreserved CF (ideally containing 1 x 10^6 - 2 x 10^6/mL cells per cryovial) in a water bath at 37 °C for approximately 2 min, until only a small amount of ice is left in the vial.

1.4. Using a 2 mL serological pipette, transfer the cell suspension dropwise into an appropriate sterile centrifuge tube containing 10 mL of FGM. For optimal cell retrieval, rinse the cryovial with 1 mL of FGM and transfer it to the centrifuge tube. As the cells are very sensitive at this stage, resuspend using a serological pipette with a bore tip to minimize cell damage by shear stress.

NOTE: If the cryopreservation medium contains a high percentage of DMSO, ensure that after cell resuspension in FGM, the DMSO content is less than 1%. Alternatively, centrifuge the resuspended cells at 300 x *g* for 5 min at 20-25 °C for the medium exchange. Then, aspirate the supernatant carefully, swirl the tube to dislodge the pelleted cells, and resuspend them in the desired volume of FGM for seeding.

1.5. Seed 0.5 x 10^6 cells in 12 mL of FGM into a T75 cell culture flask. If other labware is used, adjust the cell number to maintain a seeding density of 6.7 x 10^3/cm^2.

1.6. Replace FGM every other day for 5 days or until cells reach 80% confluency.

NOTE: The cell yield after the expansion depends mainly on the cell size and proliferation rate, which may differ between cell donors. Typically, this standard culture procedure allows the retrieval of 4 x 10^6 to 5 x 10^6 CF from a T75 cell culture flask after 5 days of culture.

2. Enzymatic dispersion of human CF (10-20 min)

NOTE: This step aims to establish a single cell suspension of human CF for both sub-culturing monolayer cells and preparation of ECT. This protocol has been optimized for human CF monolayer cultures in passages 3-4. For optimal standardization, sub-culturing CF in monolayer is recommended, at least once before ECT preparation. This protocol must be optimized for fibroblasts originating from different donors and vendors. Alternative detachment protocols may involve replacing recombinant serine protease-based dissociation reagents with, e.g., those containing proteolytic and collagenolytic enzymes.

2.1. Warm FGM, PBS (Ca^{2+}/Mg^{2+}-free), and cell dissociation reagent to ~20-25 °C.

2.2. Aspirate the medium from the cultured cells.

2.3. Wash cells with 6 mL of PBS and aspirate.

2.4. Add 6 mL of the cell dissociation reagent to the cells and incubate for 3 min at 20-25 °C until the cells start visibly detaching.

NOTE: Depending on the CF source, this may take several minutes longer. Alternatively, if cells do not detach at room temperature, incubate at 37 °C to improve enzymes' activity. To ensure

optimal cell viability, monitor cell detachment under the microscope.

2.5. Neutralize enzymatic activity by adding 5-10 mL of FGM to the dislodged cells in the cell dissociation reagent. Gently pipette up and down 4-8 times using a 10 mL serological pipette to ensure a single cell suspension and transfer cells into a fresh 50 mL collection tube. Verify the yield with the help of a microscope and a hemocytometer or an automated cell counter according to the manufacturer's instructions.

2.6. Centrifuge the cell suspension at 300 x *g* for 5 min at 20-25 °C.

NOTE: In order to reach a yield of cells sufficient for the generation of the desired amount of ECT, cells can be sub-cultured in an up to 1:6 dilution for further expansion. Let the cells grow until 80% confluency is reached (approximately 5-6 days), with the medium change every other day. Then repeat the enzymatic dispersion and proceed with step 2.7. to continue with ECT preparation.

2.7. Aspirate the supernatant and flick the tube to dislodge the pellet. Resuspend the cells in FGM at 20-25 °C to obtain a cell suspension of ≥ 15 x 10^6/mL (approximately 40% more cells than required for step 3.3.). This accounts for the cell loss due to straining in the following step.

2.8. Strain the cell suspension through a 40 µm mesh cell strainer.

CAUTION: Cell agglomerates are detrimental to the optimal formation of ECT. When using the enzymatic dispersion of human CF protocol for directly casting ECT, straining the cell suspension ensures the absence of major cell clumps that interfere with homogeneous tissue formation. Heterogeneities will compromise reliable stress-strain analyses.

2.9. Recount cell number and assess cell viability to ensure a reliable cell number in a suspension with ≥80 % viability to proceed with ECT preparation.

2.9.1. Use an automated cell counter to assess cell number and viability by electrical current exclusion.

2.9.2. Alternatively, use the trypan blue (carcinogen, hazard category 2 – take precautionary measures) dye exclusion test, with the help of a microscope and a hemocytometer for the direct identification and enumeration of live (intact cell membranes that exclude the dye) and dead (compromised cell membranes which allow binding of the dye to intracellular proteins) cells.

2.10. Reserve the collection tube with cell suspension at 20-25 °C and proceed immediately with step 3.

3. ECT preparation (1 h)

NOTE: Schematic overview of ECT generation is described in **Figure 2**.

[Place **Figure 2** here]

3.1. Prepare a 10x DMEM stock solution by dissolving DMEM powder in ddH_2O (134 mg/mL for the formulation specified in the **Table of Materials**) under a constant rotation at 37 °C for 1 h. Sterilize by filtration. The stock is stable for up to 14 days at 4 °C or -20 °C for up to 12 months.

3.2. Freshly prepare 2x DMEM by diluting a 10x DMEM stock solution and by adding 20 % (v/v) FCS in sterile ddH_2O. Optionally, use antibiotics such as 200 U/mL penicillin and 200 mg/mL streptomycin. Consult **Table 1** for pipetting volumes. The stock is stable for up to 14 days at 4 °C.

NOTE: Perform steps 3.1. and 3.2. before commencing enzymatic dispersion of cells (step 2.) for the preparation of ECT.

[Place **Table 1** here]

CAUTION: All components for the cell-collagen hydrogel mixture and centrifuge tubes must be kept on ice prior to the use. This will help to prevent collagen self-assembly from occurring before distributing the cell-collagen hydrogel mixture throughout the casting molds.

3.3. Based on **Table 2**, adjust the cell suspension to a density of 8.88 x 10^6 cells/mL by adding FGM at 20-25 °C to the cell suspension from step 2.10. Then, move the collection tube with cell suspension to ice.

3.4. To prepare the ECT hydrogel mixture, pre-chill a 50 mL centrifuge tube on ice and add to it the different components listed in **Table 2** in the following order, avoiding air bubble formation.

NOTE: The maximum number of ECT to be prepared depends on the total cell number determined in step 2.9. Use 0.3 mg of collagen per ECT, obtained from a stock solution containing 6-7 mg/mL. The concentration of the collagen stock solution determines the volume needed to obtain an optimal ECT collagen content. Volumes of the other ECT hydrogel components must be adapted accordingly. See **Table 2** for adjusted volumes according to a collagen stock solution of 6.49 mg/mL. The volumes described in **Table 2** are used in this protocol as an exemplary guideline.

3.4.1. Pipette the acid soluble-collagen type 1 hydrogel using a serological pipette with a wide bore tip.

3.4.2. Adjust the salt content of the collagen solution by adding the 2x DMEM while gently mixing by swirling the tube.

3.4.3. Neutralize the pH by adding 0.2 M NaOH while gently mixing by swirling the tube. Phenol red indicator will turn from yellow to red.

NOTE: The NaOH volume must be titrated for each individual collagen batch for the optimal pH

neutralization. Neutralization depends on factors such as buffer type and preparation, as well as absolute collagen concentration, and it affects collagen matrix assembly and cell viability[23,40]. Once the ionic content is increased by the addition of DMEM and the pH is neutralized, the self-assembly of collagen follows and must not be disrupted. Therefore, perform the following swiftly and without breaks.

3.4.4. Add the cell suspension (from step 3.3) dropwise while gently mixing by swirling the tube.

[Place **Table 2** here]

3.5. Mix the entire suspension by gently pipetting up and down only once, using a serological pipette with a wide bore tip to avoid bubble formation and minimize shear stress. Ensure complete mixture by gently swirling the tube 10 times and keep the 50 mL centrifuge tube containing ECT hydrogel mixture on ice throughout the casting process.

3.6. Pre-wet a 1 mL pipette tip with ECT hydrogel mixture and distribute 180 µL of it evenly into each mold of the 48-well casting plate, avoiding excessive shear forces that may affect the integrity of the collagen matrix assembly and ensuring that the entire plate is done in 15-20 min.

NOTE: The recommended casting volume is 180 µL, but it can be extended to 200 µL[38]. Therefore, when preferred, volumes in **Table 2** can be adapted to 200 µL in a manner that keeps the same concentrations and ratio between cells and collagen.

3.6.1. Ensure that a complete loop is formed within the mold (**Figure 3A**). If the ECT hydrogel mixture is applied discontinuously, a complete ECT ring formation will be prevented (**Figure 3B**).

3.6.2. Avoid pipetting into the inner well (**Figure 3C**) and the formation of bubbles during pipetting (**Figure 3D**), to ensure a homogeneous and functional tissue formation.

[Place **Figure 3** here]

3.7. Carefully place the 48-well casting plate inside the cell culture incubator and let the ECT hydrogel mixture reconstitute for 15-30 min. After incubation, it will appear gel-like and opaque (**Figure 3**, middle panel).

3.8. Add 600 µL of 37 °C warm FGM per well, without pipetting the culture medium directly onto the forming ECT as this can result in tissue disruption. Gently add the culture medium along the well wall, as at this point, the ECT must also not be detached from the bottom (**Figure 4**).

[Place **Figure 4** here]

3.9. Incubate for 24 h.

3.10. Replace the medium every day thereafter, with 500 µL of FGM until analysis.

NOTE: After the initial phase of cell-independent gelation, the human CF starts to further compact the ECT hydrogel mixture. Within 24 h, ECT should appear notably compacted and raised to the level where it is held on the flexible poles (**Figures 3** and **Figure 4A**).

4. Assessing ECT compaction by measuring cross-sectional area (CSA) (5 min per ECT).

NOTE: Tissue compaction starts immediately after the collagen assembly and is particularly significant during the first hours. Compaction describes changes mainly triggered by cell-driven compression of the matrix perpendicularly to the tissue's long axis. This parameter is assessed by determining the cross-sectional area (CSA) of the ECT.

4.1. At the desired time points, use a stereo microscope to record macroscopic images of the top and side views of the ECT (**Figure 5A,C**).

NOTE: ECT can be imaged inside of the culturing wells of the 48-well casting plate. Alternatively, transfer the ECT to a clear bottom multi-well plate for imaging. It is advised to image the ECT on the poles as removing those leads to the loss of preload, and consequently, within a short period, the tissue can further contract with eventual torsion due to tension release, which may hamper proper imaging for dimensions' analyses.

4.2. Use an image processing program to perform a line scan analysis. Set a scale and use the **Straight Line** tool to trace and measure the ECT diameters at a minimum of 6 positions per arm in each imaging plane (**Figure 5B,C**).

4.3. Calculate the mean diameter from top and side view planes and calculate CSA according to an elliptic area equation:

$$CSA = \frac{\pi}{2} \times \underset{(top\ view)}{averaged\ diameter} \times \underset{(side\ view)}{averaged\ diameter}$$

[Place **Figure 5** here]

5. Monitoring ECT contraction by pole deflection analysis (15 min per 48-well casting plate).

NOTE: ECT culture is typically performed for 5 days, but it can be further extended at least up to 20 days. Pole deflection occurs due to the tissue contraction driven by the cell contraction force in the direction of tension along the tissue's long axis. Assessment of ECT contraction can be performed by imaging on any day during culture.

5.1. Image the 48-well casting plate under a recording device with an integrated area scan camera placed at a fixed distance, equipped with a high resolution (≥ 5 mega-pixels) monochrome image sensor.

5.1.1. Use a near-UV (~390 nm) light source to maximize the contrast to facilitate automated

detection of the poles' tips as they contain a fluorescent dye (**Figure 6A,C**). If available, telecentric lenses are recommended for imaging as they minimize image distortions.

NOTE: Alternatively, macroscopic bright-field images from single wells or of the complete plate accompanied by a scale bar can be used for the analysis.

5.2. Measure the distance between the poles from daily records (**Figure 6C,D**) using an image processing program or automated analysis by running recorded images on software able to detect high contrast bright pixels on a dark background.

5.3. Calculate the pole deflection through the variation of poles' distance when compared to the initial distance at day zero.

[Place **Figure 6** here]

NOTE: Consider that pole deflection measured by bright tip image is only an estimative of the tissue contraction due to the difference in imaging planes. Also, note that the application of pro-fibrotic substances such as TGF-β1 during tissue culture enhances ECT compaction and contraction and can ultimately lead to early tissue disruption.

6. Assessment of stiffness and other biomechanical properties of ECT by destructive tensile measurement and stress-strain analysis (20 min per ECT)

NOTE: An optimal stress-strain curve, as the ones obtained for ECT, displays three regions: toe region, elastic region, and plastic region (**Figure 7**). The upper limit of the elastic region corresponds to the yield point, and the plastic region is comprised between the yield point and the failure point. The failure point corresponds to a sudden drop in stress due to the rupture of the tissue. The analysis of a stress-strain curve allows extracting important biomechanical parameters of the tissue such as e.g., stiffness, maximum strength, elasticity, plasticity, extensibility, resilience, and toughness.

6.1. Harvest ECT by first pulling the stretcher, including ECT, out of its well, using forceps. The stretcher can then be held on its base, and the ECT slipped over the stretcher tips using a fine hook or pipette tip.

6.2. Transfer the ECT onto two hooks clamped to the stationary arm and the transducer arm of an extensional dynamic mechanical analysis (DMA) rheometer equipped with a 37 °C tempered organ bath (custom-made) filled with PBS (**Figure 7A**).

[Place **Figure 7** here]

6.3. Set the rheometer to apply uniaxial tension at a constant linear rate of approximately 1 % of the initial distance between the hooks per second. A constant stretching rate of 0.03 mm/s can be used with the typical ECT dimensions. Initiate the stretch and record until the point of ECT

rupture.

CAUTION: Macroscopic pictures of ECT (step 4.1.) must be recorded before tensile testing, as the CSA is required for data normalization.

NOTE: The stress-strain analysis, including CSA calculation, can be processed later in time upon tensile testing. Use a spreadsheet software and a statistical analysis software for analyzing the data.

6.4. Normalize measured force values (mN) per ECT by its CSA (mm^2) to obtain stress values (kPa).

6.5. Plot stress values against strain (a geometric measure of tissue deformation given by the relative distance between the upper and lower hook) on a XY graph.

NOTE: The initial length of the tissue (distance between the upper and lower hook) immediately before the stretch ensues, L_0, must be manually adjusted and corresponds to the beginning of the toe region. Each strain point value must be calculated according to the equation, in which L_{total} is the total gap at every measuring point:

$$Strain\ point = \frac{L_{total} - L_0}{L_0}$$

When plotting the data, use the stress value at selected L_0 for background subtraction.

6.6. Determine different biomechanical parameters from the stress-strain curve (use **Figure 7B** as an example).

NOTE: A stress-strain curve display three regions: toe, elastic, and plastic regions. The upper limit of the elastic region, before the tissue starts microfracturing, corresponds to the yield point, and its strain is a measure of tissue elasticity. The plastic region is comprised between the yield point and the failure point. The later point corresponds to a sudden drop in stress due to the rupture of the tissue, defining the ultimate stain, which is a measure of tissue extensibility. The third measuring point corresponds to the maximum strength, which is defined by the highest stress that the tissue can bear without breaking during the stretch. The resilience and toughness, given by the area under the curve, corresponds to the energy absorbed by the tissue up to the yield point and to the failure point, respectively. For each obtained curve, the slope of the linear part of the elastic region corresponds to the Young's modulus, also known as elastic modulus, and is a mechanical property that measures the stiffness of the tissue.

6.6.1. Extract from each curve the XY values (strain and stress, respectively) of the yield point, failure point, and maximum stress point.

6.6.2. Assess the Young's modulus (stiffness in kPa = $mN \cdot mm^{-2}$) of each ECT from the slope of the linear part of the elastic region by plotting a linear regression of that region.

6.6.3. Use a statistical program to compute the area under the curve (AUC) to determine both resilience and toughness, up to the yield point and the failure point, respectively. Compute AUC by the trapezoidal method. Set the baseline to zero and consider only the peaks above the baseline, which are at least 10 % of the distance from the minimum to the maximum value in the Y-axis.

NOTE: The moduli of resilience and toughness are given by $\sigma \times \varepsilon$, where σ is stress (kPa) and ε is the strain (L/ΔL, mm/mm). Thus, resilience and toughness are the energy in kJ/m^3 ($kPa = kN \cdot m^{-2} = kN \cdot m \cdot m^{-3} = kJ/m \cdot m \cdot m^{-3} = kJ/m^3$) absorbed by the tissue before permanent deformation and until rupture, respectively.

REPRESENTATIVE RESULTS:

ECT reach around 95 % compaction compared to the initial cell-collagen hydrogel volume within the first 24 h. Tissue compaction and contraction under control conditions and in the presence of FCS ensues a few hours after casting and notably increases up to day 5 (**Figure 5A**). Pole deflection may further increase during the following 15 days (20 days was the longest time tested). The magnitude of pole deflection depends on cell type, cell state, and cell and tissue culture conditions. Typically, biomechanical properties are measured at day 5 of culture, but any time point can be selected. As an example of the applicability of the ECT model, it is shown how this protocol can assist in studying the impact of actin cytoskeleton integrity on the tissue function. ECT were prepared in the 48-well casting plate and treated with the actin polymerization inhibitor Latrunculin A (Lat-A, 7 ng/mL). The treatment reduced the ECT compaction as indicated by the significant increase of 1.7-fold in CSA compared to control (**Figure 8A,B**). Moreover, the contraction of the tissues was assessed during the 5 days of culture. In the absence of the drug, the contraction gradually increased up to day 5, reaching ~40 % contraction. Lat-A affected tissue contraction, resulting in only ~20 % maximum contraction (**Figure 8A,C**). Destructive unidirectional stress-strain testing was performed on day 5. From a typical stress-strain curve as the ones obtained for ECT (**Figure 7B**), several biomechanical parameters can be extracted. Exemplarily, it is shown that inhibition of actin polymerization led to a significant reduction of ~50 % in tissue stiffness over the control (**Figure 8D**). Taken together, the exemplary data show that the actin cytoskeletal integrity is essential for ECT compaction, contraction, and stiffening.

FIGURE AND TABLE LEGENDS:

Table 1: Composition of 2x DMEM.

Table 2: Preparation of ECT hydrogel (including a 10 % surplus accounting for pipetting errors).

Figure 1: Schematic representation of casting molds. (**A**) Technical drawing and dimensions of a casting mold with two flexible poles. The mold comprises an inner circumference delimited by a short wall that holds double retaining poles at the mold's main body. The flexible poles have a free horizontal distance to one another and are connected at the base. The mold allows for 180

µL casting volume. The well of each mold allows a volume capacity of at least 600 µL of culture media. Different material compositions can be used to produce poles with specific stiffnesses (e.g., TM5MED-TM9MED). (**B**) Technical drawing and dimensions of a ring-shaped mold with a single stiff rod. This is an alternative mold with distinct geometry and mechanical environment, which can be used with the ECT casting protocol[37]. The ring-shaped mold assembly method was adapted from published bigger formats[28,41]. In brief, the method includes (1) imprinting polytetrafluoroethylene (PTFE) molding spacers (8 mm diameter) in polydimethylsiloxane (PDMS, silicone) poured into glass dishes (diameter 60 mm), and (2) fixing a PDMS pole holder (1.5 mm diameter) concentrically inside of the formed hollow cavity, which serves to (3) hold a removable pole (4 mm diameter silicone tube). The hollow space resultant allows for 180 µL of casting volume. Each glass dish can comport multiple imprinted molds (exemplarily shown with 5 molds) and has the capacity for up to 5 mL of culture medium.

Figure 2: Schematic overview of ECT generation. Fibroblasts are expanded in 2D culture before use in ECT generation. After 5-10 days, cells are enzymatically dispersed and cell suspension is reconstituted in a buffered mixture containing bovine collagen type 1. The cell-collagen hydrogel mixture is pipetted into individual wells in an engineered 48-well 3D cell/tissue culture plate, designed as casting molds with two flexible poles to enable ECT suspension at a defined length and load. ECT are typically cultured for 1 to 20 days prior to measurements.

Figure 3: Casting, hydrogel formation, and ECT condensation in multi-well format. The top panels exemplify the appearance of ECT directly after casting. The middle panels exemplify the appearance of ECT after incubation for 20 minutes at 37 °C. The bottom panels exemplify the state of compaction of ECTs 24 h after preparation. (**A**) Proper ECT formation between two poles during the first 24 h. (**B-D**) Examples of pipetting errors that prevent proper ECT formation. The white and black arrows point to structural defects of ECT due to improper casting. Scale bar: 5 mm.

Figure 4: Proper and improper addition of culture medium to the freshly cast ECT. (**A**) While adding the culture medium after initial ECT solidification (20 min after casting), the condensing ECT must be left undisturbed at the bottom of the well. During the next 24 h, cell-driven matrix compaction will make the ECT slide up the ramp. The final ECT position is controlled by concave cavities at a defined pole height; this ensures that all ECT settle at the same position to allow for a comparison of pole bending activity in parallel ECT culture. (**B**) Forming ECT detached from the bottom while adding the culture medium too rapidly. Floating ECT will compact at the upper culture medium level. Pole contracting forces will not be directly comparable if ECT settle at different positions. Scale bar: 2 mm.

Figure 5: Monitoring ECT compaction over time by cross-sectional area (CSA) analysis. (**A**) ECT were generated using human CF and collagen type I and cultured around two flexible poles for 5 days. Representative images of control ECT placed in flexible molds over a time of 5 days are presented. Scale bar = 5 mm. Such bright-field images can also be used to determine pole deflection variation for estimating tissue contraction. (**B**) Schematic representation of the cross-sectional area of an ECT (top view diameter in green and side view diameter in pink).

(**C**) Macroscopic images of top and side views of an ECT obtained with a stereomicroscope and correspondent example of line scan analysis of the tissues' diameters using an image processing program. Scale bar = 2 mm. Averaged diameters are calculated from the mean of all line lengths measured on each view plan.

Figure 6: Schematic overview of the assessment of tissue contraction according to pole deflection. (**A**) Exemplary high-resolution recording of fluorescent poles in the 48-well casting plate under near-UV light excitation. This method is preferred over bright-field pictures for more precise pole tip automated tracing. (**B**) The schematic drawings demonstrate how ECT compaction leads to pole bending. (**C**) An exemplary row of the same plate records at day 0 and day 5 after casting. **D.** The close up shows how to measure the distance (pink line) between the poles using an image processing program.

Figure 7: ECT destructive tensile measurement analysis. (**A**) Rheological destructive tensile measurement on an extensional dynamic mechanical analysis (DMA) rheometer. Upper high power view: ECT after mounting at L_0 in an environmental chamber and connected to an upper and lower pole for stress-strain analyses. Bottom high power view: ECT strained at a constant rate 0.03 mm/s until the failure point at ultimate strain. Scale bars = 5 mm. (**B**) Stress-strain diagram of an ECT showing the main measured parameters. The upper limit of the elastic region corresponds to the yield point and the plastic region is comprised between the yield point and the failure point (ductility). The slope of the linear phase of the elastic region corresponds to the Young's modulus reflecting tissue stiffness. The maximum strength corresponds to the maximum tensile stress a tissue can withstand. Due to fiber microfracturing, the stress decreases until the tissue reaches the failure point. This occurs at the ultimate strain (extensibility) where a sudden drop in stress is observed due to the rupture of the tissue. Resilience corresponds to the energy (kJ/m^3) absorbed by the tissue before permanent deformation and is given by the area under the curve (AUC) up to the yield point strain. Toughness corresponds to the total energy (kJ/m^3) the tissue can absorb until rupture and is given by the AUC up to the ultimate strain.

Figure 8: Inhibition of the actin polymerization influences ECT compaction, contraction, and stiffness. ECT generated with human CF and collagen type I were cultured for 5 days around two flexible poles in the presence or absence of 7 ng/mL Latrunculin-A (Lat-A). (**A**) Representative images of the control and treated ECT placed in flexible poles after 5 days are shown. Scale bar = 2 mm. (**B**) Cross-sectional areas (CSA) were calculated from macroscopic images (n = 22). (**C**) Pole deflection was calculated over a period of 5 days. Values are given as means±SEM (n = 22). Significant changes were assessed by 2-way ANOVA with Dunnett's (*$p<0.05$ vs. Control) post hoc tests for multiple comparisons. (**D**) Tissues were subjected to rheological destructive tensile measurements and the Young's moduli were retrieved from the stress-strain analyses (n = 16). (**B** and **D**) Boxes indicate the lower and upper quartile. Horizontal line in each box represents the median CSA and stiffness, respectively. The means for each group are indicated by a +. Vertical lines extending from each box represent the minimum and maximum values measured. Significant changes in **B** and **D** were assessed by unpaired, two-tailed Student's t-test (*$p<0.05$).

DISCUSSION:

The presented protocol describes the generation of ECT from primary human CF, which allows studying the mechanical impact of these cells on their extracellular matrix environment and vice-versa.

The fibroblasts need to be expanded to yield sufficient cells for the planned ECT experiments (0.75×10^6 cells/ECT). For the best reproducibility, it is advised to pre-culture frozen or tissue-derived fibroblasts in 2D monolayer culture for a standardized duration up to 80 % confluency within each passage and prior to their use in ECT generation (Protocol step 3). For culturing primary human CF-monolayers and -derived ECT in particular, it is advised to use commercial medium and supplements appropriate for CF (see **Table of Materials**). Medium supplementation with serum is critical to ensure the expansion of CF in standard 2D cultures. Using serum-free or low serum conditions in 3D cultures, including ECT generation and further culture, can be considered depending on the selected fibroblast type. However, when using CF for ECT generation, it is advised to at least include serum in the casting hydrogel for proper initial tissue compaction.

One limitation in the procedure is associated with CF expansion in 2D culture necessary for ECT generation, which typically leads to a conversion of fibroblasts into myofibroblasts (indicated by enhanced SMA and associated stress fiber formation[4]). Due to their continuous transdifferentiation, consider that fibroblasts in different passages can give different results when used to generate ECT. In the ECT model, two processes need to be discriminated. After suspension in a collagen hydrogel and ECT formation, cells adapt to their 3D environment and the myofibroblast phenotype may be at least partially reversed. In the following culture phase, the cells might then potentially undergo a switch again in the opposite phenotypic direction, especially by using poles with increasing stiffness or by the addition of pro-fibrotic factors (such as TGF-β1). The possibility to tune the dynamic phenotypic adaptation creates the opportunity to dissect the underlying and biomechanically controlled molecular mechanisms. Such studies may ultimately allow for the modeling of fibrotic conditions and the identifications of pharmacological or gene therapy interventions targeting organ fibrosis. The use of fibroblasts of various origins may further allow for the investigation of processes underlying tissue-specific fibrosis. Application of fibroblasts or other stromal cells not only of different origin but also from different species allows for cross-species studies of mechanisms underlying fibrosis or cell-matrix interactions. Nonetheless, it needs to be noted that by using primary cells from humans, the inter-individual differences between the cells must be taken into consideration. A failure in tissue contraction (see also below) is not necessarily a cause of an experimental error but can result from the intrinsic contractile properties of the individual cell line. Therefore, it is always preferred to use cells from different donors to allow for the discrimination of general mechanisms and donor-dependent differences. Similar to the variability of the obtained results, which could arise from the individual biology of the cell, it is important to mention that all biological material can show significant variability. Therefore, parallel testing of the material from different lots is recommended, at least when a lot of change becomes necessary.

Moreover, tissues grown on the pole pairs exhibit “arm” and “pole” regions that are structurally

and biomechanically dissimilar. It remains to be determined how much the pole region contributes to stretch experiments.

The tissue preparation process must be thoroughly fast to avoid gelation at room temperature. Cell-collagen hydrogel gelation is mainly driven by collagen self-assembly, and largely cell-independent[29]. It is the first step during tissue formation, and it should occur during the first 15-30 min once placed in a culture incubator. Collagen fibrillogenesis and gelation are impacted by, e.g., hydroxylation of prolines and lysines, and highly dependent on collagen type, ionic strength, pH, and temperature, which affects fiber bundling and pore size of the collagen network[42]. That could ultimately influence the cell component and, thereupon the structure and mechanical properties of the tissues. When choosing collagen sources and the chemical composition of naturally derived collagen, it is important to identify a reliable high-quality collagen solution for tissue engineering. The use of commercial acid-solubilized bovine type I collagen is recommended at an approximate stock concentration of 6-7 mg/mL. Nonetheless, other collagen solutions with a concentration of ≥ 4 mg/mL may also be compatible with this. Several other factors such as purity, molecular integrity, solubilizing agent, and shelf-age can influence the incubation time necessary for reconstitution (solidification) of the ECT hydrogel mixture, which should under no circumstances exceed 1 h to avoid cell sedimentation. For optimal results, store and handle collagen-containing solutions at 4 ± 2 °C. Collagen integrity can be disrupted if frozen or handled at room temperature and consequently prevent fibrillogenesis and hydrogel gelation. After pH neutralization and cell reconstitution in the collagen hydrogel, pipetting during casting must be gentle as strong shear forces may affect the integrity of the collagen structure and matrix assembly. Variability between batches of collagen or different suppliers can have an impact on ECT formation. It is advisable to test collagen hydrogel to ascertain ideal condensation properties before use in ECT preparation. Moreover, to guarantee appropriate pH neutralization, NaOH volume must be titrated for each individual collagen batch. Acetic acid-solubilized collagen with a collagen concentration range between 6-7 mg/mL is advisable. In general, additional quality controls are recommended, e.g., SDS-PAGE analysis for investigating collagen integrity and concentration and shear rheology to determine the viscous properties of the collagen solution.

After the initial phase of hydrogel solidification due to collagen self-assembly, the cell component drives matrix compaction further. If ECT do not compact visibly within 24 hours after casting, this may be related to cell viability. A minimum of 80 % cell viability is recommended. Ensure proper cell viability after enzymatic detachment of input cells to obtain proper tissue compaction and functionality. In this protocol, ECT are generated with 0.75×10^6 cells in a final volume of 180 µL per tissue, but different cell numbers may be required depending on the source of cells (e.g., CF donor, vendors). Thus, it is recommended to perform a cell titration experiment in the beginning. Typically, a range of cells from 150,000 to 750,000 can be tested for optimal formation and compaction of the tissues. Generally, this protocol uses 0.3 mg of collagen per ECT corresponding to 1.67 mg/mL collagen in a final volume of 180 µL. If necessary, adjust the ratio between cell number and collagen concentration (collagen concentration from 0.14 to 0.4 mg per tissue can be tested). Moreover, ensure a correct neutralization of acetic acid-solubilized collagen during hydrogel preparation as inadequate pH may be detrimental for cell viability.

As shown in **Figure 3** (bottom panel), ECT may not form uniformly. After cell reconstitution in the pH-neutralized collagen hydrogel mixture, the gelation process ensues even at 4 °C and is accelerated at room temperature (once cast into the mold). Ensure that the casting procedure is completed within 15-20 min. Premature gelation will impede proper pipetting of the mixture due to the increased viscosity. When casting the viscous cell-collagen hydrogel, pre-wet pipette tip with hydrogel or use a low retention pipette tip, and follow using the same tip to cast multiple ECT. This practice will reduce variation in hydrogel volume and the formation of bubbles during blow-out (**Figure 3D**). Ensure to complete the loop within the mold to form a ring-shaped ECT (**Figure 3A-B**). In addition, make sure that the input cell suspension is homogeneous and free of aggregates at all stages of casting. Mix frequently the cell-collagen hydrogen mixture by swirling the tube while carrying out the casting procedure into the 48-well casting plate. Finally, the gelation during incubation at 37 °C should occur maximally within 15-30 minutes. If this process takes longer, the chance of cell sedimentation increases, producing unevenly populated tissues.

Moreover, unevenly populated tissues and uneven distribution of the cell-collagen hydrogel into the molds can lead to irregular morphology of the ECT, and ECT may not contract uniformly throughout the 48-well casting plate. The ECT position on the flexible poles can also influence the contraction levels and contribute to a similar phenomenon. If the forming ECT detaches from the bottom while adding culture medium, it might float and will compact above the anchorage point of the poles with a defined bending force (**Figure 4**). This may lead to an overestimated pole deflection and induce variability between tissues/experiments. To avoid this, the hydrogel should be carefully overlaid with the culture medium via the well wall.

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DISCLOSURES:

GLS and SL drafted the manuscript. All authors contributed to the protocol development and edited the manuscript. TM, MT, and WHZ are scientific advisors to myriamed GmbH. WHZ is the founder and shareholder of myriamed GmbH.

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Figure 1

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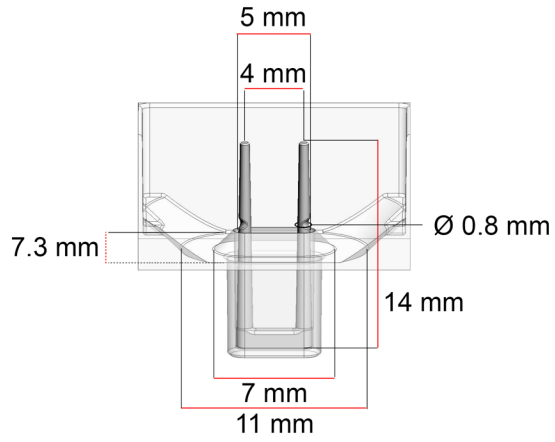

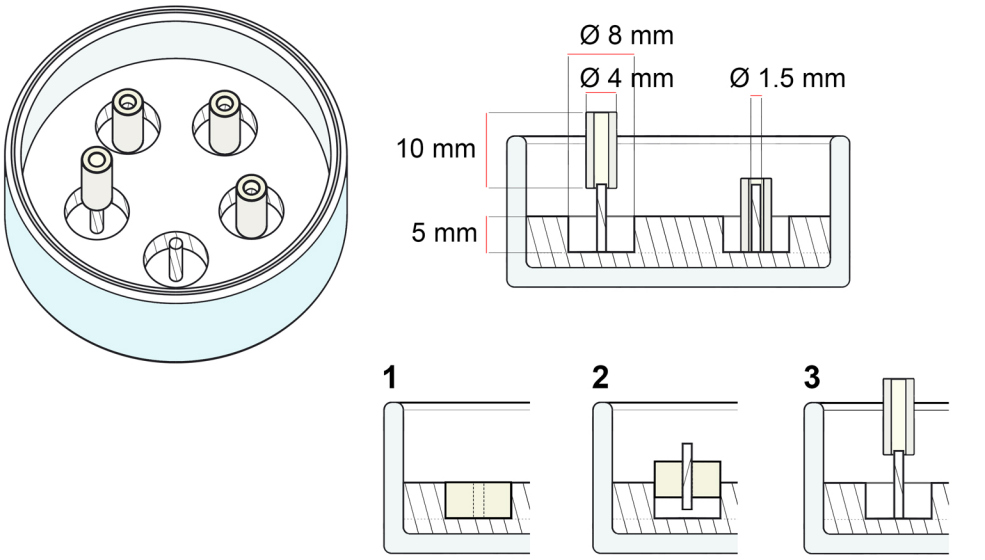

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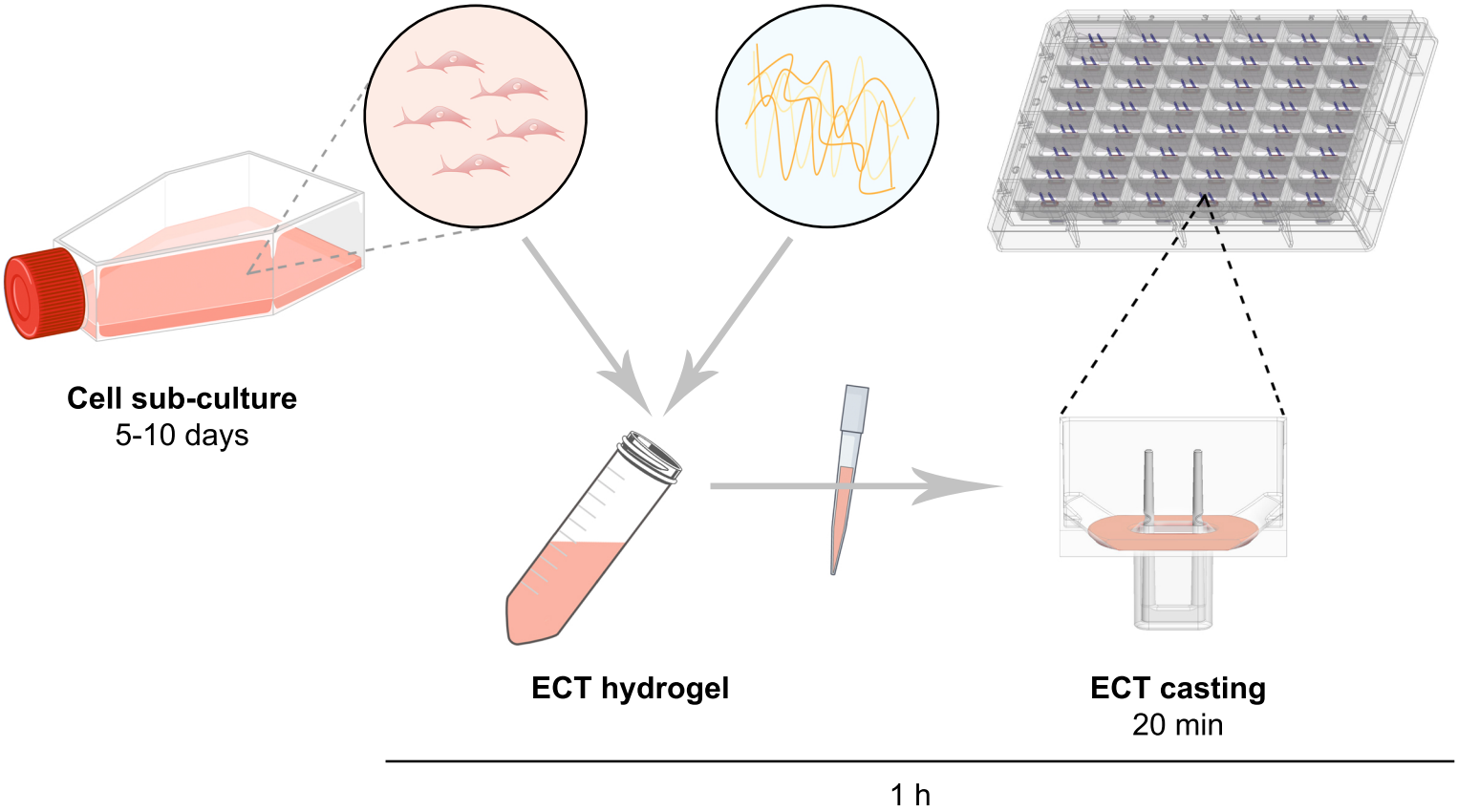

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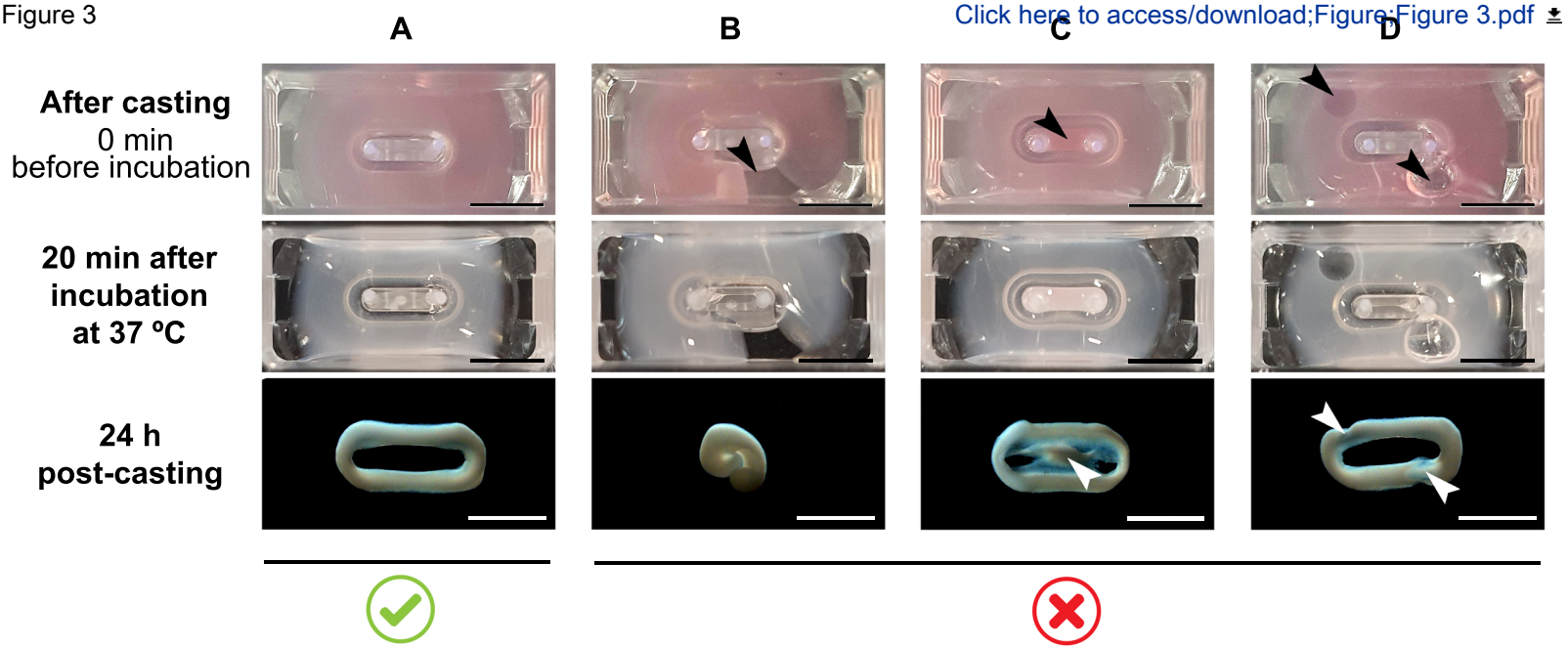

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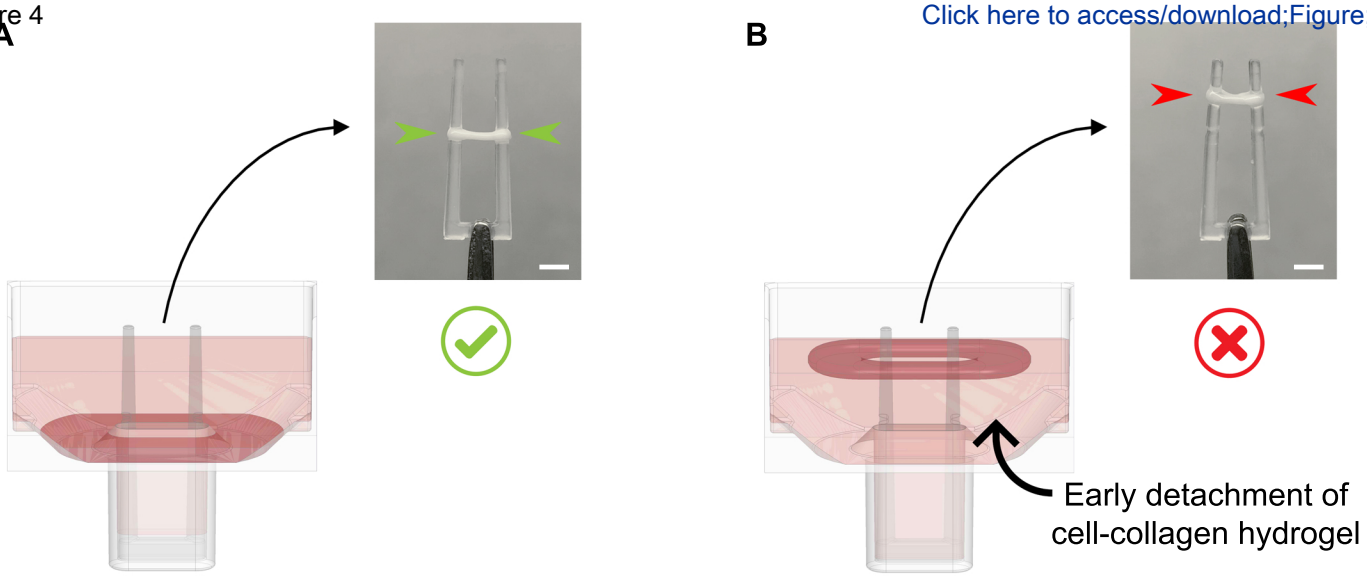

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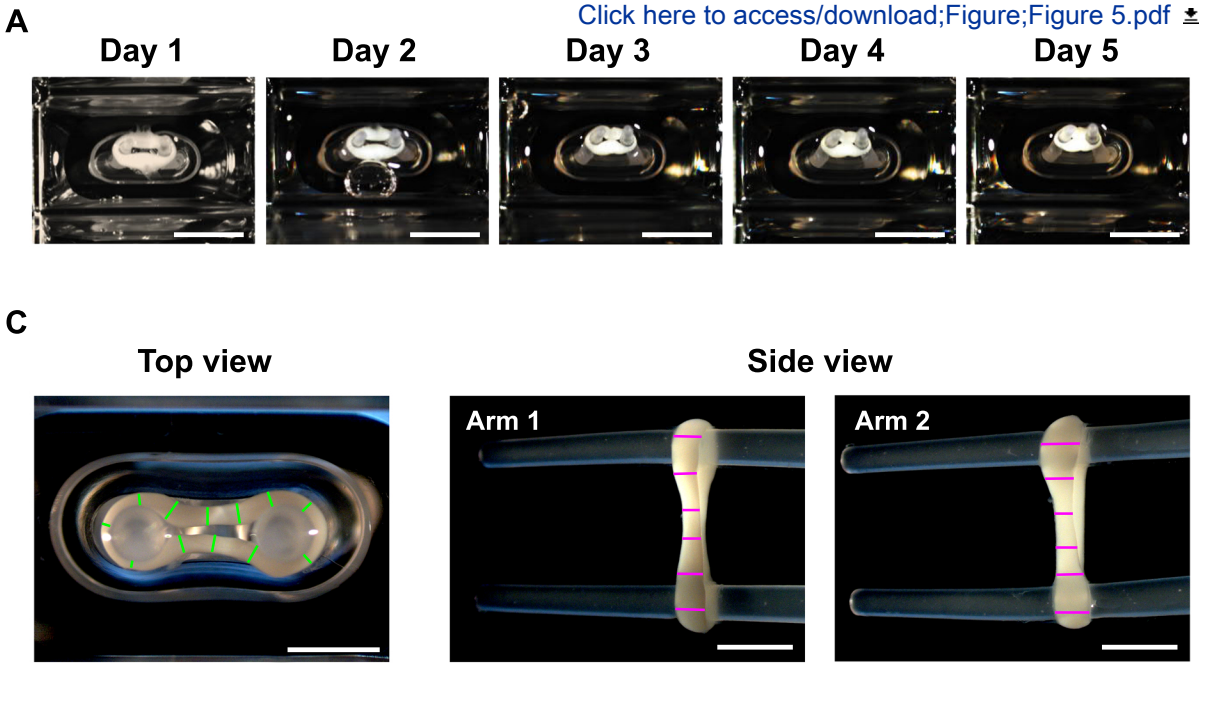

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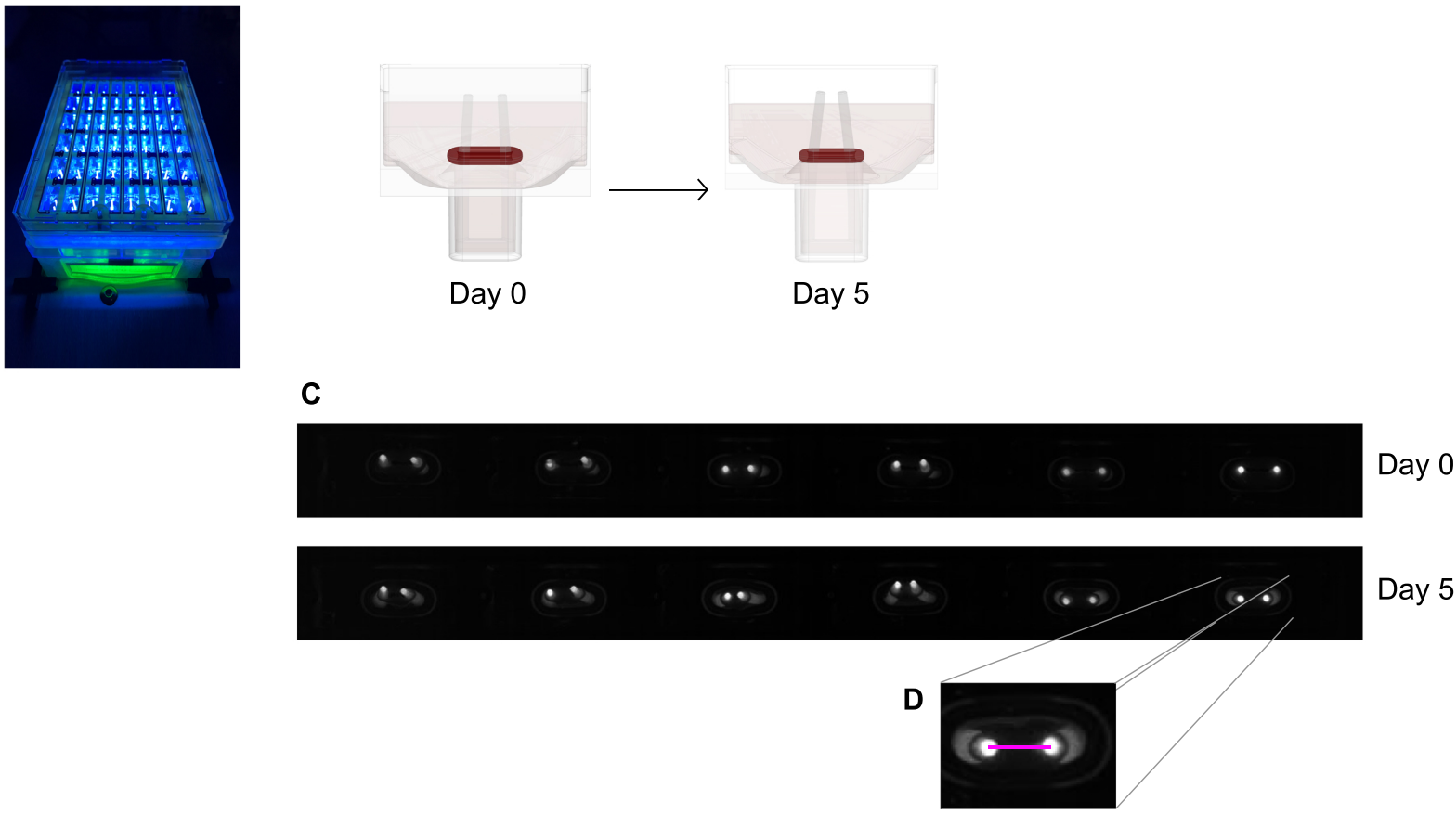

Figure 7

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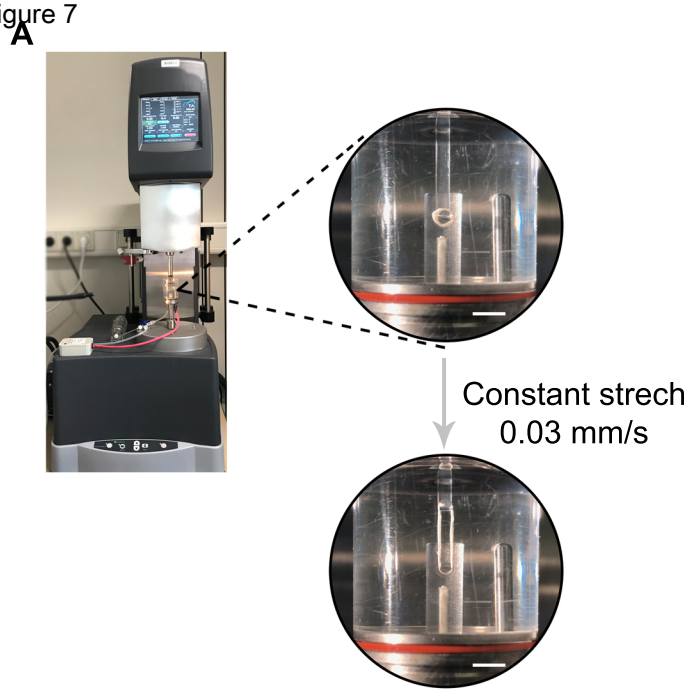


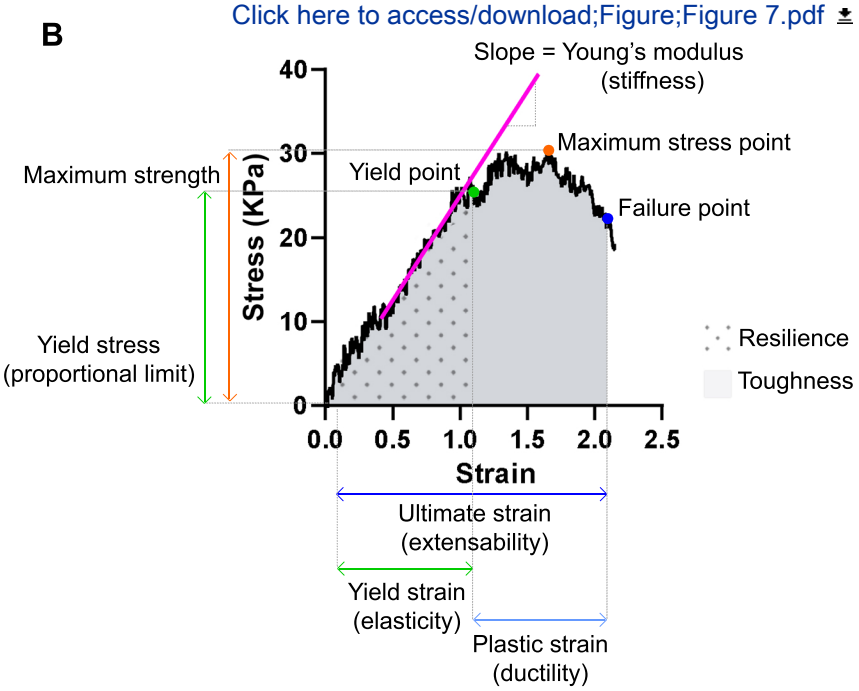
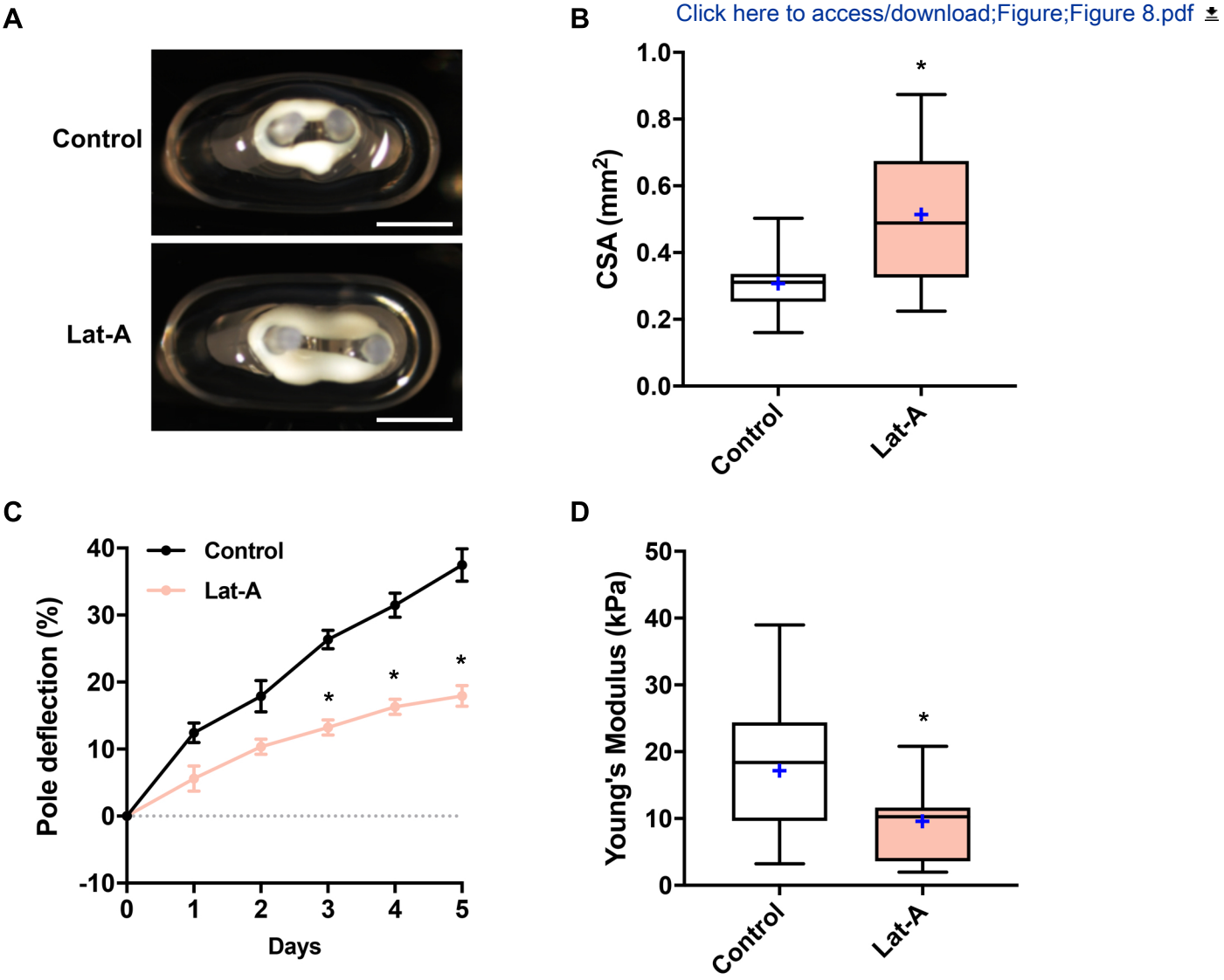


Figure 8



ECT number:	**1**	**6**	**24**	**48**
		including 10 % surplus		
Cell-collagen hydrogel components:	(µL)	(µL)	(µL)	(µL)
Collagen stock (6.49 mg/mL)	46.2	305.1	1220.2	2440.4
2× DMEM	46.2	305.1	1220.2	2440.4
0.2 M NaOH	3.1	20.5	81.8	163.7
Cell mix in FGM (8.88×10^6 cell/mL)	84.5	557.4	2229.7	4459.5
Total volume (µL)	180.0	1188.0	4752.0	9504.0

This is an exemplary table to prepare a casting volume of 180 µL per ECT, containing a total of 750,000 cells and 0.3 mg of collagen per ECT.

Table 2

Reagent	**Final Concentration**	**Volume (mL)**
10× DMEM	n/a	2
FCS	20 % (v/v)	2
Penicillin	200 U/mL	0.2
Streptomycin	200 mg/mL	0.2
ddH_2O	n/a	5.6
Total	**n/a**	**10**

Dear Dr. Iyer,

Thank you for giving us the opportunity to submit a revised version of our manuscript currently entitled "Fibroblasts-derived human engineered connective tissue for screening applications" to *JoVE*. We appreciate the time and effort that you and the reviewers have dedicated to providing your valuable and insightful feedback on our manuscript. We have been able to incorporate changes to reflect most of the suggestions provided by the editors and reviewers.

To facilitate the review process, the following is a point-by-point response to the editor's and reviewers' comments and concerns.

Editorial comments

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.
2. Reduce the word count of your summary to be 10-50 words.
3. For in-text formatting, corresponding reference numbers should appear as numbered superscripts after the appropriate statement(s), but BEFORE punctuation.
4. Please revise the following lines to avoid overlap with previously published work: 128-129; 160-163.
5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. 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 and Reagents. For example: myrPlate, myriamed GmbH; TM5 myrPlate-uniform model; TrypLE Express; Accutase; Basler acA4024; RSA-G2 (TA-Instruments); GraphPad Prism etc
6. Please revise the text, especially in the protocol, to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
7. 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.

8. The Protocol should be made up almost entirely of discrete, numbered steps without large paragraphs of text between sections. Individual steps should contain only 2-3 actions per step and a maximum of 4 sentences per step. Please rewrite the paragraphs on Cell sourcing, cell-collagen hydrogel preparation, and tissue culture platforms, culture media, primary cardiac fibroblast monolayer culture into numbered steps (levels 1, 1.1., 1.1.1., 1.1.1.1.).
9. Please consider providing solution composition as Tables in separate .xls or .xlsx files uploaded to your Editorial Manager account. These tables can then be referenced in the protocol text.
10. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.
11. After including a one line space between each protocol step, highlight up to (not more than) 3 pages of protocol text for inclusion in the protocol section of the video to clarify what needs to be filmed. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next.
12. Please include a scale bar for all images taken with a microscope to provide context to the magnification used. Define the scale in the appropriate Figure Legend.
13. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source (ital.). Volume (bold) (Issue), FirstPage–LastPage (YEAR).] For more than 6 authors, list only the first author then et al. Please include volume and issue numbers for all references, and do not abbreviate journal names.
14. Please sort the Materials Table alphabetically by the name of the material.

Response: Thank you for pointing out the necessary corrections and suggestions. We addressed all the points raised by the editors listed above, including spelling,

grammatical errors, technical and commercial language, and structure, and we hope to sufficiently have improved the manuscript.

Regarding the use of personal pronouns (*point 6.*), as an exception, in lines 85-86, we would like to keep the personal pronoun "we" as we specifically refer to our previously published work. Otherwise, all the passages with personal pronouns were rewritten.

Moreover, due to the length of the paragraphs on cell sourcing, cell-collagen hydrogel preparation, and tissue culture platforms, as raised on *point 8.* by the editors, we have re-structured the introductory section of the protocol. Those paragraphs address key aspects on which our protocol highly depend, thus we believe that it is of high importance for the *JoVE* readers to have an outline of those critical points before entering the protocol section. Therefore, we moved those key points to a new section entitle "*General considerations on materials*", which precedes the protocol section.

Comments from Reviewer #1

Santos et al MS# JoVE62700 Human engineered connective tissue for screening applications
Manuscript Summary: The manuscript describes the generation and functional assessment of 3D engineered constructs of human primary cardiac fibroblasts, termed engineered connective tissues (ECT) by the authors. The overall methodology has been widely used and validated in the literature, but Santos et al. take it a step further in this manuscript by providing a very detailed experimental protocol and the possibility of scaling it up to a 48-well format. The manuscript is very well written and the protocol easy to follow. Being a particularly 'tricky' protocol, this manuscript will be very helpful for researchers in the field of cardiac physiopathology and will also likely make the technology available to researchers currently using 2D fibroblast cultures to investigate a wide variety of topics. The manuscript provides a very good level of experimental detail and the text is well supported by seven illustrative figures. Points requiring particular attention and/or fine-tuning with regards to specific cell sources and batch-to-batch variability are well identified in the manuscript. Overall, I find the manuscript very timely and well written, and I do not have any major concerns that would preclude publication in its current form.
Major concerns: None

- **Comment 1:** *Lines 111-11 – The authors recommend the use of a commercially available casting plate for preparing the ECTs. This being a critical part of the protocol, I strongly suggest presenting additional commercial sources. If none other should exist, the authors could alternatively provide the necessary details for making appropriate custom molds, such as a simple diagram with dimensions. This information is missing in the paper by the authors' laboratories cited for that purpose (PMID: 31233754).*

  Response: Thank you for this suggestion. In fact, there are to our knowledge no alternative products on the market for parallel production of macro tissues of this structure and dimensions. So we followed the reviewer's suggestion and added a scheme with the mold dimensions, which might allow other researchers to generate this mold, e.g. by 3D printing (new Figure 1). We apologize, that the given reference does not contain detailed information on the geometry of the molds and how they are prepared, therefore, we also implemented in Figure 1 a schematic drawing of the circular molds we used in Santos et al., 2019 (PMID: 31233754). In the legend it is described how to generate these molds with simple materials like silicon tubings and glass dishes.

- **Comment 2:** *Line 305 – Please refer to Figure 2D at the end of the sentence.*

  Response: Thank you for pointing this out. We have revised the paragraph and incorporated your suggestion.

<u>Comments from Reviewer #2</u>

Manuscript Summary: G.L. Santos et al. describe in their manuscript "Human engineered connective tissue for screening applications" a protocol for the generation of fibrous connective tissue in a 48-well casting plate. They further show how these ECT can be used to determine tissue stiffness and elasticity. This technology could be of interest for researchers in the field of fibrosis and fibrotic diseases.

In general, the protocol and single steps are comprehensible. However, a number of open questions arose from reading of the manuscript. In addition, the manuscript contains several shortcomings which should be revised.

In my opinion, the current title of the manuscript is somewhat misleading, because the group of connective tissue includes different types like adipose tissue, bone or cartilage and fibrous connective tissue. The manuscript, however, focuses on fibroblasts and their function concerning the modulation of the extracellular matrix.

Response: Thank you for raising this important point here regarding the title. We agree that the title could be misinterpreted; therefore, we changed it to *"Fibroblast-derived human engineered connective tissue for screening applications"*.

- **Comment 1:** *Line 29 – ..."suitable for mechanistic studies". How can these ECT be used for mechanistic studies? It is difficult to imaging to perform gene expression or protein analysis from the cells. On the other hand, there would be no reason to use this specific 3D tissue model, probably other models might be more suitable for this purpose.*

  Response: We have demonstrated with the former version of the model that it is possible to perform mechanistic studies, as for example, deciphering the role of ROCK (Santos et al., 2019, doi: 10.1016/j.yjmcc.2019.06.015) and estrogen receptors (Dworatzek et al., 2019, doi: 10.1093/cvr/cvy185) in cardiac fibroblasts. We also have shown that these tissues can be manipulated by virally mediated gene transfer (Ongherth et al., 2015, doi: 10.1016/j.yjmcc.2015.09.009, Vettel et al., 2014, doi: 10.1152/ajpheart.00852.2013, Jatho et al., 2015, doi: 10.1371/journal.pone.0137519). In these studies, we analyzed not only gain and loss of function, but also the outcome at RNA and protein level by qPCR and immunoblotting. Although we have not yet disclosed microscopy images, this ECT model has a great potential for imaging. That can be attained by whole-mounting and sectioning via vibratome or cryostat. It is also possible to enzymatically digest ECT to re-isolate single cells. Cell number, size, and viability can be evaluated and isolated cells can further be used for flow cytometry analysis, including for cell cycle activity measurements. For details on the protocol for dissociating engineered tissues and cell re-isolation, please consult Tiburcy et al., 2017 (doi: 10.1161/CIRCULATIONAHA.116.024145).

  In general, we believe that mechanistical analyses are more informative and closer to the *in vivo* situation when performed in 3D. Especially fibroblasts are highly mechano-sensitive; therefore, mechanistic analyses should always be performed in the same model as their tissue forming abilities.

- **Comment 2:** *Line 40 – What do the authors mean with studies of "fibroblast tissue pathophysiology"? This is a rather unusual term.*

  Response: Thank you for the remark. We agree and therefore we revised the sentence to *"facilitate studies of fibroblasts in a 3-dimensional (3D) environment and pathophysiology of fibrotic tissue"* (lines 40-41).

- **Comment 3:** *Line 44 – "time-course analysis of multiple parameters, such as tissue compaction". How can the compaction be analyzed?*
- **Comment 4:** *Step 5.2. – "Perform a horizontal line scan analysis…". The description is not sufficient to be able to perform these measurements. It would be helpful to explain this procedure in more detail.*
- **Comment 5:** *The ECTs do not appear uniformly thick. Therefore, the diameters probably differ strongly which makes the calculation of the volume difficult. How can this be addressed?*

  Response: This is an important point of discussion. First, we define compaction as a reduction in ECT thickness perpendicularly to its long axis. This is a process which starts directly after casting and is very fast in the beginning. Compaction is necessary for the formation of an anisotropic tissue in which the cells eventually align longitudinally. This alignment is a prerequisite for ECT contraction. Compaction is therefore well described by a decline in the cross sectional area, whereas contraction can be determined by pole deflection (due to shortening of the arms). The ECT volume, based on its calculation by the CSA (compaction) multiplied by length (contraction), is thus a hybrid parameter. In order not to confuse our readers, we have removed it from the manuscript. Moreover, we inverted the order between steps 4. and 5., since tissue compaction precedes contraction. In the revised version of the manuscript, we present step *"4. Assessing ECT compaction by measuring cross-sectional area (CSA)"* and step *"5. Monitoring ECT contraction by pole deflection analysis"*, which include a simple explanation of each parameter.

  Secondly, the reviewer is right that the tissues do not present a perfect shape and are thinner around the poles. This is in agreement with other studies that show higher compaction/thinning of tissues around anchoring points due to increased mechanical stress in these regions (Asmani et al., 2018, doi: 10.1038/s41467-018-04336-z).

Therefore, we strongly suggest that the thickness of the ECT needs to be determined at several points. Our experience taught us that 6 measurements at each side/arm (in total 12 from top and 12 from side, see new Figure 5C) gives us a good approximation of the real tissue's dimension. Unfortunately, this was not clear from our description, so we have changed the text as follows:

"*4.2. Perform a line scan analysis, by tracing and measuring the ECT diameters at a minimum of 6 positions per arm in each imaging plane (Figure 5B,C). An image processing program can be used for these measurements.*"

- **Comment 6:** *Line 108 – "...we recommend performing a cell titration experiment in the beginning." Which concentration range of collagen and cells do the authors recommend? What parameter could be used to decide about a suitable cell number and collagen concentration?*

  Response: We agree with this and have incorporated your suggestion in the manuscript. The primary parameter to decide about suitable cell number and collagen is the tissue formation and compaction within the first 24 h after casting. This point was raised on the section of troubleshooting *"ECT do not compact visibly within 24 hours after casting"* (lines 672-673). To allow an easier understanding, we moved the statement *"...we recommend performing a cell titration experiment in the beginning"* to the troubleshooting section. Now it can be read (lines 679-682): *"Thus, it is recommend performing a cell titration experiment in the beginning. Typically, a range of cells from 150,000 to 750,000 can be tested for optimal formation and compaction of the tissues. If necessary, adjust the collagen concentration (from 0.15 to 0.4 mg per tissue)."*

- **Comment 7:** *Line 117 – Is shore A value of 46 correct? Shore A values are given as intervals of 5, 10 15 and so on.*

  Response: As you correctly indicated, shore A values are usually given as intervals of 5 to simplify the design of shore durometer conversion charts. However, shore values can be measured in a continuous scale and the material TM5MED has a shore value of 46, as indicated by the producer (https://pdb.kraiburg-tpe.com/?cid=1430).

- **Comment 8:** *By culturing the fibroblasts in conventional 2D cultures, myofibroblast differentiation will be induced especially with 10% FCS in the supplement. Would it*

be an option to use culture medium with reduced FCS concentration during the initial expansion phase and ECT generation?

Response: Thank you for pointing this out. We agree with this comment. However, fibroblasts in 2D cultures, at least primary human cardiac fibroblasts, require serum supplementation to proliferate. As a large amount of cells is required, limiting cell proliferation would penalize the cell yield for tissue production. Therefore, we do not recommend to use medium with reduced FCS during the initial expansion phase, although FCS could be reduced in the preceding hours of tissue preparation. For ECT generation, using serum-free or low-serum conditions could be an option, but it can be cell-dependent. It has been shown that using FCS in the cell-collagen hydrogel preparation and tissue culture is not strictly necessary when using HFF-1 (Schlick et al., doi: 10.1016/j.pbiomolbio.2018.11.011), but our experiments with ECT from primary human cardiac fibroblasts supplemented with low serum demonstrated an impaired compaction. As this question can help the readers designing their experiments, we have included in the new section of "General considerations on materials" the following: *"**Culture Media:** For culturing primary human CF-monolayers and -derived ECT it is strongly recommend the use of a commercial medium and supplements appropriate for CF (see Table of Materials). Medium supplementation with serum is critical to ensure the expansion of CF in standard 2D cultures. Using serum-free or low serum conditions for ECT generation and culture can be considered depending on the cell source selected. For the particular generation of primary CF-derived ECT, including serum at least in the casting hydrogel is advised for a proper initial tissue compaction."* (lines 127-133)

- **Comment 9:** *Line 213 – "...obtain a cell suspension of ≥ 15 x 10^6/ml." Why is a suspension with ≥ 15 x 10^6/ml cells needed? What is this cell concentration based on?*

  Response: The reasoning behind preparing a cell suspension of higher cell concentration (step 2.8.) than desired for tissue preparation (step 3.3.) is the cell number loss due to straining. This point was initially highlighted in step 2.9. Using a 40 µm mesh cell strainer to eliminate cell agglomerates from the suspension can lead to a loss of around 10 to 25 % of total cells, depending on extracellular matrix content and efficiency of the cell-cell dissociation during enzymatic dispersion. Thus, ≥ 15 × 10^6/ml is an empiric value that ensures a wide safety margin to guarantee that it is possible to prepare a suspension to the desired concentration after straining without

any additional steps. To better address this aspect, we have modified the section comprised between steps 2.7. and 2.9 (now lines 226-238). In the revised manuscript can be read:

"2.7. Aspirate the supernatant and flick the tube to dislodge the pellet. Resuspend the cells in 4 °C cold FGM to obtain a cell suspension of ≥ 15 × 10^6/mL (approximately 40 % more cells then required for step 3.3.). This accounts for cell loss due to straining in the following step.

2.8. Strain the cell suspension through a 40 µm mesh cell strainer.

CAUTION: Cell agglomerates are detrimental for an optimal formation of ECT. When using the enzymatic dispersion of human CF protocol for directly cast ECT, straining the cell suspension ensures the absence of major cell clumps that interfere with homogeneous tissue formation. Heterogeneities will compromise reliable stress-strain analyses.

2.9. Recount cell number by using a microscope and a hemocytometer or an automated cell counter, to ensure a reliable cell number to proceed with ECT preparation."

We hope that the changes render the steps in question more self-explanatory and that they address the point raised in a clearer way.

- **Comment 10:** *Step 3.1. – Why is there no buffer substance (like sodium bicarbonate) added to the DMEM medium?*

  Response: You have raised a much valid point here. The concentrated DMEM medium is used to adjust salt content of the acid soluble collagen to a physiologic level (step 3.4.b.), and the phenol red indicator present in the medium is used to follow the pH transition during neutralization (to pH 7) with NaOH (step 3.4.c.). The final volume of DMEM (then at 1× final concentration) constitutes in an ECT approximately 23-28 % of its volume during casting (steps 3.4.-3.6., 15-20 min) and initial consolidation (step 3.7., 15-30 min). Once the first culture media (buffered FGM) is added to the forming tissue after incubation (step 3.8.), DMEM percentage present in each tissue culture decreases to approximately 5-6 %. Thus the contribution of DMEM to the final pH of tissue culture during the first 24 h is minimal. At day 1 post casting, most DMEM is washed out during media change. Therefore, we did not require adding sodium bicarbonate to the DMEM in step 3.1.

- **Comment 11:** *Step 3.4. – Why do the authors use DMEM and not FGM for preparing the ECT hydrogel mixture?*

  Response: Similar to the previous comment, this point is also of a significant technical relevance. As referred above, DMEM medium is used to adjust salt content of the acid soluble collagen. Thus DMEM must be prepared from powder form in order to obtain 2× concentrated media which when added in a 1:1 ratio to the collagen solution forms an osmotic balanced solution. To make clearer the reasoning for this step, we edited the step 3.4.b. as follows: *"Adjust salt content of the collagen solution by adding the 2× DMEM while gently mixing by swirling the tube."* While the cell suspension (step 3.4.d) is prepared in FGM, the reason why we do not use FGM but DMEM in step 3.4.b. is the commercial unavailability, up to our knowledge, of powder form of a media optimized for fibroblast culture, such as the FGM we use.

- **Comment 12:** *Line 125 – "Allow complete mixture…" something is missing in the sentence. The sentence is somehow incomplete.*

  Response: Thank you for the kind remark. We have rewritten the sentence: *"Allow for complete mixture of all the components before use."* (current lines 157-158).

- **Comment 13:** *Reference 29 does not describe a detailed culture protocol of HFF-1. Is there another, more suitable reference available?*

  Response: We apologize for the insufficient reference given. Cell supplier, ATCC (mentioned in the Table of Materials), sources the most detailed culture protocol, which was validated with our ECT preparation protocol. Therefore, we re-wrote the passage as follows: *"As an alternative cell source to the present protocol, HFF-1 can be used following the standard sub-culture protocol advised by the supplier."* (lines 150-152).

- **Comment 14:** *Line 193 – In the case that the cells do not detach at room temperature, they could be incubated at 37°C for few minutes alternatively (see Note below).*

  Response: Thank you for pointing this out. We agree with this comment. Therefore, we have incorporated your suggestion. The text (lines 205-211) was changed to:

  "2.4. Add 5 mL of cell dissociation reagent to the cells and incubate for 3 min at 20-25 °C, until the cells start visibly detaching.

Note: Depending on CF source, this may take several minutes longer. Alternatively, if cells do not detach at room temperature, incubation can take place at 37 °C to improve enzymes' activity. To ensure optimal cell viability, it is recommend monitoring cell detachment under the microscope."

- **Comment 15:** *Line 204 and 212 – "Centrifuge the cells at 300 xg for 4 min at 4°C." and "Re-suspend the cells in 4°C cold FGM…". Working at 4°C may negatively influence the cell metabolism and therefore proliferation. Furthermore, in line 187 the authors state to warm FGM to room temperature. This is contradictory. For normal passaging of the cells, it would be better to work at room temperature.*

  Response: Thank you for raising such critical point for the protocol. We understand and agree with your concern and therefore, revised all the centrifugation steps throughout the manuscript and steps 2.6., 2.7, 2.10 and 3.3 were edited accordingly. Besides, a note was added to raise attention to the impact of temperature in the cell-collagen hydrogel mixture (lines 290-222): *"CAUTION: All components for the cell-collagen hydrogel mixture and centrifuge tubes must be kept on ice prior use. This will help prevent collagen self-assembly from occurring before distributing the cell-collagen hydrogel mixture throughout the casting molds."* We believe that the changes made to the protocol make it more coherent.

- **Comment 16:** *Within the protocol, there are two cell counting steps (2.5. and 2.9.) included. The necessity of the first cell counting step is not clear. For convenience, this step could be possibly removed.*

  Response: This point has already been addressed in *Comment 7*. The necessity to count cells at this point in the protocol (step 2.5.) relates to the fact that it is necessary to prepare a cell suspension of defined concentration in step 2.7., as outlined in response to *Comment 7*. We hope that the changes made will be sufficient to simultaneously clarify the issue raised here, without the need to remove step 2.5.

- **Comment 17:** *Line 227 – "…assess viability…". An alternative method should be described, like using the trypan blue staining e.g., because many laboratories do not have the opportunity to use an automated cell counter system.*

  Response: We appreciate the valuable suggestion, which will make the protocol more accessible. Therefore, the following passage was added *"Alternatively, the trypan blue*

(carcinogen, hazard category 2 - take precautionary measures) dye exclusion test can be used for the identification and direct enumeration of live (intact cell membranes that exclude the dye) and dead (compromised cell membranes which allow binding of the dye to intracellular proteins) cells in a cell suspension." (lines 242-245).

- **Comment 18:** *Table 1 – Cell mix in FGM: The cell concentration of 8.07x106 cells/ml cannot be related to the given final concentration of 0.75x106 cells/ECT and the volume of 92.9 µl.*

  Response: Thank you for making such an important remark. There was an error in the cell concentration of the cell mix in FGM. The value has been corrected to $8.88x10^6$ cells/mL in both table and body of the protocol. Moreover, the table was edited and re-formatted in a way that we believe it will be more self-explanatory for the *JoVE* readers.

- **Comment 19:** *The indicated volumes going from 1x to 6x to 24x and 48x contain mistakes. I assume, this is caused by rounding errors using excel?*

  Response: This question has been addressed with the modifications made in response to the previous comment.

- **Comment 20:** *Line 345 – "...recording device with an integrated camera…". Could the authors please specify the features of this camera necessary for the imaging.*

  Response: We followed the advice and edited the step 5.1., where can be read *"5.1. Image the 48-well casting plate under a recording device with an integrated area scan camera placed at a fixed distance, equipped with a high resolution (≥ 5 mega pixel) monochrome image sensor. A near-UV (~390 nm) light source maximizes contrast facilitating automated detection of the poles' tips as they contain a fluorescent dye (Figure 6A,C). If available, telecentric lenses are recommended for imaging as they minimize image distortions."* (lines 401-405). Moreover, the specific device components were added to the Table of Materials.

- **Comment 21:** *Line 456 – The given unit is not correct. kPa = mN x mm2.*
- **Comment 22:** *Line 465 – Correction: σ is stress and ε is strain. Strain is actually not unitless. In the manuscript it would be mm/mm or %.*
- **Comment 23:** *Line 466 – The translation of the units is not correct (see also above).*

- **Comment 24:** *Line 549 and 551 – The SI unit of the energy corresponding to resilience and toughness, respectively, is kJ/m³.*

  Response: We appreciate the all the remarks regarding SI unites and units conversion. We have revised the complete section (lines 483-494 from the revised version) and corrected all the points mentioned above.

- **Comment 25:** *Line 566 – "Significant changes in C. and D. …" C is wrong. I assume, it should be B. and D.*

  Response: Thank you for the kind remark. The mistake has been corrected.

- **Comment 26:** *Line 659 – The last sentence of the troubleshooting section is difficult to read and to understand. It should be rewritten.*

  Response: Thank you for the kind remark. The sentence was entirely rewritten as follows: *"Another aspect is the ECT position on the flexible poles. If the forming ECT detaches from the bottom while adding culture medium, it might float and will compact above the anchorage point of the poles with defined bending force (Figure 4). This may lead to an overestimated pole deflection and induce variability between tissues/experiments. To avoid this, the hydrogel should be carefully overlaid with culture medium via the well wall."* (lines 708-712).

- **Comment 27:** *In table of materials:*

  1. The first column is not completely visible.

  2. LC Collagen Solutions with CB-024 and Enzo Fife Sciences cannot be found on the internet.

  3. Dissociation reagent- TrypLE Express with # 12604039 is for 20 x 100ml. For a trial, it would be better to suggest a smaller amount like 100 ml (# 12604013).

  4. C-30310, C-37340 and C-31010 does not exist anymore. The kit contains all these supplements. The kit could be included instead.

  5. DPS # 14190169 (10x 500ml) is again a quite large amount. It would be better to suggest 1x 500ml with # 14190144.

  6. The same with Pen/Strep. # 15140130 (20x 100ml), alternatively 100 ml #15140122 or 20 ml # 15140148.

  7. The catalog number of the TM5 myrPlate-uniform cannot be found on the internet.

8. The stated catalog number of the serological pipettes with wide opening can also not be found on the internet.

Response: We appreciate all the remarks and suggestions made. The table of contents has been revised for formatting, typos and catalog numbers.

Comments from Reviewer #3

Overall, this is an excellent manuscript describing detail procedures for producing and testing myofibroblast tissue constructs. Below are minor points authors should consider when revising the manuscript.

- **Comment 1:** *The title and abstract are somewhat misleading. This publication is focused on fibrotic cardiac tissue produced by myofibroblasts. "Human engineered connective tissue" is too broad and even can be considered incorrect. I think the title should be "Engineered human cardiac tissue for screening applications." Or "Double Pole Tissue Culture System for Studying Cardiac Tissues." Otherwise, authors should add additional contents in the manuscript which is about non-cardiac tissues, such as skin, tendon, or other skeletomuscular tissues. If this is too much additional work, at least, the manuscript could have a separate subsection on how this culture system can be used for investigating these other non-cardiac tissues. Then the title could be a little more general such as "Double Pole Tissue Culture System for Studying Fibrotic Tissues".*

  Response: Thank you for raising an important point here regarding the title. We agree that the title could be misinterpreted; therefore, we changed it to *"Fibroblast-derived human engineered connective tissue for screening applications"*. In this way we limit the scope of connective tissues to fibrous connective tissue. Although we exemplify the protocol using primary cardiac fibroblasts, fibroblasts from other sources can be used depending on the scientific question. The compatibility of the protocol with non-cardiac fibroblasts is raised in the section *"General considerations on materials"* of the revised, where it can be read "***Cell sourcing:*** *The described prototypical procedure is based on human primary CF, obtained as cryopreserved CF from commercial vendors (see Table of Materials). Fibroblasts from other sources,*

including commercial human foreskin fibroblasts (HFF-1)[38] and fibroblasts from patient's biopsies work equally well and the choice of fibroblasts ultimately depends on the scientific question to be addressed. (...)" (lines 97-101). For example, the presented protocol (Step 3.) has been used to produce ECT with human foreskin fibroblasts to test the effect of chitosan-coated multiwall carbon nanotubes on the biomechanical properties of the tissues (Kittana et al., 2021, doi: 10.2147/IJN.S289107). We believe that by not restricting the title to *"cardiac tissues"* the manuscript will reach a larger scope of *JoVE* readers, interested on connective tissues in general, who can thus benefit from an adaptable protocol.

- **Comment 2:** *Summary section should mention the double pole culture system, since it is the most important and innovated part of the work demonstrated in this paper.*
  Response: Thank you for the insightful suggestion. We agree with the comment and the double pole system is now mentioned in the summary section: *"A robust protocol to generate engineered connective tissues (ECT) enables parallel culture of 48 tissues in a multi-well plate with double poles, suitable for mechanistic studies, disease modeling, and screening applications. The protocol is compatible with fibroblasts from different organs and species, and exemplified with human primary cardiac fibroblasts (CF)."*

- **Comment 3:** *The rest of the manuscript is very thoroughly prepared and written in high detail; however, there are some grammatical errors, awkward expressions, and typographical errors. Below are some I noted which need to be corrected.*
  ** Line 31: "here exemplified"*
  ** Line 46: "devised"*
  ** Line 97 and others: approximate symbol and degree symbol are incorrect. ~ should be ~ and should be °.*
  ** Line 227: double periods*
  ** Line 354, 381: "...as, e.g., ImageJ..." should be "...such as, Image J..."*
  ** Line 362-364: This note should be re-written entirely. It should read "...consequence to tissue compaction..." "Consider that pole deflection measured by bright tip image is only an estimation of ..."*
  ** Line 385: "Either way" should be removed.*
  ** Line 439: "me?"*

** Line 593: It should be "Similar to… which could arise from…"*

** Line 595: "latest?"*

** Line 635-647: This section is poorly written. Line 638-640, is unclear. "Secondly" should be "In addition.." since there is no mention of first. "caring" should be "carrying." And "This can result in formation of …"*

** Line 654: "on" should be "in the previous.."*

** Line 658: "induce" not "induces"*

** Line 659-661: there are about 4-5 typos.*

Response: We appreciate all the attention to detail, mismatched words, and typos. We have thoroughly reviewed the raised points and suggestions and made corrections accordingly. We hope that these changes to the manuscript now matches the journal standard.

Additional clarifications:

In addition to the above scientific comments, we attached files of the revised manuscript, new and revised figures, and tables. Spelling, grammatical, and formatting errors were corrected. Based on editor's and reviewer's concerns and suggestions, we re-wrote several passages to improve the contents and the coherency of our manuscript. We have tracked the changes within the manuscript, file 'Revised manuscript_Santos - Track Changed' version, to identify all of the edits.

We hope that our edits and the responses we provide satisfactorily address all the issues and concerns you and the reviewers have noted.

We look forward to hearing from you regarding our submission and to respond to any further questions and comments you may have.

Sincerely,

, 11.05.2021 Goettingen

(Gabriela L. Santos)

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Author(s): Gabriela L. Santos, Tim Meyer, Malte Tiburcy, Wolfram-Hubertus Zimmermann, Susanne Lutz

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