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A Multi-Cue Bioreactor to Evaluate the Inflammatory and Regenerative Capacity of Biomaterials Under Flow and Stretch --Manuscript Draft--

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TITLE:

A Multi-Cue Bioreactor to Evaluate the Inflammatory and Regenerative Capacity of Biomaterials Under Flow and Stretch

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KEYWORDS:

In situ tissue engineering, cardiovascular, tissue-engineered vascular grafts (TEVGs), macrophage, myofibroblast, co-culture, strain, shear stress, hemodynamics, scaffold, in vitro, biomechanics.

SUMMARY:

The goal of this protocol is to execute a dynamic co-culture of human macrophages and myofibroblasts in tubular electrospun scaffolds to investigate material-driven tissue regeneration, using a bioreactor which enables the decoupling of shear stress and cyclic stretch.

ABSTRACT:

The use of resorbable biomaterials to induce regeneration directly in the body is an attractive strategy from a translational perspective. Such materials induce an inflammatory response upon implantation, which is the driver of subsequent resorption of the material and the regeneration of new tissue. This strategy, also known as *in situ* tissue engineering, is pursued to obtain cardiovascular replacements such as tissue-engineered vascular grafts. Both the inflammatory and the regenerative processes are determined by the local biomechanical cues on the scaffold (i.e., stretch and shear stress). Here, we describe in detail the use of a custom-developed

bioreactor that uniquely enables the decoupling of stretch and shear stress on a tubular scaffold. This allows for the systematic and standardized evaluation of the inflammatory and regenerative capacity of tubular scaffolds under the influence of well-controlled mechanical loads, which we demonstrate on the basis of a dynamic co-culture experiment using human macrophages and myofibroblasts. The key practical steps in this approach—the construction and setting up of the bioreactor, preparation of the scaffolds and cell seeding, application and maintenance of stretch and shear flow, and sample harvesting for analysis—are discussed in detail.

INTRODUCTION:

Cardiovascular tissue engineering (TE) is being pursued as an alternative treatment option to the currently used permanent cardiovascular prostheses (e.g., vascular grafts, heart valve replacements), which are suboptimal for large cohorts of patients^{1–4}. Much sought-after applications include tissue-engineered vascular grafts (TEVGs)^{5,6} and heart valves (TEHVs)^{7,8}. Most often, cardiovascular TE methodologies make use of resorbable biomaterials (either natural or synthetic) that serve as an instructive scaffold for the new tissue to be formed. The formation of new tissue can either be engineered completely in vitro, by seeding the scaffold with cells and culturing in a bioreactor prior to implantation (in vitro TE)^{9–11}, or directly in situ, in which the synthetic scaffold is implanted without pre-culturing in order to induce the formation of new tissue directly in the body (in situ TE)^{12–14}. For both in vitro and in situ cardiovascular TE approaches, successful functional regeneration is dominantly dependent on both the host immune response to the implanted construct and appropriate biomechanical loading.

The importance of biomechanical loading for cardiovascular TE is well-acknowledged¹⁵. In the case of cardiovascular implants, the cells that populate the scaffold are exposed to cyclic stretch and shear stresses that arise as a result of the hemodynamic environment. Numerous studies have reported the stimulatory effect of (cyclic) stretch on the formation of matrix components, such as collagen^{16–19}, glycosaminoglycans (GAGs)²⁰, and elastin^{21,22}, by various cell types. For example, Huang et al. demonstrated that biaxial stretch elevated the deposition and organization of collagen and elastin in in vitro TEVGs by using a vascular bioreactor²³. While the emphasis typically lies on stretch as the dominant load, these studies often make use of flow-driven bioreactors in which the sample is also exposed to shear flow. Although relatively little is known about the isolated influence of shear stresses on tissue formation and inflammation in 3D, some data are available. For example, Hinderer et al. and Eoh et al. demonstrated that shear flow, in addition to a 3D scaffold microstructure, was important for the formation of mature elastin by human vascular smooth muscle cells in an in vitro model system^{24,25}. Altogether, these findings illustrate the relevance of both cyclic stretch and shear stress for cardiovascular TE.

Another important determinant for the success or failure of TE implants is the host's immune response to the implanted graft²⁶. This is particularly important for material-driven in situ TE strategies, which actually rely on the acute inflammatory response to the scaffold to kickstart the subsequent processes of cellular influx and endogenous tissue formation and remodeling²⁷. The macrophage is a critical initiator of functional tissue regeneration, which has been shown by multiple studies^{28–30}. Analogous to wound healing, the regeneration of tissue is governed by paracrine signaling between macrophages and tissue-producing cells such as fibroblasts and

myofibroblasts^{31–33}. In addition to coordinating new tissue deposition, macrophages are involved in the active resorption of foreign scaffold material^{34,35}. As such, the in vitro macrophage response to a biomaterial has been identified as a predictive parameter for the in vivo success of implants^{36–38}.

The macrophage response to an implanted scaffold is dependent on scaffold design features such as material composition and microstructure^{35,39,40}. In addition to scaffold properties, the macrophage response to a scaffold and their crosstalk with myofibroblasts is also impacted by hemodynamic loads. For example, cyclic stretch was shown to be an important modulator of macrophage phenotype^{41–44} and the secretion of cytokines^{43–46} in 3D electrospun scaffolds. Using a co-culture system of macrophages and vascular smooth muscle cells, Battiston et al. demonstrated that the presence of macrophages led to increased levels of elastin and GAGs and that moderate levels of cyclic stretch (1.07–1.10) stimulated the deposition of collagen I and elastin⁴⁷. In previous work, we had demonstrated that shear stress is an important determinant for monocyte recruitment into 3D electrospun scaffolds^{48,49}, and that both shear stress and cyclic stretch impact the paracrine signaling between human monocytes and mesenchymal stromal cells⁵⁰. Fahy et al. demonstrated that shear flow increased the secretion of pro-inflammatory cytokines by human monocytes⁵¹.

Taken together, the above evidence shows that an adequate understanding of and control over hemodynamic loads is crucial for cardiovascular TE, and that it is important to consider the inflammatory response to achieve this. Numerous bioreactors have been described previously for the in vitro^{52–58} or ex vivo^{59–61} culture of cardiovascular tissues. However, all these systems are designed to mimic the physiological hemodynamic loading conditions as much as possible. While this is highly valuable for the purpose of creating cardiovascular tissues in vitro or maintaining ex vivo cultures, such systems do not allow for systematic studies into the individual effects of individual cues. This is because the application of both cyclic stretch and shear stress in these bioreactors is driven by the same pressurized flow, which intrinsically links them. While microsystems that allow for accurate multi-cue mechanical manipulation have been described for 2D substrates⁶² or 3D hydrogel setups^{63,64}, such setups do not allow for the incorporation of elastomeric 3D biomaterial scaffolds.

Here, we present the application of a tubular bioreactor system that uniquely enables the decoupling of shear stress and cyclic stretch and helps to mechanistically investigate their individual and combined effects. This system allows for testing of a broad variety of tissue engineered vascular grafts (e.g., synthetic or natural origin, different micro-architecture, various porosities). To effectively decouple the application of shear stress and stretch, the key concepts that the bioreactor uses are (1) separation of the control of shear stress and stretch using distinct pump systems and (2) stimulation of the scaffolds in an ‘inside-out’ manner with computationally driven dimensions. Flow is applied on the outside surface of the tubular scaffold through the use of a flow pump, whereas circumferential stretch of the scaffold is induced by expanding a silicone tube on which the scaffold is mounted through the use of a separate strain pump. The dimensions of the silicone tube and the glass tube that contains the construct are carefully chosen and validated using computational fluid dynamics simulations, to ensure that the shear stress on the

scaffold (due to flow) and the circumferential stretch (due to tube expansion) do not significantly affect each other. This inside-out design has several practical rationales. If stretch is applied by the luminal fluid pressure (similar to physiological loading), this inherently requires the sample design to be leak-free. In addition, the pressure required to stretch the sample would be completely determined by the sample stiffness, which may vary between samples and within a sample over time, making it difficult to control the stretch. This bioreactor mounts the tissue engineered graft around a silicone tube and allows for wall shear stress (WSS) application on the outer wall of the graft and pressurizes the graft from the inside. This way, equal loading conditions between samples and within samples over time can be ensured, and moreover, the samples are allowed to be leaky, as is common for porous vascular scaffolds¹⁹. This inside-out bioreactor is specifically intended for systematic studies on the effects of shear and/or stretch, rather than the engineering of a native-like blood vessel in vitro, for which traditional vascular bioreactor setups are more suitable. See **Figure 1A–B** for the bioreactor design drawings, and its corresponding **Table 1** for a functional description and rationale behind the main components of the bioreactor.

The use of the bioreactor is demonstrated on the base of a series of recent studies by our group in which we investigated the individual and combined influences of shear stress and cyclic stretch on inflammation and tissue formation in resorbable electrospun scaffolds for in situ cardiovascular tissue^{19,43,44}. To that end, we use human macrophages and myofibroblasts either in mono- or in co-culture to simulate the various phases of the in situ regenerative cascade. We had demonstrated that cytokine secretion by human macrophages is distinctly impacted by both cyclic stretch and shear stress, affecting the matrix deposition and organization by human myofibroblasts in these scaffolds, both via paracrine signaling and direct contact^{19,43,44}. Notably, these studies revealed that in the case of combined application of shear stress and stretch, the effects on tissue formation and inflammation are either dominated by one of the two loads, or there are synergistic effects of both loads. These findings illustrate the relevance of decoupling both loads to gain a better understanding of the contribution of the mechanical environment on TE processes. This understanding can be applied to systematically optimize scaffold design parameters in relevant hemodynamic loading regimes. In addition, the mechanistic data from such well-controlled environments may serve as input for numerical models that are being developed to predict the course of in situ tissue remodeling, as recently reported for TEVGs⁶⁵ or TEHV⁶⁶, to further improve predictive capacity.

PROTOCOL:

In the studies described in this protocol, primary human macrophages isolated from peripheral blood buffy coats and human myofibroblasts isolated from the saphenous vein after coronary bypass surgery have been used⁴⁴. The buffy coats were obtained from healthy, anonymized volunteers that provided written informed consent, which was approved by the Sanquin Research Institutional Medical Ethical Committee. The use of human vena saphena cells (HVSCs) was in accordance to the “Code Proper Secondary Use of Human Tissue” developed by the Federation of Medical Societies (FMWV) in the Netherlands.

1. General Preparations and Required Actions Before Setting Up the Bioreactor

NOTE: For details on the respective isolation and culturing protocols, please refer to earlier work^{19,43,44}. All calculations in the protocol are given as examples for a co-culture experiment with monocytes and myofibroblasts, seeded in 8 hemodynamically loaded scaffolds and with 2 static controls (n=10).

1.1. Start cell isolation and cell culture. The seeding densities for the co-cultured samples of monocytes and myofibroblasts (with a seeding ratio of 2:1) are 30×10^6 monocytes/cm³ and 15×10^6 myofibroblasts/cm³, respectively.

NOTE: As the electrospun material has a high porosity (>90%), a rough estimate of the required number of cells per graft used to calculate the volume of the scaffold (the formula for volume of a hollow cylinder: $\pi \times (\text{thickness})^2 \times \text{length} \approx 0.04 \text{ cm}^3$) is 1.2×10^6 monocytes and 0.6×10^6 myofibroblasts. For 10 samples, at least 12×10^6 monocytes and 6×10^6 myofibroblasts are required; culture up to ~10–15% more cells to account for possible pipetting errors.

1.2. Degas the cell culture medium that will be used for experiments involving the bioreactor.

1.2.1. Prepare the medium for co-cultures, which consists of RPMI-1640:aDMEM (1:1), supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin. 0.5% L-glutamine, and 0.25 mg/mL L-ascorbic acid 2-phosphate (vitamin C).

1.2.2. Place the medium overnight (O/N) in an incubator in a cell culture flask with filter cap to degas.

1.2.3. Replace the filter cap with an air-tight cap and store at 4 °C.

NOTE: For calculations, amount of medium required per flow culture chamber: 50 mL. Refresh the medium three times per week; 25 mL old medium is replaced by 25 mL fresh medium. For 10 samples; after seeding, a total of 500 mL fresh medium is required, and for each subsequent medium change, a total of 250 mL fresh medium is used. Always prepare medium fresh, especially, vitamin C should be added just before changing the medium.

1.3. Prepare isotropic electrospun scaffolds (3 mm luminal diameter, 200 μm wall thickness) as described by Van Haaften et al.¹⁹ (**Figure 1G–I**). In brief, tubular polycaprolactone bisurea (PCL-BU) scaffolds are produced by electrospinning from 15% (w/w) chloroform-polymer solutions. The polymer solutions are electrospun at room temperature and 30% relative humidity, at a flow rate of 40 $\mu\text{L}/\text{min}$, 16 cm distance from the rotating cylindrical target (\varnothing 3 mm, 500 rpm), and an applied voltage of 16 kV on the electrospinning nozzle and -1 kV on the target.

NOTE: Although PCL-BU grafts were used for these experiments, a broad variety of elastomeric tissue engineered grafts can be mounted in this bioreactor (e.g., of different synthetic or natural origin, different microarchitecture, different porosities)

- 1.3.1. Remove the electrospun scaffolds from the mandrel.
- 1.3.1.1. Make a small hole in the cap of a 15 mL tube to ‘hold’ the mandrel in the center and prevent it from touching the wall of the tube.
- 1.3.1.2. Place the mandrel with the electrospun scaffold in the falcon tube and fill it with deionized water.
- 1.3.1.3. Freeze the tubes O/N at -20 °C.
- 1.3.1.4. Place the tubes at room temperature (RT) and pull out the mandrels after a few minutes, leaving the electrospun grafts in the ice.
- 1.3.1.5. Let the ice thaw completely, remove the electrospun tube from the thawed water, and ‘hang’ to dry vertically for several hours. Make sure the scaffolds do not ‘collapse’ under their own weight.
- 1.3.2. Dry scaffolds under vacuum O/N.
- 1.3.3. Image a small sample of the electrospun grafts using scanning electron microscopy (SEM) to assess their microstructure (e.g., fiber morphology, fiber diameter). The grafts in the example studies have an isotropic fiber orientation and a fiber diameter of 5 μm (**Figure 1H–I**).
- 1.4. One day before starting the experiment, place the hydraulic reservoir filled with deionized water in the incubator. Close all eight connections for flow culture chambers with white Luer caps. Connect to the compressed air system and insert pressure sensor. Run the pump (see step 4.6) O/N to allow for small expansion of the Teflon bellow.
- NOTE: Make sure all necessary materials and equipment are cleaned and/or autoclaved (see **Table of Materials**, Comments/Description column for which materials are allowed to be autoclaved), according to the manufacturer’s protocol or as described in steps 6.3–6.6.
- 1.5. Ensure sterile working conditions for the remainder of the protocol.
- 1.5.1. Perform steps 1–4.3 (setting up the system), step 5.3 (medium change), and steps 6.1–6.2 (harvest of vascular constructs) in a sterile laminar flow cabinet.
- 1.5.2. Place materials that are not directly needed for the subsequent steps in closed Petri dishes to keep everything as sterile as possible.
- 1.5.3. Clean or dry material surfaces regularly by soaking a paper tissues with 70% ethanol, and wipe the surfaces of the bioreactor components and the laminar flow cabinet.

2. Setting Up the Bioreactor

NOTE: Perform step 2 in a sterile laminar flow cabinet.

2.1. Cut the electrospun scaffolds into tubes of approximately 25 mm in length, and document them before use (e.g., photograph for the length, weigh with balance for the initial mass).

2.2. Decontaminate the electrospun scaffolds.

2.2.1. Place the electrospun scaffolds tilted in a well plate or Petri dish, with one opening facing the ultraviolet (UV) light source, to enable UV light (253.7 nm) to illuminate the inside of the scaffolds.

2.2.2. Expose the electrospun scaffolds to UV light for 5 min.

2.2.3. Turn all scaffolds and repeat the UV illumination for the other opening.

NOTE: After this step, only touch the electrospun scaffold when needed. Always use clean tweezers or clean gloves.

2.2.4. Wash the glass tubes of the flow culture chamber in ultrapure water, dry, and place in a large, closed Petri dish.

NOTE: The following steps, especially steps 2.3–2.5, are ideally performed by two experimenters.

2.3. Mount the electrospun scaffolds onto the silicone tubing.

2.3.1. Attach the 5-0 prolene suture to one end of the silicone tubing by taking the suture through one side of the tube and out of the other, leaving two opposite taut sutures spanning the cross section of the tubing. Make a small knot on both sides of the tube and leave approximately 10 cm of wire on both knots. Make a third knot at the end of the two 10 cm left-over wires.

2.3.1.1. Cut off the suture needle and all free threads that might stick out and damage the inside of the electrospun scaffold. Cut away the edges of the silicone tubing into a triangular shape to aid in pulling the silicone tubing through the electrospun scaffold.

2.3.2. Dip the electrospun scaffold in 30% ethanol (this serves as extra decontamination step and aids in sliding the electrospun scaffold over the silicon tubing) and place the electrospun scaffold over the free 10 cm wire. Experimenter A stretches the silicone tubing by pulling gently on both the silicone tubing and the knot of the 10 cm suture wire, while experimenter B gently slides the electrospun scaffold over the silicone tubing using tweezers with a smooth inner tip to prevent damaging of the scaffolds.

2.3.3. Slowly release the stretch on the silicone tubing, while simultaneously smoothening the electrospun scaffold with tweezers. Dip the electrospun scaffold on the silicone tubing in ultrapure water two times.

NOTE: It is possible that some wrinkling of the electrospun scaffold occurs. This wrinkling will disappear during the applied pre-stretch right before fixing the scaffolds to the culture chambers at step 2.5.3.

2.3.4. Repeat steps 2.3.2 and 2.3.3 for the other electrospun scaffolds. Depending on the length of the silicone tubing, multiple electrospun scaffolds can be mounted on the same silicone tubing.

2.3.5. When all electrospun scaffolds are mounted on the silicone tubing, cut the silicone tubing around the scaffolds, all to the same length (5.5 cm); at one side, close to the end of the electrospun scaffold, at the other side, leaving ~2–3 cm of free silicone tubing.

2.4. Construct the bottom compartment of the flow culture chamber (**Figure 1A–B**).

2.4.1. Take the upper part of the bottom compartment containing the flow outlet, and close the flow outlet with a male Luer plug.

2.4.2. Push the pressure conduit with holes through the bottom compartment, and place a silicone O-ring around the lower end of the pressure conduit to prevent leakage. Screw the lower part of the bottom compartment to the upper part of the bottom compartment to secure the pressure conduit. Make sure that the lower engraved groove of the pressure conduit is approximately 3–5 mm above the edge of the adapter bushing of the bottom compartment; this will later ‘hold’ the tight knot of the suture wire, fixing the electrospun scaffold over the silicone tubing.

NOTE: If the pressure conduit can be easily maneuvered up and down, it indicates that the bottom compartment is not well secured. Repeat step 2.4.2 to prevent leakage in later stages (**Figure 2D**).

2.5. Secure the silicone tubing with the electrospun scaffold to the pressure conduit.

2.5.1. Pull the silicone tube with the electrospun scaffold over the pressure conduit.

2.5.2. Make a knot with the suture wire at the lower end of the electrospun scaffold at the location of the engraved groove on the pressure conduit. Make a second knot at the opposite side to tightly secure the silicone tubing with the electrospun graft.

CAUTION: This is a critical step. Make sure that the knot exactly ‘falls’ into the engraved groove of the pressure conduit to prevent leakage of the water from the hydraulic reservoir to the flow culture chambers. If not sure, try to tighten the suture wire at several positions, above or below the expected location of the groove, to ensure that the final knots are exactly at the groove

(Figure 2A).

2.5.3. Place the scissor clamp at the upper end of the silicone tube, and stretch the silicone tubing upwards (this will directly test the first knot, if it is possible to move the silicone tubing with the electrospun scaffold over the pressure conduit, it was not tightened well enough). With the pulling force, the silicone tubing is pre-stretched. To ensure that the silicone tubing is consistent among the different samples, attach a ruler to the scissor clamp. Pull the scissor clamp upwards until the lower end of the ruler reaches the height of the lower end of the scaffold.

NOTE: It is important to keep the pre-stretch in each sample roughly the same (~5%) for two reasons: (1) if silicone tubing is pre-stretched, it will result in more homogeneous expansion along the length of the sample when pressurized; (2) the pre-stretch will impact the mechanical properties of the silicone, therefore it should be the same across all samples to ensure equal stretch conditions between the samples.

2.5.4. Remove wrinkles in the electrospun scaffold by gently pulling on the electrospun scaffold. Again, make two knots at both sides with a suture wire on the upper end of the scaffold at the location of the upper engraved groove on the pressure conduit.

2.5.5. Release the scissor clamp, and cut away the excess of silicone tubing with a knife, leaving 20–30% of the screw thread covered with silicone tubing, to prevent leakage when the nose cone is mounted on the screw thread.

NOTE: Repeat steps 1.4 and 1.5 for all dynamic samples.

2.5.6. For the static control samples, secure the electrospun scaffold mounted on the silicone tubing on pressure conduits without holes. These conduits can be kept separately in a 15 mL tube until seeding (step 3) and do not need to be fixed in the flow culture chamber compartments.

2.6. Decontaminate the partly constructed flow culture chambers with electrospun scaffold by exposing it to UV light for 10 min. Turn the flow culture chambers with electrospun scaffolds to the other side, and repeat UV light exposure for 10 min.

2.7. Screw the nose cones on the screw thread of the pressure conduits with holes for the dynamic samples.

2.7.1. Make sure the top end of the silicone tubing fits into the nose cone to prevent leakage in later stages. If there is too much silicone tubing, cut the excess of tubing away with a knife.

2.7.2. Place the partly constructed flow culture chambers in a large Petri dish, and point the nose cone towards the UV light source. Apply UV illumination for 5 min.

2.8. Complete construction of the flow culture chamber with the glass tube and top compartment of the flow culture chamber (Figure 1A–B).

2.8.1. Pre-wet the electrospun scaffolds by dipping the pressure conduit with the silicone tubing and electrospun scaffold in 30% ethanol, followed by a dip in ultrapure water two times.

2.8.2. Place the glass tube over the pressure conduit, and push gently in the bottom compartment and gently secure it.

2.8.3. Take the top compartment containing the flow inlet, place a silicone O-ring, a flow straightener, and the adapter bushing in the correct order (**Figure 1A–B**), and place over the open end of the glass tube and gently secure it.

2.8.4. Screw a white Luer cap on the flow inlet of the top compartment.

2.8.5. Remove the male Luer plug from the flow outlet of the bottom compartment, and clean the surface around it with an ethanol-soaked tissue.

2.8.6. Place a syringe with 10 mL of ultrapure water in the flow outlet, open the white Luer cap on the top compartment, and fill the chamber with ultrapure water. Close the white Luer cap again, remove the syringe, clean again with ethanol, and close the flow outlet with a male Luer plug.

NOTE: Repeat steps 2.6–2.8 for all flow culture chambers.

2.8.7. For the static controls, add 10 mL of ultrapure water to the 15 mL tubes holding the pressure conduits without holes for the static samples.

2.9. Place all flow culture chambers in the incubator. Replace the ultrapure water with culture medium one day before cell seeding on the same way as described in steps 2.8.5 and 2.8.6 (make sure to collect the ‘old’ ultrapure water with an ethanol-soaked paper towel placed directly on the flow outlet).

[The protocol can be paused here]

3. Preparations for the pump setup

NOTE: Perform step 3 in a sterile laminar flow cabinet.

3.1. Collect all pump setup materials and prepare for usage.

NOTE: Experimenters are referred to the manufacturer’s protocol for a detailed description of setting up the pump, the fluidic units, and medium tubing through the valves of the fluidic unit.

3.1.1. Set the pump to 200 mbar capacity.

3.1.2. Screw the reservoir holders for 60 mL reservoirs to the fluidic units.

3.1.3. Clean the re-usable rubber air filters with a tissue soaked in ethanol, make sure the air filter stays dry.

3.2. Place the 60 mL reservoirs in the reservoir holders, and place the standard medium tubing through the valves of the fluidic unit. Connect the medium tubing with a larger inner diameter with female Luer lock couplers into an enclosed loop.

3.3. Clamp the medium tubing with a hose clip, directly below the reservoirs.

3.4. Fill the reservoirs with 25 mL of culture medium per 60 mL reservoir. Release the hose clip, and let the medium enter the tubing.

3.5. Close the medium reservoirs with the rubber air filters, and place in the incubator till step 4.

4. Cell Seeding Using Fibrin as a Cell Carrier

NOTE: Perform step 4 in a sterile laminar flow cabinet.

4.1. Prepare the fibrin gel for the cell seeding step. For details, see Mol et al.⁶⁷ For the fibrin gel, the fibrinogen solution should have a final concentration of 10 mg/mL (correct for the purity of the protein stock), and the thrombin solution should have a final concentration of 10 U/mL.

4.1.1. Thaw fibrinogen to RT, before weighing ~50 mg (enough for 10 samples) in a plastic container with a red lid.

4.1.2. Add cell culture medium to prepare the fibrinogen solution (at a concentration of 10 mg/mL, correct for the purity of the protein stock). Mix well and filter to sterilize the fibrinogen solution with a 0.2 µm syringe filter into a sterile 15 mL tube. Keep the filtered fibrinogen solution on ice.

NOTE: Avoid preparing the fibrinogen solution in advance, otherwise the fibrinogen may clot spontaneously.

4.1.3. Thaw thrombin and make a thrombin solution (at a concentration of 10 U/mL) in cell culture medium and place on ice. Prepare 20 µL thrombin + cells solution per sample. For n=10 samples, 200 µL is needed; therefore, prepare 250 µL thrombin solution to account for possible pipetting errors.

4.2. Collect and count the cells from the culture flasks. Mix the cells in the desired ratio and amount (1.2×10^6 monocytes and 0.6×10^6 myofibroblasts per scaffold). Make sure there are enough cells for n+1 samples to correct for pipetting errors. Centrifuge at $350 \times g$ for 10 min at

RT. Remove the supernatant.

4.3. Make a mixture of the suspended cells and thrombin.

4.3.1. For each sample, use 20 μL of the thrombin solution. For $n=10$ samples, add 200 μL thrombin to the cell pellet and mix. Measure the volume of the cell suspension (cells + thrombin), and calculate how to divide evenly over all 10 scaffolds (e.g., if the thrombin + cell suspension has a volume of 260 μL , each electrospun sample will receive $260\ \mu\text{L}/10\ \text{samples} = 26\ \mu\text{L}$ thrombin + cell suspension).

4.3.2. As the seeding of the scaffolds is performed in two steps, prepare two 1.5 mL microfuge tubes that will hold half of the cell suspension for each scaffold (in the example calculation of the previous step: prepare two tubes with 13 μL of thrombin + cell suspension). Place on ice.

NOTE: The following steps, especially step 4.4, are ideally performed by two experimenters.

4.4. Dry the pre-wetted electrospun scaffolds with vacuum to prepare for cell seeding.

4.4.1. Connect a glass Pasteur pipet to the vacuum system of the laminar flow cabinet, and place in an empty 50 mL tube for sterile temporary storage.

4.4.2. Take the flow culture chambers from the incubator, remove the male Luer plug from the flow outlet, and remove the medium after opening the white Luer cap and placing an ethanol-soaked tissue in front of the flow outlet.

4.4.3. Take off the top compartment and the glass tube, and place in a sterile Petri dish for temporary storage.

4.4.4. Place the vacuum Pasteur pipet on the electrospun scaffold, and remove as much medium as possible.

CAUTION: Vacuum dry the electrospun scaffold very gently. Instead of a back-and forth linear motion over the scaffold, place the vacuum pipet at multiple locations. Clamp the vacuum tubing on top of the Pasteur's pipet in between the fingers for better control.

4.4.5. Mix the fibrinogen solution in a 1:1 ratio with the thrombin + cell suspension (e.g., mix 13 μL of fibrinogen with 13 μL of thrombin + cell suspension). To make sure that the fibrin polymerizes in the scaffold and not in the microfuge tube, pipet the fibrinogen, turn the pipet wheel for the 'extra volume' of the thrombin + cell suspension, and pipet up and down once in the microfuge tube with cell suspension to mix.

4.4.5.1. Directly homogeneously drip the solution over the full length of the electrospun scaffold. It is advised that Experimenter A drips the fibrin mixture, while experimenter B holds the bottom compartment with the electrospun scaffold

529 mounted to the pressure conduit.

530
531 4.4.6. After the fibrin with the cells is dripped over the electrospun scaffold, Experimenter B
532 slowly moves the scaffold, from left to right and up and down, to further divide the cells evenly
533 over the scaffold.

534
535 4.4.7. Repeat step 4.4.5 and step 4.4.6 on the other side of the electrospun scaffold.

536
537 4.4.8. Mount the flow culture chamber again by carefully placing the glass tube (prevent fibrin
538 sticking and clotting to the inner side of the glass tube), and push back the top compartment of
539 the flow culture chamber. Directly place the seeded construct without any medium or
540 phosphate-buffered saline (PBS) in the flow culture chamber in the incubator.

541
542 4.4.9. Repeat steps 4.4.1–4.4.8 for all dynamic samples. For the static samples mounted to
543 pressure conduits without holes, seed according to steps 4.4.1–4.4.6, and place in a 15 mL tube
544 afterwards.

545
546 4.4.10. Let the fibrin polymerize for 60 min in the incubator.

547
548 [The protocol can be paused here for 30–60 min.]

549
550 4.5. After polymerization, fill the flow culture chambers (dynamic samples) or the 15 mL tubes
551 (static samples) with medium.

552 **5. Coupling of the bioreactor and pump systems before starting experiment**

553
554
555 NOTE: Perform steps 5.1–5.3 in a sterile laminar flow cabinet.

556
557 5.1. Take the tray carrying the flow culture chambers and the fluidic units with filled reservoirs
558 and connected medium tubing inside the laminar flow cabinets.

559
560 5.2. Position the flow culture chambers on the bioreactor base for the experimental groups
561 loaded with cyclic stretch and with combined hemodynamic loads (**Figure 1E**).

562
563 5.2.1. Tilt the flow culture chamber upside down, and fill the pressure conduit from below with
564 ultrapure water using a syringe with thin tubing (this can be of any type, as long as it is flexible
565 and thin. In this experiment, attach a 10 cm long, 0.15 mm inner diameter wire to the needle.

566
567 5.2.1.1. Place the thin tubing inside the pressure conduit, and while the pressure conduit
568 is filled with ultrapure water by gradually pushing the water out of the syringe, pull
569 the wire out of the pressure conduit simultaneously, to make sure that there are no
570 air bubbles inside the pressure conduit.

571
572 5.2.2. Place the flow culture chamber on one of the eight screw threads on bioreactor base.

Place a silicone O-ring between the bioreactor base and the white Luer connector to prevent possible leakage, and tighten the white Luer connector from the bottom compartment.

5.2.3. Repeat steps 5.2.1 and 5.2.2 for all cyclically stretched samples.

5.3. Connect the flow culture chambers for all experimental groups, except the static control, to the pump system.

5.3.1. Place a hose clip on the medium tubing. Remove the white Luer cap covering the flow inlet of the top compartment of the flow culture chamber. Remove the female Luer coupler of the medium tubing, and connect the medium tubing on one side with the flow inlet on the top compartment, and the other side of the medium tubing with flow outlet at the bottom compartment.

5.3.2. Repeat step 5.3.1 for all flow culture chambers. At this point, the bioreactor and the flow culture chambers are filled with medium and connected to the flow systems.

5.3.3. For the static control samples, place the samples vertically in a cell culture flask with filter cap by using the scissor clamp. Fill the cell culture flask with medium, and place in the incubator.

5.4. Transfer the complete setup from the laminar flow cabinet to the incubator, and connect the fluidic units to the air pressure tubing and the electric cable.

5.5. Start the software, and initialize the pumps. Start the medium flow for the samples one by one.

5.5.1. Check if the valves of the fluidic unit are clicking.

5.5.2. Remove the hose clamp from the medium tubing.

5.5.3. Start the pump with 100 mbar and 10 s switching time.

5.5.4. Carefully check the flow direction for possible leakage or air bubbles. Any entrapped air bubbles can be removed by turning the flow culture chamber upside down.

NOTE: Make sure that the medium levels in the medium reservoirs are balanced. To prevent suction of air into the system and air bubbles in the flow culture chambers, do not allow the reservoirs to run dry (**Figure 2C**).

5.5.5. Repeat step 5.5 for all fluidic units and flow culture chambers one-by-one.

5.6. Initialize the strain pump.

5.6.1. Connect the pneumatic actuated pump via the air inlet on the pneumatic cylinder to the

compressed air. Connect the lower air outlet with the blue tubing for air out (**Figure 1F**).

5.6.2. Open LabVIEW software, run the LabVIEW script and compressed air pressure application system, as described by Van Kelle et al.⁶⁸, enter displacement and frequency (start with low frequency of 0.2 Hz). Pause the pump when the Teflon bellow is at its lowest level.

5.6.3. Place the pressure sensor in the pressure sensor inlet on the hydraulic reservoir.

5.7. Change the pump settings to the desired settings (for 1.5 Pa, use 150 mbar, 10 s switching time).

5.8. Start the strain pump, and apply the preferred setting (e.g., 0.5 Hz, 1.05 stretch).

6. Running Experiment for Multiple days; Monitoring of Shear and Stretch During Culture and Medium Replacement

6.1. Calculate the WSS at the scaffold wall.

6.1.1. Record the flow magnitude every other day (see the manufacturer's manual for details). In short, observe the change in liquid levels (in mL) in the medium reservoirs in between the switching of the fluidic unit reservoir for 10 s. Conduct at least five measurements, calculate the mean value, and multiply by 6 to get the flow rate Q in mL/min.

6.1.2. The flow is described by a Poiseuille flow through an annular channel. Assuming culture medium as a Newtonian fluid, calculate the WSS at the scaffold wall, r_1 , by Equation 1.

$$\tau_w = \frac{1}{4} \left(-\frac{\partial p}{\partial z} \right) \left[\frac{1}{r_1 \ln\left(\frac{r_2}{r_1}\right)} * (r_2^2 - r_1^2) - 2r_1 \right] \quad (1)$$

where the WSS τ_w at the scaffold wall (r_1 ; here $r_1 = 1.7$ mm), resulting from a steady state flow, is determined by the applied pressure p and the inner radius of the glass tube r_2 (here $r_2 = 2.3$ mm). The pressure gradient in the axial direction is assumed to be uniform between the flow inlet and flow outlet and is given by Equation 2 (**Figure 1J**).

$$-\frac{\partial p}{\partial z} = \frac{8\mu Q}{\pi(r_2^2 - r_1^2)} \left[r_2^2 + r_1^2 - \frac{r_2^2 - r_1^2}{\ln\left(\frac{r_2}{r_1}\right)} \right]^{-1} \quad (2)$$

with μ the dynamic viscosity (here medium viscosity was assumed constant, $\mu = 0.7 \times 10^{-3}$ Pa·s at 37 °C) and Q the applied flow rate.

6.2. Monitor the stretch applied to the scaffolds every other day.

6.2.1. Place a dark background behind the flow culture chamber to increase the contrast

between the scaffold and the background. Position the LED light lamps, pointing towards the scaffold, to help the visualization of the scaffold.

6.2.2. Take time-lapse photographs of the scaffold at a frequency of 30 Hz for 6 s (i.e., 3 stretch cycles) with a high-speed camera.

NOTE: A lower recording frequency may suffice if the camera permits. However, the minimally required frequency was not determined.

6.2.3. Manually determine the minimum and maximum diameter of the scaffold from the images.

6.2.4. Calculate the minimum and maximum outer diameter of the electrospun scaffold to calculate the maximum stretches according to Equation 3.

$$\lambda_{\theta} = \frac{d_1}{d_0} \quad (3)$$

where the circumferential stretch (λ_{θ}) is given by the ratio between the outer diameter of the scaffold, d_1 , and its initial diameter, d_0 .

6.3. Correct for medium evaporation, and refresh medium three times per week.

6.3.1. Stop and decouple the cables for the systems and the strain pump.

6.3.2. Place hose clips on the medium tubing.

6.3.3. Determine how much medium evaporated based on the volume indicator marks on the medium reservoirs.

6.3.4. Transfer the tray with the bioreactor and the fluidic units to the laminar flow cabinet.

6.3.5. Remove the rubber air filters of the medium reservoirs; add autoclaved ultrapure water to compensate for the evaporated volume of medium. Close the medium reservoirs again, and connect to the pump again to mix the medium with the ultrapure water.

6.3.6. Repeat steps 5.3.1–5.3.5. Remove the rubber air filters again, take out 25 mL of culture medium, and spin down at $300 \times g$ for 5 min at RT.

6.3.6.1. Collect 1.5 mL supernatant, and store at -30°C for analysis of secretory profiles (for analysis with enzyme-linked immunosorbent assay (ELISA)).

6.3.6.2. Collect the desired volume of supernatant for paracrine signaling studies, by using the supernatant as conditioned medium⁴³.

699
700 6.3.7. Add 25 mL of fresh medium to the medium reservoirs.

701
702 6.3.8. Place rubber air filters back on the medium reservoirs.

703
704 6.3.9. Place the complete setup back in the incubator; connect all cables and air tubing to the
705 pump and strain pump. Release the hose clips and repeat steps 4.4–4.8.

706
707 6.4. Check if silica drying beads in the drying bottles connected to the pump are moist (white
708 appearance), and replace with dry silica beads if required (orange appearance).

709
710 **7. Ending Experiment, Sample Collection, and Equipment Cleaning and Storage**

711
712 7.1. On the last day of the experiment, correct for medium evaporation as described in steps
713 5.3.1–5.3.5, and harvest the samples one-by-one.

714
715 7.1.1. To harvest the samples one-by-one, pause the pump and strain pump several times. Place
716 a hose clip on medium tubing. Temporarily stop the pump and the strain pump. Disconnect one
717 flow culture chamber from the bioreactor base; replace by a white Luer cap on the bioreactor
718 base. Take the flow culture chamber and fluidic unit to the laminar flow cabinet. Start the pump
719 and strain pump again to apply the hemodynamic load to the other samples until harvesting.

720
721 7.1.2. Collect medium from the medium reservoirs for paracrine cytokine production analysis
722 via ELISA.

723
724 7.2. Decouple flow units and harvest tubular construct. Section according to the desired
725 cutting scheme. Parts of the construct can be stored at 4 °C (after 15 min fixation in 3.7%
726 formaldehyde and 3 x 5 min washing in PBS) or -30 °C (after snap-freezing in liquid nitrogen) until
727 further analysis.

728
729 7.3. Clean the bioreactor and pump components. Additionally, the advised cleaning method
730 per item is mentioned in the **Table of Materials**.

731
732 7.3.1. Clean the rubber air filters with 70% ethanol. Be very careful to not moisten the inner
733 filter!

734
735 7.3.2. Collect all separate components: medium tubing, medium reservoirs, glass tubes, male
736 Luer plugs and female Luer locks, white Luer caps, pressure conduits, nose cones, silicone O-rings,
737 adapter bushing, flow straighteners (excluding pumps, fluidic units, rubber air filters, the
738 bioreactor base), and rinse in running tap water.

739
740 7.3.3. Place O/N in 0.1% sodium dodecylsulfate in deionized water.

741
742 NOTE: Do not use ultrapure water as the parts might rust.

743
744 7.3.4. Rinse with tap water and dishwashing soap.

745
746 7.3.5. Immerse in deionized water, followed by 70% ethanol two times, followed by deionized
747 water.

748
749 7.3.6. Place all materials separately on tissues and let them dry. Use pressured air to dry tubing.

750
751 7.3.7. Clean all non-autoclavable materials with a paper tissue soaked in 70% ethanol. This
752 includes the rubber air filter (bear in mind that the air filter should stay dry) and the bioreactor
753 base (Teflon bellow and pneumatic cylinder).

754
755 7.3.8. Autoclave the components of the fluidic chamber (including the silicone O-ring), the
756 medium tubing, the medium reservoirs (without the rubber air filter), male Luer plugs and female
757 Luer couplers, white Luer caps, hose clips, and standard equipment (e.g., tweezers, clamping
758 scissors)

759
760 7.3.9. For convenient use during next experiments, combine the separate components for one
761 complete fluidic chamber in an autoclavable box.

762
763 7.4. Remove water from the hydraulic reservoir. Clean with 70% ethanol, followed by
764 deionized water. Let it dry. Refill with deionized water and a few drops of water-bath-preserving
765 disinfectant.

766
767 7.5. Store the glass tubes for the flow culture chamber in 70% ethanol.

768
769 7.6. Place the moist silica drying beads (white appearance) in the oven O/N at 120 °C to let
770 them dry (orange appearance), and store in an air-tight flask.

771
772 **REPRESENTATIVE RESULTS:**

773 This bioreactor was developed to study the individual and combined effects of shear stress and
774 cyclic stretch on vascular tissue growth and remodeling in 3D biomaterial scaffolds. The design
775 of the bioreactor allows for culturing up to eight vascular constructs under various loading
776 conditions (**Figure 1A**). The vascular constructs are positioned in a flow culture chamber
777 (**Figure 1B**) in which both the circumferential stretch and WSS can be independently controlled.
778 The top compartment of the flow culture chamber holds a flow straightener to stabilize the flow
779 in a relatively short settling length (**Figure 1C**). Directly downstream of the flow straightener, the
780 nose cone distributes the flow evenly through the annular channel (**Figure 1D**). When all steps of
781 the protocol are performed in the correct manner, the vascular scaffolds in the flow culture
782 chamber can be subjected to a continuous unidirectional flow through the annular channel
783 between the scaffold and the glass wall and are circumferentially stretched by the pneumatic
784 pump (**Figure 1E–F**). Before the scaffold is mounted, the electrospun tube should be cut into
785 25 mm tubes (**Figure 1G**) and can be examined with SEM to analyze the microarchitecture
786 (**Figure 1H–I**). It is important to note that the PCL-BU grafts in this example can be replaced by

any other elastomeric tissue engineered grafts (natural or synthetic origin, different microarchitecture or porosity). The inside-out design allows testing of highly porous grafts as it does not necessarily have to be leak-free. The schematic image of the flow culture chamber shows the physical interpretation of the parameters used in the equations to describe the WSS (equation 1), the pressure gradient (equation 2), and the circumferential stretch (equation 3) (**Figure 1J**).

Incorrect execution of the critical steps of the protocol can result in a few scenarios. For example, leakage from the hydraulic reservoir can occur as a result of incorrectly mounted knots, leading to leakage of hydraulic fluid from the pressure conduits with holes passing the silicone tube and entering the flow culture chamber (**Figure 2A**). Leakage of the hydraulic fluid at the connection between the screw threads and the bioreactor base can also occur when the silicone ring is not well placed or if the Teflon bellow was not allowed to slightly expand O/N one day before the experiment (**Figure 2B**). Furthermore, when medium is not degassed, or if the medium levels in the medium reservoirs are not well balanced and one medium reservoir runs dry, resulting in air being sucked into the system, air bubbles can arise in the flow culture chamber (**Figure 2C**), which disturbs the WSS patterns, compromising cell viability and subsequent tissue growth. Lastly, when the silicone O-ring in the bottom compartment is not correctly placed, medium spill may be observed below the flow culture chamber (**Figure 2D**).

As this bioreactor setup allows for the application of individual and combined hemodynamic loads, multiple hemodynamically loaded experimental groups can be included in one experiment (**Figure 3A**). Previously, different hemodynamic loads (i.e., two shear stress regimes and two stretch regimes) were validated by applying various possible system settings (**Figure 3B**). When stretch (**Figure 3C**) and WSS (**Figure 3D**) were monitored over long-term culture periods, it was validated that these can be maintained at relatively constant levels over a period of up to 20 days.

The bioreactor is especially well suited to study the influence of hemodynamic loading on growth and remodeling in an in situ vascular TE context. The stages of in situ TE are hypothesized to mirror the stages of the natural wound healing response (**Figure 4A**). The co-cultures of monocyte-derived macrophages and myofibroblasts derived from human saphenous veins, as described here, were established as an in vitro mimic of the proliferative phase. Three days after seeding, immunofluorescent staining showed a homogenous distribution of both cell types throughout the scaffold (**Figure 4B**). After 20 days of co-culture, cyclic stretch alone resulted in the deposition of more numerous and thicker collagen type I fibers, while in the group with combined hemodynamic loads, this effect of cyclic stretch was overruled by shear stress, resulting in less pronounced collagen type I deposition, illustrated here by immunofluorescent staining (**Figure 4C**). For successful in situ tissue regeneration, a tight balance between tissue production and scaffold resorption is required. In addition to tissue formation, the bioreactor allows for the induction of cell-driven scaffold resorption. For example, when culturing a monoculture of macrophages for 8 days on the electrospun grafts, fiber erosion and fiber cleavage were observed in all hemodynamic loading regimes, with the most pronounced resorption in the static group and least pronounced resorption in the shear stress group (**Figure 4D**). Together,

these results show the impact of the different hemodynamic loading regimes on both growth and remodeling. These insights are helpful in optimizing the design parameters for newly developed in situ TEVGs.

Another important determinant of the tissue regeneration process is the presence of pro- and anti-inflammatory cytokines. As the bioreactor is a closed-loop system, the cells in the system will be continuously exposed to the paracrine stimuli of secreted factors. The cytokine secretion profiles in the medium of the dynamically loaded co-cultures of human peripheral blood mononuclear cell (PBMC)-derived macrophages and human myofibroblasts from saphenous veins were analyzed in early and later stages (**Figure 5A**). These representative results illustrate the impact of both cyclic stretch and shear stress on the cytokine secretion profile in the co-culture setup. Interestingly, the combined effects of both loads showed either dominance of one of the two loads (e.g., cyclic stretch for interleukin-6 (IL-6) and monocyte chemoattractant protein-1 (MCP-1)) or synergistic effects of both loads (e.g., for IL-10) (**Figure 5B**). These insights, gathered by using this in vitro testing platform, give valuable information for the development of in situ TEVGs that are based on the rationale of macrophage-driven in situ tissue regeneration.

Co-culture experiments of macrophages and myofibroblast showed that the mechanical environment and the resulting loading-dependent inflammatory environments modulated the phenotype of the myofibroblasts. After 20 days of hemodynamic loading, gene expression of myofibroblast markers showed clear differences in the individual and combined impact of the loading and in direct and paracrine signaling of macrophages on myofibroblasts (**Figure 6A**). Furthermore, the gene expression patterns of contractile marker alpha smooth muscle actin correlated with protein synthesis (**Figure 6B**). Furthermore, cyclic stretch stimulated collagenous and elastic matrix gene expression and attenuated matrix metalloproteinase 1/tissue inhibitor matrix metalloproteinase 1-mediated collagen remodeling, whereas a stabilizing effect of shear stress was observed in the co-culture (**Figure 6C**). These long-term co-culture experiments show the possibility of studying the later stage tissue remodeling phase (**Figure 4A**) in various hemodynamic loading regimes in tissue engineered vascular grafts with this bioreactor. As the TEVG are mounted “inside out” on a silicone tube, the circular stretch and WSS can be applied for longer culture periods.

FIGURE AND TABLE LEGENDS:

Figure 1: Design and overview of the bioreactor. (A) Construction drawing of the bioreactor base and (B) exploded view of the fluid culture chamber with all parts indicated (*steps 2.4–2.8*). The top compartment of the flow culture chamber holds a flow straightener. (C) The spherically blunted nose cone is positioned after the flow straightener to distribute the flow through the annular channel (flow direction indicated in pink). (D) Together, these components control and guide flow directionality. See **Table 1** for a functional description of the individually mentioned parts. (E) Photograph of complete bioreactor setup (*step 5.2*) and (F) a close-up view of the flow culture chambers (*step 5.6.1*). (G) Gross appearance of the electrospun PCL-BU scaffold before seeding (ruler ticks 1 mm). Scanning electron microscopic images of tubular electrospun PCL-BU scaffold with 3 mm inner diameter and 5 μ m average fiber diameter at different magnifications, scale bars (H) 100 μ m and (I) 10 μ m (*step 1.3*). (J) Schematic image of the culture chamber

consisting of a tubular electrospun scaffold, with outer radius (r_1) centered in a glass tube of inner radius (r_2). The flow (Q) inlets and outlets are connected to the annular ring, with channel height h for applying wall shear stress (t). The pressure/stretch (P) inlet is connected to the silicone-mounted scaffold for applying a circumferential stretch ($\lambda(t)$) to the mounted electrospun scaffolds from the inside (*step 6.1*). Abbreviations: polycaprolactone bisurea (PCL-BU). Panels C, D and G–J were adapted from Van Haaften et al.¹⁹.

Figure 2: Results of inaccurate execution of the critical steps in the protocol. Photographs of some occurrences that can be observed in the bioreactor setup when critical steps are not performed in the correct manner. **(A)** If the knot is not tight enough or not exactly placed in the engraved groove (indicated by arrow), a slight leakage of hydraulic fluid into the flow culture chamber may occur (*step 2.5*). **(B)** If the Teflon bellow was not allowed to expand O/N one day before the experiment or when the silicone ring is not well placed, hydraulic liquid leakage from the hydraulic liquid at the connection between the screw threads and the bioreactor base might occur (indicated by arrow) (*step 1.4*). **(C)** Air bubbles in the flow culture chamber (indicated by arrow) will result in disturbed shear stress patterns. Always degas the culture medium and make sure that the medium levels in the medium reservoirs are balanced to prevent one reservoir running dry and air getting sucked into the flow chamber system (*steps 1.2 and 5.5.4*). **(D)** When the silicone ring in the bottom compartment is not placed correctly, spillage of the medium may be observed (indicated by arrow) (*step 2.4.2*).

Figure 3: Control of shear stress and stretch. As the bioreactor allows for independent and combined application of stretch and shear, **(A)** multiple experimental groups can be included in one experiment. **(B)** Examples of variations in maximum stretches and shear stresses at a specific point in time tested under four distinct system settings (indicated by the colors). Black rectangles represent mean \pm standard deviation of the measurements for each setting. The dotted lines are computed as the mean of the stretches (*horizontal line*) and the shear stresses (*vertical line*) to indicate the four distinct loading conditions. **(C)** Cyclic circumferential stretches in the cyclic stretch and combined groups over the course of the experiment of 20 days, based on outer diameter measurements of the scaffold constructs monitored with a time lapse of high-speed camera (*step 6.2*). **(D)** Monitored wall shear stresses in the shear stress and combined groups over the course of the experiment, based on the changing medium levels in the syringe (*step 6.1*). Panels A, C, and D were adapted from Van Haaften and Wissing et al.⁴⁴; panel B was adapted from Van Haaften et al.¹⁹.

Figure 4: Concept of in situ vascular tissue engineering, cell distribution, tissue production, and scaffold degradation. A schematic illustration depicting the hypothesized phases of scaffold-driven tissue regeneration at the host's functional site. **(A)** The results shown are derived from experiments that focused on the proliferative phase, in which macrophages and tissue-producing cells have colonized the scaffold material. **(B)** Myofibroblasts from human saphenous vein (*red*) and PBMC-derived macrophage (*green*) distribution at the outer side of the electrospun scaffold (*grey*) at day 3. Scale bar 200 μm . Cross-section of the co-culture construct at day 20 stained for collagen type I (*green*), collagen type III (*red*), and DAPI (*white*). **(C)** Scale bar 100 μm , *indicates

outer side of the construct, corresponding to the flow side. **(D)** SEM images of decellularized grafts of 8 days macrophage monoculture showing macrophage degradation; scale bar 20 μ m. Abbreviations: human saphenous venous cells (HVSC); peripheral blood mononuclear cell (PBMC); 4',6-diamidino-2-phenylindole (DAPI); scanning electron microscopy (SEM). Panel A was adapted from Wissing and Bonito et al.²⁷; panels B and C were adapted from Van Haaften and Wissing et al.⁴⁴; and panel D was adapted from Wissing et al.⁴³.

Figure 5: Inflammatory environment in hemodynamically loaded co-culture constructs at 3 days and 20 days. Co-culture of human PBMC-derived macrophages and human myofibroblasts from saphenous veins. **(A)** Heat map of total cytokine secretion measured in supernatant via multiplex ELISA. **(B)** Boxplots for a selection of cytokines at day 20, normalized to total DNA content. P-values were calculated using Kruskal-Wallis test with a Dunn's multiple comparison test; * $p < 0.05$, ** $p < 0.01$, *** Abbreviations: tissue inhibitor of metalloproteinase (TIMP), matrix metalloproteinase (MMP), interleukin (IL), monocyte chemoattractant protein 1 (MCP-1), transforming growth factor beta 1 (TGF- β 1), connective tissue growth factor (CTGF), tumor necrosis factor alpha (TNF- α), platelet-derived growth factor (PDGF), static (ST), cyclic stretch (CS), shear stress (SS). Panels A and B were adapted from Van Haaften and Wissing et al.⁴⁴.

Figure 6: Changes in myofibroblast phenotype and markers of matrix growth and remodeling in response to hemodynamic loading in the vascular constructs at day 20. **(A)** Relative gene expression of myofibroblast-specific phenotypic markers. **(B)** Cross-sections stained for α SMA (green) and DAPI (blue). **(C)** Relative expression of genes related to collagenous matrix, elastic matrix, proteoglycans, and remodeling genes. P-values were calculated using Kruskal-Wallis test with a Dunn's multiple comparison test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Abbreviations: S100 calcium binding protein A4 (S100A4), alpha-smooth muscle actin (α SMA or ACTA2), calponin 1 (CNN1), smoothelin (SMTN), vimentin (VIM), collagen I (COL1A1), elastin (ELN), versican (VCAN), matrix metalloproteinase (MMP), tissue inhibitor of metalloproteinase (TIMP), static (ST), cyclic stretch (CS), shear stress (SS), 4',6-diamidino-2-phenylindole (DAPI). # measured at or below the detection limit. Panels A, B, and C were adapted from Van Haaften and Wissing et al.⁴⁴.

Supplementary Figure S1: Protein expression myofibroblast- and macrophage-monocultures subjected to individual and combined hemodynamic loads. **(A)** Representative confocal images of myofibroblasts, cultured for 10 days with actin fibers (green), nuclei (red), and scaffold (blue), show a clear actin fiber orientation in the loaded samples, when compared to the static samples in which no preferential actin fiber direction can be observed. Scale bar 50 μ m. **(B)** Confocal images of same myofibroblast monoculture stained for collagen (green) and nuclei/scaffold (white). Scale bar 50 μ m. **(C)** Boxplots of protein secretion profiles of statically and dynamically cultured THP1-derived macrophages for 8 days, with calculated M1/M2 ratios based on the cytokine secretion levels of IL-6, TNF- α , MCP-1 (pro-inflammatory) and IL-10, IL-13, MMP-9 (anti-inflammatory). The dot in the MCP-1 graph represents a statistical outlier. **(D)** ELISA data of the relative protein secretion levels of statically and dynamically cultured macrophages at day 8 compared to the average secretion of pro-inflammatory, anti-inflammatory, growth, and remodeling proteins (protein levels were corrected for the average DNA content per group). The dots and shaded areas indicate, respectively, the 50th and 25th–75th percentiles. Abbreviations:

monocyte chemoattractant protein 1 (MCP-1), interleukin (IL), transforming growth factor beta (TGF- β), matrix metalloproteinase (MMP), platelet-derived growth factor (PDGF), connective tissue growth factor (CTGF), tumor necrosis factor alpha (TNF- α), enzyme-linked immunosorbent assay (ELISA). * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$. Panels A and B were adapted from Van Haaften et al.¹⁹. Panels C and D were adapted from Wissing et al.⁴³.

DISCUSSION:

The bioreactor described herein allows for the systematic evaluation of the contributions of the individual and combined effects of shear stress and cyclic stretch on inflammation and tissue regeneration in tubular resorbable scaffolds. This approach also enables a large variety of analyses to be performed on vascular constructs, as exemplified in the representative results section. These results show the distinctive impact of the different hemodynamic loading regimes (i.e., different combinations of shear and stretch) on both growth and remodeling of the TEVG construct. These insights, collected via this in vitro platform, aid in the optimization of scaffold design parameters for newly developed in situ TEVGs. To ensure a proper experimental workflow, an understanding of the critical steps and the limitations of this protocol is important.

The most critical steps in the protocol are related to the application of stretch to the samples. For stretch application, it is essential that the setup is leak-free. There are two weak points in the system: the knots mounting the electrospun grafts to the pressure conduits and the connection between the bioreactor base and the flow culture chambers. As described in steps 1.5.2 and 1.5.4, multiple, tight knots have to be positioned exactly at the engraved groove. If the knot is not tight enough or is placed slightly above or below the groove, a slight leakage of hydraulic fluid into the flow culture chamber may occur. This leakage may be detected as a pressure drop in the hydraulic reservoir and a steadily increasing voltage on the strain pump to reach the set pressure value. Besides, this leads to a disturbed flow in the flow culture chamber, an increased risk of contamination of the cell culture, and a dilution of the medium. Once this happens, the flow culture chamber has to be taken out of the experiment, and the screw thread on the hydraulic reservoir should be closed with a white Luer cap. This major measure of taking out a complete sample, which is needed to ensure proper continuation of the experiment for the other seven flow culture chambers, emphasizes the importance of accurately placing tight knots, exactly in the engraved grooves. Only then can proper separation be ensured between the water in the hydraulic reservoir and the medium in the flow culture chambers. To improve robustness of the mounting of the scaffolds, the grooves in the pressure conduits will be made slightly deeper in a revised version of the bioreactor, allowing for easier and better knot placement and thus, secure separation of the hydraulic fluid from the medium.

Another potential source of leakage is between the screw threads of the bioreactor base and the white Luer connectors of the flow culture chamber. Because of possible wear and tear of the Teflon material, an extra silicone O-ring can be added to prevent leakage (*step 5.2.2*). Moreover, the Teflon bellow of the strain pump should be allowed to slightly expand O/N one day before the experiment (*step 1.4*). If leakage occurs, the lost hydraulic fluid should be compensated by adding a small amount of ultrapure water via one of the eight screw threads (use a syringe with needle and flexible wire). Place the flow culture chamber back with a small piece of parafilm

between the screw thread and the white Luer connector on the bottom compartment of the flow culture chamber. To overcome this leakage issue in future experiments, the current Teflon screw threads on the bioreactor base will be replaced by stainless steel threads in the next-generation version of the bioreactor to prevent wear-out of the system.

The variation in stretch (**Figure 3C**) is larger than the variation in WSS (**Figure 3D**), as stretch is more difficult to control. Nevertheless, besides the measures to prevent leakage, there are other measures that limit the variation in stretch: (i) avoiding air bubbles in the flute (*step 1.4 and 5.2.1*), (ii) ensuring consistent pre-stretch among the different samples (*step 2.5.3*), and (iii) ensuring consistent scaffold properties among different samples (*step 1.3*).

Finally, extra caution is needed when mounting the electrospun scaffold onto the silicone tubing (*step 2.3*). Specifically, when the fragile electrospun scaffold needs to be pulled over the stretched silicone tubing, it is important not to apply too much force to prevent the electrospun graft from being damaged, especially on the inside of the electrospun graft. If damage occurs on the inside of the electrospun graft, either from forceful pulling or from too weak stretching of the silicone tube, the extent of the damage to the electrospun fibers only becomes visible after the construct is harvested and analyzed. In the current setup, the success of the seeding can only be confirmed upon immunofluorescent or immunohistochemical analysis, after sacrificing the sample. However, the seeding procedure with fibrin as the cell carrier is a well-established method⁶⁷ that typically leads to a homogenous cell distribution for scaffolds with a sufficiently large pore size. One of the most important aspects with respect to cell seeding is to ensure that the scaffold is dry prior to seeding (*step 4.4*), to prevent the fibrin gel with the cells from passing through the wet scaffold, resulting in inhomogeneous seeding. Finally, although the decontamination method of the electrospun graft by UV radiation and dipping in 30% ethanol is not as stringent as the sterilization of electrospun grafts prepared for in vivo studies, which are often sterilized by ethylene oxide or gamma irradiation, it is sufficient for in vitro culture experiments that can last up to 20 days without any signs of contamination (for examples, see **Figure 3–6** and Van Haaften and Wissing (2020)⁴⁴). Moreover, the PCL-BU material that is used here, does not allow for long exposure to high ethanol concentrations. The most suitable sterilization method can be chosen depending on the material used.

In addition to the results of previously performed co-culture studies, a broader variety of studies can be performed with the same system. The system was previously employed to perform dynamic monocultures of myofibroblasts (**Supplementary Figure 1A–B**) and macrophages (**Supplementary Figure 1C–D**) to investigate the effects of hemodynamic loading on individual cell types and their paracrine signaling^{19, 43}. Different hemodynamic loading regimes resulted in clear actin fiber orientation in myofibroblasts (**Supplementary Figure 1A**) and distinctly different collagen deposition (**Supplementary Figure 1B**) after 10 days. The cytokine production by THP1-derived macrophages was drastically different between the different hemodynamic loads (**Supplementary Figure 1C**) and showed a more pro-inflammatory profile when loaded (**Supplementary Figure 1D**). Other validated possibilities include the application of oscillatory flow, by using an extra pump and fluidic unit. The viscosity of the medium can be increased towards the range of blood viscosity (e.g., by adding xanthan gum)⁶⁹. Modulating the medium

viscosity represents an additional variable to broaden the range of applicable shear stresses. Lastly, although the described protocol employs the flow-conditioning setup, setups from other manufacturers can be used as well, as long as similar flow regimes can be applied.

One of the major advantages of using this system is the relatively large construct (approximately 15 mm x 10.5 mm) that can be hemodynamically loaded, allowing for a wide variety of possible readout parameters to be extracted from a single sample. At the same time, the construct size may be viewed as a limitation as well, as this setup requires a relatively large amount of (sometimes costly) material, especially if primary cells are used, or if the culture medium requires costly additives. Moreover, the throughput of the setup is relatively low. Consequently, the current setup is particularly suited for hypothesis-driven research in which a limited number of variables is comprehensively tested, rather than the screening of a large number of variables with limited readout. For future experiments, small improvements on the current setup are being made to enable the option for mounting smaller scaffolds and downscaling the size of the medium reservoirs. With respect to the latter, the current volume of the medium reservoirs is required to enable sufficient volumetric flow rates to achieve the desired shear stresses. The required flow rates—and with that, the volume of the medium reservoirs—can be reduced by increasing the viscosity of the medium (e.g., by adding xanthan gum, as previously established⁶⁹).

To conclude, this bioreactor allows for the quantification of the individual and combined effects of shear stress and cyclic stretch on tissue growth and remodeling on a wide variety of elastomeric 3D biomaterial scaffolds. The bioreactor can culture up to eight vascular constructs under various loading conditions. Owing to its design, the bioreactor is especially suited to study the interplay between hemodynamics and in situ vascular TE processes.

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The authors have nothing to disclose.

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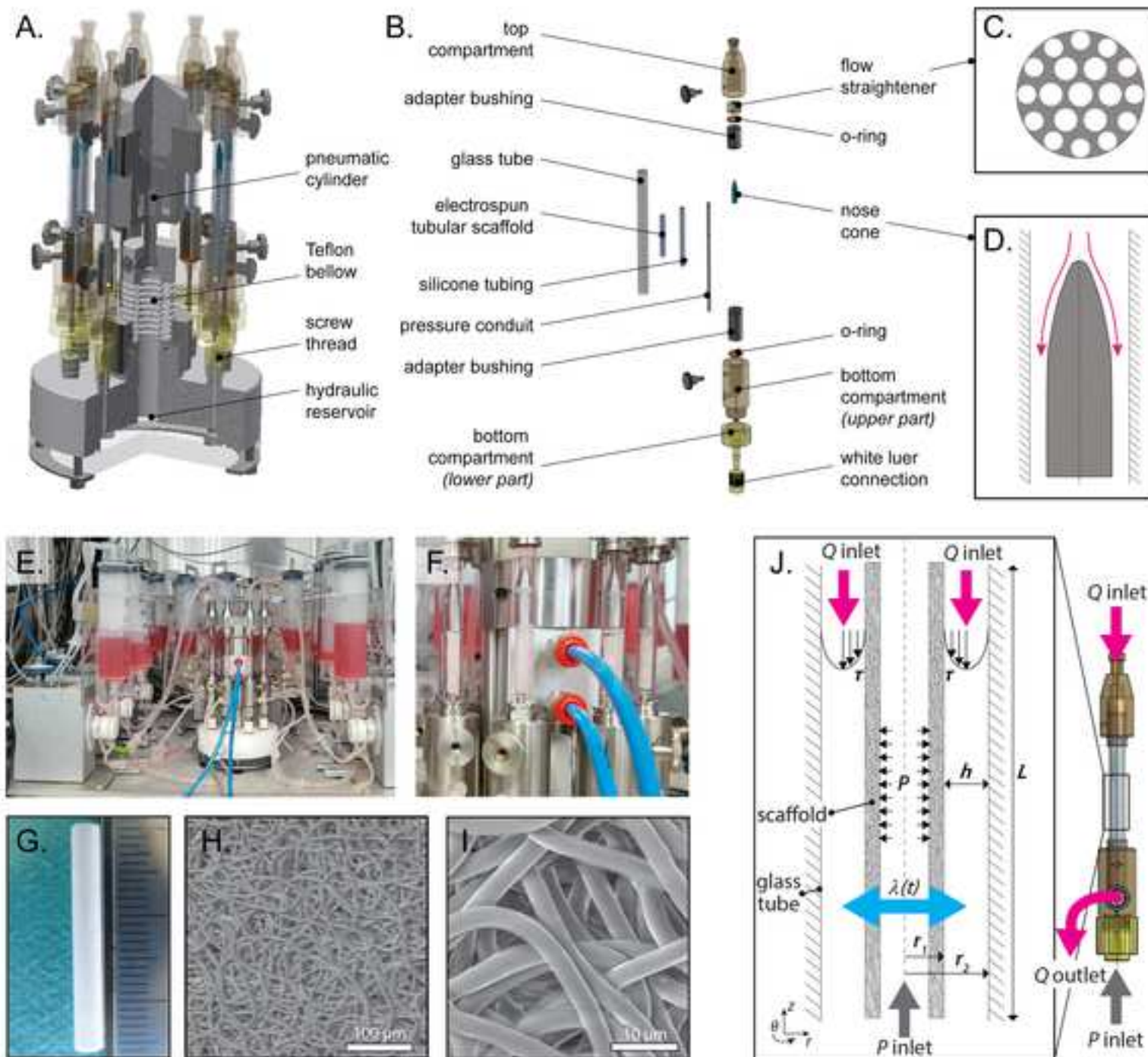
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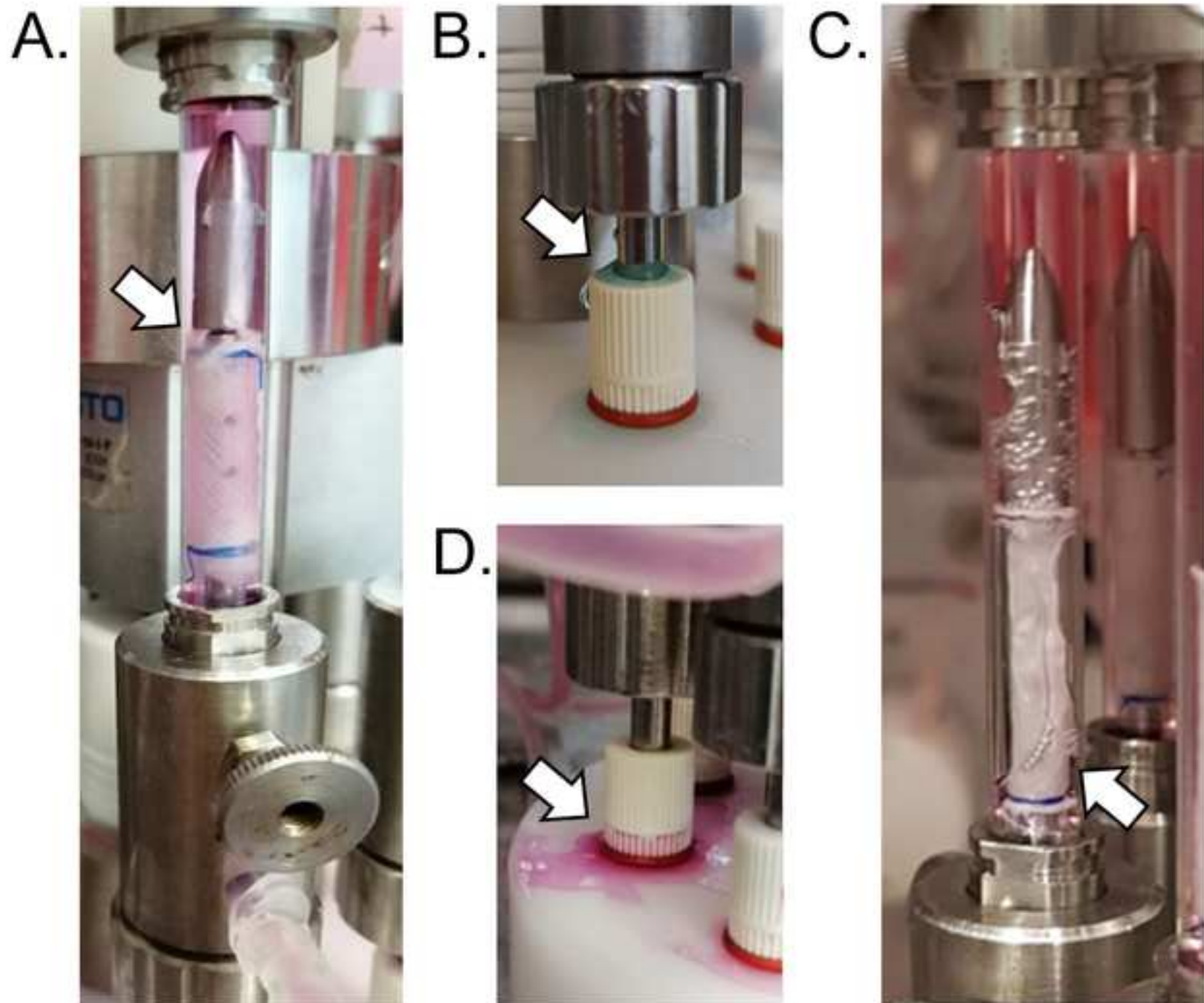
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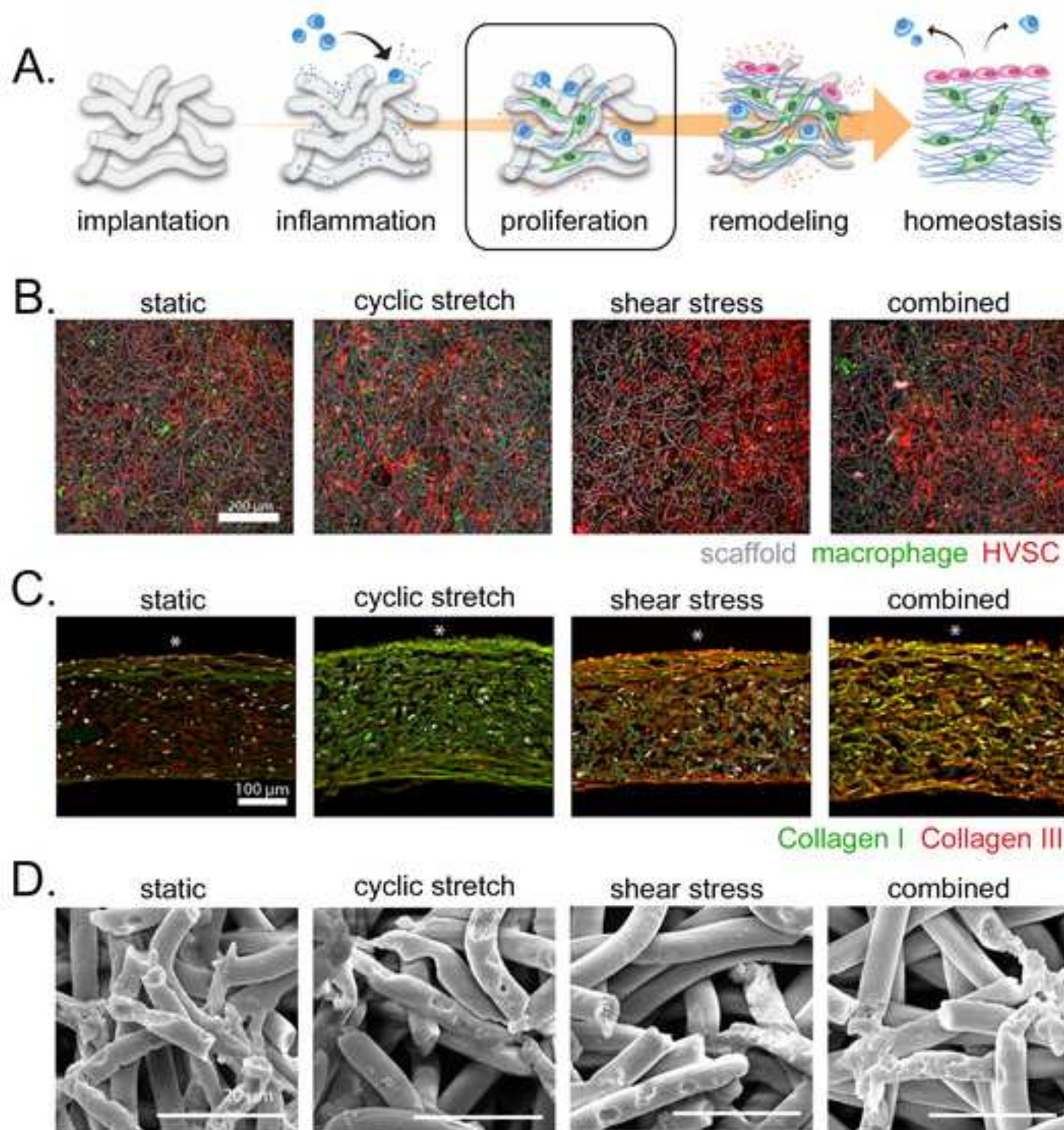
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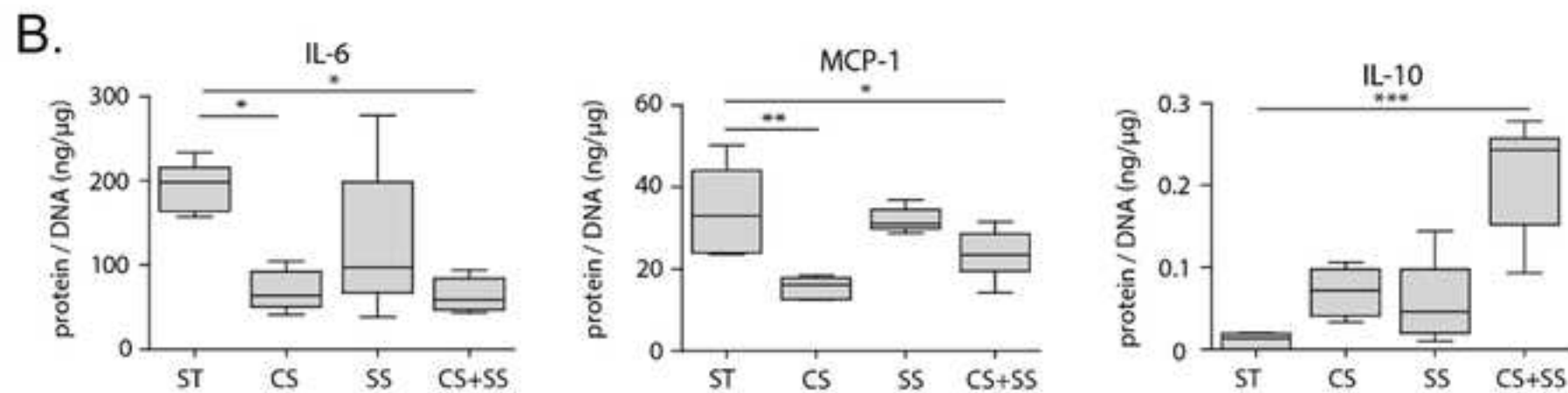
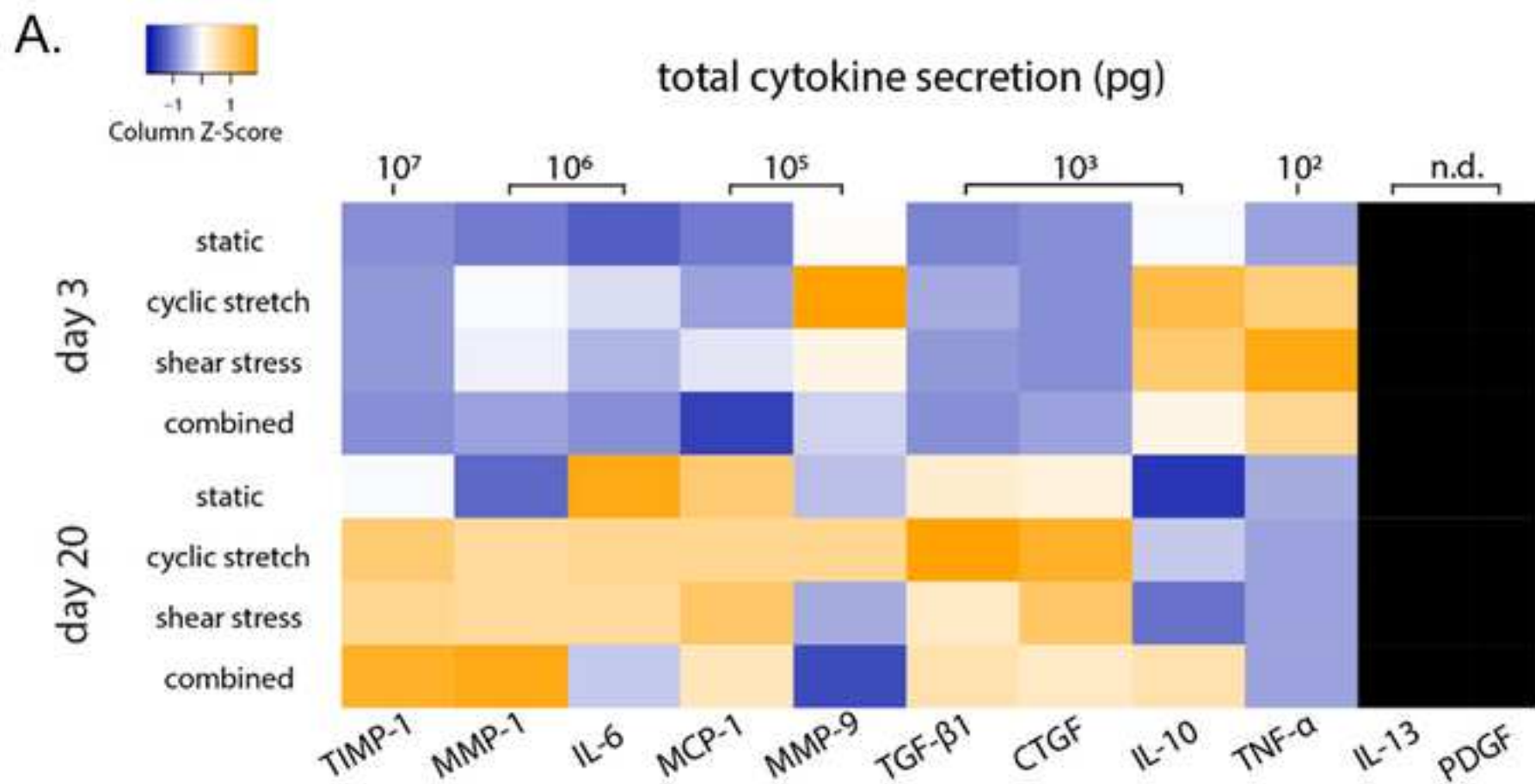
Figure 1

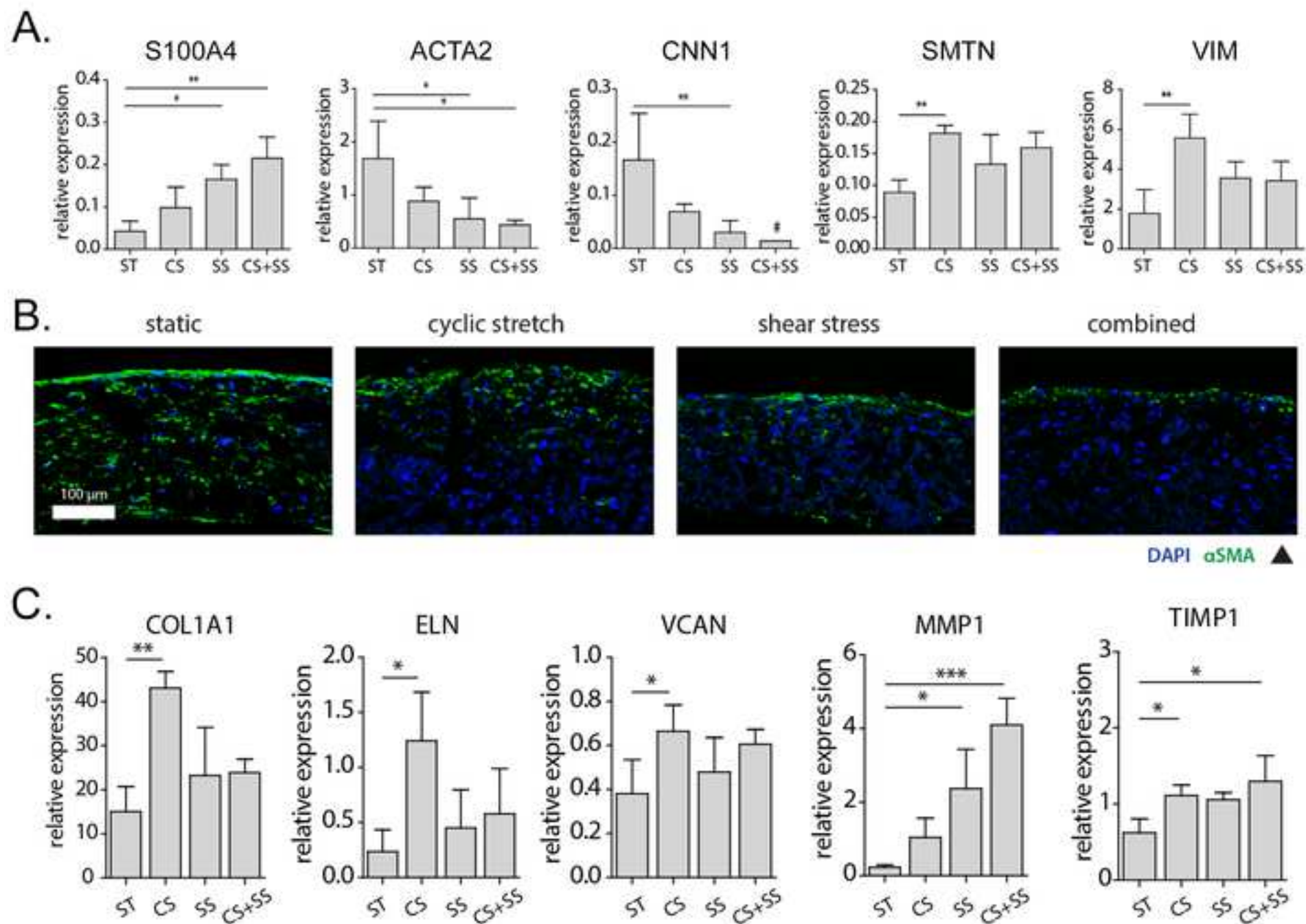
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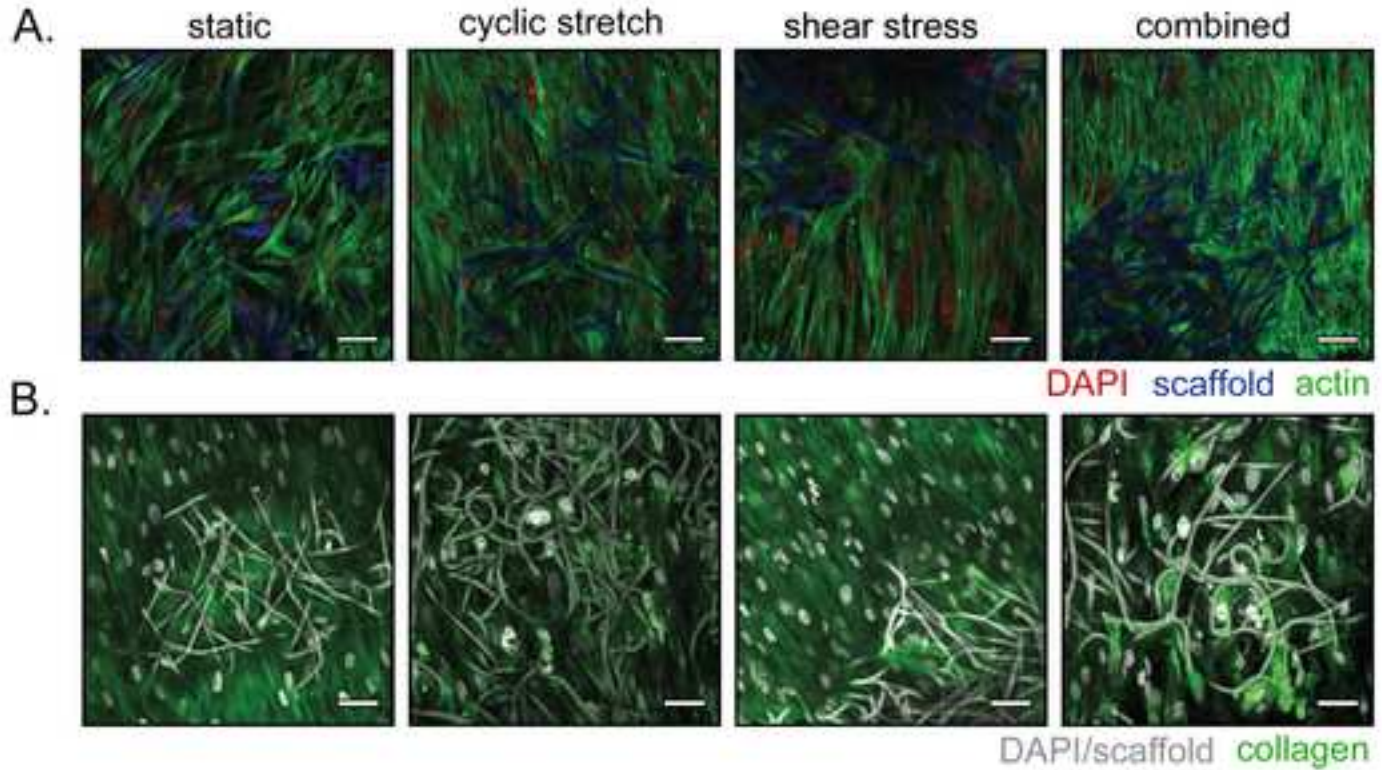








Myofibroblast monocultures



Macrophage monocultures

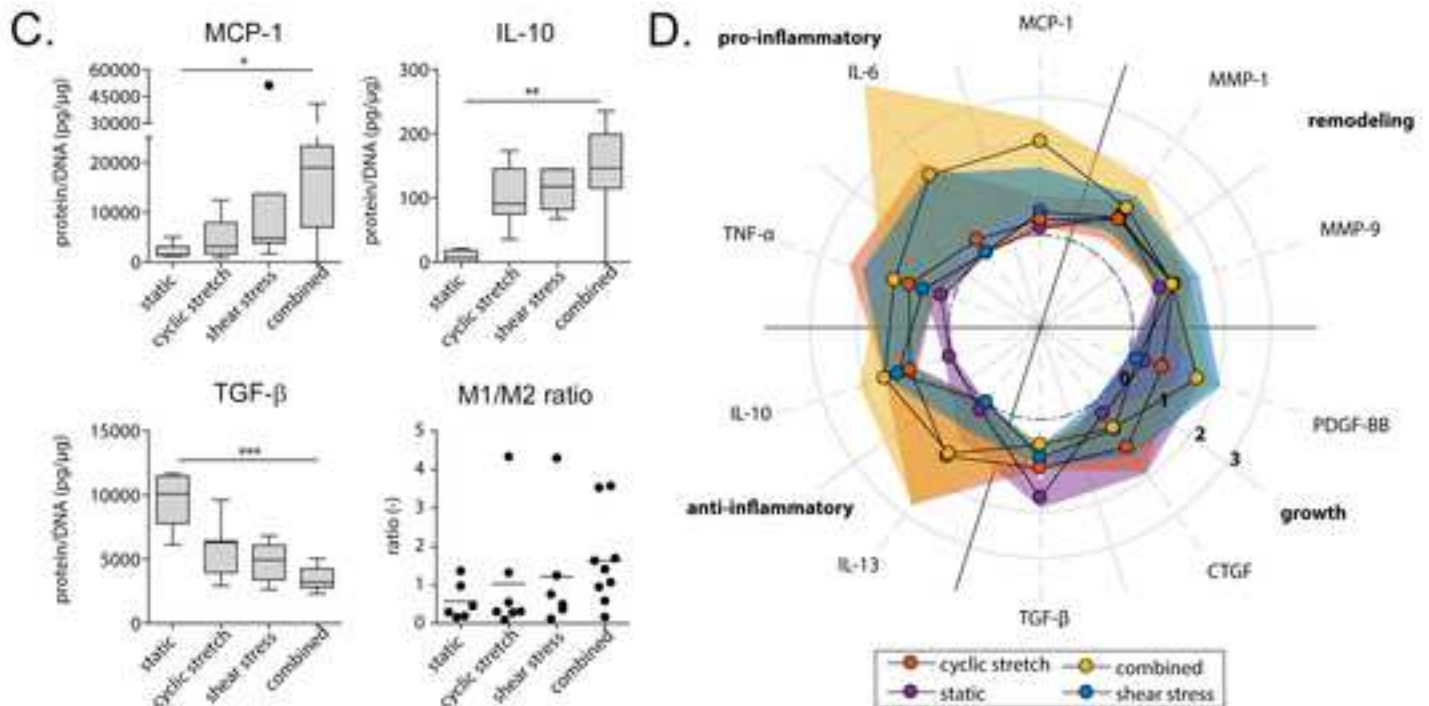


Table 1: Functional des parts in Figure 1 A-D.

Bioreactor parts

Stretch application

Pneumatic cylinder

Teflon bellow

Hydraulic reservoir

Screw thread

White luer connector

Pressure conduit with small holes

Silicone tubing

Flow application

Ibidi pump system

Top and bottom compartment with flow inlet and outlet

Glass tube

Flow straightener

Nose cone

Adapter bushing

Silicone o-ring

Description of bioreactor main features, corresponds with indicated

Functional description

Actuates Teflon bellow.

Loads the hydraulic reservoir.

Can be connected with up to 8 flow culture chambers, is filled with demi water, applies pressure to the silicone mounted constructs.

Connection between the flow culture chamber and hydraulic reservoir. The pressure inlet for the silicone mounted constructs.

Used to screw the flow culture chamber tight to one of the eight screw threads on the hydraulic reservoir/bioreactor base.

Directly connected with water in hydraulic reservoir. When the hydraulic reservoir is pressurized, the pressure conduits fill the space in between the silicone tube (that is mounted on the pressure conduit) with water, pushing the silicone tubing outwards, resulting in a circumferential stretch on the silicone mounted graft from inside.

To mount the electrospun graft on the pressure conduit, is circumferentially stretched from inside.

Used to control the flow in the flow culture chambers.

Connects the flow culture chamber into the flow loop.

Contains the pressurized scaffold in the center and allows perfusion of the scaffolds.

Stabilizes the flow in a relatively short settling length.

Distributes the flow evenly.

To fixate the glass tube.

To prevent leakage of medium.

Table 1: Functional des parts in Figure 1A-D.

Bioreactor parts
<i>Stretch application</i>
Pneumatic cylinder
Teflon bellow
Hydraulic reservoir
Screw thread
White luer connector
Pressure conduit with small holes
Silicone tubing
<i>Flow application</i>
Ibidi pump system
Top and bottom compartment with flow inlet and outlet
Glass tube
Flow straightener
Nose cone
Adapter bushing
Silicone O-ring

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Stabilizes the flow in a relatively short settling length.

Distributes the flow evenly.

To fix the glass tube.

To prevent leakage of medium.

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
advanced Dulbecco's modified EagleMedium (aDMEM)	Gibco	12491-015	cell culture medium for fibroblasts
Aqua Stabil	Julabo	8940012	prevent microorganism growth in bioreactor-hydraulic reservoir
Bovine fibrinogen	Sigma	F8630	to prepare fibrinogen gel
Bovine thrombin	Sigma	T4648	to seed the cells on the electrospun scaffold
Centrifuge	Eppendorf	5804	to prepare fibrinogen gel
Clamp scissor - "kelly forceps"	Almedic	P-422	to seed the cells on the electrospun scaffold
CO2 cell culture incubators	Sanyo	MCO-170AIC-PE	to spin down cells and conditioned medium
Compressed air reservoir	Festo	CRVZS-5	clamp the silicone tubing and apply pre-stretch to the scaffold so the scaffold can be sutured into the engraved groove (autoclave at step 0, step 6)
Custom Matlab script to calculate the maximum stretches	Matlab	R2017. The Mathworks, Natick, MA	for cell culturing
Data acquisition board	National Instruments	BNC-2090	smoothing air pressure fluctuations and create time delays for pressure build-up
Ethanol	VWR	VWRK4096-9005	calculate the minimum and maximum outer diameter of the electrospun scaffold
Fetal bovine calf serum (FBS)	Greiner	758087	data processing in between amplifier system and computer
Flow culture chamber compartments, consisting of a pressure conduit with engraved grooves and small holes to apply pressure on silicone tubing, a screw thread, nose cone, top compartment with flow inlet and bottom compartment flow outlet, adapter bushing	Custom made, Department of Biomedical Engineering, Eindhoven University of Technology	n.a.	to keep sterile working conditions
Glass pasteur pipet	Assistant	HE40567002	cell culture medium supplement; serum-supplement
Glass tubes of the flow culture chamber	Custon made, Equipment & Prototype Center, Eindhoven University of Technology	n.a.	flow culture chamber compartments (autoclave at step 0, step 6)
GlutaMax	Gibco	35050061	apply vacuum on electrospun scaffold (autoclave at step 0)
			part of the flow culture chamber (clean and store in 70% ethanol, at step 0 and 6)
			cell culture medium amino acid supplement, minimizes ammonia build-up

High speed camera	MotionScope	M-5	to monitor the stretch during culture; time-lapse photographs of the scaffolds are captured at a frequency of 30 Hz for 6 sec (i.e. 3 stretch cycles)
High speed camera lens - Micro-NIKKOR 55mm f/2.8 - lens	Nikon	JAA616AB	to monitor the stretch during culture; time-lapse photographs of the scaffolds are captured at a frequency of 30 Hz for 6 sec (i.e. 3 stretch cycles)
Hose clip	ibidi GmbH	10821	block medium flow (autoclave at step 0, step 6)

Hydraulic reservoir with 8 screw threads for 8 flow culture chambers	Custom made, Department of Biomedical Engineering, Eindhoven University of Technology	n.a.	to apply pressure to the silicone mounted constructs (clean outside with a paper tissue with 70% ethanol, rinse reservoir with 70% ethanol followed by demi water, at step 0 and 6)
Ibidi pump system (8x) including ibidi pump, PumpControl software, fluidic unit, perfusion set (medium tubing), air pressure tubing, drying bottles with orange silica beads	ibidi GmbH	10902	set up used to control the flow in the flow culture chambers. Note 1: the ibidi pumps were modified by the manufacturer to enable 200 mbar capacity. Note 2: can be replaced by pump system of other manufacturer, as long as same flow regimes can be applied.
Knives (no.10 sterile blades, individual foil pack) and scalpel handle (stainless steel, individually wrapped)	Swann Morton	0301; 0933	to cut the silicone tubing in the correct size for the scaffold and to cut the suture material
LabVIEW Software	National Instruments	version 2018	to control the stretch applied to the scaffolds
Laminar flow biosafety cabinet with UV light	Labconco	302310001	to ensure sterile working conditions. The UV is used to decontaminate everything that cannot be autoclaved, or touched after autoclaving
Large and small petri dishes	Greiner	664-160	for sterile working conditions
L-ascorbic acid 2-phosphate (vitamin C)	Sigma	A8960	cell culture medium supplement, important for collagen production
LED light cold source KL2500	Zeiss	Schott AG	to aid in visualization for the time lapse of the scaffolds during monitoring of the stretch
Luer (female and male) locks and connectors, white luer caps	ibidi GmbH	various, see (https://ibidi.com/26-flow-accessories)	to close or connect parts of the bioreactor and the ibidi pump (autoclave at step 0, step 6)
Measuring amplifier (PICAS)	PEEKEL instruments B.V.	n.a.	to amplify the signal from the pressure sensor and feedback to LabView
Medium reservoir (large syringes 60 mL) and reservoir holders	ibidi GmbH	10974	medium reservoir (autoclave at step 0, step 6)

Medium tubing with 4.25 mm outer diameter and 1 mm inner diameter	Rubber BV	1805	to allow for a larger flow rate, the ibidi medium tubing with larger diameter is used. Note: the part of medium tubing guided through the fluidic unit valves are the same as the default ibidi medium tubing
Motion Studio Software	Idtvision	2.15.00	to make the high speed time lapse images for stretch monitoring
Needle (19G)	BD Microlance	301700	together with thin flexible tubing used to fill the hydraulic reservoir with ultrapure water without adding air bubbles
Needle driver	Adson	2429218	to handle the needle of the nylon suture through the silicone tube (autoclave at step 0, step 6)
Paper tissues	Kleenex	38044001	for cleaning of the equipment with 70% ethanol
Parafilm	Sigma	P7793-1EA	quick fix if leakage occurs
Penicillin/streptomycin (P/S)	Lonza	DE17-602E	cell culture medium supplement; prevent bacterial contamination
Phosphate Buffered Saline (PBS)	Sigma	P4417-100TAB	for storage and washing steps (autoclave at step 0)
Plastic containers (60 mL) with red screw caps	Greiner	206202	to prepare the fibrinogen solution
Pneumatic cilinder	Festo	AEVC-20-10-I-P	to actuate the Teflon bellow (clean with a paper tissue with 70% ethanol at step 0 and 6)
Polycaprolactone bisurea (PCL-BU) tubular scaffolds (3 mm inner diameter, 200 μ m wall thickness, 20 mm length)	SyMO-Chem, Eindhoven, The Netherlands	n.a.	produced using electrospinning from 15% (w/w) chloroform (Sigma; 372978) polymer solutions. See Van Haaften et al <i>Tissue Engineering Part C (2018)</i> for more details
Pressure conduit without holes (for static control)	Custom made, Department of Biomedical Engineering, Eindhoven University of Technology	n.a.	to mount electrospun tubes on silicon tubing (autoclave at step 0, step 6)
Pressure sensor and transducer	BD	TC-XX and P 10 EZ	the air pressure going to the pneumatic actuated pump is raised until it reaches the set pressure
Proportional air pressure control valve and pressure sensor	Festo	MPPES-3-1/8-2-010, 159596	provides compressed air to the pneumatic actuated pump

Roswell Park Memorial Institute 1640 (RPMI-1640)	Gibco	A1049101	cell culture medium for monocyte/macrophage
Safe lock eppendorf tubes (1.5 mL)	Eppendorf	0030120086	multiple applications (autoclave at step 0)
Silicone o-rings	Technirub	1250S	to prevent leakage (autoclave at step 0, step 6)
Silicone tubing (2.8 mm outer diameter, 400 um wall thickness)	Rubber BV	1805	to mount the electrospun tubes on the pressure conduits (autoclave at step 0)
Sterile tube (15 mL)	Falcon	352095	multiple applications
Suture, 5-0 prolene with pre-attached taperpoint needle	Ethicon, Johnson&Johnson	EH7404H	Prolene suture wire 5-0 (75cm length, TF taperpoint needle, 1/2 circle, 13 mm needle length)
Syringe (24 mL)	B. Braun Melsungen AG	02057932	to add the ultrapure water or medium to the hydraulic reservoir or flow culture chamber
Syringe filter (0.2 µm)	Satorius	17597-K	to filter the fibrinogen solution
T150 cell culture flask with filter cap	Nunc	178983	to degass culture medium
T75 Cell culture flask with filter cap	Nunc	156499	to culture static control samples
Teflon bellow	Custom made, Department of Biomedical Engineering, Eindhoven University of Technology	n.a.	to load the hydraulic reservoir (clean outside with a paper tissue with 70% ethanol at step 0 and 6)
Tray (stainless steal)	PolarWare	15-248	for easy transport of the fluidic culture chambers and the bioreactor from incubator to laminar flow cabinet and back (clean with a paper tissue with 70% ethanol before and after use)
Tweezers	Wironit	4910	sterile handling of individual parts (autoclave at step 0 and 6)
Ultrapure water	Stakpure	Omniapure UV 18200002	to correct for medium evaporation, mixed with aqua stabil mixed and used as hydraulic fluid. (autoclave ultrapure water at step 0)
UV light	Philips	TUV 30W/G30 T8	for decontamination of grafts and bioreactor parts before seeding

*Journal of Visualized Experiments*Dr. Fengxun Li, Guest Editor
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Dear Dr. Li,

We respectfully submit the revision of our manuscript, entitled '**A multi-cue bioreactor to evaluate the inflammatory and regenerative capacity of biomaterials under flow and stretch**'. We would like to thank the reviewers for their assessment of our manuscript and their helpful comments and suggestions, and we thank you for allowing us to submit a revised version of our manuscript.

We have addressed all reviewer's comments as specified in the detailed point-by-point response in the rebuttal document, in which our replies are *written in blue and italic*. All edits in the manuscript have been marked via track changes as instructed.

As all reviewers commented on the complex design of the bioreactor, and reviewer #3 suggested to include a more detailed description of the main components of the bioreactor, we added a new table. This table provides a functional description of the main features of the bioreactor and corresponds with the indicated parts in the construction drawing of the bioreactor in Figure 1. With this new table, in combination with Figure 1 and, most importantly, the JoVE video, we believe that this protocol is useful for the scientific community and hope to inspire other biomedical engineers with this bioreactor design.

Besides the details on the bioreactor, more specific details on the electrospun grafts that are used in the described protocol and shown in the results section, were missing. We have now included these details in the manuscript and the Table of Materials, and we stress the possibility of testing a broad variety of elastomeric tissue engineered vascular grafts within the same bioreactor system.

Finally, as suggested by reviewer #3, we have expanded a little on the overall impact of our results, giving more relevance to the bioreactor and its use, always keeping in mind the actual focus of the JoVE manuscript that is the methodology.

All authors have approved submission of the revised manuscript to *JoVE*.

With this, we are confident that the manuscript will form a valuable contribution to *JoVE*. We look forward to your kind reply.

Yours sincerely,
On behalf of all authors,

Anthal Smits

Reviewer(s) comments to Authors and *reply by Authors*

Editorial comments:

Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling (line 71) or grammar issues, and that all abbreviations have been defined.

The spelling error in line 71 is corrected. Spelling and grammar issues, as well as abbreviations have been checked throughout the manuscript and were corrected if applicable.

2. If human blood or tissue was used, please clarify whether the study design was approved by an institutional ethics committee.

Human myofibroblasts and primary human macrophages are used in this study. A statement regarding the origin and ethical approval of the used tissues is added in the protocol section ("step 0. General preparations and required actions before setting up the bioreactor."). Conform the Dutch medical scientific research with human subjects act (WMO), secondary usage of patient material does not require assessment by a Medical Ethics Examination Committee (see line 176-182).

5. Are the dimensions and other details of the electrospun scaffolds unambiguously described by Van Haaften et al.(lines 187-188)? If not, please provide those details.

The fabrication of the PCL-BU tubular grafts are extensively described by Van Haaften et al. In addition, for clarification, we have added the PCL-BU grafts to the Table of Materials, the basic dimensions to the text (step 0.3, line 217-222) and the fabrication to the preparation part of the protocol (step 0.3 and 0.3.3, line 217-249). In Figure 1 G-H, both a macroscopic view and a microscopic view of the electrospun scaffolds are shown.

6. Please specify if any precautions need to be taken while sterilizing the electrospun scaffolds (line 243) in 1.2.

After decontamination of the electrospun scaffold with UV light, it is advised to work extra careful and as clean as possible (e.g. only touch the electrospun scaffold with a clean tweezer, or with clean gloves when needed). The be sure we have added a second decontamination step right before the seeding; in which we dip the scaffold in 30% ethanol (step 1.3.2, line 310-315). We have added a note stressing the importance of minimizing touching the graft after 1.2.3 (the UV contamination step, line 288-289).

7. Please provide details about the needle (line 254, 1.3.1).

The taperpoint needle is pre-attached to the suture wire. To prevent confusion, we have removed the words 'needle' and 'needle driver' and replaced these by 'suture' (line 298). In the Table of Materials, more details of the suture with the pre-attached needle are added.

8. Please provide details-how much of the solution is to be made etc (lines 425-426, 3.1.3).

We have added the volume of solution that needs to be prepared in step 3.1.3 (line 490-493).

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The authors provide a thorough, yet difficult to follow protocol detailing the design and operation of a complex bioreactor used to decouple the effects of shear stress and stretch on electrospun synthetic vascular grafts.

We have indeed tried to describe the design and operation of the bioreactor thoroughly and, thanks to the detailed comments from the reviewers, we have now better clarified parts of the protocol that might have been too tersely explained.

Major Concerns:

The protocol is overly specific for this complex bioreactor setup, which limits usefulness for other researchers. The bioreactor design is complex and not adequately described in the protocol, which makes the protocol format of this manuscript less impactful and useful to the scientific community.

While the concept and motivation of the bioreactor – to allow decoupling of shear stress and stretch in tubular scaffolds – is broadly relevant for the community, this manuscript is indeed focused on this particular bioreactor setup, to facilitate new users in building a similar setup and benefiting from it in their research. To make the overall idea behind the bioreactor design more clear, we added Table 1, in which we give a functional description and rationale behind the main features of the bioreactor. In combination with Figure 1 and, most importantly, the JoVE video, we believe that this protocol is useful for the scientific community and that our bioreactor design can hopefully inspire other biomedical engineers.

Minor Concerns:

General Comments

Table of Materials: There is no mention of the polymer used for scaffold fabrication.

Good point. We have added the details of the polymer and a reference to the paper (Van Haaften et al, 2018, Tissue Engineering Part C) in which we previously described the fabrication method in the Table of Materials.

-Throughout the protocol please be more descriptive when pointing to certain figures. Instead of just stating 'Figure 1', consider 'Figure 1a'.

We thank the reviewer for his/her suggestions and added the specific panel-letter to all figure references in the text.

Specific Comments

Page 2, Introduction, Line 56 - Please briefly provide reasoning or include references that detail why these prostheses are suboptimal for patients.

We have added references to papers which describe in detail the suboptimal functioning of permanent cardiovascular prosthesis in large cohorts of patients (line 57). And we added references to reviews elaborating on the development of TEVGs and TEVHs in the next sentence (line 58).

Page 4, Note Section, Line 155 - Please provide justification for using different macrophage sources for either a mono- or co-culture.

The choice for using THP1 cells arose from a practical consideration, to limit donor-to-donor variation (which is commonly observed in PBMC-derived macrophages), and as these THP1 cells are very metabolically active, which is especially interesting in the context of scaffold degradation studies. However, to keep the described protocol clear, we now only describe the co-culture experiment (with PBMC-derived macrophages and myofibroblasts) in the revised document. The THP1 monoculture is now only mentioned in the figure description (see line 971)

Page 4, Step 0.1, Lines 161-166 - Cell densities and graft size are reported in terms of volume (cm³ or mm³). For these values, is this the volume of material in the graft? Or based on a more simple calculation of the total volume of the graft as a cylinder, i.e. including the hollow center of the graft in the volume calculation. Please clarify in the text.

To calculate the volume of the scaffold we use the formula for a hollow cylinder: $\pi \times \text{thickness}^2 \times \text{length} \approx 0,04 \text{ cm}^3$. The electrospun material has a high porosity (>90%). We have added the calculation to step. For clarity of the protocol, we have adjusted all volumes to cm³. (line 190-191)

Page 4, Step 0.1, Lines 165-66 - Rather than stating "a little bit more than these numbers" please provide the factor by which you increased your number of cells in culture.

We advise to culture up to 10-15% more cells to account for possible pipetting errors. It is adjusted in the text (line 193).

Page 6, Step 1.2, Line 238 - Please revise to remove "the of the".

The typo is removed (line 279)

Page 6, Steps 1.2.1-1.2.3, Lines 240-245 - For electrospun fiber scaffolds it is common to use sterilization methods that are effective at permeating into porous materials such as ethylene oxide. It is surprising to see that UV exposure is the only method used to sterilize the scaffolds. Is this accurate?

First of all, as recommended by reviewer #3, we have replaced 'sterilization' by 'decontamination' (line 279), which is a more accurate term in this study. The reviewer correctly points out that more stringent methods (e.g., gamma irradiation or EtOH) are typically used to sterilize porous scaffolds, especially for in vivo experiments. In our in vitro set-up, we take all precautions which we regard as essential for the culturing of the grafts. Besides the UV irradiation, we also dip the scaffolds in 30% ethanol (see step 1.3.2, line 311), to decontaminate the scaffolds again before seeding. The UV-radiation and 30% ethanol dip are also the decontamination method of choice because our used material PCL-BU might dissolve/change properties when placed in high enough ethanol concentrations or for long periods. In our laboratory, we perform(ed) multiple in vitro studies in which PCL-BU electrospun scaffolds are decontaminated via this UV radiation technique before cell seeding, either in a tubular shape or in strips/punches of scaffold. Some experiments lasted up to 20 days (for example see Van Haaften and Wissing et al. Adv Biosyst 2020), and all in vitro cultures stayed free of contamination. From these experiences we conclude that our method of decontamination/sterilization is adequate for in vitro cell culture experiments.

Page 6, Step 1.3.1, Lines 254-259 - This step is very difficult to follow. When "taking the needle with a needle clamp through the end of the silicon tubing twice and making a small knot on both sides", was the suture run through one end of the tube and out of the other - leaving a taut suture spanning the cross section of the tubing? Or was the suture just run through each side of the tube and tied so that there were knots and 10 cm of wire on opposite walls of the tubing? Please clarify in the text.

We agree that this step could have been written more clearly. As this is a difficult step, we highlighted this part in yellow, so this will be featured in the JoVE video to enable reproducibility. This step aims to describe the best way to pull the electrospun scaffold over the silicon tubing. For this, the silicon tubing needs to be stretched a bit, without tearing apart the silicon tubing end with the suture wire. That is why we make a knot on both sides of the one end of the silicon tubing and, subsequently, make a third knot from the two wires coming from the knots, so we can first pull the wires through the electrospun scaffold, stretch the silicon tubing a bit and gently slide the electrospun tubing over the silicon tubing. We appreciate the reviewer's suggestion for clarifying the text and we have adjusted the text accordingly (step 1.3.1, line 298-308).

What do the authors mean by a tapered edge shape? Why leave 10 cm of wire if this will all be cut away per the final line in this step?

This is indeed not directly clear from the text. We cut away the loose wires around the 3 knots, and then cut the silicone tubing end into a more triangle-like shape to easily guide the silicone tubing through the electrospun tubes (first over the suture and subsequently over the silicon tubing). We have adjusted this in the text (line 305-308).

Page 6, Step 1.3.2, Lines 261-265 - Why dip the scaffold in 30% ethanol?

Although we decontaminated the electrospun grafts with UV light exposure, the steps following this decontamination involve a lot of (almost) touching the graft with tweezers or gloves. To minimize any risk of contamination, we dip the electrospun grafts in 30% ethanol shortly before cell seeding. Furthermore, we experienced that this pre-wetting aids in sliding the electrospun grafts over the silicon tubing. We have added this explanation in the text (step 1.3.2, line 310-311)

The previous step advises to "cut away all free threads and wires", but this step instructs the reader to place the scaffold over the free 10 cm wire. It needs to be made clear that that 10 cm length of wire should not be cut in the previous step.

This is indeed confusing. We have adjusted the text (step 1.3.1, line 301)

Page 9, Step 1.5.3, Lines 315-319 - Since pre-stretch consistency among the different samples is an important part of the setup, it should be a larger focus. Wouldn't be more accurate to pre-cut the silicon tube to a set length so that way all samples can be stretched the same amount and tied at the same positions more easily? The way that this step is currently described seems like it can become a large source of error without improving the degree of control.

The pre-stretch on the silicone tubing ensures that the circumferential stretch on the silicone tubing, and therefore the surrounding scaffolds, is more homogenous across the length of the tube. Pre-stretch might indeed affect the mechanical properties, and therefore the amount of stretch delivered to the scaffold. So the reviewer is correct that the pre-stretch should be consistent among the different samples. We thank the reviewer for the suggestion of pre-cutting the silicon tubes to a set length. To make sure that the silicon tubes are all stretched to the same height, a ruler can be mounted on the scissor clamp and used to check the amount of stretch on the scaffold (e.g. the scissor clamp can be pulled upwards until the lower end of the ruler has the same height as the lower end of the scaffold). We have added these instructions to step 1.3.5 and 1.5.3 (line 328-330, line 366-369).

Page 13, Step 4.2.1, Lines 505-506 - How is the thin wire used to fill the pressure conduit in this step?

We agree that "thin wire" is a confusing term. We replaced it by 'thin tubing' and added extra explanation on why and how we use the thin wire to fill the pressure conduit with ultrapure water

without adding air bubbles. We've corrected the confusing sentence (step 4.2.1, line 575-581) added the syringe and needle details to the Table of Materials.

Page 15, Step 5.3.6, Lines 628-629 - This step refers to steps 5.4.1-5.4.4, which do not exist in this protocol.

This should be 5.3.1-5.3.5, it is corrected in the text (line 707).

Page 15, Step 6.1, Lines 649-650 - Same as previous comment.

This should be 5.3.1-5.3.5, it is corrected in the text (line 729).

Reviewer #2:

Manuscript Summary:

This manuscript describes a bioreactor system, in which tubular tissue engineered structures can be circumferentially stretched while the outer surface of the tubular tissue is exposed to shear stress on axial direction, at the same time and independently. The authors claim that it is not possible to decouple mechanical stretch and shear stress in currently available ex vivo models or other bioreactor models that tries to mimic the physiological mechanical conditions. Furthermore, they state that their system provides ability to test affect of mechanical cues that are beyond physiological limits, separately. The manuscript is written and analyses were done in a professional fashion.

We thank the reviewer for carefully reading the manuscript.

Major Concerns:

The engineering aspect of the system is pretty complicated. Even only giving the scaffold a tubular shape takes a very long and many-step process. In the discussion part they can also mention that their system can be used even with the freshly isolated native vessels (similar to doi: 10.1177/0963689718792201). It can be interesting to mount animal or human vessels (with suitable sizes) onto their silicon tubing and apply shear and stretch in different ranges.

We agree that making tubular grafts is a highly complex and multistep process. However, as that is not the main theme of this article, we refer to previous published papers, in which the electrospinning process is described in more detail. The suggestion of adding freshly native vessels, as elaborately described by Kural et al (2018) is an interesting suggestion, however, as our system works via an 'inside-out principle'; in which the stretch is applied from inside and the shear applied on the 'outside'; it would implicate that the ex vivo material could be perfused from outside or that the ex vivo material should have to be pulled inside out. However, we missed this reference in our introduction section, in which we refer to other bioreactors that have been described. We added this reference (line 112).

Reviewer #3:

Manuscript Summary:

The manuscript presented by Smits group is a detailed and well written description of a protocol for culturing tubular grafts under controlled hemodynamic conditions aimed at investigating inflammatory response and growth capacity of electrospun scaffolds. The work reported is valuable and useful for experimenters dealing with vascular tissue regeneration even if some points should be better clarified and commented.

We thank the reviewer for his/her careful evaluation of the manuscript and the useful comments. We have included a point-to-point response to the raised questions and useful suggestions of the reviewer.

Major Concerns:

- The abstract should be better focused on the actual aim of this manuscript. I suggest the author to revise some parts of the text of the abstract in lines 44-51 in order to focus on the use of the bioreactor and on the purpose of Jove manuscript.

We agree that we should mention the goal of the JoVE manuscript –to describe the use of the bioreactor - earlier in the abstract. We have adjusted this (see line 44-46).

- In the first part of the introduction the authors introduce the in situ cardiovascular TE, the importance of mechanical loading and of immune response for cell growth and tissue remodeling, proposing also a brief overview of the literature. In the last part of the introduction the authors introduce their scaffolds and their bioreactor describing their already done experiments and main results related to influence of shear stress and cyclic strain on inflammation and tissue formation. In this last part I would suggest the authors to add a more specific description about scaffolds used in the work (which material, electrospun structure, sizes, ...) in paragraph 108-119.

The reviewer is correct that the scaffold description was missing. We have added the material to the Table of Materials. And we have given more details on the dimensions of the graft (see step 0.3, line 217)). However, a broad variety of tissue engineered scaffolds can be tested within the system. From different synthetic or natural origin, different micro-architecture, various porosity etc. We have added this in the introduction (line 124-126) and discussion section (line 1090) and added a NOTE after the electrospun scaffold information in step 0.3 (line 224-226).

More importantly I noticed the lack of basic information about the bioreactor in the introductory part (main features, actuated by ibidi pumping units ...), which in the reviewer opinion is mandatory to understand the protocol steps related to the bioreactor setting and the scaffold mounting. I would suggest the authors to add the main characteristics of their bioreactor and also some details about its components, useful for the protocol reading, in the paragraph 121-132, where some general aspects are already introduced. As an example, step 0.4 (line 212) of the protocol is not very clear without having in mind the bioreactor configuration.

We agree with the reviewer that the overall description of the bioreactor design was missing. Therefore we added Table 1 that we introduce in the introduction section (see line 144), in which we give a functional description and rationale behind the main features of the bioreactor. In combination with Figure 1 and most of all the JoVE video, we believe that this protocol will be much easier to follow and understand.

- In the bioreactor used for the experiments the fluid flow and the shear stress is applied outside the scaffold instead of in the lumen. Could the authors discuss this point and how this kind of stimulation can affect the results, with respect to bioreactors stimulating the luminal side of a vascular graft?

We agree with the reviewer that the original manuscript did not comment on this design choice enough. We have added a short explanation on why we chose this inside-out principle to load the samples in the introduction section (see line 135-145). With respect to the implications of having shear stress on the outside of the scaffold as compared to the luminal side we refer the reviewer to our corresponding methods paper in which this is extensively discussed (Van Haaften et al. Tissue Eng Part C 2018). It is important to note in this respect that this inside-out bioreactor is intended for systematic studies on the effects of shear and/or stretch, not to engineer a native-like blood vessel in the lab, for which a traditional vascular bioreactor is more suitable.

- In the protocol it is not always clear which operations have to be done under hood cabinet and which not, especially in the protocol parts related to scaffolds preparation and scaffold mounting (e.g., step 1.9). I would suggest the author to specify this aspect very clearly at the beginning of the protocol or in the main steps described.

In the beginning of the protocol, at step 0.6.1 (line 263) "perform step 1-4.3 (setting up the system), step 5.3 (medium change) and step 6.1-6.2 (harvest of vascular constructs) in a sterile laminar flow cabinet" the reader is advised to work in a hood cabinet. An extra reminder/note is added below the headers of these steps (line 274, 441, 473) or it is described in the step that e.g. the set-up should be transferred from the incubator to the laminar flow cabinet or vice versa.

- An additional figure more related to the mounting steps of the protocol involving scaffolds and bioreactor parts would be useful for the protocol visualization. As an example, steps 1.3.1 and 1.3.2 are difficult to follow and they would benefit of a panel in a dedicated Figure.

We agree that this is hard to follow with only the text. We feel that the mounting of the flow chamber parts would need a lot of photo's to give a complete visualization. We therefore highlighted this text in yellow as we would like to demonstrate this in the JoVE video.

- Step 1.2 of the protocol: I would use "decontaminate" instead of "sterilize". Using UV is not an actual sterilization of the scaffold.

We agree that, in this context, the term 'decontamination' is more appropriate and accurate than 'sterilization'. The reviewer correctly points out that more stringent methods (e.g., gamma irradiation or EtOH) are typically needed to sterilize porous scaffolds, especially for in vivo experiments. In our in vitro set-up, we take all precautions which we regard as essential for the culturing of these PCL-BU grafts. Besides the UV irradiation, we also dip the scaffolds in 30% ethanol (see step 1.3.2 and 1.8.1), to decontaminate the scaffolds again right before seeding. As the PCL-BU material might dissolve/change properties when placed in high enough ethanol concentrations or for long periods, the UV-radiation and short dip in 30% ethanol are applied in this experiment. If in another experiment, a graft material is used that allows for the more stringent sterilization methods, these types of sterilization methods can be used as our bioreactor does not prevent the use of these techniques. In our laboratory, we perform(ed) multiple in vitro studies in which electrospun scaffold are decontaminated via this UV radiation technique before cell seeding, either in a tubular shape or in strips/punches of scaffold. Some experiments lasted up to 20 days (for example see Van Haaften and Wissing et al. Adv Biosyst 2020), and all in vitro cultures stayed free of contamination. From these experiences we conclude that our method of decontamination is adequate for in vitro cell culture experiments.

In the revised manuscript, the term sterilization is replaced by decontamination. Furthermore, we comment on this decontamination technique in the discussion section (line 1046-1054).

- Step 1.42. pressure conduits: state how they are made especially concerning the presence of some holes. It was clear to me that there are holes in the conduits later in the protocol. Just state that there are conduits with holes for dynamic sample and conduits without holes for static ones.

Pressure conduits with and without holes for respectively the dynamic and the static samples, are mentioned separately in the Table of Materials. To prevent confusion, we adjusted the naming of both types of pressure conduits throughout the protocol.

- Step 1.5 lines 310-319: how the experimenter can apply the same pre-stretch to the samples and verify it? Please add some advice to the readers about this issue.

The pre-stretch on the silicone tubing ensures that the circumferential stretch on the silicone tubing, and therefore the surrounding scaffolds, is more homogenous across the length of the tube. Pre-stretch might indeed affect the mechanical properties, and therefore the amount of stretch delivered to the scaffold. So the reviewer is correct that the pre-stretch should be consistent among the different samples. Some advice to the reviewers on this point is indeed helpful. Reviewer #1 suggested to pre-cut all silicon tubes to a set length. Besides, to make sure that the silicon tubes are all stretched to the same height, we mount a small ruler on the scissor clamp. This allows to keep a consistent amount of stretch on the scaffold (e.g. the scissor clamp can be pulled upwards until the lower end of the ruler has the same height as the lower end of the scaffold). We have added these instructions to step 1.3.5 and 1.5.3 (line 328-330, line 366-369)..

- Line 391 and line 402: the reservoirs are 60-ml or 50-ml reservoir?

The medium reservoirs are 60 mL. We've corrected this in step 2.4 (line 462).

- Line 487-88, line 512 and line 523: specify the steps to repeat more specifically

We have added the specific steps in the text (line 554-555 and line 587).

- Line 584, Equation 2: the pressure gradient is not correct here (teta instead of delta). Please correct and also verify description of equation 1, which is not clear in my opinion.

We noted that the descriptions for equations 1 and 2 were indeed not clear, because the sentences were not correctly ordered. We have therefore move them in the correct order and believe that this will resolve the unclarity. We indeed noted that the symbol of the differential in equation 2 was incorrect, which we corrected (step 5.1.2, line 652-667).

- Line 639: rubber air filters are mentioned at this point for the first time. I suggest to mention them also before in the mounting procedure steps.

The 'rubber air filters' are mentioned in the part describing the preparations for the ibidi set-up in step 2.1.3 (line 453). We've changed the naming of these filters throughout the text to "rubber air filters".

- Step 5.4: please check the description of this step, which is not clear.

Indeed, due to a typo this sentence was not clear. It should be: "drying bottles" instead of "drying beads". We have adjusted this in the manuscript (line 723).

- Lines 652-655, Step 6.1.1: it is not clear to me why after the experiment is stopped, the pump systems for flow and strain are needed to be re-started. If this procedure is due to the fact that you could have some different time points, please clarify better this point in the protocol, considering this possibility.

We agree with the reviewer that it was not clearly described. We've added extra explanation to the text (line 731-736). As the samples need to be harvested one-by-one, to make sure that the samples are under hemodynamic loads till right before harvesting, the ibidi pump and strain pump are stopped and re-started multiple times.

- Line 685, step 6.3.7. What about the non autoclavable components? The author should better specify the components, which have not to be autoclaved and give indication for their sterilization or decontamination.

The cleaning procedure of the non-autoclavable components is now added in step 6.3.7 (line 769-771) and also added in the Comments section in the Table of Materials.

- Line 729: Air babbles can disturb WSS, but also compromise the cell viability and tissue growth. Please add also this issue.

This is added to the text (820-821).

- Text describing results reported in Figure 2 (lines 720-731) is redundant with the Figure 2 caption itself. I would suggest the authors to use the caption to recall the protocol steps, the warning associated to key operations and to the system handling. In general I think that the captions should better describe the figures and the panels reported in the figure rather than repeat what is already stated in the main text. A careful revision is needed on this point. I suggest the authors to refer to protocol steps, when appropriate and useful, in the figure captions.

We thank the reviewer for the excellent practical suggestion to include a reference to the protocol steps in the figure captions (line 878-910). With regard to the troubleshooting steps in Figure 2, we indeed realize the redundancy with the main text, however, we believe that this is necessary to ensure that the figures are standalone.

- Figure 4 to 6: I suggest the authors to expand a little bit the comments about their results giving more relevance to their impact.

As we understand it, the JoVE guidelines state that the main focus should be on the protocol. Nevertheless, we have now added some overall conclusions of the impact of the results to both the results section (line 847-850, 860-862, 873-876) as well as the discussion section (line 989-992).

- From Figure 3 it is worth to notice that stretch is not stable during culture. The authors should comment this result and they should give some advice to the readers about how to limit such variations. I suppose they have had some experience that is useful to state for the reader benefit.

In general, indeed, the variation in stretch (Figure 3C) is larger compared to the variation in WSS (Figure 3D), because it is simply more difficult to control. The most important tips to limit variations are (in order from highest to lowest priority): prevent air bubbles in the flutes (step 0.4 and 4.2.1), prevent leakage at coupling between hydraulic reservoir and flow chamber (step 4.2.2), ensure consistent pre-stretch among different samples (step 1.5.3), ensure consistent scaffold properties among different samples (step 0.3). We have added these words of advice to minimize the variation in stretch to the discussion section (line 1027-1032).

- In Fig. 3 caption specify the time related to the short term experiments.

These measurements were performed to indicate the variation of stretch and shear stress between samples at a specific point in time under distinct bioreactor settings. It indicates how the variation increases when the magnitude of shear stress and, especially, stretch increase. We have rephrased the caption to clarify (line 914-915).

In Fig. 4 better clarify that HVSC are myofibroblasts from human SVs.

This is added in both the figure caption (line 930-931) and the main text (line 835).

In Fig. 5 state which kind of cells are used in the experiment.

This is added in both the figure caption (line 942-943) and the main text (line 855-856).

- Supplementary fig. S1. I suggest the authors to consider to add the Supplementary figure as main figure introducing the mono-cultures also in the main text of the results and not only at the end of the discussion. The authors should also provide a better explanation of results in panel D highlighting their impact.

We have thought about making the supplementary figure one of the main figures. However, we decided in the end that it would be confusing to have both monocultures and co-cultures in the protocol description. Therefore we only introduce the mono-culture at the end. We agree with the reviewer that the description of figure S1 was a bit short. We have briefly mentioned all panels separately in the main text (line 1058-1065) and we added extra information on how to interpret panel D in the figure description (line 973-975).

- Line 888: step 1.5.2 is not in my opinion the only protocol step involved in this procedure. Please refer to all the related steps.

Indeed, this is a good point. We added 1.5.4 too, in which the second knot (on the upper end of the graft) is made and has to be exactly at the engraved ring (line 999).

- Lines 949-951: Together with the possibility to host smaller scaffolds the authors should also consider the possibility to reduce the reservoirs dimensions to obtain also a reduced priming volume so that to reduce reagents used for one experiment. I invite the authors to consider also this issue.

This is a good suggestion, as the required volume of medium is relatively high. The size of the 60 ml ibidi syringes is needed because of the required flow. Only if the required flow can be reduced, we can downscale the syringe sizes (i.e., the smaller 13 ml ibidi syringes). Several options to reduce the flow would be: Downscaling the channel height (not preferred, because it is a trade-off with WSS homogeneity) (1); reducing the WSS (depends on research question) (2); increasing the medium viscosity (to make it more blood-like anyway this I think is a worthwhile option and reduces the required flow linearly) (3). With the latter one being the preferred option. We added this option to the discussion section (line 1082-1087) .

Minor Concerns:

- Introduction, lines 145-146. This sentence seems contradictory; decoupling the shear and the strain is not a biomimicking situation. I suggest the authors to change the sentence to explain better what they mean.

We agree with the reviewer that the sentence was not clear. We have adjusted this sentence (line 158-161).

- Supplementary figure S1: state also in the Fig panels about the cell (macrophage or myofibroblast) under investigations.

We added the the cell type referring to the specific mono-cultures in the figure as per the reviewer's suggestion.

- I suggest to use mount instead of reconstruct in the protocol (e.g., line 482)

We agree with the reviewer and used the term 'mount' instead of 'reconstruct' (line 549).

- Line 236 I'm not sure that the word balance is correct in this context. Maybe scale? Please check.

We agree that the words scale and balance are often used interchangeably. However, we prefer to use the term 'balance' in this context, as we measure the mass of our electrospun scaffold on a dedicated balance in our laboratory.

- Line 238 check the sentence: "the of the"

The typo is removed (line 297).

- Line 770: myofibroblasts instead of fibroblasts

Adjusted to myofibroblasts (line 868).

- Line 931: remove with before can be

The typo is removed (line 1057).