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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 theses 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: π*thickness^2*length ≈ 0,04 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 cm^3. (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 precut 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 precutting 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 electrosun 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 biorector 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 precut 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 carful 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 suggests 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 biomimiking 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).