**We are grateful to the reviewers for their thoughtful comments. We believe the modified manuscript is stronger for their efforts. Below is a point-by-point response to their critiques. Responses are indicated by bold text in this document. Altered text is indicated in the modified manuscript using track changes. All line references are in reference to the Display for Review setting “Final: Show Markup” with the track changes.**

**Editorial comments:**  
  
The manuscript has been modified by the Science Editor to comply with the JoVE formatting standard. Please maintain the current formatting throughout the manuscript. The updated manuscript (52971\_R0\_120814.docx) is located in your Editorial Manager account. In the revised PDF submission, there is a hyperlink for downloading the .docx file. Please download the .docx file and use this updated version for any future revisions.   
  
Changes made by the Science Editor:  
  
1. There have been edits made to the manuscript.

**We have changed the reference to our Biofabrication paper (Hald et al. 2014) back to the initial language used. This language is required by IOP Publishing for any reproduction/modification included in this manuscript. All three figures contain parts that are reproduced/modified directly from this paper.**  
  
Changes to be made by the Author(s):  
  
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

**We have made edits throughout the manuscript to correct grammar issues.**  
  
2. The highlighted protocol length is >2.75 pages. Please revise the highlighted protocol text to 2.75 pages or less to ensure that the videography can occur in a single day.

**We have removed highlighting in sections of the protocol. It is now ~2.75 pages or less.**   
  
3. There is unnecessary branding in steps 1.4.1 and 2.1.3.2 that must be removed: Sylgard.

**The unnecessary branding has been removed in both steps (lines 190 and 290).**  
  
4. More detail is required in a number of areas:   
-[2.1.3.4](http://2.1.3.4/): About how long does this take?

**We have added the phrase “approximately 30 min” to the end of the step (line 295).**

-[2.2.3.6](http://2.2.3.6/): Under what conditions are coverslips incubated after seeding?

**We have added the sentence “Incubate the seeded coverslips in a sterile incubator at 37 °C and 5% CO2.” to the beginning of the next step, 2.2.3.7 (line 420).**

-[2.2.4.1](http://2.2.4.1/): How much medium is added?

**We have added clarification to this step. At this point, any reducers used are removed and 4 mL of serum-free medium are added to each well (lines 426-427).**

-3.1.7-3.1.8: Are images captured during treatment?

**We have added clarification to both steps. Images should be acquired throughout the treatment assay at desired time intervals (lines 466-467). Treatments are applied at a single time and application should be done between image acquisitions (lines 470-473).**

5. Journal titles not formatted correctly in the references, and Ref 6 has no title. JoVE reference format requires that DOIs are included, when available, for all references listed in the article. This is helpful for readers to locate the included references and obtain more information. Please note that often DOIs are not listed with PubMed abstracts and as such, may not be properly included when citing directly from PubMed. In these cases, please manually include DOIs in reference information.

**All journal titles now have proper formatting. Reference 6 has been corrected. Missing DOIs have been added with the exception of Ref 3, as we could not determine a DOI for this reference.**  
  
**Reviewers' comments:**  
  
**Reviewer #1:**   
*Manuscript Summary:*   
The manuscript covers a new protocol of creating patterned ECM that does not degrade over long periods of cell culture because of the extra layer of genipin. The protocol is well developed and described, and the proof of principle experiments are well explained and demonstrate the feasibility of this approach. The new method addresses an outstanding need in tissue engineering of maintaining engineered tissues in culture for long periods of time without the loss of functionality.   
  
*Major Concerns:*  
On lines 487-489 the authors explain that the reduction of stress at day 14 is due to the loss of cells. It is clear that the cells are not proliferating, but it is not clear why they are dying as compared to day 10. As the premise of the new method is to maintain the cells in culture for long periods of time, it would be very helpful if the authors could expend the discussion of this point.

**To improve the clarity and importance of this explanation, we have added the following to the text: “The addition of a minimal basal level of serum to culture medium may alleviate this problem in future work.” (lines 510-511)**  
  
*Minor Concerns:*  
It might be better to have figure 3 go before figure 2, as the latter is never mentioned in the protocol portion.

**To correct this inconsistency, we have added reference to Figure 2 in the protocol (Step 2.2.3.7, lines 421-422).**  
  
**Reviewer #2:**  
*Manuscript Summary:*   
The manuscript details a new way of microfluidic deposition of a cushion layer of a crosslinking agent between culture substrate (PDMS) and ECM proteins (FN) for prolonged culture of muscular tissue. In addition, the manuscript details a method to quantify contractile output (MTF)  
  
*Major Concerns:*   
None  
  
*Minor Concerns:*   
1. The manuscript title, abstract and discussion focus exclusively on the microfluidic genipin deposition technique. However, the focus should also be on the MTF technology. Even though MTFs have been widely published in the past, JoVE would benefit from a detailed MTF technology paper as well.

**We have changed the title of the manuscript (lines 2-3) and added more details regarding MTF technology throughout the text to draw more focus to the MTF technique. (lines 55-56, 69-70, 559-561, 590-591)**  
  
2. There are also some hanging phrases and sentences in the final two pages of the manuscript (but that might be an artifact of the PDF compilation process).

**We believe this was an artifact of the PDF compilation process, as we see no such issues in the modified word document received for revision.**  
  
**Reviewer #3:**   
*Manuscript Summary:*   
In Hald et al, the authors describe a new system for micropatterning fibronectin for vascular muscular thin film (vMTF) constructs, which consist of vascular smooth muscle tissues engineered on glass coverslips selectively coated with PIPAAM and PDMS silicone elastomer. Microcontact printing has traditionally been used for fibronectin patterning, but tissues tend to delaminate after several days in culture due to the hydrophobic nature of PDMS. In this manuscript, the authors fabricated a microfluidic PDMS device with 10 um channels that is placed on top of a coverslip spin-coated with PIPAAM and PDMS. Genipin, a cross-linking compound, is first flowed through the device to reduce the hydrophobicity of PDMS, followed by fibronectin. The microfluidic device is removed and the coverslip is seeded with vascular smooth muscle cells. The authors show that vascular smooth muscle cells patterned and remained attached for 2 weeks on their fluidically patterned constructs. They also measured contractility in constructs cultured for up to 2 weeks using their vMTF assay, indicating that tissues also maintained their function.   
  
*Major Concerns:*  
In the Introduction, the authors states "Arterial lamellae are concentric, circumferentially-aligned sheets of contractile VSMCs separated by sheets of elastin." This is not completely accurate, as the endothelial lining is another important feature of arteries. This should be mentioned and the authors should note that their artery model only includes the muscle layer.

**Strictly speaking, the lamellae make up the tunica media and do not include the endothelial cells. However, the reviewer’s broader point is well taken, so we have added text to elucidate the importance of the endothelium in artery and clarify that our model does not incorporate endothelial function: “While the endothelium is also critical in overall vascular function, engineered arterial lamella provide a useful model system for assessing changes in vascular contractility during disease progression.” (lines 83-85)**

The introduction could benefit from a basic definition of genipin - it is described only as a "desirable candidate for substrate modification due to its relatively low toxicity compared to similar crosslinking agents and its increasing use as a biomaterial in the fields of tissue repair and ECM modification."

**We have added the following to the text “a natural hydrolytic derivative of the gardenia fruit” to better define genipin (lines 100-101).**  
  
In 3.1.8), the authors should include more details about how they added drugs to their experiment bath. For example, did they dilute a more concentrated solution directly into the bath? This is important since adding drug solutions by aspirating the bath completely will likely ruin the assay.

**This detail has been added to 3.1.8): “Add concentrated solutions of each treatment to the experimental dish containing 5 mL of sterile 1X Tyrode’s solution at specified time points, yielding the desired treatment concentration in the 5-mL volume. Make treatment additions during the interval between time-lapse image acquisition to avoid capturing pipette in images.” (lines 470-473)**  
  
The statement in lines 487-489 "The slight drop in tissue contractility at the end of the assay is the direct result of the reduced number of cells composing the tissue, since serum-starved  
VSMCs do not proliferate." seems to conflict with the data in Fig. 2C. If VSMCs are naturally apoptosing over time and not being replaced, wouldn't that be reflected as a drop in confluence as well? Is the loss of cells potentially reflected by a drop in tissue thickness, if confluence is not changing? Or do the authors have a measurement of cell number (based on nuclei) as a function of time in culture to back up their claim?

**We agree that these questions relating to tissue integrity and function at the later time points are valid and report in our Biofabrication a decrease in cell number as a function of time. We chose to not include this result here, as we felt it was not as relevant to a protocol-centric publication, but it is cited (line 510, reference 19). At this time, we are not certain why there is no drop in confluence. There could be changes in cell morphology and/or phenotype that could be the cause of maintained confluence during cell loss.**  
  
As a suggestion, a useful addition to the Discussion section would be a reference to Agarwal et al, Lab on a Chip 2013. The authors could mention that a laser engraving system can also be used to automate and standardize the tape application step and manual cutting of cantilevers. A second suggested addition is McCain et al, Biomaterials 2014, as another example of altering the culture substrate to extend the culture lifetime of engineered tissues compared to traditional microcontact printing on PDMS.

**We have added this discussion and these references (lines 559-561 and 590-591).**

*Minor Concerns:*  
In several instances (lines 113, 309), the authors refer to genipin as a crosslinker protein. Genipin is not a protein.

**This error has been corrected throughout the manuscript (lines 63, 64, 96, 110, 115, 123, 316, 319, 340, 494, and 557).**   
  
Wafers for photolithography and tridecafluro-trichlorosilane are missing from the materials list.

**We have added the necessary information for both of these materials to our materials list (see spreadsheet lines 9 and 13).**  
  
In 2.1.3.6) and 2.1.3.9), the authors should specify that cutting is done with a razor blade.

**We have added the additional detail of using a razor blade for cutting to the requested steps (lines 300-301 and 309).**  
  
In 2.2.2.3), is the user supposed to aspirate and add PBS at the inlet or outlet?

**We have clarified this step to read: “After 5-10 min, carefully aspirate the excess ethanol at the inlet, immediately replacing it with 1X phosphate buffered saline (PBS) at the inlet.” (lines 352-353)**  
  
In 2.2.2.9) and 2.2.2.14) should the dish be covered to maintain sterility and prevent evaporation? This should be explicitly stated.

**We performed our incubations in a non-sterile incubator. Treatment with the penicillin/streptomycin solution sterilizes the samples prior to seeding [2.2.3.5)]. We have added clarifying statements (lines 372-374 and 390).**  
  
In 2.2.3.6) the authors should better explain what a reducer is.

**We added an example of a reducer to the text in this step as follows: “One example of a reducer is the cut top of a 15-mL conical tube attached to the coverslip with sterile vacuum grease prior to seeding.” (lines 416-418)**  
  
It would be useful to reference Fig. 1D in the appropriate section of the Protocol (not just in Representative Results).

**We have added multiple references to Fig. 1D in the Protocol (2.2.2.6, 2.2.2.7, 2.2.2.12, 2.2.2.13, and 2.2.3.6; lines 363, 366, 384, 387, and 414, respectively).**  
  
Sample sizes need to be indicated for Fig. 2C-D and Fig. 3E-F.

**We have added n-values to the captions for Figs. 2C-D (lines 531-532) and 3E-F (line 548).**  
  
  
**Reviewer #4:**   
*Manuscript Summary:*   
This manuscript describes a protocol to modify PDMS surfaces with genipin and fibronectin to increase the longevity of 2D engineered smooth muscle tissues in vitro. The protocol is well described and clearly presented. Results demonstrate the approach works. However, the description as to why it works and some details on the surface chemistry are not supported by data in this JoVE article, the 2014 paper in Biofabrication on which this work is predicated, or the other papers referenced by the authors. Specific comments and suggestions are detailed below.   
  
*Major Concerns:*  
1. Abstract, 1st paragraph, "…has been used to fabricate the basic functional unit of arteries…" is not quite what the authors have done previously or here. Rather, they have engineered a 2D smooth muscle tissue that responds to vasoactive compounds. This not the same as a 'basic functional unit of the artery" unless the authors have head-to-head data, perhaps in another publication, that validates this.

2. In line with comment 1, it is suggested that the authors rephrase statements throughout the paper that this is a system that "recapitulates structure and function of native artery." It does not do this; there are multiple structural and functional features that are not present that are important for disease modeling. Rather, this is an engineered 2D vascular smooth muscle tissue that has uniaxial alignment and generally responds to vasoactive compounds as expected, providing an improved in vitro model system over other alternatives, but in no way recreates function or structure equivalent to native artery.

**We changed the wording in the first paragraph of the abstract. It now reads: “…has been used to fabricated two-dimensional functional arterial mimics…” (lines 53-54)**

**We agree that our initial phrasing was inaccurate and have thus changed the phrasing throughout the manuscript as suggested** **(lines 69-70, 85-87, 113-114, 525 and 593).**  
  
3. Abstract, 2nd paragraph, "…new proteins cannot attach to hydrophobic PDMS…" is not correct based on existing literature, in fact the opposite is true. It is not an issue of attachment. Proteins, especially ECM protein, stick to hydrophobic PDMS. Rather, the issue being addressed by the authors is the stability of the ECM protein layer, the protein bioactivity and its attachment to the PDMS. ECM proteins adhere to most surfaces by partial denaturation and conformational change where some hydrophobic amino acid residues adhere to the PDMS and the hydrophilic residues are oriented towards the aqueous media. The genipin changes both the configuration of the attached fibronectin to the PDMS and thus potentially also its bioactivity. These are likely the factors that provide the enhanced culture duration and not the issue of hydrophobic recovery stated by the authors.

**The paragraph in question has been modified to reflect the proper explanation of genipin’s role in supporting extended culture of vascular smooth muscle cells (lines 57-61).**  
  
4. In the intro, the statement about hydrophobic recovery in paragraph 2 is not supported by the data or presented literature, as stated in comment 3.

**We have omitted the statement about hydrophobic recovery in paragraph 2 (lines 93-95) and paragraph 3 (lines 106-107).**  
  
5. How is the genipin attached to the PDMS? Simple adsorption? Other researchers have used APTES to covalently link the genipin to PDMS, but that was not done here. Why or why not?

**The genipin is attached via simple adsorption. Genchi et al. 2013 did have an intermediary APTES step in their genipin attachment technique. We initially included this step, but found limited success. This led us to try simple adsorption of genipin onto PDMS. Thus, we have eliminated the APTES step, simplifying the protocol and yielding similar results to Genchi et al. 2013. We added a statement clarifying that our genipin simple adsorption technique is similar to the technique used by Genchi et al. 2013 (line 99).**  
  
6. It is stated that the presented technique enables studying over disease-relevant time courses. Which disease cannot be assessed at 1 week per the previous microcontact printing approach versus 2 weeks enabled by this new technique?

**We have added reference to the persistence of cerebral vasospasm up to 14 days post-injury (Weir et al. 1978) to the Discussion (line 566).**   
  
7. How long does the tissue last? Data is presented for 2 weeks, but what about 3 or 4 weeks or even longer?

**We have cultured the VSMCs for up to four weeks in lab. But this was a one-time test that we do not feel comfortable including here. We can consistently culture for two weeks.**   
  
8. How is the PDMS in-between the genipin/fibronectin modified lines treated? Is it just bare PDMS?

**We have clarified that it is bare PDMS between the genipin/fibronectin modified lines in the Representative Results section of the manuscript (line 495).**  
  
*Minor Concerns:*  
Discussion, 3rd paragraph, "…Genchi et al showed extended viability of isotropic cardiac muscle…" is incorrect. Genchi et al used myoblasts differentiated into myotubes, which is skeletal muscle.

**We have corrected this mistake (line 581).**  
  
**Reviewer #5:**   
*Manuscript Summary:*   
The manuscripts presents a new technique for long-term culture of patterned vascular smooth muscle cells. The techniques presented are a modification of existing microcontact printing approaches and add significant value and knowledge to the field.

*Major Concerns:*  
None.  
  
*Minor Concerns:*  
None.