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Microfluidic genipin deposition technique for extended culture of micropatterned vascular muscular thin films --Manuscript Draft--

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Abstract:	<p>The chronic nature of vascular disease progression requires the development of experimental techniques that simulate physiologic and pathologic vascular behaviors on disease-relevant time scales. Previously, microcontact printing has been used to fabricate two-dimensional functional arterial mimics through patterning of extracellular matrix protein as guidance cues for tissue organization. Vascular muscular thin films utilized these mimics to assess functional contractility. However, the microcontact printing fabrication technique used typically incorporates hydrophobic PDMS substrates. As the tissue turns over the underlying extracellular matrix, new proteins must undergo a conformational change or denaturing in order to expose hydrophobic amino acid residues to the hydrophobic PDMS surfaces for attachment, resulting in altered matrix protein bioactivity, delamination, and death of the tissues.</p> <p>Here, we present a microfluidic deposition technique for patterning of the crosslinker compound genipin. Genipin serves as an intermediary between patterned tissues and PDMS substrates, allowing cells to deposit newly-synthesized extracellular matrix protein onto a more hydrophilic surface and remain attached to the PDMS substrates. We also show that extracellular matrix proteins can be patterned directly onto deposited genipin, allowing dictation of engineered tissue structure. Tissues fabricated with this technique show high fidelity in both structural alignment and contractile function of vascular smooth muscle tissue in a vascular muscular thin film model. This technique can be extended using other cell types and provides the framework for future study of chronic tissue- and organ-level functionality.</p>

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Dear Dr. Veien,

We are pleased to submit our manuscript entitled "Microfluidic Genipin Deposition Technique for Extended Culture of Micropatterned Tissues" for consideration for publication in *the Journal of Visualized Experiments*.

This manuscript details a novel fabrication technique for extending viable culture time of engineered tissues. We utilize microfluidic protein deposition to modify and pattern PDMS substrates with genipin for extended culture of vascular smooth muscle. Visual depiction of this technique will allow others to more readily extend it to other systems where long-term function is important.

Author Contributions:

Study conception and design: ESH and PWA
Acquisition of data: ESH, KES, JAR, and ZW
Analysis and interpretation of data: ESH, JAR, and PWA
Drafting of manuscript: ESH and PWA
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We believe that this work will be well-served by the unique multimedia format used by the *Journal of Visualized Experiments* and is consistent with the kind of scientific advancement and novelty that typify research protocols published in the journal. We appreciate your consideration.

Sincerely,



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Microfluidic genipin deposition technique for extended culture of micropatterned vascular muscular thin films

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SHORT ABSTRACT:

We present a method for microfluidic deposition of patterned genipin and fibronectin on PDMS substrates, allowing extended viability of vascular smooth muscle cell-dense tissues. This tissue fabrication method is combined with previous vascular muscular thin film technology to measure vascular contractility over disease-relevant time courses.

LONG ABSTRACT:

The chronic nature of vascular disease progression requires the development of experimental techniques that simulate physiologic and pathologic vascular behaviors on disease-relevant time scales. Previously, microcontact printing has been used to fabricate two-dimensional functional arterial mimics through patterning of extracellular matrix protein as guidance cues for tissue organization. Vascular muscular thin films utilized these mimics to assess functional contractility. However, the microcontact printing fabrication technique used typically incorporates hydrophobic PDMS substrates. As the tissue turns over the underlying extracellular matrix, new proteins must undergo a conformational change or denaturing in order to expose hydrophobic amino acid residues to the hydrophobic PDMS surfaces for attachment, resulting in altered matrix protein bioactivity, delamination, and death of the tissues.

Here, we present a microfluidic deposition technique for patterning of the crosslinker compound genipin. Genipin serves as an intermediary between patterned tissues and PDMS substrates, allowing cells to deposit newly-synthesized extracellular matrix protein onto a more hydrophilic surface and remain attached to the PDMS substrates. We also show that extracellular matrix proteins can be patterned directly onto deposited genipin, allowing dictation of engineered tissue structure. Tissues fabricated with this technique show high fidelity in both structural alignment and contractile function of vascular smooth muscle tissue in a vascular muscular thin film model. This technique can be extended using other cell types and provides the framework for future study of chronic tissue- and organ-level functionality.

INTRODUCTION:

Vascular diseases, such as cerebral vasospasm^{1,2}, hypertension³, and atherosclerosis⁴, develop slowly, are typically chronic in nature, and involve dysfunctional force-generation by vascular smooth muscle cells (VSMCs). We aim to study these slow-progressing vascular dysfunctions using *in vitro* methods with finer control of experimental conditions than in *in vivo* models. We have previously developed vascular muscular thin films (vMTFs) for measuring functional contractility of *in vitro* engineered cardiovascular tissues⁵, but this method has been limited to relatively short-term studies. Here, we present a substrate modification technique that expands our previous vMTF technique for long-term measurements.

While the endothelium is also critical in overall vascular function, engineered arterial lamellae provide a useful model system for assessing changes in vascular contractility during disease progression. To engineer a functional vascular disease tissue model, both the structure and function of the arterial lamella, the basic contractile unit of the vessel, must be recapitulated with high fidelity. Arterial lamellae are concentric, circumferentially-aligned sheets of contractile VSMCs separated by sheets of elastin⁶. Microcontact printing of extracellular matrix

(ECM) proteins onto polydimethylsiloxane (PDMS) substrates has been previously used to provide guidance cues for tissue organization to mimic aligned cardiovascular tissue^{5,7-10}. However, tissues patterned using microcontact printing can lose integrity after 3-4 days in culture, limiting their applicability in chronic studies. This protocol provides a solution to this issue by replacing previous microcontact printing techniques with a new microfluidic deposition technique.

Genchi et al. modified PDMS substrates with genipin and found prolonged viability of myocytes up to one month in culture¹¹. Here, we use a similar approach to extend culture of patterned vascular smooth muscle cells on PDMS. Genipin, a natural hydrolytic derivative of the gardenia fruit, is 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^{12,13} and ECM modification^{14,15}. In this protocol, fibronectin is utilized as a cell guidance cue, as in previous microcontact printing methods; however, genipin is deposited onto PDMS substrates prior to fibronectin patterning. Thus, as cells degrade the patterned matrix, newly synthesized ECM from attached VSMCs can bind to the genipin-coated PDMS substrate.

This protocol utilizes a microfluidic delivery device for two-step genipin and ECM deposition. The design of the microfluidic device mimics microcontact printing patterns used for engineered arterial lamellae in previous studies¹⁶. Thus, we expect this protocol to yield arterial lamellae mimics that successfully recapitulate the highly-aligned *in vivo* structure and contractile function of arterial lamellae. We also evaluate tissue contractility to confirm that genipin is a suitable substrate modification compound for long-term *in vitro* vascular disease models.

PROTOCOL:

Note: The goal of this protocol is to construct and utilize a vascular muscular thin film (vMTF) with the structure shown in **Figure 1** to assess contractility during extended culture of vascular smooth muscle cells (VSMCs) on PDMS substrates. To prolong VSMC viability, we utilize the crosslinker compound genipin. The substrates for these vMTFs are designed to analyze tissue contractility as developed by Grosberg et al.⁸ Other vMTF methods⁵ may also be used, with subtle changes to the presented substrate fabrication protocol.

1) Substrate Fabrication

1.1) Coverslip Cleaning

1.1.1) Place 25 mm diameter glass coverslips into a coverslip staining rack. Place the rack into a large beaker or container (e.g. an empty 100-1000 μ L pipet tip container).

1.1.2) Add 70% ethanol to the container to fully immerse the coverslips. Sonicate them for at least 30 min.

1.1.3) Remove the coverslip rack from the ethanol solution. Allow the coverslips to air dry by hanging the rack in a sterile culture hood (to prevent particle accumulation on coverslips) for 1-2 h.

Note: The coverslips must be completely dry prior to the following steps.

1.2) Poly(N-iso-propylacrylamide) (PIPAAm) Strip Isolation on Coverslip

1.2.1) Using adhesive tape, tape off the sides of a cleaned coverslip, leaving an exposed strip centered on the coverslip (**Figure 1A**). Modify the width of this exposed strip based on application and/or microfluidic design.

1.2.2) Mark the edges of tape strips on the coverslip using a lab marker for later reference (**Figure 1A**).

1.2.3) Cut around the perimeter of the coverslip to release from the dish (**Figure 1A**, dotted red line).

1.3) Poly(N-iso-propylacrylamide) (PIPAAm) Coating

1.3.1) Using an analytical balance, weigh 1 g of PIPAAm powder. Do not use unpolymerized N-iso-propylacrylamide, which is a carcinogen.

1.3.2) Transfer the PIPAAm to a 50 mL centrifuge tube. Add 10 mL of 1-butanol inside a chemical hood to yield a 10% w/v solution. CAUTION: The flash point of 1-butanol is 37 °C. Store the resulting solution in a flammable cabinet and avoid heating.

1.3.3) Allow the PIPAAm to dissolve for 10 min. If powder is still visible, mix the solution using a vortex-mixer until all powder dissolves.

Note: The following steps require the use of a spin coater. For each coverslip:

1.3.4) Place the taped coverslip on the spin coater chuck with forceps.

1.3.5) Transfer 150 µL of PIPAAm solution onto the coverslip by placing droplets along the exposed glass in the center of the coverslip. Ensure complete coverage of the exposed area.

1.3.6) Spin coat PIPAAm using the following recipe:

1.3.6.1) Ramp 10 s to 3000 RPM. Dwell for 5 s.

1.3.6.2) Ramp 10 s to 6000 RPM. Dwell for 60 s.

1.3.6.3) Ramp 10 s to 3000 RPM. Dwell for 5 s.

1.3.7) Place the coverslip into a covered Petri dish with the PIPAAm facing up. Allow to air dry for at least 15 min.

1.3.8) Carefully remove the adhesive tape from all coverslips, leaving the full coverslip exposed with a thin layer of PIPAAm coating in a center strip.

1.4) PDMS Coating

1.4.1) Mix and degas 15 g of PDMS in a 10:1 base:crosslinker ratio. Add 7-8 drops of sonicated 0.2 μm fluorescent microbeads prior to mixing. Cover the cup of PDMS with aluminum foil when not in use to prevent dust and other particles from contaminating the PDMS.

Note: The following steps require the use of a spin coater. For each coverslip:

1.4.2) Place a PIPAAm-coated coverslip on the spin coater chuck with forceps.

1.4.3) Transfer PDMS onto the coverslip, covering at least one-third of the coverslip area.

1.4.4) Spin coat using the following recipe:

1.4.4.1) Ramp 5 s to 500 RPM. Dwell 5 s.

1.4.4.2) Ramp 5 s to 1000 RPM. Dwell 5 s.

1.4.4.3) Ramp 10 s to 3000 RPM. Dwell 10 s.

1.4.4.4) Ramp 10 s to 4000 RPM. Dwell 60 s.

1.4.4.5) Ramp 10 s to 2000 RPM. Dwell 15 s.

1.4.4.6) Ramp 10 s to 1000 RPM. Dwell 10 s.

1.4.4.7) Ramp 5 s to 500 RPM. Dwell 5 s.

1.4.5) Place the coverslip into a covered Petri dish with the PDMS facing up. Record the time when the coverslip was spin-coated. Keep track of the time associated with each coverslip throughout the experiment for later use in determining the PDMS substrate thickness.

1.4.6) Place the Petri dish containing the coverslips in a 90 °C oven for at least 1.5 hr to ensure proper PDMS curing. If an oven is not available, let the coverslips cure for at least 48 hr at room temperature.

1.4.7) Remove the coverslips from the oven and store them in a dark drawer until ready to use.

1.4.8) Set aside every fourth coverslip for later measurement of substrate thickness, as a function of spin coating time, with a profilometer.

2) Microfluidic Patterning for Engineering Tissues

2.1) Fabrication of Microfluidic Devices

2.1.1) Design of Tissue Microfluidic Photomask

2.1.1.1) Use any appropriate computer-aided design program to design microfluidic patterns. For arterial lamellae composed of human umbilical cord artery vascular smooth muscle cells, use an alternating pattern of 10 μm channels with 10 μm walls.

2.1.1.2) Use binary channel branching if possible¹⁷, but other branching designs may be used. Decrease the width and length of channels for each branching iteration until attaining the desired tissue pattern spacing (walls and channels, **Figure 1B**).

2.1.1.3) Design the device to have a single inlet for surface treatment solution placement and a single outlet for application of vacuum.

2.1.1.4) Fabricate a photomask containing the microfluidic design(s), as previously described¹⁸.

2.1.2) Photolithographic Wafer Fabrication

Note: Perform photolithography in a suitable cleanroom or similar facility. To make silicon wafers with patterns for soft lithographic fabrication of tissue microfluidic devices (~20-25 μm channel height) using photolithography:

2.1.2.1) Clean a silicon wafer in acetone, methanol, and isopropyl alcohol for 1 min each. Dry the wafer with a nitrogen gun.

2.1.2.2) Prebake the wafer on a hot plate for 5 min at 115 °C to remove excess moisture.

2.1.2.3) Spin coat the wafer with SU-8 3025 photoresist using the following recipe to yield features 20-25 μm in height:

2.1.2.3.1) Ramp 5 s to 500 RPM. Dwell 5 s.

2.1.2.3.2) Ramp 15 s to 4000. Dwell 15 s.

- 2.1.2.4) Soft bake the wafer on a hot plate at 95 °C for 15 min.
- 2.1.2.5) Load a photomask, and expose the wafer for 16 s using a vacuum contact program on a contact mask aligner.
- 2.1.2.6) Hard bake the wafer on a hot plate at 95 °C for 4 min.
- 2.1.2.7) Develop the wafer for 6 min in developer. Then, wash the wafer twice for 2 s in fresh developer and rinse the wafer with isopropyl alcohol.
- 2.1.2.8) Silanate the patterned wafer overnight by placing 2-3 drops of tridecafluoro-trichlorosilane in an empty dish within a vacuum desiccator. Prop the wafer up using Petri dishes so that both the bottom and top of the wafer are exposed.

CAUTION: Tridecafluoro-trichlorosilane is a flammable and corrosive liquid. Proper personal protective equipment and local exhaust is necessary for use.

2.1.3) Tissue Microfluidic Device Fabrication

- 2.1.3.1) Place a silanated, patterned wafer feature-side up in a Petri dish.
- 2.1.3.2) Mix and degas 100 g of PDMS with a 10:1 base:crosslinker ratio. Pour the PDMS into the dish, completely and evenly covering the wafer.
- 2.1.3.3) Place the dish in a vacuum desiccator until all air bubbles are removed from the uncured PDMS, approximately 30 min. Cure the PDMS in the dish at 90 °C for at least 1.5 h. Time and temperature can be adjusted as dictated by manufacture guidelines to obtain a complete cure.
- 2.1.3.4) Once the PDMS has cured, cut out the PDMS around the wafer with a razor blade and carefully release the PDMS-covered wafer from the dish. Remove excess PDMS underneath wafer and slowly peel PDMS away from the top of wafer.
- 2.1.3.5) Place the PDMS disk feature-side up in a clean dish and store the wafer away from light after use.
- 2.1.3.6) Cut away the excess PDMS from around the patterns using a razor blade. Cut devices into rectangular shapes (**Figure 1C**) to ease peeling of device from substrates in later steps. Precise cuts are not necessary, as long as ample space exists for the inlet, outlet, and tissue pattern area (**Figure 1C**).
- 2.1.3.7) Punch inlet and outlet holes (**Figure 1C**) using a 1 mm surgical biopsy punch.

2.2) Microfluidic Device Deposition

Note: In this protocol, microfluidic delivery is used to deposit patterned genipin, the key crosslinking agent for long-term tissue culturing, as well as fibronectin. Steps prior to penicillin/streptomycin sterilization (2.2.3) do not need to take place in sterile conditions, but limiting contamination and dust collection is encouraged throughout the protocol. All steps occurring after coverslip sterilization with penicillin/streptomycin (2.2.3) should utilize sterile technique. Note: This portion of the protocol must be started one day prior to cell seeding.

2.2.1) Substrate and Microfluidic Device Preparation

2.2.1.1) Sonicate the microfluidic devices in 70% ethanol for at least 30 min.

2.2.1.2) Dry the sonicated microfluidic devices using pressurized air or nitrogen, and place them in a Petri dish with channel features face-up to prevent unnecessary wear on the features.

2.2.1.3) Place up to 10 vMTF substrate coverslips in a UVO cleaner (cover removed on dish so the surface is functionalized) for 8 min.

2.2.1.4) Remove the UVO-treated coverslips, and place the microfluidic devices feature-side down onto each slip one at a time (orientation should be similar to **Figure 1C**). Press down firmly on the devices to ensure a tight seal to the PDMS-coated coverslips.

2.2.2) Deposition of Genipin and Fibronectin

2.2.2.1) Prepare a 5 mg/mL genipin solution by adding 1 mL of sterile ddH₂O to a 5 mg container of lyophilized genipin. Mix the solution using a vortex-mixer. Set aside at room temperature for at least 30 min.

Note: The powder is difficult to solubilize at room temperature, so repetitive mixing for at least a minute is often necessary.

2.2.2.2) Quickly, place a drop of 70% ethanol at the inlet of each device for device priming. Note: The ethanol should wick through the devices.

2.2.2.3) After 5-10 min, carefully aspirate the excess ethanol at the inlet, immediately replacing it with 1X phosphate buffered saline (PBS) at the inlet. **From this point forward, be sure to not allow inlet to become completely dry to avoid introduction of air to the device.**

2.2.2.4) Place a vacuum aspirator tip at the outlet of each device. Draw 1X PBS through devices to rinse ethanol away. Leave a small amount of 1X PBS at the inlet. Note: If inlet appears nearly dry, add more 1X PBS.

2.2.2.5) Aspirate excess 1X PBS so that only a small amount remains at the inlet prior to application of genipin solution.

2.2.2.6) Place 60 μL of the 5 mg/mL genipin solution at each inlet (**Figure 1D**). Draw the genipin solution through the devices by placing a vacuum aspirator tip at the outlet (**Figure 1D**). Be sure not to draw all of the solution through, leaving a small amount of solution at the inlet.

2.2.2.7) Place drops (approximately dime-sized) of 1X PBS at both the inlet and outlet to maintain wetting during incubation. Move the dish containing devices to a humidified oven or incubator (sterile environment is not necessary) set to 37 °C, and incubate for 4 h. The dish does not need to be covered.

2.2.2.8) During incubation, resuspend fibronectin to a concentration of 50 $\mu\text{g}/\text{mL}$ in sterile ddH₂O on ice for at least 30 min prior to application to microfluidic device.

2.2.2.9) After the incubation of genipin, aspirate all remaining 1X PBS at the device outlets. Continue to apply a vacuum aspirator at each device outlet, pulling through the remaining 1X PBS at the inlet.

2.2.2.10) Place 100 μL of 50 $\mu\text{g}/\text{mL}$ fibronectin solution at each inlet, adding to a minimal amount of remaining 1X PBS at inlet (**Figure 1D**).

2.2.2.11) Draw the fibronectin solution through the devices using a vacuum aspirator tip at the outlet (**Figure 1D**). Be sure to not draw all of the solution through. Note: Fibronectin drop will look different than the 1X PBS and genipin due to differences in surface tension.

2.2.2.12) Move the uncovered dish containing the devices to an oven or incubator set to 37 °C, and incubate for 24 h. Note: The fibronectin step does not require wetting of the inlet and outlet with 1X PBS. The remaining pool of fibronectin at the inlet will dry out. This is expected.

2.2.3) Sterilization and Preparation for Cell Seeding

2.2.3.1) Prepare a solution of penicillin/streptomycin for sterilization of patterned vMTF coverslips. Add 5 mL of penicillin/streptomycin (10,000 units/mL; 10,000 $\mu\text{g}/\text{mL}$) to 500 mL of sterile 1X PBS.

2.2.3.2) Place the dish containing the devices in a sterile biosafety hood.

2.2.3.3) Carefully remove the devices from the coverslips by slowly peeling the device at a corner, while lightly grasping the coverslip in opposite hand. Note: This step requires practice to reduce coverslip damage in the removal process. An alternative is using a syringe to inject 1X PBS at the inlet and/or outlet to aid in device release.

2.2.3.4) Place the coverslips in sterile six-well dishes. Add at least 5 mL of penicillin/streptomycin solution to each well. Place the dishes in a sterile incubator at 37 °C for at least 30 min.

2.2.3.5) After sterilization, aspirate the penicillin/streptomycin solution, and seed the coverslips with cultured human umbilical artery vascular smooth muscle cells¹⁹ (**Figure 1D**). The seeding concentration for VSMCs is ~80,000 cells per cm². To reduce the number of cells needed for each sample, use a reducer to decrease the seeding area. 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.

2.2.3.6) Incubate the seeded coverslips in a sterile incubator at 37 °C and 5% CO₂ and allow the seeded cells to attach and form aligned arterial lamellae mimics overnight (**Figure 2A-B**).

2.2.4) Long-term vMTF Tissue Culture

2.2.4.1) One day after seeding, remove the cell medium and reducers. Rinse the tissues with 1X PBS. Add 4 mL of serum-free cell medium to induce a contractile phenotype in the VSMCs²⁰.

2.2.4.2) Repeat the 1X PBS rinse and addition of fresh serum-free medium every other day as desired for long-term culture.

[Place Figure 1 here]

3. Tissue Function Analysis with vMTF Contractility Assay

Note: The MTF contractility assay presented here is modeled after the technique developed in Grosberg *et al.*⁸

3.1) vMTF Contractility Experiment

3.1.1) Place a tissue sample in a 100 mm dish. Add sterile 1X Tyrode's solution⁸ at pH 7.4 warmed to 37 °C to cover the sample.

3.1.2) Viewing through a stereomicroscope, use a razor blade to make two straight, parallel cuts in the middle of the tissue, parallel to the strip of PIPAAm (**Figure 3A, end cuts**). To make clean cuts, place a razor blade in contact with sample and firmly drag to the side. Remove and dispose of the loose strip of tissue between the cuts.

3.1.3) Rotate the dish 90° and make several parallel cuts perpendicular to the PIPAAm edge. Make cuts in a manner that yields wider tissue sections that will be the vMTFs (with width of ~2 mm) alternating with thin strips (**Figure 3A, side cuts**). Remove the thin strips in between

vMTFs to prevent adjacent films from making contact.

3.1.4) Allow the sample to rest at room temperature for 10 min, or until all the PIPAAm has dissolved. Note: If PIPAAm remains in future steps, sample may be returned to the cutting dish to dissolve residual PIPAAm. A gentle scraping of the underside of vMTF can aid in PIPAAm removal, as needed.

3.1.5) Place a small dot of vacuum grease in a clean 35-mm Petri dish. Add 5 mL of fresh, sterile 1X Tyrode's solution at 37 °C. Transfer the coverslip with cut films from the 100 mm dish to the 35 mm dish, and press onto vacuum grease to prevent movement of the coverslip.

3.1.6) Place the dish in a temperature-controlled platform on the stereomicroscope stage.

3.1.7) Capture time-lapse transmitted and fluorescent light images at desired intervals (e.g. 30 s) throughout treatment assay.

3.1.8) Serially treat vMTFs with 50 nM endothelin-1 for 20 min (induced contraction) and 100 μ M HA-1077 for 30 min (tissue relaxation). 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 acquisitions to avoid capturing pipette in images.

3.2) vMTF Contractility Analysis

3.2.1) Using coverslips set aside in 1.4.8, measure PDMS substrate thickness with a profilometer²¹. Create a thickness vs. spin time curve for each set of coverslips. Use this curve to estimate the vMTF thickness for each coverslip used in a contractility experiment.

3.2.2) Measure the vMTF projection lengths for each time point during the experiment, and calculate the associated radii of curvature (**Figure 3B**) using previously reported methods⁸.

3.2.3) Calculate vMTF stress at each time point using previous vMTF methods⁵.

Note: Use the estimated vMTF thickness calculated from 3.2.1. Measure VSMC thickness using confocal images, as previously reported⁹. Obtain PDMS Young's modulus from company data sheets.

REPRESENTATIVE RESULTS:

The primary goal of this work was to extend the viability of micropatterned VSMCs on hydrophobic PDMS substrates. This was accomplished by incorporating a microfluidic delivery system to deposit patterned genipin and fibronectin on PDMS (**Figure 1**). Deposition of ECM proteins using microfluidic delivery yielded high fidelity transfer of the channel pattern with bare PDMS between lines of genipin and fibronectin (**Figure 1D**). The attached cells (**Figure 1E**) form confluent monolayers mimicking the *in vivo* structure of arterial lamellae (**Figure 2**),

similar to previous microcontact printing methods^{5,10,16}. These tissues yielded responsive, contractile constructs, whose stress was measured using vMTF technology (**Figure 3**).

Qualitative assessment of tissue viability over the course of two weeks showed minimal deterioration on genipin-modified substrates (**Figure 2A**). Tissue confluence and alignment were maintained over two weeks (**Figure 2B, 2C and 2D**). Two key stress values were calculated for every vMTF: 1) basal tone and 2) induced contractility (**Figure 3C and 3D**). Basal tone is the stress maintained by unstimulated VSMCs at equilibrium. Induced contractility is the additional stress induced by stimulation with endothelin-1. Both basal tone and induced contractility showed consistent behavior over the two-week time course, demonstrating vasoactive tissues throughout (**Figure 3E and 3F**). 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 proliferate¹⁹. The addition of a minimal basal level of serum to culture medium may alleviate this result in future work.

Figure 1: Microfluidic protein delivery device. A. Taped off coverslip for PIPAAm coating. Red dotted circle: cutting path to release coverslip. B. Representative AutoCAD drawing of tissue microfluidic mask pattern. Inset: Detail of binary branching to alternating 10 μm x 10 μm tissue pattern. C. Placement of microfluidic device on a coverslip substrate with inlet and outlet indicated. D. Schematic of microfluidic protein patterning and delivery. Left-to-right: scanning electron microscope image of microfluidic channels (scale bar: 50 μm); Detailed schematic of method for protein deposition; Immunohistochemistry stained fibronectin (scale bar: 50 μm); Cell seeding with vascular smooth muscle cells. E. Schematic of fabricated tissue. 1st inset: Detail of layered construct. 2nd inset: Detail of genipin modification of PDMS substrate after microfluidic deposition. © IOP Publishing. Reproduced and/or modified with permission. All rights reserved.¹⁹

Figure 2: Tissues remain viable and successfully mimic *in vivo* arterial lamellar structure for two weeks on genipin-modified substrates. A. Representative phase contrast images of tissues at sacrifice time points throughout the course of two weeks (scale bar: 200 μm). B. Representative immunohistochemistry images of tissues fabricated on genipin-modified substrates fixed at Day 1, Day 4, and Day 10 after serum starvation (green: f-actin filaments, blue: nuclei (shown to establish presence of cells), scale bar: 100 μm). C. Percent confluence measured by f-actin coverage (error bars: standard error, n=3-7). D. Tissue alignment measured by f-actin orientation order parameter (OOP)²² (error bars: standard error, n=3-7). © IOP Publishing. Reproduced and/or modified with permission. All rights reserved. All rights reserved.¹⁹

Figure 3: Tissue contractility is maintained over the course of two weeks. A. Representative vMTF cutting scheme. B. Schematic of cut vMTF. Upon cooling below 32 °C, PIPAAm dissolves, releasing vMTF. Stress in the active cell layer causes the passive PDMS layer to bend. Measurement of projection length can be converted to a radius of curvature according to methods in Grosberg et al.⁸ Radius of curvature is used to calculate the average cross-sectional stress in the tissue. C. Sequential transmitted light images of representative contractility assay

(scale bar: 1 mm). Tissues reach equilibrium, are stimulated with endothelin-1, then treated with HA-1077 to allow complete relaxation. Bottom: Schematic of side view of idealized tissue during the course of the assay. D. Representative stress curve for the contractility assay. Two key stress values are calculated. Basal tone is the difference between the equilibrium stress state and the relaxed stress state. Induced contractility is the change in stress from the equilibrium state to the endothelin-1 stimulated state. E. Basal tone (error bars: standard error, n=5-12). F. Induced contractility (error bars: standard error, n=5-12). © IOP Publishing. Reproduced and/or modified with permission. All rights reserved.¹⁹

DISCUSSION:

Here, we present a protocol that builds upon previously developed vMTF technology, allowing extended experiment times more typical of chronic vascular disease pathways^{1,23,24}. To accomplish this, we micropattern genipin, which has previously been shown to provide long-term functionalization of PDMS substrates¹¹, using a microfluidic deposition technique to yield engineered arterial lamellae with improved vascular tissue viability for use in MTF contractility experiments. McCain et al. developed an alternative micromolded gelatin hydrogel substrate for extended culture of engineered cardiac tissues for several weeks in a related MTF model²⁵.

This protocol yielded vMTFs that successfully mimicked arterial lamella structure (**Figure 2**) and function (**Figure 3**) over the course of two weeks. While successful completion of the presented protocol yields the desired result of extended culture times for disease-relevant time course (e.g. the pathologic effects of cerebral vasospasm persisting for up to 14 days²⁶), a few common pitfalls arise. Repeated use of PDMS microfluidic devices results in deleterious damage that can result in partial or complete blockage of branches in the device. Thus, new devices must be used for each experiment. Another issue, while not as common, is unexpected and inconsistent delamination of tissues. These occurrences seemed to be random in nature and rare in occurrence. By observation, tissues form an arch- or lumen-like structure before delaminating. We also observed this behavior in tissues fabricated using microcontact printing techniques. Thus, we believe this issue to be the result of either over-seeding or a phenotypic switch in the cultured VSMCs that results in abnormal behavior and not a direct result of the fabrication methods presented here.

The current microfluidic design requires linear flow patterns and as such, is limited to tissues with alignment in a single principal direction. Application to tissues requiring more complex patterning, such as the “brick wall” pattern of ventricular myocardial structure²⁷, will require more elaborate microfluidic design. We have not seeded genipin-modified substrates with other cell types. However, Genchi et al. showed extended viability of isotropic skeletal muscle on genipin-modified PDMS¹¹. Thus, we feel confident that a minimally-modified version of this protocol has widespread applicability to other organ systems in the future development of organ-on-a-chip technologies.

The stress calculation method for vMTFs based on previous methods⁸ is limited by the relative contractility in the tissues and corresponding cut length of the thin films. If cut too long, films will curl on themselves. If cut too short, films will not bend. Either case prevents proper analysis

of contractile properties due to model limitations. Proper cut length should be refined through repeated experiments. Implementation of a laser engraving system, as in Agarwal et al., can improve upon repeatability and quality of MTF cutting²⁸.

The ability to better recapitulate native tissue structure and function in a tightly-controlled, self-contained experimental system is a key pursuit in the field of bioengineering. This pursuit has led to the development of several *in vitro* tissue mimics and multi-functional, yet simplified model organs-on-a-chip, advancing the understanding of fundamental physiologic and pathologic behaviors of organ systems^{29,30}. Using deposition of genipin onto PDMS substrates, we have demonstrated extended cell viability and maintenance of vascular smooth muscle function over two weeks. This is a significant improvement over previous fabrication techniques, which can lead to delamination of tissues and cell death after 4-7 days in culture¹⁹, and may aid development of more robust artery-on-a-chip methods. Due to the chronic nature of most vascular diseases, this advance provides the framework for a variety of future investigations into the contractile mechanisms involved in specific vascular pathologies.

DISCLOSURES:

The authors have nothing to disclose.

ACKNOWLEDGMENTS:

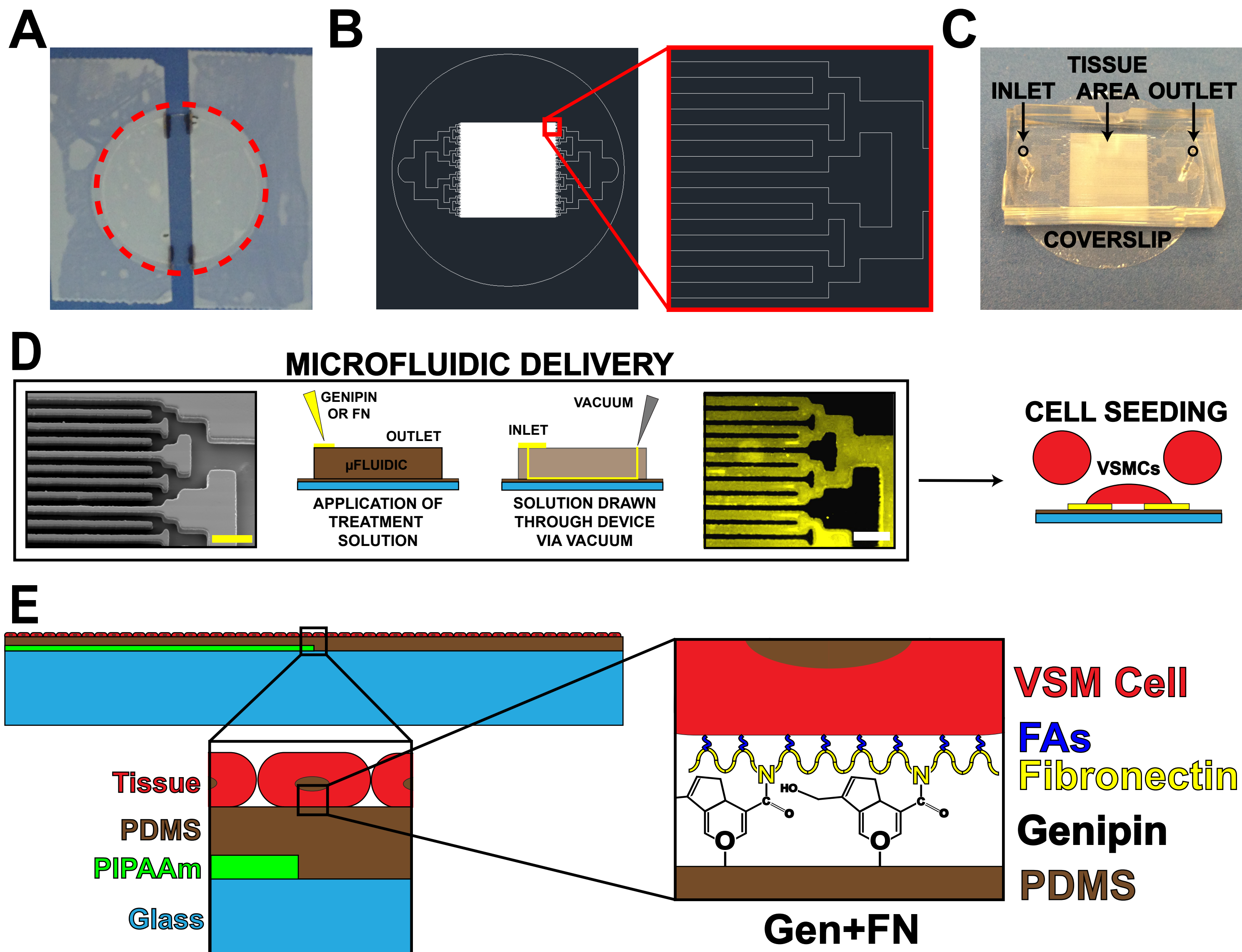
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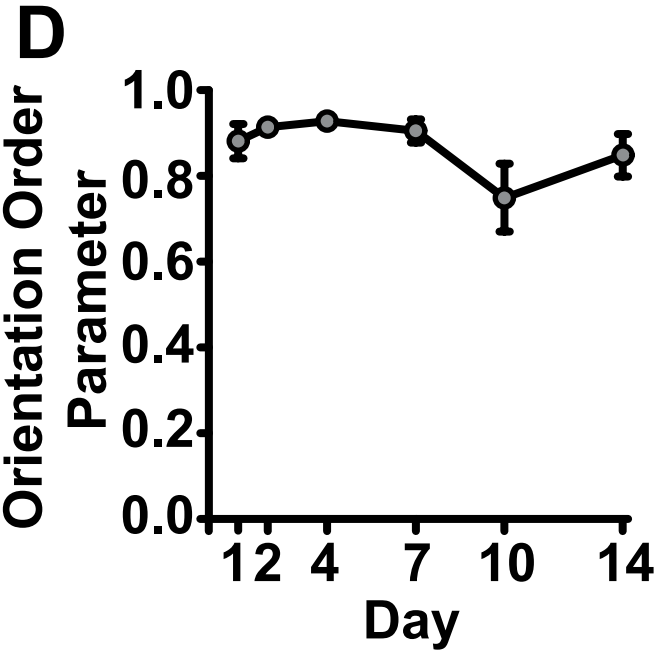
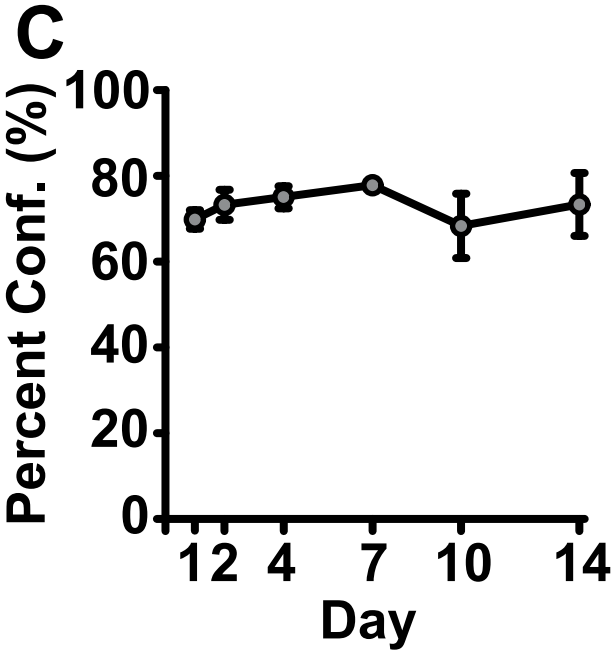
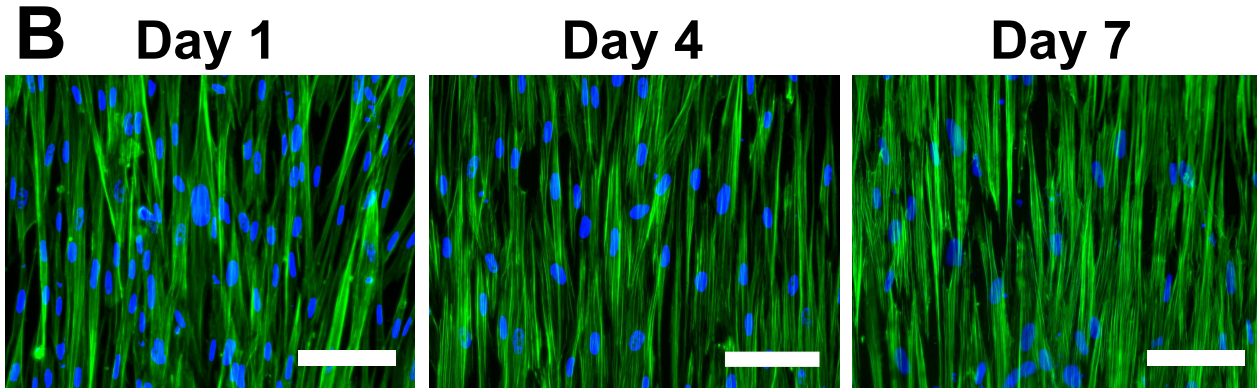
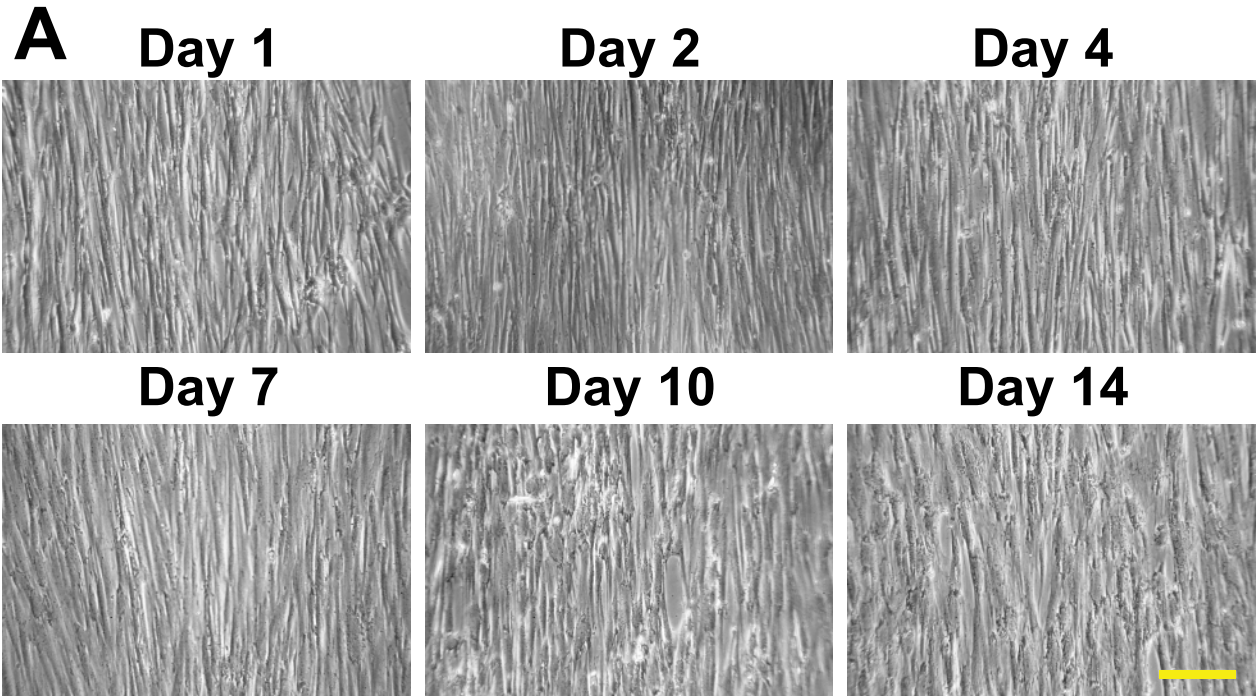
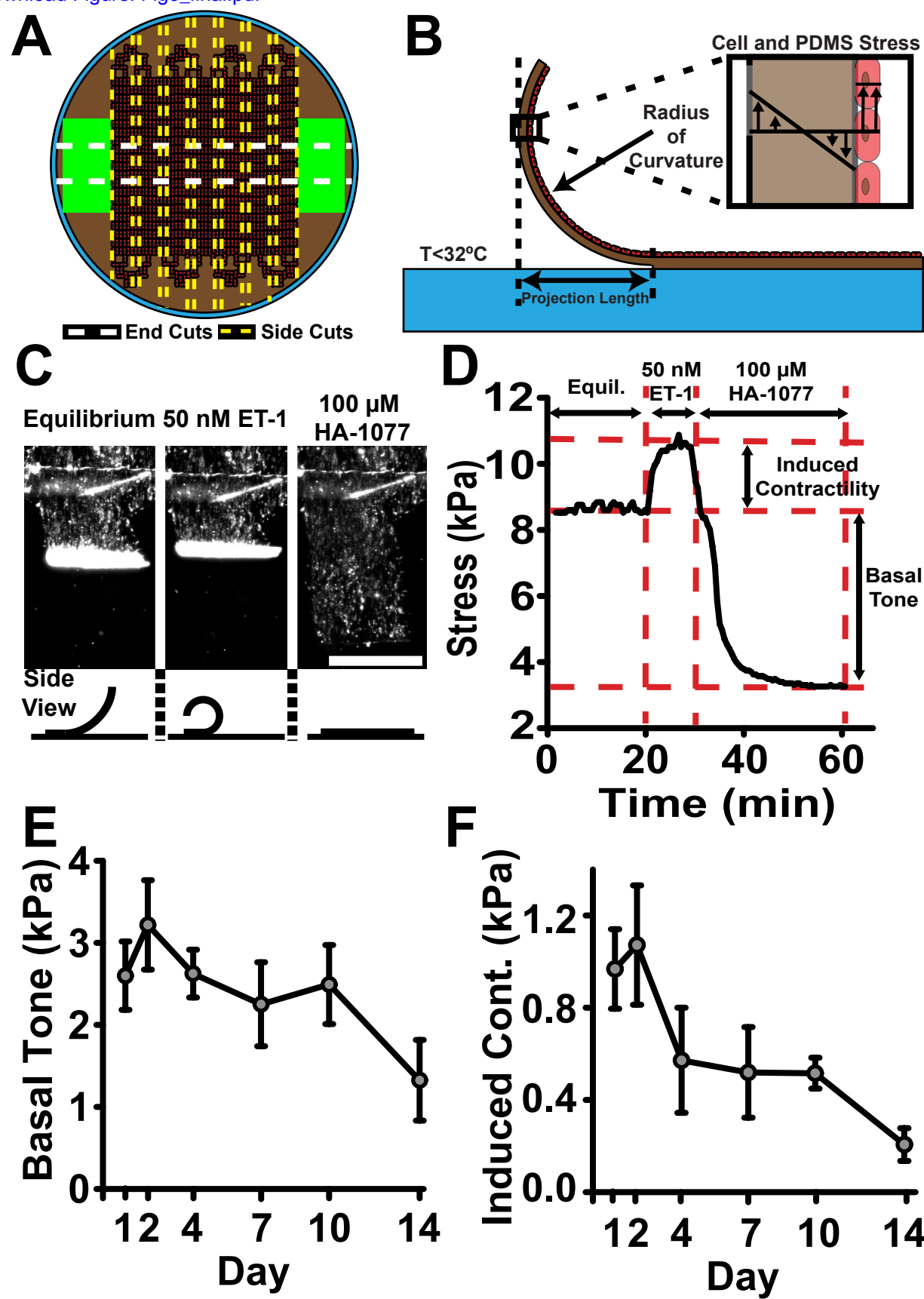


Figure 3
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Name of Material/ Equipment	Company	Website	Catalog Number
Coverslip staining rack	Electron Microscopy Sciences	www.emsdiasum.com/	72239-04
Microscope cover glass - 25 mm	Fisher Scientific, Inc.	www.fishersci.com	12-545-102
Poly(N-iso-propylacrylamide) (PIPAAm)	Polysciences, Inc.	www.polysciences.com/	#21458
1-butanol	Sigma-Aldrich	www.sigmaaldrich.com	360465
Spincoater	Specialty Coating Systems, Inc.	www.scscoatings.com	
	Ellsworth Adhesives (Dow		184 SIL ELAST KIT
Polydimethylsiloxane (PDMS)	Corning)	www.ellsworth.com	0.5KG
Fluorescent microbeads	Polysciences, Inc.	www.polysciences.com/	17151
Silicon wafers	Wafer World, Inc.	www.waferworld.com	2398
Photoresist	MicroChem Corp.	www.microchem.com	
Contact mask aligner	Suss MicroTec	www.suss.com	
Developer	MicroChem Corp.	www.microchem.com	
Tridecafluoro-trichlorosilane	UCT Specialties, Inc.	www.unitedchem.com	T2492
Surgical biopsy punch	Integra LifeSciences Corp.	www.miltex.com	33-31AA-P/25
Genipin	Cayman Chemical	www.caymanchem.com	10010622
1X phosphate buffered saline	Mediatech, Inc.	www.cellgro.com	21-031-CV
Fibronectin	Corning, Inc.	www.corning.com	356008
Penicillin/streptomycin	Life Technologies, Inc.	www.lifetechnologies.com	15140-122
Umbilical artery smooth muscle cells	Lonza	www.lonza.com	CC-2579
Tyrode's solution components	Sigma-Aldrich	www.sigmaaldrich.com	various 4350020000000000
Stereomicroscope	Zeiss	www.zeiss.com	0 641659; 640352; 641922
Temperature-controlled platform	Warner Instruments	www.warneronline.com	641922
Endothelin-1	Sigma-Aldrich	www.sigmaaldrich.com	E7764-50UG
HA-1077	Sigma-Aldrich	www.sigmaaldrich.com	H139-10MG

Comments/Description

Alternative coverslip rack may be used

Alternative brand and size may be used; Microscope slides may also be substituted as substrate base

Sigma-Aldrich makes an alternate compound, but we have not tested it for use with this protocol; Compound gives strong odor, use |
Hazard: flammable (store stock solution in flammable cabinet); flash point is 37 °C, avoid heating; alternative product may be used

SCS G3P8 Model; Alternative brand and/or model may be used

Alternative distributor may be used

Alternative brand and/or larger size may be used

Alternative brand and/or size may be used

SU-8 3025 allows 20-25-µm feature height

MA6 contact mask aligner; alternative brand and/or model may be used for wafer exposure

SU-8 Developer; Hazard: flammable

Silane for non-stick coating of patterned silicon wafers (CAUTION: Tridecafluoro-trichlorosilane is a flammable and corrosive liquid. Pre

Alternative brand and/or size may be used

Sigma-Aldrich (G4796-25MG) makes an alternate compound, but we have not tested it for use with this protocol

Alternative brand may be used

Sigma-Aldrich (F1056) makes an alternate compound, but we have not tested it for use with this protocol

Alternative brand and/or size may be used, as long as concentration is the same

Alternative cell types may be used for alternative applications. Media should be modified accordingly

Alternative brand may be used for mixing solution

SteREOLumar V12; Alternative brand/type of stereomicroscope may be used

Alternative amount may be purchased, as long as treatment concentration is maintained

Alternative amount may be purchased, as long as treatment concentration is maintained

proper ventilation

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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](#): 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](#): 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](#): 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 expand 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 μ m 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 tridecafluoro-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.