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TITLE:

Stepwise Cell Seeding on Tessellated Scaffolds to Study Sprouting Blood Vessels

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SUMMARY:

Engineered tissues heavily rely on proper vascular networks to provide vital nutrients and gases and remove metabolic waste. In this work, a stepwise seeding protocol of endothelial cells and support cells creates highly organized vascular networks in a high-throughput platform for studying developing vessel behavior in a controlled 3D environment.

ABSTRACT:

The cardiovascular system is a key player in human physiology, providing nourishment to most tissues in the body; vessels are present in different sizes, structures, phenotypes, and performance depending on each specific perfused tissue. The field of tissue engineering, which aims to repair or replace damaged or missing body tissues, relies on controlled angiogenesis to create a proper vascularization within the engineered tissues. Without a vascular system, thick engineered constructs cannot be sufficiently nourished, which may result in cell death, poor engraftment, and ultimately failure. Thus, understanding and controlling the behavior of engineered blood vessels is an outstanding challenge in the field. This work presents a high-throughput system that allows for the creation of organized and repeatable vessel networks for studying vessel behavior in a 3D scaffold environment. This two-step seeding protocol shows that vessels within the system react to the scaffold topography, presenting distinctive sprouting behaviors depending on the compartment geometry in which the vessels reside. The obtained results and understanding from this high throughput system can be applied in order to inform better 3D bioprinted scaffold construct designs, wherein fabrication of various 3D geometries cannot be rapidly assessed when using 3D printing as the basis for cellularized biological environments. Furthermore, the understanding from this high throughput system may be utilized for the improvement of rapid drug screening, the rapid development of co-cultures models, and

the investigation of mechanical stimuli on blood vessel formation to deepen the knowledge of the vascular system.

INTRODUCTION:

The field of tissue engineering is rapidly progressing towards the fabrication of engineered constructs to replace missing or damaged organs and tissues[1]. However, fully functional constructs have yet to be achieved, in part, since generating operational vascular networks for tissue nourishment remains an outstanding challenge. Without proper vascularization, engineered tissues are limited to a passive diffusion transport of oxygen and nutrients, constraining the maximum viable tissue thickness to the diffusion limit, approximately 200 μm^2. Such thicknesses are not suitable to repair large tissue defects or for full organ fabrication, which renders the presence of functional vascular network a mandatory characteristic for functional and implantable tissues[3].

The vascular system is comprised of a wide variety of blood vessels, with different sizes, phenotypes, and organization, tightly related to the host tissue. Understanding the behavior, response and migration decisions made by the developing and sprouting vessels can instruct their integration in engineered tissues[4]. Currently, the most common approach for creating in vitro vascular networks is combining endothelial cells (ECs) with support cells (SCs, with the capability to differentiate into mural cells), seeded within a three-dimensional micro-environment. This environment provides chemical and physical cues to allow the cells to attach, proliferate and self-assemble into vessel networks[2,5–8]. When co-cultured, SCs secrete extracellular matrix (ECM) proteins while providing mechanical support to the ECs, which form the tubular structures. Furthermore, a cross-interaction between both cell types promote tubulogenesis, vessel sprouting and migration, in addition to the SCs maturation and differentiation into α-smooth muscle actin-expressing (αSMA) mural cells[4]. Vessel network development is most commonly studied in 3D environments created using hydrogels, porous polymeric scaffolds, or a combination thereof. The latter option equally provides a cell-friendly environment and the required mechanical support for both the cells and the ECM[9].

A great amount of work has been carried out to study vascular development, including co-culturing the cells on hydrogels[10], hydrogels-scaffold combinations[11,12], 2D platforms, and microfluidic devices[13]. However, hydrogels can be easily deformed by the cell-exerted forces[14], while 2D and microfluidics systems fail to recreate a closer-to-nature environment to obtain a more extrapolatable response[15,16]. Understanding how forming vessels react to their surrounding environment can provide critical insight that might allow for the fabrication of engineered environments with the capability of guiding the vessel development in a predictable manner. Understanding vascular formation phenomena is especially critical to keep pace with the rapid emergence of submicron-to-micron scale fabrication techniques, such as stereolithography, digital projection lithography, continuous liquid interface production, 3D melt-electro jetwriting, solution based 3D electro jet writing, and emerging bioprinting techniques[17–21]. Aligning the control of these micromanufacturing techniques with a deepened understanding of vascular biology is key to the creation of an appropriate engineered vasculature for a target tissue.

Here, we present a 3D system to study the response of new forming and sprouting vessels to the surrounding scaffold geometry, observing their sprout origin and subsequent migration[22]. By utilizing 3D scaffolds with tessellated compartment geometries, and a two-step seeding technique, we succeeded to create highly organized vascular networks in a clear and easy to analyze fashion. The tessellated geometries provide a high throughput system with individual units containing vessels that respond to their local environment. Using multicolored ECs, we tracked sprout formation origins and subsequent migration patterns, correlated to the compartment geometry and the SCs location[22].

Although the proposed protocol has been prepared to analyze the effects of geometrical cues on vascularization behavior, this approach can be expanded and applied to a variety of new applications. The tessellated scaffold and the easily imageable networks allow for the straightforward analysis of different ECs and SCs interaction, the addition of specific organ cells and their interaction with the vascular networks, drug effect on vascular networks, and more. Our suggested system results very versatile and of simple fabrication and processing.

PROTOCOL:

1. Tessellated scaffold fabrication

NOTE: Photolithography is a widespread technique with many resources accessible to researchers. It requires specialized equipment typically housed within a nanofabrication facility/laboratory. The method laid out in this protocol was generalized as much as possible for the audience; however, slight changes to procedures may be necessary depending on equipment available to the reader. We recommend performing these procedures in a clean room at a nanofabrication facility (or with an experienced laboratory) to ensure the highest process quality. If photolithography has never been performed before, it would be good to seek advice from an experienced group. Before beginning, obtain access to a mask-aligner (or some UV-exposure set-up), a spin coater, hot plates, a solvent washing station, a photomask, and potentially a plasma cleaner. The solvents and chemicals used in procedure are hazardous, so please take the upmost care to avoid any chemical exposure. When designing the photomask, identify what sized silicon wafers and photomasks are compatible with the spin-coater and mask-aligner. Additionally, the photoresist located toward edges of the silicon wafer is typically deformed from handling; hence, make the designs toward the center area of the wafer.

1.1. Prepare scaffolds using a photolithography technique with the selected geometry of interest.

1.1.1. Before spin coating, clean the silicon wafer. This can be done with plasma cleaning or a solvent cleaning technique. If plasma cleaning, follow the standard operating procedure of the instrument for operational details. In either case, ensure the wafer is free of defects/contamination. Spray with compressed nitrogen gas and inspect the wafer to ensure it is free of debris before spin-coating. This wafer will serve as the substrate in which the scaffolds are created.

NOTE: For best results start with a fresh wafer. Wafers can be re-used but should be cleaned and free of old photoresist, surface defects and debris. Be sure to never touch the surface of the wafer, except toward the outer edge where you do not have any design features. It is helpful to use wafer-tweezers when handling. Silicon wafers vary in size, and what size wafer used will be depend on the accessible equipment.

1.1.2. Center a silicon wafer onto the spin-coater chuck using a guide. Briefly spin the wafer to ensure it has been properly centered on the chuck, adjust as necessary until properly centered. This must be done every time a wafer is placed on the spin-coater. Dispense 1-4 mL of the lift-off reagent onto the wafer (this will depend on the size of the wafer). Approximately 2 mL of lift-off reagent works well for a 4-inch wafer.

1.1.2.1. Set spin-coater spread speed at 500 rpm for 5 seconds, and the spin-speed to 1000 rpm for 30 seconds, then spin the lift-off reagent. Inspect the wafer to ensure it has an even coating across the wafer. Any debris left on the wafer will be very obvious at this point. Any scaffolds in that area will likely be unusable. After spin coating, transfer to a hot plate and bake for 1 minute at 200 °C.

1.1.3. Repeat the previous step two more times for a total of three coatings.

1.1.4. Spin-coat the Omnicoat-coated silicon wafer with SU-8 2050 photoresist until obtaining a thickness of approximately 100 μm.

1.1.4.1. Dispense 1 mL of resist for every 25 mm of substrate diameter.

NOTE: Be careful to avoid bubbles while pouring the resist. For SU-8 2050, pouring approximately a 2-inch diameter circle, when using a 4-inch silicon wafer works well.

1.1.4.2. Spin-coat the SU-8 2050. Spread at a speed of 500 rpm for 5 seconds, followed by a spin speed between 1700 to 1800 rpm to achieve approximately 100 μm thickness.

1.1.4.3. **Optional**: After spinning, leave the spin coated wafers to de-gas overnight on a level surface protected from light prior to the pre-bake. This may help to rid the resist of any bubbles and allow defects to level out.

NOTE: The actual thickness of the resist and the resultant scaffolds can vary with user error and equipment parameters. Therefore, the thickness of the scaffolds should be verified later, in step 1.6. Modify spin coating procedures accordingly to achieve the desired thickness.

1.1.5. Prior to exposure, pre-bake the wafers at 65 °C for 10 min and then 95 °C for 40-50 min. Prior to exposure, test the surface of the resist by pressing on the edge with a pair of tweezers to ensure that it is no longer tacky/viscous. It is helpful to pull the wafer off of the hot plate and allow it to cool for a 0.5-1 min before assessing the tackiness.

NOTE: Slow temperature ramp times (heating and cooling) may help to prevent warping of the scaffolds.

1.2. Expose the photoresist to UV light (350-400 nm) through a photomask with an exposure energy of 215-240 mJ/cm^2 for a resist thickness of 85 – 110 µm. Refer to photoresist manufacturer guidelines for recommended exposure energy-thickness correlations.

1.2.1. Ensure that the exposure is properly calibrated such that the energy is verified. Adjust the exposure time as necessary to achieve desired exposure energy. Furthermore, mask aligners may allow for different exposure modes: vacuum contact, hard contact, soft contact, and proximity contact (specific terms may vary). Generally, these will impact resolution and feature alignment. The authors typically used a "hard contact" mode; however, with the large feature sizes (>30 µm), it is unlikely to significantly impact the results.

1.2.2. For small features or multiple layers, where alignment will matter, consider exposure modes and mask aligner procedures more carefully. Be sure to consider the height of the resist when adjusting the thickness value. Refer to the standard operating procedures of your mask-aligner for operation details.

NOTE: The design of the photomask will determine the scaffolds size, compartment geometry, and number of scaffolds obtained per batch. The use of a hard glass or quartz photomask will yield the highest resolution; however, a soft polymer transparent film photomask generally can be used for these large feature sizes (>10 µm). The use of a film-photomask may lead to topographical features along the z-axis of the scaffold pores as a result of poorer feature resolution. This could potentially have an influence on cell behavior. Verify the desired resolution and consult with whomever is producing the photomask before deciding because this will change depending on how the photomasks are produced.

1.3. Bake the wafer immediately after UV exposure at 65 °C for 2-5 minutes and then at 95 °C for 8-10 minutes.

1.4. Develop the scaffolds to remove undeveloped resist.

1.4.1. Immerse the scaffolds using a low volume of SU-8 developer solution for 7-10 minutes to dissolve any undeveloped resist.

1.4.2. Rinse with isopropyl alcohol (IPA).

1.4.3. Dry wafer with compressed nitrogen.

NOTE: Be careful during step 1.4 to not cause premature release of the scaffolds. Low volumes of developer and gentle handling are necessary to achieve this.

1.5. Following development, hard bake the wafers at 150 °C for 15 min.

NOTE: After hard bake, turning off the hotplate to allow the wafers to cool very slowly may be advantageous in preventing warping/curling of the final scaffolds.

1.6. **Optional:** Following the hard bake and prior to lift-off, assess scaffold thickness since the scaffolds should still be gently adhered to the surface of the wafer. For this procedure, contact profilometry works well; however, any appropriate method could be employed.

1.7. Lift the scaffolds off the wafer.

1.7.1. Submerge the scaffolds in SU-8 developer to cause them to immediately lift off the wafer. If scaffolds do not lift off, gently push the scaffolds with a pair of wafer-tweezers.

1.7.2. Remove excess developer.

1.7.3. Transfer the scaffolds into new container and submerge in isopropyl alcohol. Rinse in IPA for a minimum of 6 times to ensure all developer has been removed.

1.8. Air dry the scaffolds for a minimum of a week before use.

2. Scaffold fibronectin coating

2.1. For disinfection, submerge the scaffolds in 70% ethanol (v/v) for a minimum of 15 minutes, and wash 2 times in phosphate buffered saline (PBS) before use.

CAUTION: The SU-8 scaffolds are frail and can break easily. Handling is recommended using blunt and curved-tip forceps, and they should be handled with upmost care. Easier handling can be achieved by submerging the scaffolds in liquid, to avoid electrostatic interactions between the scaffolds and the surface.

2.2. Prepare a dilution of 50 µg/mL of human fibronectin in PBS and cover the scaffolds by protein adsorption.

2.2.1. Mix 1.5 µL of fibronectin stock solution (1 mg/mL) with 28.5 µL of PBS per each scaffold to be seeded. Preferably, prepare only one fibronectin dilution to be used for all scaffolds to avoid pipetting errors.

2.2.1.1. For 10 scaffolds, mix 15 µL of stock fibronectin solution in 285 µL of PBS, amounting to a total of 300 µL of 50 µg/mL fibronectin dilution.

2.2.2. Place the scaffolds sparsely on top of a hydrophobic surface (i.e., non-tissue culture (nonTC) 10 cm dish) and cover each scaffold with 30 µL of the fibronectin dilution prepared in step 2.2.1.

CAUTION: Make sure that no bubbles are trapped in the scaffold compartments. If bubbles are present, they can be removed by gently shaking the scaffold within the droplet or light pipetting.

2.2.3. Replace the plate's lid and place the fibronectin-covered scaffolds into an incubator with 37 °C and 100% humidity for a minimum of an hour.

2.2.4. After incubation, lightly rinse the scaffolds in PBS to remove fibronectin remnants. The scaffolds can be kept in PBS at 4 °C for up to a week before use.

3. Endothelial cells seeding

3.1. Prepare EC medium by mixing the basal medium with its correspondent medium kit components, including an antibiotic solution (Pen/Strep), fetal bovine serum (FBS), and endothelial cell growth supplements, as indicated by the manufacturer.

3.2. Make a human adipose microvascular endothelial cell (HAMEC) suspension in EC medium with a concentration of 4 x 10^6 cells/mL.

NOTE: Real time imaging can be performed if the used endothelial cells are previously transfected to express a fluorescent protein, or pre-stained using a non-toxic cytoplasmic membrane dye (furtherly referred as labeled ECs).

3.3. Using forceps, place one fibronectin-coated scaffold per well on a nonTC 24-well plate well.

3.4. Cover each scaffold with 25 µL droplets of the HAMEC suspension. Beware not to let the suspension flow away from the scaffold, since it can hinder cell adhesion.

3.5. Put the lid on the plate and place it in an incubator at 37 °C, 5% CO_2 and 100% humidity. Let incubate for a minimum of 60 minutes and a maximum of 90 minutes.

NOTE: During incubation, placing the plate on an orbital shaker within the incubator improves cell attachment onto the scaffold's walls. The orbital shaker should be set at no more than 5 rpm.

3.6. After incubation, fill each well with 700 µL of EC medium using a pipette.

NOTE: Expect to see ECs at the bottom of the well, since some of the cells do not attach to the scaffold and fall through the scaffold's voids.

3.7. Incubate the endothelialized scaffolds until EC confluence can be observed using fluorescent microscopy on the scaffold's walls (when using labeled ECs), or for 3 days (when using non-labeled ECs), which provides enough time to achieve EC confluence. Change the medium every other day.

4. Support cell seeding and co-culture

4.1. Prepare dental pulp stem cell (DPSC) medium (DPSCm) by mixing 500 mL of low-glucose Dulbecco's modified Eagle medium (low-glucose DMEM), 57.5 mL of FBS, 5.75 mL of non-essential amino acids (NEAA), 5.75 mL of GlutaMAX, and 5.75 mL of penicillin-streptomycin-nystatin solution.

4.2. Transfer the endothelialized scaffolds into a new nonTC 24-well plate to discard ECs attached to the wells' bottom using forceps.

4.2.1. Discard all media from the current plate using a 1 mL pipette, or vacuum suction. Be careful not to apply vacuum straight on the scaffold.

4.2.2. Place one scaffold per well, preferably at the center of the well to avoid running liquid toward the walls due to surface tension effects. Dry the area surrounding the scaffold with light vacuum but avoid complete drying of the scaffold.

4.3. Prepare a DPSC suspension in fibrin pre-gel solution.

4.3.1. Dilute thrombin and fibrinogen stock solutions with PBS until obtaining a final concentration of 5 U/mL and 15 mg/mL, respectively.

4.3.2. Prepare an 8 x 10^6 DPSC/mL suspension in the 5 U/mL thrombin dilution and distribute in individual eppendorfs 12.5 µL of said suspension per scaffold to be seeded.

4.3.3. Set a 5-50 µL pipette (or similar) to 12.5 µL and fill it with the 15 mg/mL fibrinogen solution.

4.3.4. Without removing the tip, set the pipette to 25 µL. The material in the tip should rise and leave an empty volume towards the tip opening.

4.3.5. Slowly press the plunger button until the liquid reaches the tip opening but does not leak out. Hold the plunger in this position and put the tip into one of the microcentrifuge tubes containing the cells in thrombin suspension, making sure the tip is in contact with the liquid.

CAUTION: The fibrin crosslinking will begin immediately after the thrombin and fibrinogen come into contact. The following step should be done quickly.

4.3.6. Gently release the plunger button and draw the cell suspension into the tip. Thoroughly mix both materials, avoiding bubble formation.

4.4. Quickly dispense the mixed materials on top of an endothelialized scaffold. Repeat the previous steps for each scaffold, making sure to always change tips between uses to avoid

unexpected fibrin gel formation within the tip.

4.5. Replace the plate lid and incubate the scaffolds at 37 °C, 5% CO_2 and 100% humidity for 30 minutes.

4.6. After incubation, fill each well with 1 mL of 1:1 DPSC and EC medium.

4.6.1. Culture for 1 week, changing medium every other day.

4.6.2. During culture, remove the medium from the well and image the constructs using a confocal microscope at different time points to study the vascular development or any other parameter of interest.

NOTE: This step can only be performed if the experiment is performed using labeled ECs. See Step 6 - NOTE for further clarification.

5. Immunofluorescent staining for characteristic vascular markers

NOTE: The following steps can be performed in the same wells where the constructs were cultured. It is critical to always make sure that the solutions completely cover the scaffolds. Moreover, when possible, performing the steps on an orbital shaker is recommended, although not mandatory.

5.1. At day 7, discard the media from the wells and rinse the scaffolds by adding PBS to the wells, and removing it using vacuum.

5.2. Fix the scaffolds by adding 4% paraformaldehyde for 20 minutes. Wash in PBS 3 times, 5 minutes per wash.

5.3. Permeabilize the fixed cells using 0.3% Triton-X in PBS (v/v) for 15 minutes. Wash in PBS 3 times, 5 minutes per wash.

5.4. Prepare a 5% bovine serum albumin (BSA) in PBS (w/v) blocking solution and cover the scaffolds. Leave at room temperature for 1 hour.

NOTE: All the required solution amounts will depend on the number of scaffolds to be stained. Prepare the solutions as needed keeping the stated concentrations.

5.5. Prepare and apply the primary antibodies staining solution .

5.5.1. Dilute rabbit anti-von Willebrand factor (vWF) antibody 1:150 and mouse anti-SMA antibody 1:50 in fresh blocking solution.

NOTE: Other endothelial cells markers, such as CD31 or VE-Cadherin can be used.

5.5.2. Cover the scaffolds with the primary antibodies solution and keep at 4 °C overnight. Wash in PBS three times, 5 minutes per wash.

5.6. Prepare and apply the secondary antibodies staining solution.

5.6.1. Dilute goat anti-mouse Cy3 antibody 1:150 and goat anti-rabbit Alexa-Fluor 488 1:400 in PBS.

5.6.2. Cover the scaffolds with the secondary antibodies solution and incubate for a minimum of 3 hours at room temperature, or at 4 °C overnight. Wash 3 times in PBS, 5 minutes per wash. Keep stained scaffold in 0.3% PFA in PBS (v/v) for up to a month.

6. Scaffold confocal imaging and vessel development analysis

NOTE: The following steps can be performed on the fixed and stained scaffolds at the chosen final time point or, if fluorescent cells were used, during the cell culture period without the need to terminate the experiment. For the latter, it is recommended to set specific time points; this work shows day 0 (before SCs seeding), and days 1, 3, 5 and 7 after SCs seeding (**Figure 3A**).

6.1. Image the scaffolds using a confocal microscope with a 5X lens and a zoom of 0.5 and the full z range of the scaffold. This will allow to capture 9 separate compartments for the squared and circular geometries, or 8 full compartments for the hexagonal geometry. Image the fluorescent signal and the transmitted light, to get both the vessel network structure and the compartment geometry (**Figure 3A**).

6.2. Using ImageJ (or any other image processing software), crop each individual compartment; for this process, only the vascular networks markers are relevant. **Remove any vessel not located inside the compartment area**. Create a maximum intensity projection and save each individual cropped compartment as a TIFF image.

6.2.1. In ImageJ, press **File > Open** and select the desired TIFF image containing several compartments. The image should have at least two channels, the fluorescent vessel network, and the transmitted light image.

6.2.2. From the toolbar, select the **Polygon selection** tool. Select the transmitted light channel. Click on one of the corners of the hexagonal compartment, at the interface between the compartment area and the scaffold wall, this will start the mask definition. Continue clicking on the next corners in a sequential order until the initial corner is selected, closing the mask.

NOTE: This explanation applies to a hexagonal compartment. When the compartment is either squared or circular, use the **Rectangle** or **Oval** tools, respectively, to create an appropriate mask. Regardless the shape, always fit the mask to follow the inner wall of the scaffold.

6.2.3. Click on **Analyze > Tools > ROI manager**. In the ROI manager (ROI, region of interest), click the **Add** button to save the created mask. The new mask will show up in the left side list. This step allows the analysis to be consistent between different images.

NOTE: In the ROI manager, it is possible to save a file with all the created masks by clicking on **More > Save**. In the **Save selection** window, choose a name and location, and save the .roi file. To retrieve the mask, in the ROI manager, click on **More > Open** and choose the desired .roi file.

6.2.4. Select the fluorescent vessel network image and click on **Edit > Clear outside.** Only the image contained in the mask will remain, while the rest will be eliminated.

6.2.5. With the cleared network image open, click on **Image > Duplicate**. In the **Duplicate** pop-up window, write the name of the image. If the **Duplicate hyperstack** is selected, unselect it and click **OK**. A new image containing only the cropped fluorescent vessel networks will be created.

6.2.6. Save the cropped image by clicking on **File > Save as > Tiff**. Choose the name and directory and click **OK**.

6.3. Analyze the vascular development on the cropped images.

6.3.1. Download and install the analytical free software AngioTool, which provides quantitative tools to quantify a variety of vascular parameters. The software can be obtained from the following address **https://ccrod.cancer.gov/confluence/display/ROB2/Home.**

6.3.2. Set the scale value of the images to obtain results in millimeter units and load an image.

6.3.2.1. Click on the **Setting** tab and fill the **Distance in pixels** and **Distance in mm** with representative values from the taken images. Using the imaging parameters mentioned above, the values to fill are 400 pixels per 1 mm.

6.3.2.2. Press the **Open image** button and browse for the image of interest.

6.3.3. Choose the analysis parameters to quantify vessel development in the **Analysis** tab.

6.3.3.1. Under the **Vessel diameter and intensity** field, deselect any present marker from the top range selector, and activate the marker representing the number 3.

6.3.3.2. Under the **Vessel diameter and intensity** field, on the bottom range selector, move the lower range marker to 60, and leave the high ranger maker at 255.

6.3.3.3. Activate the **Remove small particles** box and set the marker to 100.

6.3.3.4. Under the field **Saving preferences**, select the name and location of the spread sheet file in which the results will be saved and press the **Run analysis** button. If the **Save result**

image box is selected, Angiotool will generate and save a new jpeg image showing the vessel network quantified parameters.

NOTE: For each performed analysis without changing the spread sheet file name, a new row will be added at the bottom of the file, with the newly acquired results.

6.4. Organize the data and run a statistical analysis.

6.4.1. For all the processed images, choose the parameter of interest from the spread sheet (e.g. **Total vessel length and Vessel area**) and input them into a statistical analysis software to properly study the obtained results (**Figure 3C**).

7. Time lapse imaging for sprouting origin detection and migration tracking

7.1. Alternatively, to culturing the scaffolds in the incubator as described in step 4.6, set a confocal microscope culture chamber to 37 °C, 5% CO_2 and 100% humidity.

7.1.1. Put the plate containing the fully seeded scaffolds in the confocal microscope chamber and set the imaging parameters to the desired values.

7.1.2. Set the image taking interval to 30 minutes, and the total imaging time of 72 hours. This will enable to run the time lapse completely without the need for a medium change.

CAUTION: In case a longer time lapse imaging is desired, a careful position calibration should be performed to be able to retrieve the original imaging location, since medium changes require removing the plate from the confocal incubator and into the sterile biological hood.

NOTE: ECs detachment from the scaffold walls and tubular structures formation will start within the first hour after SCs seeding (step 4) and may take up to 50 hours to complete. After initial tube formation, new sprouts will start migrating into the compartment from the surrounding vessels. Sprouting and subsequent network remodeling will continue throughout the experiment approximately until day 10, when the vessel networks become stable[22]. Use this information to choose the time lapse starting point and time step accordingly.

7.2. Save the complete time lapse movie file as a TIFF sequence.

7.3. Open ImageJ and click **File > Import > Image sequence** and select your files. This will open your TIFF sequence as a movie.

7.4. Open the **Manual Tracking** plugin by clicking on **Plugins > Manual Tracking**. This will open the **Tracking** window.

7.5. Observe the movie and select the vessel to be tracked. Pay attention to its origin and migration.

7.6. Activate the **Show parameters** box and input the 30 minutes for **Time interval**. The **x/y calibration** will depend on the pixel amount selected during imaging.

7.7. Proceed to manually track the sprouting vessels from the scaffold contour.

7.7.1. Press the **Add track** button. On the movie's first frame, press the location of the vessel to be tracked. After this, the movie will change to the next frame automatically.

7.7.2. Click on the image of the second frame where the vessel is located. Again, the movie will change to the next frame automatically.

7.7.3. Proceed with the same vessel until the full migration tracking has been completed.

7.7.4. On the Tracking window, click on the **Overlay Dots & Lines** to imprint a visual path of the vessel migration on the movie. This will open a copy of the movie with a dot representing the current position of the tracker, and a line showing the previous path.

7.7.5. Click on the **End Track** button to finalize recording the current vessel movement.

7.7.6. Click on the **Add track** button again to start over with a new vessel. When doing this, the line and dot markers will automatically change their color for the new vessel. Repeat until all desired vessels are tracked.

NOTE: The x/y location, vessel velocity and moved distance can be retrieved from the pop-up window **Results**. Each vessel will be identified by its track number in the leftmost column. This data can be copied and pasted into a spread sheet to perform further analysis.

REPRESENTATIVE RESULTS:

The presented protocol, using stereolithography techniques, allows for the fabrication of tessellated scaffolds made of SU-8 photoresist. Scaffolds with distinct compartment geometries (squares, hexagons, and circles), and highly accurate and repeatable features were obtained (**Figure 1**).

[Place **Figure 1** here]

With a stepwise cell seeding (steps 2 to 4), the fabricated scaffolds were used to create highly organized vascular networks. When using a traditional simultaneous seeding of both ECs and SCs, the resulting vessels lacked a clear organization. For this, the scaffold fibronectin coating was performed (step 2), the scaffold endothelialization step was skipped (step 3), and the DPSC and HAMEC were simultaneously co-seeded in fibrin gel (step 4). In this fashion, the cells are homogeneously distributed over the scaffold (**Figure 2**, top row), resulting in unpredictable and disorganized developed vascular networks that do not seem to interact with the surrounding scaffold. Contrarily, firstly seeding the ECs on the scaffold walls provides an accurate initial

endothelial cell patterning. The later addition of SCs within a fibrin gel results in a predictable tubulogenesis phenomenon, with forming vessels closely following the shape of the scaffold wall, and sprouting new vessels migrating into the compartment space (**Figure 2**, bottom row).

[Place **Figure 2** here]

When using fluorescent ECs, either transfected or dyed, the vessels can be imaged in real time without the need to fix and terminate the experiment for each time point. Red fluorescent protein expressing ECs (RFP-ECs) were cultured on hexagonal scaffolds and imaged after seeding (**Figure 3A**, day 0). At day 1, the SCs were added and the vascular networks were imaged every other day to quantify the vessel development (**Figure 3A**, days 1, 3, 5 and 7). For each time point, wide images of the whole scaffold were taken (**Figure 3B**). For every compartment, the vessels mainly organized and interacted with cells located within their confinement. Hence, each compartment was isolated and the superfluous vessels outside the compartment were removed using ImageJ. The clean, single-compartment images were then analyzed using Angiotool. Angiotool returned a spread sheet file containing several vessel parameters, and a visual representation of the main network characteristics, such as skeleton, intersection points, and vessel surface. The obtained data was analyzed using the statistical analysis software Prism, and a clear vessel growth was observed for total vessel length and area during the experiment time frame (**Figure 3C**). During a 1-week experiment, vessels are expected to further develop and extended as shown in **Figure 3A** and **Figure 2C**. Decreasing vessel length or area, failure to form vessels by day 3 or vessels forming as shown in the top row of **Figure 2** can be interpreted as a failed experiments.

[Place **Figure 3** here]

Using multicolored ECs to facilitate single cell identification, a confocal imaging time lapse was performed to allow single vessel tracking (**Supplementary Video 1**). The vessels were observed and tracked using the **Manual Tracking** ImageJ plugin. The tip cell was selected for each frame of the movie (**Figure 4A**) until the vessel anastomosed with the surrounding vasculature. As a result, the **Manual Tracking** plugin generated the vessel path in real time, which allowed to observe the vessel migration (**Figure 4B**).

[Place **Figure 4** here]

Vessel maturation, represented by the presence of SMA+ SCs, can be easily observed in the proposed platform. Vasculatures presenting higher numbers of SMA+ SCs represent a more mature network, since SMA expression correlates with vessel stabilization over time[23]. For the circular, hexagonal and squared compartments, the amount of SMA+ SCs increases over time (**Figure 5A**). By day 3, all shapes showed scattered SMA+ SCs and uncomplex vessels with few or no sprouts whatsoever. By day 7, all shapes showed a rich and complex vascular network, with a higher presence of SMA+ SCs surrounding the vessels. Furthermore, higher magnification images reveal a denser SMA+ SCs presence co-localized with formed vessels, evidencing the SCs recruitment and differentiation surrounding vascular structures (**Figure 5B**).

[Place **Figure 5** here]

FIGURE AND TABLE LEGENDS:

Figure 1: Representative scanning electron microscopy images of the tessellated square, circular, and hexagonal scaffold geometries (scale bar = 50 μm).

Figure 2: Vascularization comparison between simultaneous vs. stepwise cell seeding. Representative images of vascular development in tessellated scaffolds for a simultaneous (top row) cell seeding of ECs (red) and SCs (green), and step-wise cell seeding (bottom row) at days 1 and 5. The stepwise seeding results in organized vascular networks that follow the scaffold walls and sprout into the compartment space (scale bar: 100 μm).

Figure 3: Representative development images and analysis of organized vascular networks. (**A**) ECs (red) reach confluence on the scaffold wall on which SCs are afterwards seeded; the SCs addition represents day 0 of the experiment. At day 1 after the SCs seeding, the ECs detach to the compartment space and start forming vessels that will continue sprouting and connecting at further days. (**B**) Confocal image processing steps for vascular network analysis (i) A wide confocal image containing several compartments is taken, (ii) a single compartment is cropped (demarked by the white dashed hexagon), (iii) then the vascular network channel is separated, and all vessels outside the compartment walls are cropped out. The single compartment image is analyzed using Angiotool, returning a list of vascular parameters complemented with visual markers, such as the vessel area (outlined in yellow), the vessels length (displayed with green lines), and the intersection points (marked as blue dots). (**C**) Comparative results of the total vessel length and the total vessel area within hexagonal compartments at different time points (results are presented as mean ± SD, $n > 6$; all scale bars: 200 μm).

Figure 4: Representative sprouting vessel tracking. (**A**) A multicolored ECs (green, red, and blue) time lapse is used to facilitate single vessel identification. A sprouting vessel is identified and tracked using the ImageJ plugin **Manual Tracking**. The end side of the vessel is marked for every time point to track in real time; the black-in-white marker was added to show the selected vessel end point. (B) The resulting 2D tracking of the vessel, as processed by the ImageJ plugin, showing the farthest point with a dot, and the formed path with a line (scale bar = 200 μm).

Figure 5: SMA+ SCs and blood vessels increase over time. A) Smooth muscle actin (red) and vWF (vessels, green) are shown for vascular networks in circular, squared and hexagonal compartments at day 3 and day 7. Both vasculature extension and SMA-expressing support cells (SMA+ SCs) increase over time, signifying a higher vessel maturation and complexity (scale bar = 200 μm). **B**) Representative images of the SMA+ SCs denser accumulation around vessels at day 7. The nuclei (blue) in the composite image reveal the presence of SCs not expressing the SMA protein (scale bar = 50 μm).

Supplementary Video 1: multicolored ECs time lapse for vessel migration tracking.

DISCUSSION:

The need for a rich vasculature within embedded in engineered tissues is critical for construct survival and proper function[1]. Although engineering the vascular system has been the focus of a vast amount of research, much is left to investigate and understand[24]. In particular, when recreating a specific tissue, the microvasculature should behave and organize ~~a~~ccordingly[12]. The most common approach for microvessels generation is co-seeding endothelial and support cells within a suitable 3D environment compatible for cell attachment, proliferation, and vessel formation[25]. This methodology often results in greatly unorganized networks, making it difficult to study the microvascular behavior[22]. Here, the presented protocol provides a new tool that generates highly organized vascular networks in a high-throughput system, with vessels that can be easily tracked and monitored through time, to study their development and behavior**.** The stepwise seeding of the ECs (step 2-3), followed by the SCs (step 4), are critical steps to achieve such organized networks[22]. Additionally, this technique presents a real 3D environment for vascular models, in which vessels can migrate in the three dimensions and create relevant structures. In contrast, more popular methods for vascular modelling, such as microfluidics systems, only offer a 2.5D tissue representation[26].

The proposed method can be easily modified and applied to study many different factors affecting the vessel network development and behavior. Photolithographic SU-8 resin scaffold fabrication is a common technique with an impressive versatility that enables creating a wide variety of shapes[26,27], with the potential to design structures resembling complex native constructs, such as the alveoli or the nephrotic unit. Nonetheless, a drawback of using SU-8 is its low bioabsorbability[27], making the platform mainly a research tool, instead of an implantable tissue. However, this could be improved by utilizing biocompatible materials which can crosslink under UV/visible light illumination, and 3D printed using accurate techniques like stereolitography[29]. The resulting vascularized constructs could then be implanted in an animal model[30]. Another limitation of the system is the scaffold maximum achievable thickness with high detail accuracy, limited by the stereolithographic technique[31]. The proposed protocol is suitable for creating thin scaffold which might represent native tissue sizes.

This platform offers the potential to investigate several variables affecting the vascularization phenomena[4,32]. First, the interactions between different types of ECs and SCs, and their vessel formation, development and maturation capabilities, which are known to differ when using cells from different tissue origins[33]. Second, the effect of growth factors, small molecules, and inhibitors on the vasculature, allowing for a clear visualization of their impact in real time[34,35]. Third, the addition of other cell types, spheroids, or organoids and their communication and interaction with the forming vessels[36]. For this, the proposed system represents a powerful tool for investigating the microvascular system.

Future steps will take advantage of the proposed system to investigate the effect of mechanical stimuli, such as interstitial flow and mechanical stretching[37,38], on the developing vasculature. This will hopefully shed light on new aspects that will expand the current knowledge and state-of-the-art research of the vascular mechanobiology.

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

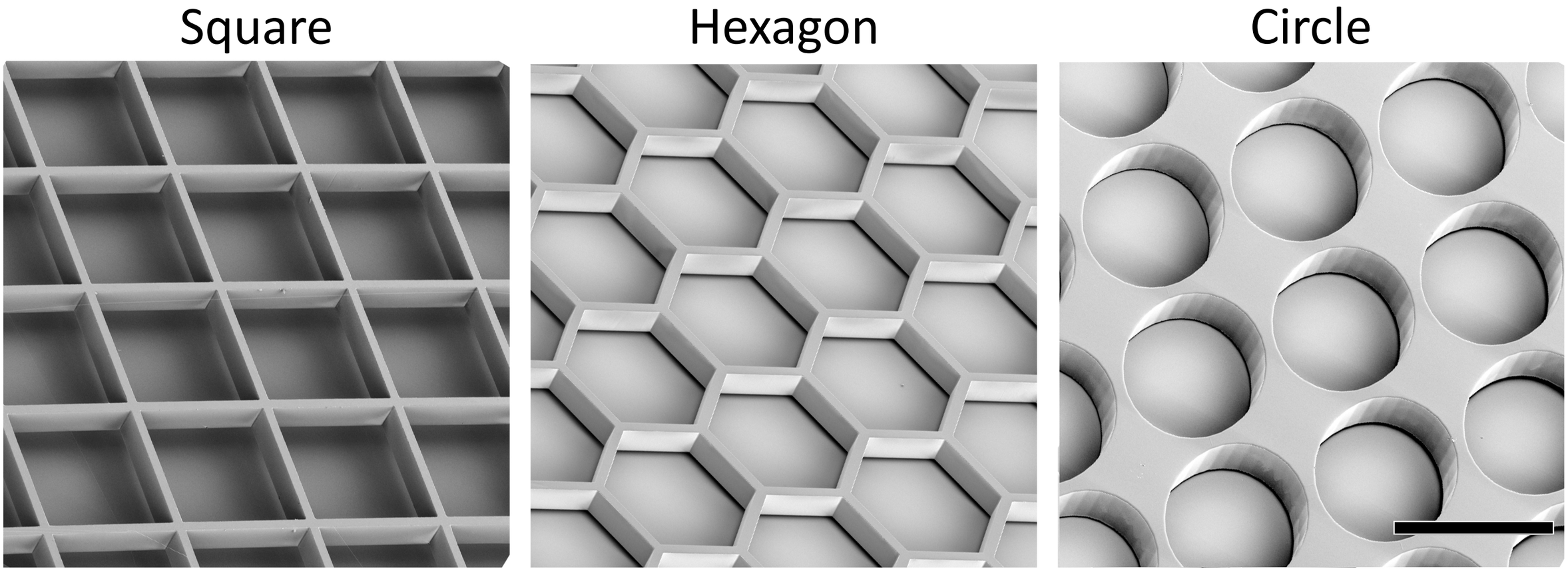

Figure 2

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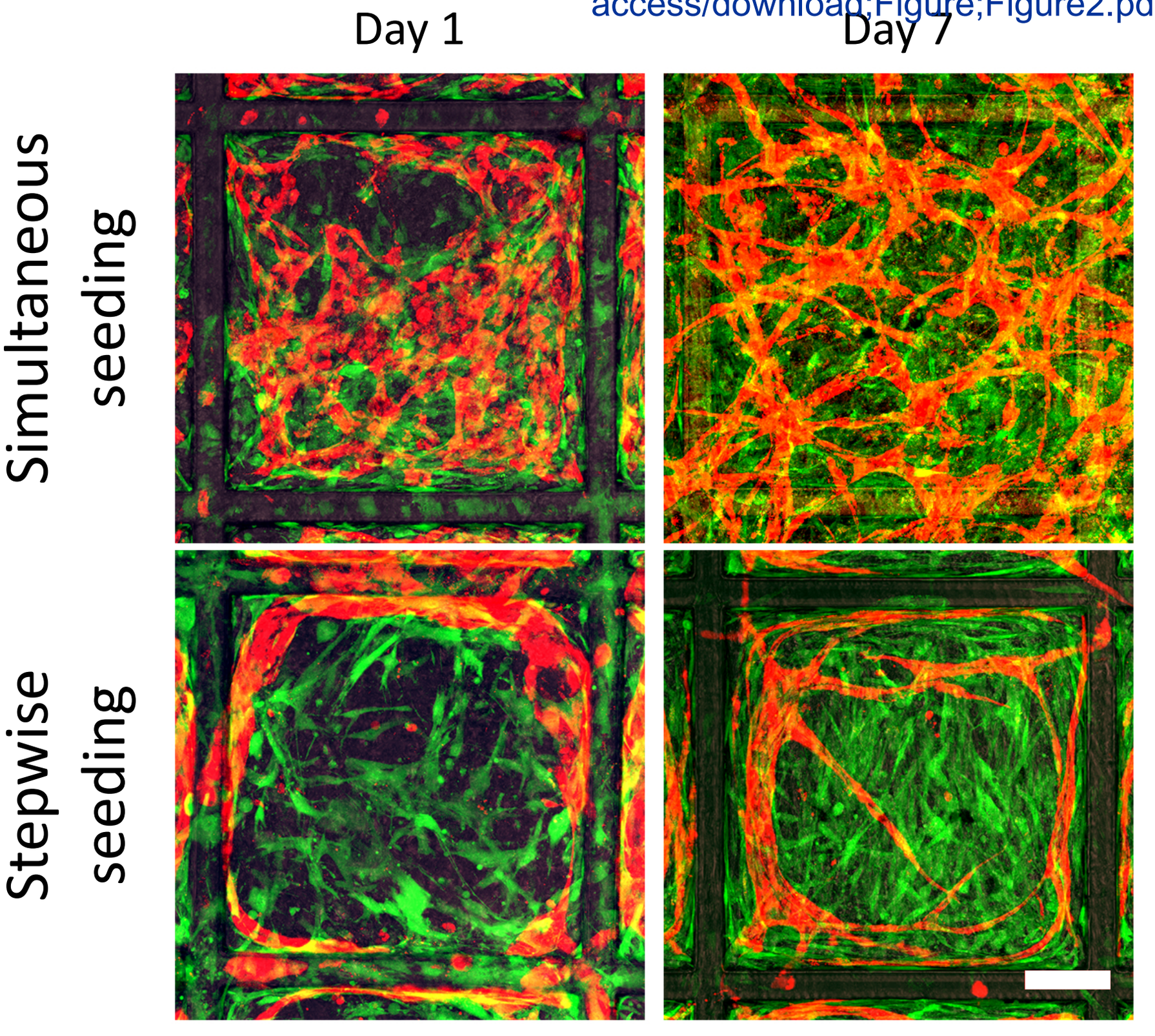

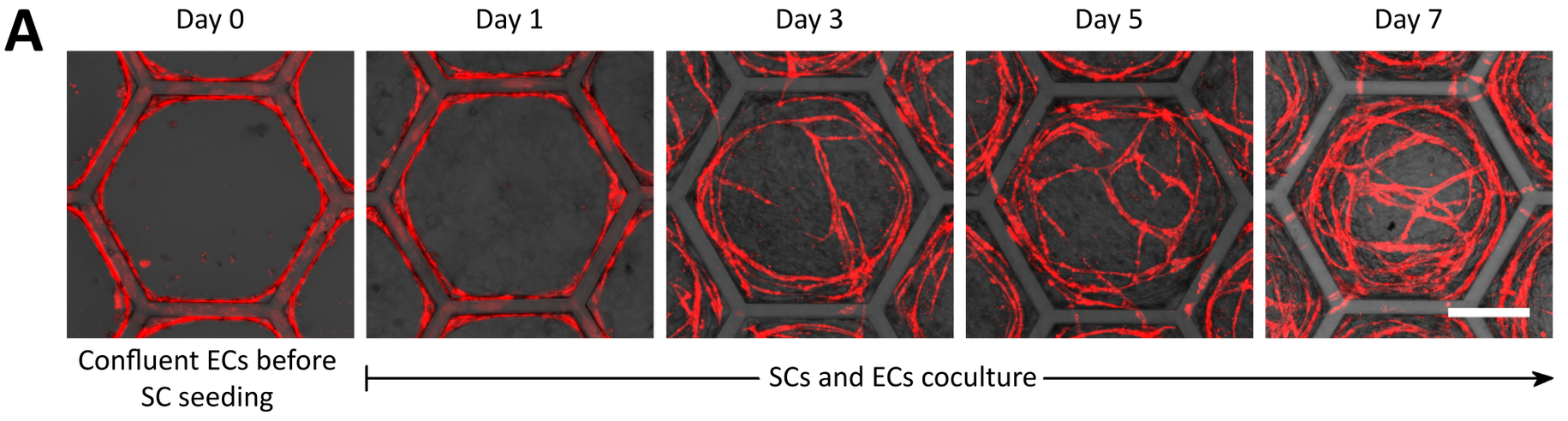

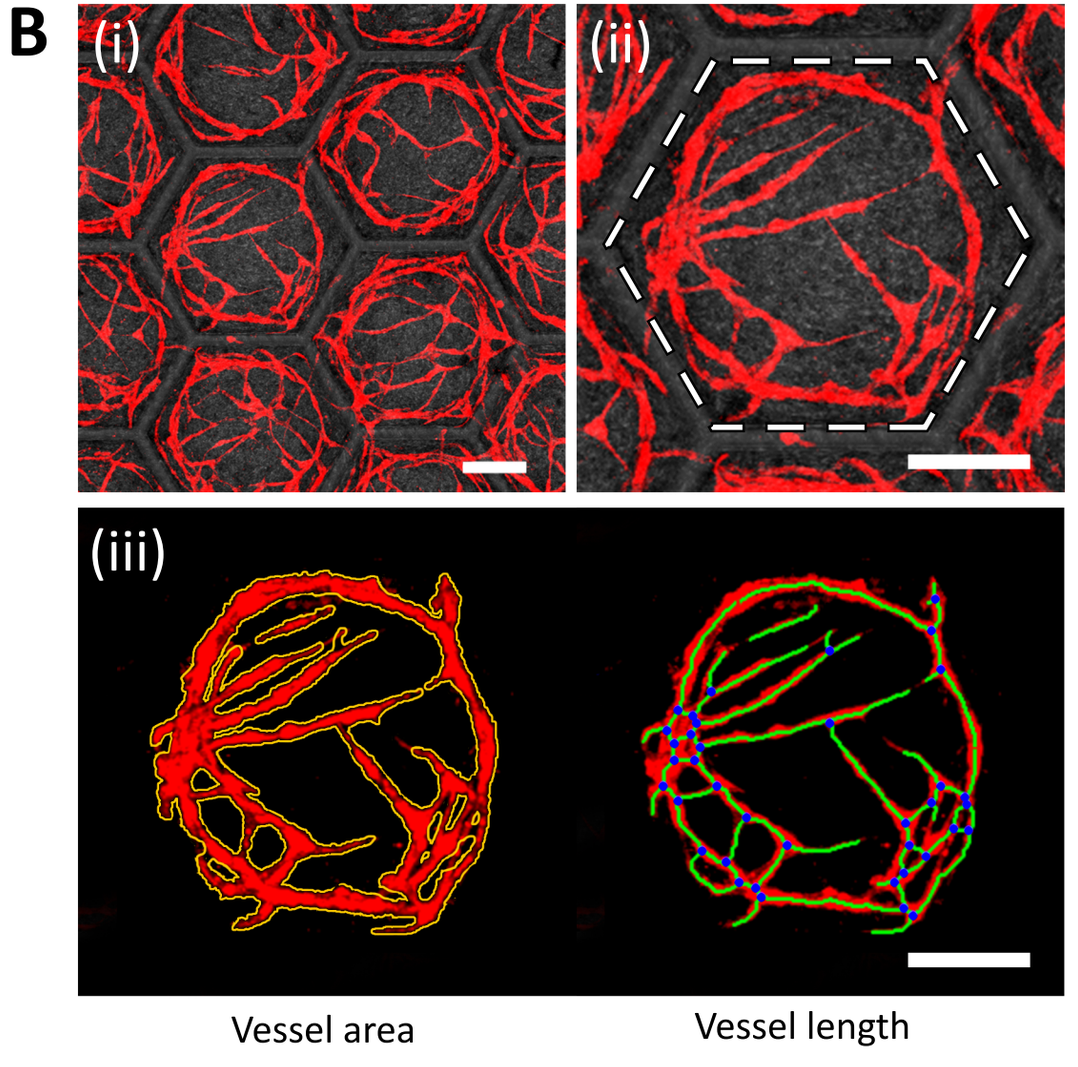

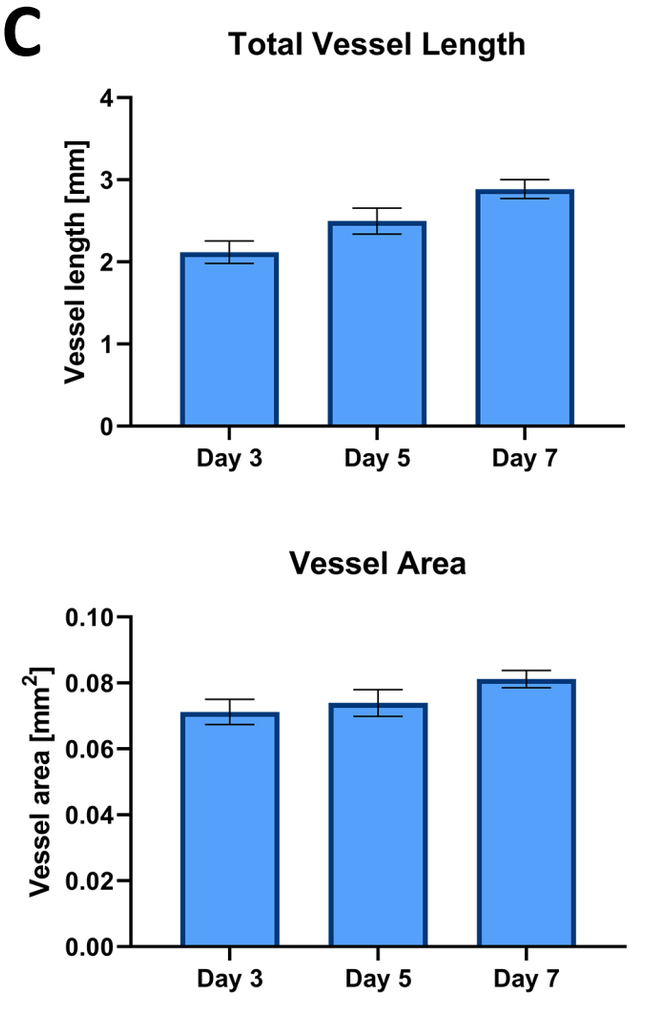

Figure 4

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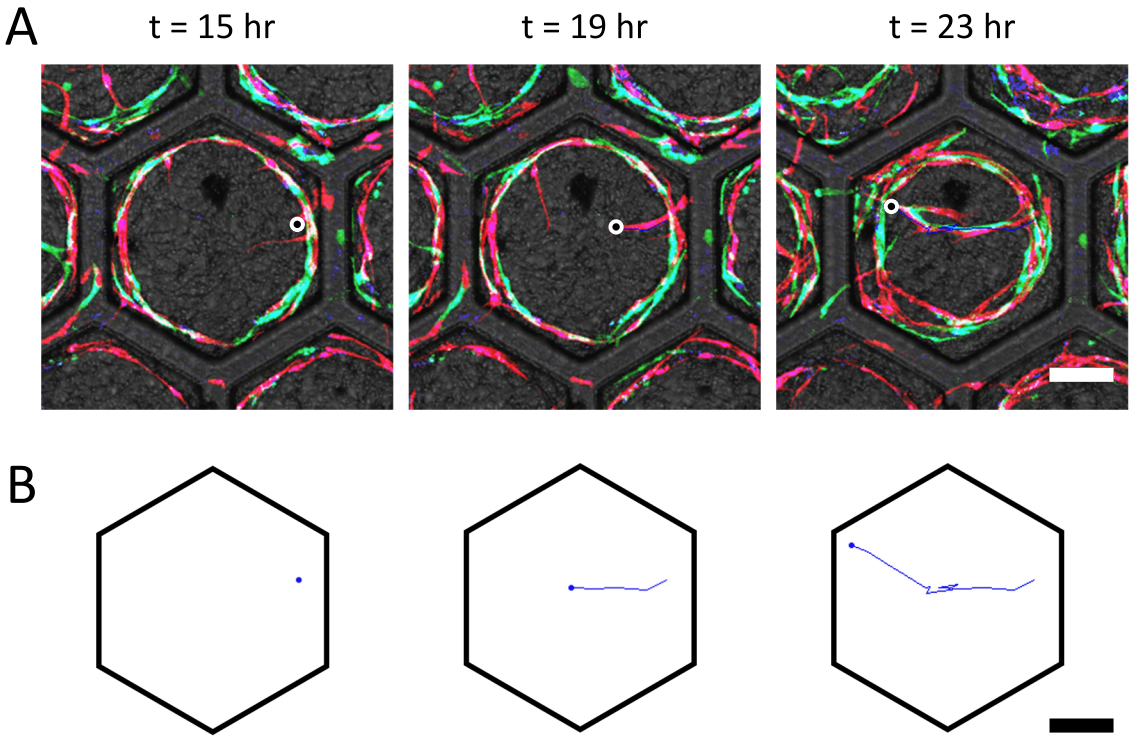

Fugure 5

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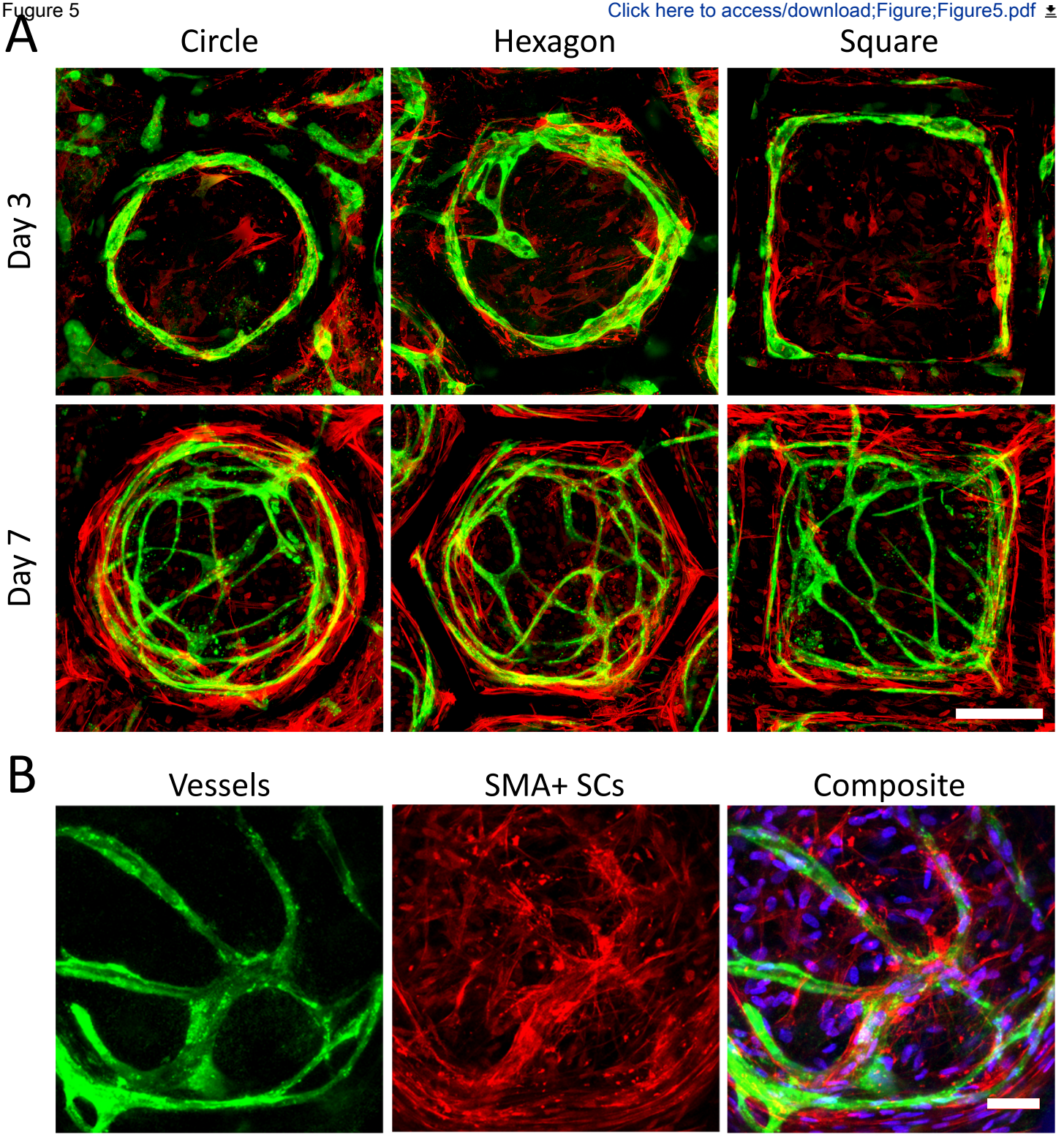

Supplementary video 1

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Video or Animated Figure

Supplementary Video 1.mp4

Table of Materials

Click here to access/download;Table of Materials;List of materials Szklanny_resubmission.xls

Name of Material/ Equipment	Company	Catalog Number
Angiotool freeware	NIH-CCR	
Bovine albumin serum Probumin	Millipore	82-045-1
Dental pulp stem cells	Lonza	PT-5025
ECM media + bullet kit	Sciencell	#1001
Ethanol 96%	Gadot-Group	64-17-5
Evicel fibrin sealant	Johnson&Johnson	EVB05IL
GlutaMAX	Gibco	35050061
Goat anti-mouse Cy3 antibody	Jackson	115-166-072
Goat anti-rabbit Alexa-Fluor 488	Thermo- Fisher Scientific	A11034
Human adipose microvascular cells	Sciencell	#7200
Human fibronectin	Sigma	F0895-5MG
ImageJ	NIH	
Isopropyl alcohol	Gadot-Group	67-63-0
Lift-off reagent	Kayaku Advanced Materials, Inc	G112850
Low-glucose DMEM	Biological Industries	01-050-1A
Mouse anti-SMA antibody	Dako	M0851
NEAA	Gibco	11140068
Paraformaldehyde solution 4% in PBS	ChemCruz	SC-281692
Penicillin-Streptomycin-Nystatin Solution	Biological Industries	03-032-1B
Phospate buffered saline (PBS)	Sigma	P5368-10PAK
Rabbit anti-vWF antibody	Abcam	ab9378
Silicon wafer	Silicon Valley Microelectronics (SVM)	
SU-8 2050 photoresist	Kayaku Advanced Materials, Inc	Y11058
SU-8 developer	Kayaku Advanced Materials, Inc	Y020100
Tryton-X 100	BioLab LTD	57836

Comments/Description
Free download at https://ccrod.cancer.gov/confluence/display/ROB2/Home
Provides both thrombin and fibrinogen (BAC2) solutions
Stock concentration: 1 mg/mL Free download at https://imagej.nih.gov/ij/download.html
Commercial name Omnicoat
Wafers 4", Type N-1-10, 500-550 microns thick

Editorial comments:

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. Please define all abbreviations at first use.

2. Please make the title concise: Stepwise Cell Seeding on Tessellated Scaffolds to Study Sprouting Blood Vessels.

The title has been changed to the proposed new title.

3. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

We have provided more details to the protocol to be clearer to follow.

4. 1.1: Please cite a reference to help readers replicate your protocol. Is the silicon wafer in 1.1.2 to be plasma cleaned in 1.1.1? Please clarify this. Is this wafer the basis of your scaffold? Please add a note in the beginning so the readers can follow the sequence.

Yes, the silicon wafer in 1.1.3 is to be cleaned in 1.1.1, this has been clarified in the revised manuscript. Additionally, the wafer is the basis of the scaffolds, which has been clarified as well.

5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: Omnicoat, BAC2, FIJI,

The terms Omnicoat, BAC2 and FIJI were replaced in the manuscript for "lift-off enhancing material" (LOEM), fibrinogen, and ImageJ, respectively. The fibrinogen was updated also in the materials table.

6. 1.1.2, 1.1.4.2: Please convert centrifuge speeds to centrifugal force (x g) instead of revolutions per minute (rpm).

The device used in this procedure is a spin-coater, not a centrifuge. Spin-speeds are not reported in "x g," as it is not a centrifuge where a force is dependent on the radius of the rotor. The spin-coater is only controlled by the rotor spinning at "X" RPM, and this is what the manufacturer's protocols define for similar procedures. The authors have clarified the use of a "spin-coater" in the revised manuscript.

7. 1.4.2: Please define IPA at first use if you will use it more than once, but use the abbreviation consistently after definition (ref: 1.8.3).

This has now been corrected.

8. 2.2.1: What is the concentration of the fibronectin solution?

To clarify this issue, the following text (marked in yellow) was added:

2.2.1. Mix 1.5 µl of fibronectin stock solution (1 mg/ml) with 28.5 µl of PBS per each scaffold to be seeded. Preferably, prepare only one fibronectin dilution to be used for all scaffolds to avoid pipetting errors.

2.2.1.2. E.g. For 10 scaffolds, mix 15 µl of stock fibronectin solution in 285 µl of PBS, amounting to a total of 300 µl of 50 µg/ml fibronectin dilution.

2.2.2. Place the scaffolds sparsely on top of a hydrophobic surface (i.e. non-tissue culture (nonTC) 10 cm dish) and cover each scaffold with 30 µL of the fibronectin dilution prepared in step 2.2.1.

9. 3.2: What is the EC medium composition?

The text has been modified to indicate exactly how to prepare the medium.

3.1. Prepare EC medium by mixing the basal medium with its correspondent medium kit components, including an antibiotic solution (penstrep), fetal bovine serum (FBS), and endothelial cell growth supplements, as indicated by the manufacturer.

10. 4.2: What is a non24-well plate?

*Thank you for identifying this error. It was meant to be a **nonTC 24-well plate**, and it has now been corrected in the manuscript.*

11. As we are a methods journal, please add to the Discussion with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods

We have included more details on limitations, critical steps, modifications and significance to the discussion.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The manuscript describes the production by photolithography of very thin scaffolds - termed tessellated scaffolds of various patterned types either repeating circles, squares or hexagons. The scaffolds are seeded with human adipose microvascular endothelial cells (HAMEC) and cultured for a number of days before dental pulp stem cells (support cells -SMactin+) in a fibrin gel are seeded over the HAMAC. A staining technique to identify the two cell types is described. The pattern of growth formed by the ECs with and without the support cells is observed and described for several methods including live confocal microscopy, and the growth of EC sprouts quantitated and various vessel parameters quantitated using the AngioTool software.

Major Concerns:

There are no major concerns. There manuscript is well written and clearly describes in a step-wise fashion the techniques used, with some helpful hints included. The figures are generally very clear.

Minor Concerns:

1) The cell types used for the model are human adipose microvascular endothelial cells (HAMEC) (Section 3) and dental pulp stem cells (DPSC) (Section 4). Although both cell types are reasonably well known there is absolutely no detail on how cell suspensions of these cell types are derived. The authors may consider this outside the scope of the manuscript, but references to methods describing how to obtain these two cell types should be included.

We appreciate this comment from the reviewer. As it is stated in the list of materials, both cell types were bought from the ScienCell company and not obtained from ex-vivo tissues, and no part of this protocol is related to obtaining the cells. Furthermore, by working with bought cells, we do not feel confident to provide a protocol on obtaining said cells that was not tried by our lab staff first-hand with proven results.

2) In Section 4 Support Cell Seeding and Co-Culture section, point 4.3.1 the manuscripts states: 'Dilute thrombin and BAC2 stock solutions with PBS until obtaining a final concentration of 5U/mL and 15mg/mL, respectively'. Its not clear from this description that BAC2 is fibrinogen. In the consumables list at the end of the manuscript it is part of an Evicel Fibrin

Sealant (Human) kit. Using only the BAC2 terminology is confusing. It should be clarified in the text that BAC2 is fibrinogen.

Thank you for this comment, the proper clarifications were made to make the protocol more understandable:

4.3.1. Dilute thrombin and fibrinogen stock solutions with PBS until obtaining a final concentration of 5 U/mL and 15 mg/mL, respectively.

4.3.3. Set a 5-50 µL pipette (or similar) to 12.5 µL and fill it with the 15 mg/ml fibrinogen solution.

CAUTION: The fibrin crosslinking will begin immediately after the thrombin and fibrinogen come into contact. The following step should be done quickly.

3) Figure 2B iv) In the high resolution image the 'blue' labelling can be seen, although its not that obvious - could this label be made 'brighter' or more pronounced?

Thank you, we fixed this to make the blue dots be more visible.

4) In Figure 2C, a graph of total vessel area is presented. Is this a measure of the thickness of the (red) vessels by their length? Or is it some other measure? Please explain in more detail.

To clarify this matter, we added to Figure 3C the contour that delineates the vessels after segmentation, a visual aid given by Angiotool to show the included surface by the freeware. In short, the surface calculation works with the intensity segmentation, which considers a vessel any object big enough with a pixel intensity over a certain threshold. After the segmentation is done, anything within this boundaries is considered ***vessel surface.***

5) In Figure 4 (red) support cells (DPSC) are seen throughout the images, but close attachment of the support cells is not particularly obvious. Please provide a higher power image of the support cells attaching closely to the abluminal surface of the endothelial cells of the blood vessels.

Figure 4 (now Figure 5) has been expanded to include an image of the close relation between SMA+ SCs and vessels (ECs). The image (Figure 5B) presents 3 different images: vessels in green, SMA+ SCs in red, and a composite image containing the two first images plus a nuclei staining in blue to show the existence of SMA- SCs. In these images, it is very clear to see that the SMA+ SCs concentrate where vessels are found.

The figure caption now includes the following text:

Figure 5: SMA+ SCs and blood vessels increase over time. A) Smooth muscle actin (red) and vWF (vessels, green) are shown for vascular networks in circular, squared and hexagonal compartments at day 3 and day 7. Both vasculature extension and SMA-expressing support cells (SMA+ SCs) increase over time, signifying a higher vessel maturation and complexity (scale bar = 200 µm). B) Representative images of the SMA+ SCs denser accumulation around vessels. The nuclei (blue) in the composite image reveal the presence of SCs not expressing the SMA protein (scale bar = 50 µm).

6) Why is Section 2: Scaffold Fibronectin Coating, highlighted in yellow - is this a formatting oversight?

To create a video protocol from the provided written protocol, the editors ask the authors to highlight the video parts in yellow. We are not aware of if the reviewers are meant to see this formatting or not, since it is part of the editorial process.

Reviewer #2:
Manuscript Summary:
The authors developed a novel 3D scaffold with tessellated compartment geometries using photolithography technique, which was applied for vascularization of ECs. The article also investigated effect of the two-step seeding technique on vascular networks.

Major Concerns:
None

Minor Concerns:
1) Please simply explain why to choose the ratio of ECs and SCs (1:1).

We appreciate the reviewer's question. The ratio was chosen after experimenting with different cell concentrations, having in mind that better results are achieved when the cell ratio approaches 1:3 ECs:SCs when using HAMECs+DPSCs, as shown by Landau et al (2018). Since not all of the ECs attach to the scaffold surface during seeding, the number of cells remaining is approximately 30.000 to 40.000 ECs after reaching confluence; this was quantified by trypsinizing the cells after reaching confluence and counting them. Since the gel entraps most of the seeded cells, adding 100.000 SCs per scaffold keeps the preferred 1:3 ECs:SCs ratio.

2) What are the standards for the preparation of the endothelialized scaffolds? Or just let the ECs confluence on the surface of scaffolds? Please make it clear.

The endothelialized scaffolds should be cultured until confluence can be observed using fluorescent microscopy, or for 3 days when the used ECs are not fluorescent. The following changes were made in the manuscript to clarify the matter:

3.7. Incubate the endothelialized scaffolds until ECs confluence can be observed using fluorescent microscopy on the scaffold's walls (when using labeled ECs), or for 3 days (when using non-labeled ECs), which provides enough time to achieve EC confluence. Change the medium every other day.

3) In Figure2, is the scaffold only seeded with ECs? If it is, the ECs can also forms vessel without SCs. May you please compare the results of ECs + fibrin (without SCs) with the ECs + fibrin (with SCs)?

We thank the reviewer for bringing this into our attention. Figure 2 shows, at day 0, the confluent endothelial cells on the scaffold BEFORE SCs seeding, also happening at day 0. For the rest of the days, the images show the ECs+SCs co-culture. We have not shown results of ECs + fibrin, since it has been shown in the literature that this system does not work for vessel formation. Nonetheless, to clarify this, we improved figure 2 to make explicit the presence of the SCs. Also, we modified the Figure 2's legend:

Figure 2: Representative development images and analysis of organized vascular networks. *(A) ECs (red) reach confluence on the scaffold wall on which SCs are seeded; the SCs addition represents day 0 of the experiment. At day 1 after the SCs seeding, the ECs detach to the compartment space and start forming vessels that will continue sprouting and connecting at further days.*

4) It is suggested to add more characterizations of Tessellated scaffold, for example, SEM images.

We have added Figure 1 to the manuscript which shows SEM images for the three different compartments discussed in this manuscript, with the caption:

Figure 1: Representative scanning electron microscopy images of the tessellated square, circular, and hexagonal scaffold geometries (scale bar = 50 µm).

Reviewer #3:
Manuscript Summary:
The manuscript describes a procedure for generating neovasculatures in a controlled, defined setting using prefabbed (via photolithography), defined frames (or scaffolds as called by the authors) as 1) a depot for cells and 2)

to form a simple tissue compartment for neovessel tubes to form. This is a format/approach that could prove useful in studying cell dynamics associated with forming cell structures, such as EC cords as presented in this paper.

Major Concerns:
In general, the protocols are clearly written and provide good detail. While there is a fair amount of method details related to making the scaffolds, the authors should revisit that section of the writeup from the perspective that a user with little to no experience in photolithography would be doing the work.

We appreciate the reviewer's concerns and revised the manuscript to be as detailed as possible with as many generalized pieces of advice possible regarding the photolithography procedures. It is worth noting, that unlike the cell-seeding protocol, photolithography is a wide-spread technique with many resources available to an interested reader. In the author's perspective, this protocol should be sufficient for an inexperienced researcher to get started. If this comment was not sufficiently resolved, please provide the author's more specific feedback.

Line 489. The authors refer to "vessel maturation". While the addition of perivascular cells to the endothelial cell cord is part of the maturation process, the biology of vessel maturation is more involved. Besides, it's not clear that there is significant SMA+ cell association with the EC cords. The authors should qualify their conclusions concerning vessel maturation to better reflect their findings.

We appreciate the author's concern for this matter. Indeed, vessel maturation is a complex process that involves many factors that have not been explained in this manuscript. We focused on the SMA+SCs due to the possibility of assessing their presence using the proposed platform. The platform offers the possibility to observe the SCs differentiation into SMA+ cells within a 3D system with clear imaging that is not achievable in other system. A further verification of vessel maturation (such as an angiogenic cytokine array) is beyond the scope of this paper. Furthermore, we provided references to papers that discuss vessel maturation, and recruitment of perivascular cells is certainly a maturation milestone (Potente and Mäkinen, 2017; Welti et al. 2013).

We agree that the SMA+ SCs and ECs cords association in the provided images is not completely clear, so we expanded Figure 5 (previously Figure 4) to include a higher magnification image that evidences the close relation between SMA+SCs and vascular structures. The caption was also expanded, as follows:

Figure 5: SMA+ SCs and blood vessels increase over time. A) Smooth muscle actin (red) and vWF (vessels, green) are shown for vascular networks in circular,

squared and hexagonal compartments at day 3 and day 7. Both vasculature extension and SMA-expressing support cells (SMA+ SCs) increase over time, signifying a higher vessel maturation and complexity (scale bar = 200 µm). B) Representative images of the SMA+ SCs denser accumulation around vessels. The nuclei (blue) in the composite image reveal the presence of SCs not expressing the SMA protein (scale bar = 50 µm).

Line 507 Figure 2. Typically, vessel lengths are reported as the sum of vessel lengths divided by the area measured. While it's appreciated that the area examined is likely the same across all samples (given the scaffold pattern), reporting of the length density as opposed to just the sum of vessel lengths accounts for more or less area being measured between samples which could lead to more or less vascularity simply being including more or less vessels. Also, in the area plot, the Y axis label is "vessel area", yet the legend states "total vessel area for vascularization". These are two different measurements that require clarification in their presentation.

We thank the reviewer for this comment, which gave us perspective on an overlooked topic. As the reviewer notes, the vessel length values are reported as the total of all the elements' length (in mm) within the region of interest, which remains the same due to the scaffold geometry. These results are presented as ***Total vessel length*** *since no normalization was done using the region of interest area (this would be the* ***vessel length density****, with units of mm/mm^2). Indeed, for comparing between different geometries, an area-based normalization should be done, but it is not the case for the results presented in this work. Regarding the vessel area, we agree with the reviewer and removed the "for vascularization" from the caption. Furthermore, in Figure 2, we included a yellow contour around the vessels which explicitly shows the way the vessel area is considered. The appropriate changes to the caption were made as follows:*

Figure 3: Representative development images and analysis of organized vascular networks. *[…] (B) Confocal image processing steps for vascular network analysis (i) A wide confocal image containing several compartments is taken, (ii) a single compartment is cropped (demarked by the white dashed hexagon), (iii) then the vascular network channel is separated, and all vessels outside the compartment walls are cropped out. The single compartment image is analyzed using Angiotool, returning a list of vascular parameters complemented with visual markers, such as the vessel area (outlined in yellow), the vessels length (displayed with green lines), and the intersection points (marked as blue dots). (C) Comparative results of the total vessel length and the total vessel area within hexagonal compartments at different time points (results are presented as mean ± SD, n > 6; all scale bars: 200 µm).*

Line 543. Throughout the paper, the authors describe the neovessel tubes as "highly organized vascular networks". Can the authors define better what is meant by "highly organized"? Are they referring to the network architecture, the distribution of neovessel tubes within the space, the formation of neovessel tubes, etc.?

We included the following text in the Representative results section, to clarify the meaning of highly organized vascular networks:

When using a traditional simultaneous seeding of both ECs and SCs, the resulting vessels lacked a clear organization. [...] *In this fashion, the cells are homogeneously distributed over the scaffold (Figure 2, top row), resulting in unpredictable and disorganized developed vascular networks that do not seem to interact with the surrounding scaffold. Contrarily, firstly seeding the ECs on the scaffold walls provides an accurate initial endothelial cell patterning. The later addition of SCs within a fibrin gel results in a predictable tubulogenesis phenomenon, with forming vessels closely following the shape of the scaffold wall, and sprouting new vessels migrating into the compartment space (Figure 2, bottom row).*

Minor Concerns:
Line 112, 1.1.1 Plasma clean wafer. Details or a reference should be provided.

The authors have provided additional detail in the revised manuscript.

Line 117-119, 1.1.2 how is the spinning performed? When inspecting, what are you looking for to indicate complete coating?

This has been clarified in the updated manuscript.

Line 124, how do you determine when 100 um thickness has been obtained?

1.1.4 is the header of that step. It was noted under 1.1.4.3 that "The actual thickness of the resist and the resultant scaffolds will be dependent on many factors. Therefore, the thickness of the scaffolds should be verified later, in 1.7. Modify spin coating procedures accordingly to achieve the desired thickness."

1.7 reads: "Following the hard bake, and prior to lift-off is the most advantageous time to assess scaffold thickness since the scaffolds should still be gently adhered to the surface of the wafer. For this procedure, contact profilometry works well; however, any appropriate method could be employed."

Line 155, is it clear to readers how to make a photomask?

There are many differences in photolithography apparatuses, which make it difficult to provide a "one size fits all" method for preparation of photomasks. If a reader is interested in following this procedure, there are ample resources available, including companies, to support the design and production of the necessary photomask. The use of terms like "hard glass or quartz" and "soft polymer transparent film photomask" in the context of "photolithography" should be the key words readers need to start their process. More importantly, the authors noted under 1.2 that glass photomasks are higher resolution than transparencies, as the choice a cheaper film photomask vs a more expensive glass photomask will likely cause topographical features on the scaffolds as a function of poorer feature resolution.

Line 245, 3.7. Is visualization done by phase or is the fluorescence-based method described earlier needed?

A clarification was added as follows:

3.2. Make a human adipose microvascular endothelial cell (HAMEC) suspension in EC medium with a concentration of 4·106 cells/ml.

NOTE: Real time imaging can be performed if the used endothelial cells are previously transfected to express a fluorescent protein, or pre-stained using a non-toxic cytoplasmic membrane dye (furtherly referred as labeled ECs).

[…]

3.7. Incubate the endothelialized scaffolds for up to 3 days (when using non-labeled ECs), or until cell confluence can be observed with a confocal or fluorescent microscope on the scaffold's walls (when using labeled ECs). Change the medium every other day.

Line 251, 4.1. Is there a need to lot test the fbs, as some cell types are fbs lot-sensitive.

We do not think that an FBS lot test is necessary. For culturing HAMECs, we have always used the mentioned ECM from ScienCell, which provides its own FBS. As for the DPSC, we have used two different FBS brands and several lots from each and we have not observed significant differences in their growth and functionality.

Line 261, 4.2.2. The phrase "…running liquid towards the wall…". Is this referring to surface tension effects?

Indeed, the phrase refers to the surface tension effects, and thanks to this comment, we modified the text to clarify this matter:

4.2.2. Place one scaffold per well, preferably at the center of the well to avoid running liquid toward the walls due to surface tension effects. Dry the area surrounding the scaffold with light vacuum but avoid complete drying of the scaffold.

Line 285, 4.3.6. Perhaps instead of "gently" use the term "slowly".

We have modified the text as follows:

4.3.5. Slowly press the plunger button until the liquid reaches the tip opening but does not leak out. Hold the plunger in this position and put the tip into one of the eppendorfs containing the cells in thrombin suspension, making sure the tip is in contact with the liquid.

Line 299, 4.6.2. With the confocal imaging, is there a need to fluorescently label the cells or is the expectation that the cells are pre-tagged as described earlier? Either way, the authors should indicate in this entry the expectation.

At step 4.6.2. we added the following note:

NOTE: This step can only be performed if the experiment is performed using labeled ECs. See Step 6 for further clarification.

This will refer the reader to the following note which was included in the original manuscript:

NOTE: The following steps can be performed on the fixed and stained scaffolds at the chosen final time point or, if fluorescent cells were used, during the cell culture period without the need to terminate the experiment. For the latter, it is recommended to set specific time points; this work shows day 0 (before SCs seeding), and days 1, 3, 5 and 7 after SCs seeding (Figure 2A).

Line 312, 5.2. Is the paraformaldehyde prepared in PBS?

In the materials list, it can be found that the proposed PFA is obtained as a 4% solution with no need for dilution.

Line 318, 5.4. Can other blocking agents besides BSA be used?

There is a wide variety for staining protocols that use an extensive range of different reagents (different primary and secondary antibodies, different Tryton-X concentrations, and so on). We have only referred to what was used for the current experiment, and verifying other reagents is outside the scope of this work. Nonetheless, we are confident that other known staining protocols using other blocking agents (e.g. FBS) will work without noticeable changes.

Line 351, 6.2. The inclusion of a schematic or diagram (or labeling of provided images) would be useful in indicating the different compartments the authors are referring to in this description.

Thank you for this comment. We greatly expanded step 6.2 to make the process clearer, which also allows to understand the compartments better. We also added in figure 2C a frame representing the area to be cropped, following the shape of the compartment. The following text was added to the figure caption to further clarify the matter:

Figure 2: Representative development images and analysis of organized vascular networks. […] (B) Confocal image processing steps for vascular network analysis (i) A wide confocal image containing several compartments is taken, (ii) a single compartment is cropped (demarked by the white dashed hexagon).

Moreover, we included a new figure (Figure 1) with SEM images of the resulting scaffolds in which the compartments can be easily seen.

Line 402, 7.1.3. The authors describe imaging intervals based on practical considerations. Based on their experience with the model, the authors should also present temporal information related to the time course of relevant biological events (e.g. cell migration occurs over hours vs days, etc.) to help others set imaging intervals, as needed.

Thank you for this comment. We included the following note after step 7.1.3 to explain the critical time points, so readers can choose the time step and starting time point according to their needs:

NOTE: ECs detachment from the scaffold walls and tubular structures formation will start within the first hour after SCs seeding (step 4) and may take up to 50 hours to complete. After initial tube formation, new sprouts will start migrating into the compartment from the surrounding vessels. Sprouting and subsequent network remodeling will continue throughout the experiment until day 10, in which the vessel networks become stable[22]. Use this information to choose the time lapse starting point and time step accordingly.

Line 501 Figure 1. The authors should refer to the specific protocol steps in which simultaneous vs step-wise seeding was performed.

We thank the reviewer for this comment. To address this, we added the following text in the Representative results section:

Using a stepwise cell seeding (steps 2 to 4), the fabricated scaffolds can be used to create highly organized vascular networks. […] To compare the step-wise seeding vessel organization capabilities, a traditional simultaneous seeding of both ECs and SCs was done. For this, the scaffold fibronectin coating was performed (step 2), the scaffold endothelialization step was skipped (step 3), and the DPSC and HAMEC were simultaneously co-seeded in fibrin gel (step 4).

Line 519 Figure 3. Are the different colors used to identify individual ECs. If so, this should be stated in the legend.

We appreciate the reviewer comment. We agree that there is a need to clarify the purpose of the multicolored ECs, and the following changes were made on the manuscript body to address this matter:

Using multicolored ECs to facilitate single cell identification, a confocal imaging time lapse was performed to allow single vessel tracking (Supplementary Video 1).