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TITLE:

An In Vitro 3D Model and Computational Pipeline to Quantify the Vasculogenic Potential of iPSC-Derived Endothelial Progenitors

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SUMMARY:
Endothelial progenitors derived from induced pluripotent stem cells (iPSC-EPs) have the potential to revolutionize cardiovascular disease treatments and to enable the creation of more faithful cardiovascular disease models. Herein, the encapsulation of iPSC-EPs in three-dimensional (3D) collagen microenvironments and a quantitative analysis of these cells' vasculogenic potential are described.

ABSTRACT:
Induced pluripotent stem cells are a patient-specific, proliferative cell source that can differentiate into any somatic cell type. Bipotent endothelial progenitors, which can differentiate into the cell types necessary to assemble mature, functional vasculature, have been derived from both embryonic and induced pluripotent stem cells. However, these cells have not been rigorously evaluated in three-dimensional environments, and a quantitative measure of their vasculogenic potential remains elusive. Here, the generation and isolation of iPSC-EPs via fluorescent-activated cell sorting are first outlined, followed by a description of the encapsulation and culture of iPSC-EPs in collagen hydrogels. This extracellular matrix (ECM)-mimicking microenvironment encourages a robust vasculogenic response; vascular networks form after a week of culture. The creation of a computational pipeline that utilizes open-source software to quantify this vasculogenic response is delineated. This pipeline is specifically designed to preserve the 3D architecture of the capillary plexus to robustly identify the number of branches, branching points, and the total network length with minimal user input.

INTRODUCTION:

Human umbilical vein endothelial cells (HUVECs) and other primary endothelial cell types have been utilized for two decades to model blood vessel sprouting and development in vitro[1]. Such vascular platforms promise to illuminate molecular and tissue-level mechanisms of cardiovascular disease and may present physiological insight into the development of primitive vascular networks[2,3]. Though the field of vascular modeling has witnessed significant advances, a "gold standard" assay that can quantitatively model and assess physiological vascular development remains elusive. Most published protocols do not adequately recapitulate the vascular niche to encourage the formation of mature, functional blood vessels or do not have a method to quantitatively compare the vasculogenic potential of the assessed cell types in three dimensions (3D).

Many current vascular models are limited in their ability to mimic the physiological vascular niche. One of the most commonly employed in vitro platforms is the gelatinous protein mixture-based tube formation assay. Briefly, HUVECs are seeded as single cells on a thin layer of gel that consists of proteins harvested from murine sarcoma extracellular matrix (ECM); within one to two days, the HUVECs self-assemble into primitive tubes[4]. However, this process occurs in two-dimensions (2D) and the endothelial cells (ECs) utilized in this assay do not form enclosed, hollow lumens, thereby limiting the physiological significance of these studies. More recently, ECs and supporting cells (e.g., mesenchymal stem cells (MSCs) and pericytes) have been co-cultured in 3D microenvironments that simulate the fibrous architecture of the native ECM, such as collagen or fibrin hydrogels[5]. To model vascular development in these microenvironment, polymeric beads coated with ECs are typically employed[6]. The addition of exogenous growth factors and/or growth factors secreted by other cells interstitially embedded in the hydrogel can induce the ECs, coating the polymeric beads, to sprout and form single lumen; the number and diameter of sprouts and vessels can then be computed. However, these sprouts are singular and do not form an enclosed, connected network as is seen in physiological conditions and thus is more reminiscent of a tumor vasculature model. Microfluidic devices have also been utilized to mimic the vascular niche and to promote the formation of vasculature in EC-laden hydrogels[7,8]. Typically, an angiogenic growth factor-gradient is applied to the circulating cell culture medium to induce EC migration and sprouting. ECs that constitute the lumen of developed vessels are sensitive to the shear stress induced by the application of fluid flow through the microfluid device; thus, these microfluidic devices capture key physiological parameters that are not accessible in the static models. However, these devices require costly microfabrication abilities.

Most importantly, all three vascular models (2D, 3D, microfluidic) overwhelmingly utilize primary ECs as well as primary supporting cell types. Primary cells cannot be developed into an effective cardiovascular therapy because the cells would engender an immune response upon implantation; furthermore, HUVECs and similar primary cell types are not patient-specific and do not capture vascular abnormalities that occur in patients with a genetic disposition or pre-existing health conditions, e.g., diabetes mellitus. Induced pluripotent stem cells (iPSCs) have emerged in the past decade as a patient-specific, proliferative cell source that can be differentiated into all somatic cells in the human body[9]. In particular, protocols have been published that outline the generation and isolation of iPSC-derived endothelial progenitors (iPSC-EPs)[10,11]; iPSC-EPs are bipotent and can, therefore, be further differentiated into endothelial cells

and smooth muscle cells/pericytes, the building blocks of mature, functional vasculature. Only one study has convincingly detailed the development of a primary capillary plexus from iPSC-EPs in a 3D microenvironment[12]; though this study is critical to an understanding of iPSC-EP assembly and differentiation in natural and synthetic hydrogels, it did not quantitatively compare the network topologies of the resulting vasculature. Another recent study has used the polymeric bead model to compare the sprouting of HUVECs and iPSC-derived ECs[5]. Therefore, there is a clear need to further elucidate the physical and chemical signaling mechanisms that regulate iPSC-EP vasculogenesis in 3D microenvironments and to determine if these cells are suitable for ischemic therapy and cardiovascular disease modeling.

In the past decade, different open-source computational pipelines and skeletonization algorithms have been developed to quantify and compare vascular network length and connectivity. For example, Charwat et al. developed a Photoshop-based pipeline to extract a filtered, binarized image of vascular networks derived from a co-culture of adipose-derived stem cells and outgrowth ECs in a fibrin matrix[13,14]. Perhaps the most widely used topology comparison tool is AngioTool, a program published online by the National Cancer Institute[15]; despite the program's widespread adoption and well-documented fidelity, the program is limited to analyzing vessel-like structures in two dimensions and other programs, including AngioSys and Wimasis, share the same dimensionality limitation[16]. Powerful software suites such as Imaris, Lucis, and Metamorph have been developed to analyze the network topology of engineered microvasculature; however, these suites are cost-prohibitive for most academic labs and limit access to the source code, which may hinder the ability of the end-user to customize the algorithm to their specific application. 3D Slicer, an open-source magnetic resonance imaging/computed tomography package, contains a Vascular Modeling Toolkit that can effectively analyze the topology of 3D vascular networks[17]; however, the analysis is dependent on the user manually placing the end-points of the network, which may become tedious when analyzing a large dataset and can be influenced by the user's subconscious biases. In this manuscript, a computational pipeline that can quantify 3D vascular networks is described in detail. To overcome the above-outlined limitations, this open source computational pipeline utilizes ImageJ to pre-process acquired confocal images to load the 3D volume into a skeleton analyzer. The skeleton analyzer uses a parallel medial axis thinning algorithm, and was originally developed by Kerschnitzki et al. to analyze the length and connectivity of osteocyte networks[18]; this algorithm can be effectively applied to characterize the length and connectivity of engineered microvasculature.

Altogether, this protocol outlines the creation of microvascular networks in 3D microenvironments and provides an open-source and user-bias free computational pipeline to readily compare the vasculogenic potential of iPSC-EPs.

PROTOCOL:

1. Preparation of culture media and coating solutions

1.1. To prepare vitronectin coating solution, dilute vitronectin 1:100 in Dulbecco's phosphate-buffered saline (DPBS).

CAUTION: Once diluted, it is not recommended to store this solution for future use.

1.2. Upon receiving phenol red-free, growth factor-reduced gelatinous protein mixture (see **Table of Materials**) from the manufacturer, thaw on ice at 4 °C until the mixture is transparent and fluid. Keeping the mixture on ice in a laminar flow hood, pipette 75 µL of the mixture into a 1.8 mL microcentrifuge tube and freeze at -20 °C immediately. These aliquots can be stored for up to one year.

CAUTION: This gelatinous protein mixture becomes very viscous when kept at room temperature and will solidify at higher temperatures. Keep this solution ice-cold.

1.3. To prepare this mixture for coating, thaw a 75 µL aliquot on ice and dilute with 6 mL of ice-cold Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12). This prepared volume is enough to coat a 12 well plate.

1.4. Prepare a 100 mg/mL stock solution of ascorbic acid magnesium-2-phosphate (a white lyophilized powder) in ultrapure water by adding 500 mg of powder to 5 mL of water in a 10 mL glass scintillation vial. Agitate with a stir bar until the solution is completely transparent (this may take up to an hour). This solution can be stored at -20 °C for up to one year.

1.5. Create a 10 mM stock of CHIR99021 (CHIR) by dissolving 10 mg of lyophilized powder in 1.9928 mL of dimethyl sulfoxide (DMSO) in a 15 mL conical centrifuge tube and warm in a 37 °C bead (or water) bath until the solution is transparent. Aliquot into 1.8 mL microcentrifuge tubes and store at -20 °C for up to one year.

1.6. Create a 10 mM stock of Y-27632, a ROCK inhibitor (ROCKi), by dissolving 10 mg of lyophilized powder in 3.1225 mL of dimethyl sulfoxide (DMSO) in a 15 mL conical centrifuge tube and warm in a 37 °C bead (or water) bath until the solution is transparent. Aliquot into 1.8 mL microcentrifuge tubes and store at -20 °C for up to one year.

1.7. To prepare Essential 8 (E8) medium, thaw frozen supplements, add to the 500 mL bottle of basal medium, and filter sterilize. This solution can be stored at 4 °C for up to two weeks.

CAUTION: It is very important not to warm this medium at 37 °C, as the proteins in the medium formulation may degrade with prolonged use. Instead, keep this medium at room temperature for 15 to 30 min before use.

1.8. To prepare sorting buffer, add 1.33 mL of 7.5% bovine serum albumin (BSA) in DPBS and 200 µL of 0.5 mM ethylenediaminetetraacetic acid (EDTA) to 48.5 mL of DPBS to create a final solution of 0.5% BSA and 2 mM EDTA.

1.9. To prepare LaSR Basal, add 300 µL of 100 mg/mL ascorbic acid magnesium-2-phosphate and 5 mL of GlutaMAX to 500 mL of Advanced DMEM/F12. This solution can be stored at 4 °C for up to one month.

1.10. To prepare Endothelial Cell Growth Medium-2 (EGM-2), thaw supplements and add to 500 mL bottle of basal medium. This solution can be stored at 4 °C for up to one month.

1.11. To prepare blocking buffer, add 500 mg of lyophilized bovine serum albumin (BSA) and 50 µL of an emulsifying reagent (see **Table of Materials**) to 48 mL of DPBS. Incubate at 37 °C for at least 15 min to ensure that the BSA does not clump and subsequently separate from the solution.

2. Thawing, maintenance, and passaging of iPSCs

2.1. Before thawing a cryopreserved iPSC vial, coat a single well of a 6-well plate with 1 mL of vitronectin solution (prepared as described in step 1.1). Incubate for one h at room temperature. Do not let this coating air dry before adding E8 medium to the plate.

2.2. To thaw iPSCs, retrieve a cryopreserved vial of iPSCs from its liquid nitrogen storage container and thaw at 37 °C until a small crystal of ice remains. Carefully (dropwise) transfer the content of the thawed vial to a 15 mL conical centrifuge tube containing 8 mL of ice-cold DMEM/F12. Wash the thawed vial with an addition 1 mL of ice-cold DMEM/F12 to minimize cell loss. Centrifuge for 5 min at 300 x *g*.

2.3. While waiting for the centrifuge, aspirate the vitronectin solution and add 1 mL of E8 medium (prepared as described in step 1.6) to the well. Then, remove the DMEM/F12 supernatant from the centrifuged conical tube and re-suspend the pellet in 1 mL of E8 medium. Transfer the cells to the newly coated plate, being sure to agitate the suspension to prevent the colonies from clumping upon seeding.

2.4. There is usually substantial cell death after thawing. The next morning, remove the medium and replace with fresh E8 medium.

NOTE: Even under optimal conditions, iPSCs tend to recover poorly from cryopreservation. It is recommended to cryopreserve a near-confluent 6-well for future seeding into a single 6-well. Presence of cell debris is normal for the first 2-3 days during cell recovery.

2.5. Replace E8 medium daily until the cells are ready for passaging (70%–80% confluency).

2.6. To passage, first prepare vitronectin-coated wells (as described in step 2.1).

2.6.1. Once vitronectin-coated wells are ready (about 1 h), aspirate E8 medium from well to be passaged and wash twice with 1 mL of DPBS. Incubate with 1 mL of 0.5 mM EDTA for 5–7 min at 37 °C. While incubating, remove the vitronectin solution from the newly coated well and replace with 1–2 mL of E8 medium.

2.6.2. Remove the EDTA solution from the wells to be passaged and detach the colonies by gently washing once or twice with 1 mL of E8. Transfer 75-200 µL of cell suspension to the freshly coated E8-containing wells, agitate the plate to ensure even coverage, and place in the incubator immediately.

CAUTION: The incubation time for iPSC detachment is cell-line dependent; it is recommended that the user of this protocol tests a range of times upon sufficiently expanding a received/generated iPSC line. 75 µL of cell suspension generally results in 4 days between passages; 150 µL or more leads to 2–3 days between passages.

3. Generation of iPSC-derived endothelial progenitors (Figure 1).

3.1. When maintaining iPSCs, make sure the colonies are well compacted and that there are a low number of differentiated cells in culture. If the colonies begin to contact one another, the cells are most likely too confluent and need to be passaged immediately.

3.2. When iPSCs are 70%–80% confluent, coat a 12-well plate with the gelatinous protein mixture (prepared as described in 1.2/1.3). Pipette 500 µL of this mixture into each well and keep for 1 h at room temperature.

3.3. During the above 1-h incubation, remove the E8 medium and Y-27632 from the refrigerator and freezer, respectively. Once E8 medium and Y-27632 reach room temperature, prepare E8 + ROCKi by adding 13 µL of 10 µM Y-27632 to 13 mL of E8 in a 15 mL conical centrifuge tube.

3.4. Remove the E8 medium from the iPSC wells, wash twice with 1 mL of DPBS, and then incubate with 0.5 mM EDTA for 5-7 min at 37 °C. After the incubation period, remove the EDTA and rapidly shear the cells into a single-cell suspension with a P1000 pipette tip with 1 mL of E8 + ROCKi medium.

3.5. Count the cells with a hemocytometer. For a suspension that may contain millions of iPSCs, add 20 µL of cell suspension to 180 µL of trypan blue. Determine the number of total cells in the single-cell suspension.

3.6. Transfer 0.432×10^6 to 1.296×10^6 cells (10^5 to 3×10^5 cells/cm^2) to the remaining E8 + ROCKi medium. Remove the gelatinous mixture coating from the newly-coated 12-well plate and add 1 mL of cell suspension to each well, ensuring that the cells are well-distributed and do not form small clumps.

NOTE: This range of densities is optimal for differentiation of CD34 positive cells; however, these seeding densities are cell line-dependent and may need to be optimized by the user of this protocol.

3.7. After 24 h of culture, replace medium with 1 mL of fresh E8 medium. Incubate for an additional 24 h under the same conditions.

3.8. Remove the E8 medium and replace with LaSR Basal supplemented with 6-12 µM of CHIR99021. This timepoint is defined as D0 of differentiation. After 24 h of culture, replace with the same cell culture medium.

NOTE: As described previously, the amount of CHIR99021 added to this medium will depend on the iPSC line utilized. Please test a range of CHIR99021 concentrations to ensure optimal results.

3.9. After 48 h of incubation with CHIR99021, replace with 2 mL of LaSR Basal.

3.10. Replace the medium daily with 2 mL of LaSR Basal for 2-3 additional days. At this time point (D5 of differentiation), a significant proportion of these cells will express CD34 and can be characterized as endothelial progenitors. A schematic of this protocol is outlined with representative results in **Figure 1** and described in full by the investigators who first published this method [11, 19].

NOTE: These endothelial progenitors can be further differentiated along an endothelial lineage (35%–99%) or a smooth muscle lineage (1%–65%). Differentiation efficiencies vary depending on the type of ECM coating and the cell culture medium used during differentiation [20].

4. FACS of endothelial progenitors

4.1. Before dissociating the D5/D6 differentiated cells, prepare sorting buffer as described in step 1.5 and keep on ice.

4.2. Incubate the differentiated cells with 500 µL of cell detachment solution per well (see **Table of Materials**) for 10 min at 37 °C.

4.3. Dissociate the cells with a P1000 pipette tip into a single cell suspension in cell detachment solution. Consolidate the cell-cell detachment solution mixture in a 15 mL conical centrifuge tube and centrifuge for 5 min at 300 x *g*.

4.4. Remove the supernatant and resuspend in 200 µL of ice-cold sorting buffer (prepared as described in step 1.7. Add 5 µL of the concentrated CD34-PE antibody to the cell-sorting buffer suspension and incubate for 10 min at 4 °C.

4.5. Re-suspend in 5 mL of ice-cold sorting buffer. Filter this suspension through a cell strainer with a 40 µm cap before placing on the sorter.

4.6. On the appropriate fluorescent channel, sort 10,000 cells that have not been labeled with a fluorescent antibody. Gate a region at a high fluorescent intensity that does not contain any of these 10,000 cells (**Figure 1D**). This will serve as a negative control.

4.7. Run the sample containing iPSC-EPs labeled with a fluorescent solution and begin the sort. CD34 is highly expressed in these endothelial progenitors and is easily separated from the main population. After sorting, transfer the solution to a 15 mL conical centrifuge tube and centrifuge for 5 min at 300 x *g*. Remove the supernatant.

NOTE: If the solution is in a tube larger than a microcentrifuge tube (e.g., a 5 mL polystyrene tube commonly employed in many FACs protocols), re-suspend the sorted cell pellet in 1 mL of sorting buffer and transfer this solution to a microcentrifuge tube. Centrifuge at 300 x *g* for 5 min and remove the supernatant.

4.7.1. Optional: if further iPSC-EP characterization is desired, add other primary antibodies (e.g., CD31-APC) simultaneously with CD34-PE. Ensure that crosstalk between different fluorescent channels is minimized.

5. Encapsulation and long-term culture of iPSC-EP-laden collagen hydrogels

5.1. Prepare seeding medium by adding 40 µL of 10x medium 199 to 400 µL of endothelial growth medium (EGM-2) supplemented with 10 µM Y-27632 in a 1.8 mL microcentrifuge and place tube on ice.

NOTE: While the use of antibiotics is discouraged for stem cell culture and the maintenance of differentiated cells, dosing with Pen/Strep is recommended after sorting because it is difficult to guarantee sterility in the sorting environment (this is more imperative if the sorter is shared among many laboratories).

5.2. Remove all the supernatant from the cell-suspension and add 200 µL of EGM-2 supplemented with 10 µM Y-27632. Transfer the cell suspension to seeding medium and mix vigorously.

5.3. Add 350 µL of collagen to the solution prepared in step 5.2 and mix well. The solution will become pale yellow.

NOTE: The final concentration of collagen can have a significant impact on the formation of the capillary plexus. This protocol assumes that the collagen has been supplied at 10 mg/mL and that the hydrogels have a final concentration of 3.5 mg/mL. Adjust these volumes to achieve their final collagen concentration; it is recommended to restrict the final collagen concentration from 2 mg/mL to 4 mg/mL.

5.4. Add 5 µL of 1M NaOH to the solution prepared in 5.3 and mix well, avoiding the introduction of air bubbles. The solution will become bright pink.

5.5. Pipette 56 µL of neutralized collagen-cell solution prepared in 5.4 into individual wells of a 96-well ultra-low attachment U-bottom cell culture plate. Incubate at 37 °C for 30 min. Before

adding media, check that the cells have been evenly distributed by examining the samples with a brightfield microscope.

5.6. Prepare 1 mL of culture medium comprised of EGM-2 supplemented with 10 µM Y-27632 and 50 ng/mL vascular endothelial growth factor (VEGF) by adding 1 µL of each stock solution to a microcentrifuge tube. After mixing well, pipette 100 µL of this cell culture medium onto the cell-laden hydrogels. Transfer the plate to 37 °C for long-term culture.

5.7. Replace the culture medium daily, adding additional angiogenic growth factors or small molecule inhibitors as dictated by the goal of the study. To optimally remove the media, tilt the plate and use a P100 tip to gently aspirate medium without disturbing the hydrogel.

6. Fixing, immunostaining, and visualization of EP-based vascular networks

6.1. After one week of culture, add 250 µL of 4% paraformaldehyde (PFA) solution to a 48-well plate. Fill as many wells as there are hydrogels. Remove the medium from the hydrogels (PFA) and use fine-tipped tweezers to transfer the hydrogels to PFA containing wells. Incubate for 10 min at room temperature and remove the PFA by washing rapidly with PBS.

6.2. Permeabilize by adding 250 µL of 0.5% of a nonionic surfactant (see **Table of Materials**) for 5 min at room temperature. Wash twice with 250 µL of PBS supplemented with 500 mM glycine. Incubate at room temperature for 5 min for each washing step to ensure the removal of excess detergent. Block by immersing the hydrogels in 250 µL of blocking buffer for 30 min.

6.3. Incubate with the desired primary antibodies diluted in blocking buffer overnight at 4 °C. For example, if immunostaining with phalloidin and VE-cadherin, dilute 1:40 and 1:200, respectively, in blocking buffer.

6.4. The next day, remove the primary antibody solution and wash twice with 0.5% emulsifying reagent in DPBS. Cover with aluminum foil and incubate with a species-specific secondary antibody (e.g., 1:200 Alexa Fluor 488 in PBS) for 2 h at room temperature.

6.5. Remove the secondary antibody solution and wash twice with 0.5% emulsifying reagent in DPBS. Incubate at room temperature for 5 min for each washing step to ensure the removal of free fluorophores.

6.6. To visualize cell nuclei, dilute 4', 6-diamidino-2-phenylindole (DAPI) 1:10000 in PBS and add to the sample(s).

6.6.1. Incubate for 2 min at room temperature and wash twice with PBS. Incubate at room temperature for 5 min for each washing step to ensure the removal of free fluorophores.

6.7. Transfer the samples to an angiogenesis µ-Slide or a glass bottom petri dish using fine-tipped tweezers. Ensure that no air bubbles are trapped underneath the hydrogel.

6.8. Image on a confocal microscope by acquiring z-stacks that extend from the bottom to the top of the sample. Ensure that the detector is not saturated and that the lowest magnification available is in use (a large field-of-view is desirable).

NOTE: For future processing, ensure that the z-stacks are saved with minimal compression (e.g., .czi).

7. Using the computational pipeline to analyze and compare vascular network topologies

7.1. Inspect each z-stack to ensure that slices only contain vessels. Open the z-stack in ImageJ and enhance the contrast by clicking **Image** > **Adjust** > **Brightness/Contrast**. The vessel borders are now clearly demarcated, and the background level is minimized.

CAUTION: ECs will migrate towards the edges of the gel and will form small cobblestone colonies. While these are useful to estimate the boundaries of the hydrogel, they will interfere with the final image analysis and should be deleted.

NOTE: ImageJ is a Java-based open-source image analysis software developed in concert by the National Institutes of Health and the Laboratory for Optical and Computational Instrumentation. It is recommended to download Fiji, which is simply ImageJ bundled with useful plugins (https://fiji.sc/).

7.2. In ImageJ, blur the image in 3-dimensions clicking **Process** > **Filters** > **Gaussian Blur 3D** and then setting the sigma values in all 3 dimensions to 2.0 (this value might need to be adjusted by the end user).

7.3. In ImageJ, click **Process** > **Binary** > **Make Binary** and then select an appropriate thresholding algorithm. The cross-entropy thresholding algorithm developed by Li et al. is effective in separating vessels from the background[21]. Calculate the threshold for each image and set the background to “Default”.

7.4. In ImageJ, remove spurious noise and fill in “holes” in lumens by clicking **Process** > **Noise** > **Remove Outliers**.

NOTE: Removing “bright” outliers will fill in small holes in connected vessels; removing “dark” outliers will remove dead cells. The size of the removal radius will vary based on the magnification and size of the vessels. For images acquired with a wide-field objective that are 512 x 512 pixels, the radii will typically range from 4-6 pixels.

7.5. Process all raw (e.g., czi extension) files and convert into .tif files before proceeding to step 7.6. To aid in this processing, an ImageJ script, “Binarize and Filter.ijm” has been attached to this manuscript.

7.6. Save the processed .tif files in the same folder as the file "BatchProcessSkeleton.m", available for download in the manuscript. This script, developed by the authors, calls the functions published by Phillip Kollmannsberger[22] and conducts some additional file manipulation.

7.7. Download and unzip all files associated with the two main functions "Skel2Graph3D" (https://www.mathworks.com/matlabcentral/fileexchange/43527-skel2graph-3d) and "Skeleton3D" (https://www.mathworks.com/matlabcentral/fileexchange/43400-skeleton3d). Save all the functions into the same folder as the opened processed z-stack.

7.8. Open a multi-paradigm numerical computing environment (see **Table of Materials**) and navigate to the folder where all the files described above have been saved.

7.9. Run "BatchProcessSkeleton.m" either by typing **BatchProcessSkeleton** in the Command Window or by opening the script and hitting the Run button (right-facing green arrow) in the Editor.

NOTE: To analyze a large dataset with a single command, ensure that the string inside the *dir* function in line 6 contains an asterisk (e.g., '*.tif'). Otherwise, replace the asterisk with the file name if the analysis of a single file is desired.

7.10. The length of time that this script will take to execute will vary substantially based on the computing power available. It is recommended to conduct this analysis on a computer with at least 8 GB of RAM so that the user can access other programs while the script runs.

CAUTION: While not strictly necessary, graphing the original confocal acquisition (in gray) and overlaying with this image matrix with the skeleton matrix (in red) can aid in evaluating the performance of the skeletonization algorithm. In addition, the reader can create a nodal graph, as implemented by Kollmannsberger et al., to evaluate the accuracy of the network analysis. Creating both graphs, while useful, will dramatically increase the runtime of the script and require additional memory; if the user simply requires a final topology analysis and does not want to visualize the data set, simply comment out lines XX to YY (skeleton graph) and lines YY to ZZ (topology graph).

7.11. Upon completion, the **Data** matrix, visible in the Workspace, now contains the processed data. Double click **Data** and open this cell in the Variable editor.

7.11.1. Note that this matrix will contain, from left to right: 1) the file name, 2) the number of nodes (branch points + end points), 3) branch points, 4) links (i.e., vessels), 5) network length (including isolate vessels), and 6) the number of slices contained in the z-stack. Save this data as a .csv file and export for further analysis to any software of choice.

REPRESENTATIVE RESULTS:

After differentiation (**Figure 1**), FACS, and encapsulating of iPSC-EPs in collagen hydrogels, the cells will typically remain rounded for 24 h before beginning to migrate and form initial lumens.

After about 6 days of culture, a primitive capillary plexus will be visible in the hydrogel when viewed with brightfield microscopy **(Figure 2**). After imaging the fixed, stained cell-laden hydrogels on a confocal microscope (**Movie 1, Supplemental Movie 1**), the pre-processed images are converted to a skeleton which enables an analysis of the overall length and connectivity of the network. These quantitative measures can then be used to determine which set of conditions are optimal for producing robust vascular networks.

This protocol allows for the development of a robust, three-dimensional capillary plexus that is responsive to local physical and chemical cues. In previous work, this network formation has been shown to be sensitive to matrix density, matrix stiffness, matrix metalloprotease inhibition, and the type and concentration of various angiogenic mitogens[20,23].

FIGURE AND TABLE LEGENDS:

Figure 1: Generation of iPSC-EPs from pluripotent stem cells. (**A**) WiCell 19-9-11 iPSCs, which stained positive for Oct4, were cultured in E8 medium supplemented with 10 µM Y-27632 ROCK inhibitor for 48 h. (**B**) The iPSCs were then induced with 6 µM of CHIR99021 in LaSR Basal medium for 48 h, at which point the cells were positive for Brachyury, a mesoderm marker. (**C**) The cells were further cultured in LaSR Basal media until they expressed CD34, a marker for endothelial progenitors. (**D**) Roughly 15%–25% of the differentiated cells expressed CD34. All scale bars represent lengths of 200 µm.

Figure 2: Generation and analysis of iPSC-EP vascular networks in collagen hydrogels. (**A**) A cross-section of the 3D microenvironment used in this assay to promote vascular network formation from iPSC-EPs. A floating collagen hydrogel is seeded with iPSC-EPs and exposed to EGM-2 supplemented with 50 ng/mL VEGF and a temporal dose of Y-27632. (**B**) The resulting capillary plexus is highly branched and interconnected, as visualized via staining with F-actin (cyan). The binarized image, shown on the left, is generated by pre-processing with ImageJ. This z-stack is then analyzed via a previously developed algorithm, which skeletonizes the network (shown in a collection of thin red lines) and then analyzes the network topology for branches (yellow), end points (blue), isolated vessels (black), and connected vessels (red). The scale bar represents a length of 100 µm. (**C**)Morphological changes of iPSC-EPs-laden collagen hydrogels: 24 h after seeding, the iPSC-EPs remain spherical and within 96 h gradually take on a more elongated phenotype. Further culture results in assembly of lumen-containing VE-Cadherin network, as shown in the inset at the 144-h time point. The scale bars represent lengths of 400 µm; green = VE-Cadherin, red = F-actin, and blue = DAPI.

Movie 1: **Z-stack of VASCULATURE GENERATED FRom iPSC-EPs.** Vascular networks were fixed, stained with F-actin, and visualized by acquiring z-stacks on a confocal microscope. Slices were acquired at 17 µm intervals.

Supplemental Movie 1: 3D rendering of vessels. Vascular networks were fixed, stained with F-actin (red) and VE-cadherin (green), and visualized by acquiring z-stacks on a confocal microscope.

DISCUSSION:

This protocol involves the long-term culture of cells in three types of cell culture media: E8, LaSR Basal, and EGM-2. Therefore, great care should be taken to appropriately sterilize all materials. Additionally, lab coats and ethanol-cleaned gloves should always be worn when working in the laboratory's laminar flow hood. It is recommended to frequently test for mycoplasma contamination; if a large amount of debris is observed during iPSC culture or a sudden drop in differentiation efficiency is noted, mycoplasma contamination is a likely cause. iPSC-EPs generated with this protocol tend to deposit a significant amount of ECM, which lengthens the dissociation time and causes the single cell suspension to separate into small clumps. Do not use Trypsin-EDTA (0.25%), as the solution may disrupt CD34 epitopes on iPSC-EPs. However, treatment with collagenase/dispase solution may remove deposited ECM and ease dissociation with a standard cell detachment solution. After extensive dissociation, some small clumps of cells and ECM may remain; remove these clumps with a P100 pipette tip, as they are likely to clog the cell strainers or interfere with the FACS.

Pluripotent stem cells are sensitive to dissociation and will undergo apoptosis in a single-cell suspension unless a ROCK inhibitor (commonly Y-27632) is added to the medium[24]. iPSC-EPs are also sensitive to dissociation; including Y-27632 at 10 µM for the first 24 h of culture is imperative to increase cell survival and proliferation. Therefore, it is critical that a ROCK inhibitor is included in both the hydrogel and surrounding medium immediately following FACS. The seeding density of iPSC-EPs also significantly impacts vessel development and total network size. Generally, a concentration of 500,000 cells/mL to 3 million cells/mL is an appropriate range of seeding densities. A further increase in seeding density often leads to hydrogel compaction and cell death.

The density of ECM structural proteins has been found to have a significant impact on the development of engineered microvasculature[25,26]. Generally, increasing the density of an ECM-based hydrogel (often collagen or fibrin) limits vascular network length and connectivity. Therefore, it is critical that careful attention is paid to the collagen matrix density; concentrations below 2 mg/mL promotes the premature proteolytic degradation of collagen hydrogels, which results in an irreparable loss of the hydrogel's structural integrity. In contrast, concentrations above 4 mg/mL induce the formation of short, wide lumen that exhibit poor connectivity; hydrogel deformability and a change in pore size are primarily responsible for this abrogation[20].

Acellular and cell-laden collagen hydrogels do not bind to the ultra-low attachment plates; the hydrogels tend to remain suspended in the media and will occasionally float to the top of the well. If tissue culture-treated plates are employed in this protocol, the embedded progenitors will migrate to the bottom of the well and will form a near confluent monolayer at the bottom of the plate. The resulting decrease in cell seeding density inhibits the assembly of these progenitors into 3D networks. Additionally, if hydrogels are cast into the wells of a tissue culture treated plate, they will weakly bind the inner surface of the well; this binding applies strain to the hydrogel. Since this platform was developed to isolate the importance of physical and chemical

cues, it is critical that no extraneous forces are imparted to the cells[27]. The floating hydrogels may be difficult to feed because most of the cell culture media lies below the hydrogel; to overcome this, tilt the plate and use a P100 pipette to remove media from the side of the wall.

When imaging the vascular networks on a confocal microscope, it is critical to follow all washing steps to ensure that excess fluorophore does result in a low signal to noise ratio. Excess fluorescence may confuse default thresholding algorithms and make the z-stack difficult to binarize. To overcome this issue, use phalloidin, a fungal toxin that selectively labels F-actin and displays limited off-target binding. In general, use primary antibodies that have been pre-conjugated to a secondary antibody to limit the concentration of free fluorophore that diffuses through the gel. When generating the z-stacks a slice depth of 10-20 microns is recommended to balance the time needed for acquiring a z-stack against the need for a high-resolution image.

Here a quantitative assay to assess the vasculogenic potential of iPSC-EPs in 3D microenvironments is described. This assay utilizes open source software and is unaffected by user biases. Still, it represents an oversimplified model of the physiological vascular niche. While iPSC-EPs can differentiate into the cells needed for mature, functional vasculature, this assay neglects the contributions of other cell types (e.g., fibroblasts and macrophages) that participate in vasculogenic processes. Furthermore, this system is static and does not expose the developing vessels to flow. Finally, while collagen I is one of the dominant proteins in the ECM[28] and maintains its fibrillar architecture in vitro, it is lot-dependent and is limited to weak physical crosslinking when neutralized at high temperatures. Despite these limitations, this assay represents a significant step forward in the quest to engineer functional vasculature for cardiovascular disease therapy and modeling.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure

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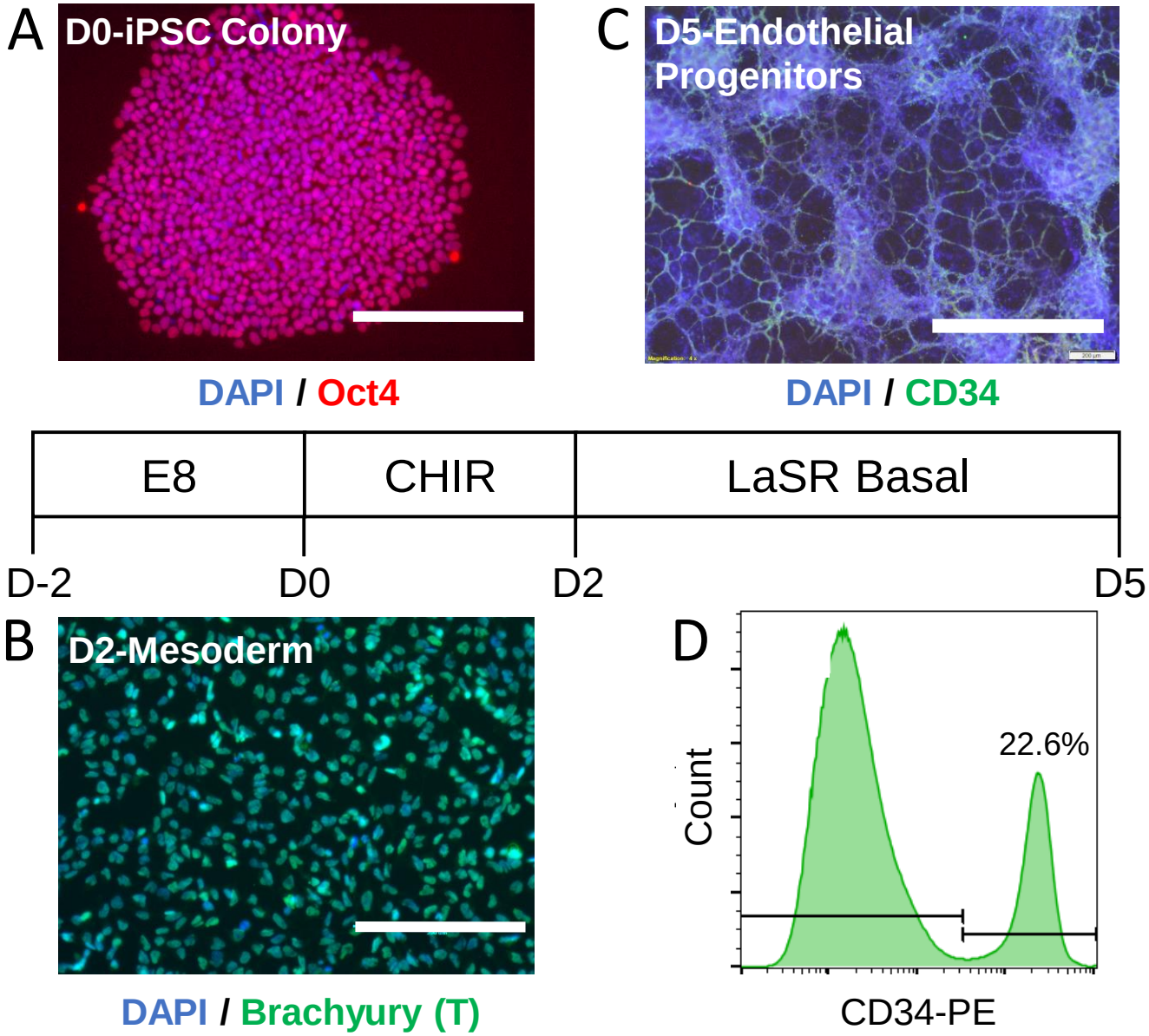

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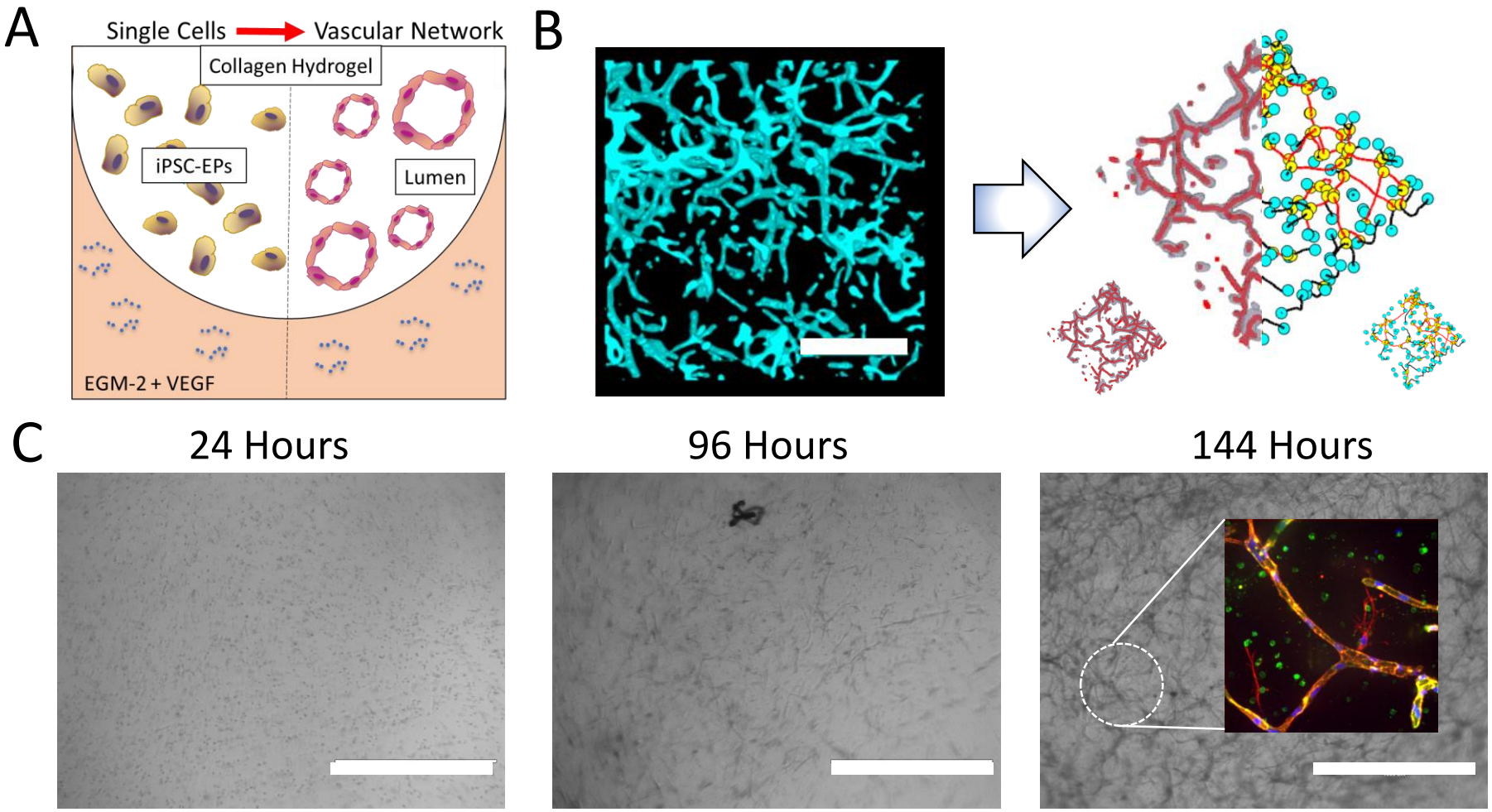

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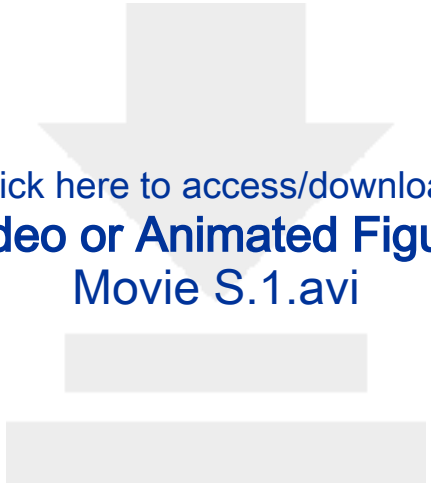

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Table of Materials

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Name of Material/Equipment	Company	Catalog Number
µ-Slide Angiogenesis	Ibidi	N/A
96 well, round bottom, ultra low attachment microplate, sterile	Corning	7007
Accutase	STEMCELL Technologies	7920
Advanced DMEM/F12	Thermo Scientific	12634010
Barnstead GenPure xCAD Plus	Thermo Fisher Scientific	50136165
Bovine Serum Albumin solution,7.5% in DPBS, sterile-filtered, BioXtra, suitable for cell culture	Fisher Scientific	A8412
CD34-PE, human (clone: AC136)	Miltenyi Biotec	130-098-140
CHIR99021	LC Laboratories	C-6556
Collagen I Rat Tail High Protein 100 mg	VWR	354249
Conical centrifuge tubes (15/50 mL)	Fisher Scientific	14-959-49D/A
DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride)	Thermo Fisher Scientific	D1306
DMEM/F12	Thermo Fisher Scientific	11320-082
Dulbecco's phosphate-buffered saline (DPBS)	ThermoFisher	14190-250
EDTA	Sigma-Aldrich	E8008
Endothelial Cell Growth Medium 2	PromoCell	C-22011
Essential 8 Medium	Thermo Fisher Scientific	A1517001
Glycine,BioUltra, for molecular biology, >=99.0% (NT)	Sigma-Aldrich	50046
L-Ascorbic acid 2-phosphate sesquimagnesium salt hydrate,>=95%	Sigma-Aldrich	A8960
MATLAB	MathWorks	1.8.0_152
Matrigel Matrix GFR PhenolRF Mouse 10 mL (gelatinous protein mixture)	Fisher Scientific	356231
Medium-199 10X	Thermo Fisher Scientific	1825015
Microcentrifuge tubes (1.7 mL)	VWR	87003-294
Phosphate-buffered saline (PBS)	Sigma-Aldrich	P3813
Penicillin-Streptomycin (10,000 U/mL)	Thermo Fisher Scientific	15140122
Recombinant Human VEGF 165 Protein	R&D Systems	293-VE

Rhodamine phalloidin	Themo Fisher Scientific	R415
Triton X-100 (nonionic surfactant)	Sigma-Aldrich	X-100
Tween-20 (emulsifying reagent)	Fisher Scientific	BP337
VE-Cadherin (F-8)	Santa Cruz Biotechnology	sc-9989
Vitronectin	ThermoFisher	A14700
Y-27632	Selleck Chemicals	S1049

Comments/Description
A flat, glass bottom tissue-culture plate with side walls enables facile confocal imaging
Prevents the binding of cell-laden collagen hydrogels to the cell culture dish
Gentle cell detachment solution; does not degrade extracellular epitopes vital for FACS
The base media for iPSC-EP differentiation.
Water purification system; others can be readily substituted
To preserve cell viability when FACs sorting
Antibody used for FACs isolation of iPSC-EPs
Induces the formation of mesoderm from pluripotent stem cells
Main component of the 3D microenvironment
Used to store and mix relatively large volumes of reagents and cell culture media
To counterstain and visualize cell nuclei
For dilution of Matrigel and thawing of pluripotent stem cells
To wash monolayer cultures
For passaging of pluripotent stem cell colonies and to prevent cell aggregation when FACs sorting
Promotes endothelial cell viability and proliferation
For maintenance of pluripotent stem cells
Neutralizes remaining detergent
Component of iPSC-EP differentiation medium
Multi-paradigm numerical computing environment (free available at most academic institutions)
Diluted in DMEM/F12 to coat plates for iPSC-EP differentiation
Used to balance final hydrogel osmolarity and pH
Stores small volumes of reagents
The main ingredient of the immunostaining solutions
Antibiotic used after sorting to remove possible contamination from FACS instrument
Mitogen that stimulates endothelial cell proliferation and tubulogenesis

To identify F-actin deposition and therfore outline the borders of the vascular networks
Detergent used to gently permeabilize cells
Increases the binding specificity of the added antibodies
To identify 3D endothelial lumen in collagen hydrogels
For maintenance of pluripotent stem cells
Preserves pluripotent stem cell and iPSC-EP viability when dissociated and re-seeded

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Dear Dr. Steindel,

Thank you for considering our manuscript JoVE59342 "Evaluating the vasculogenic potential of iPSC-derived endothelial progenitors in 3D collagen microenvironments" for publication in JoVE. We hope that our responses (outlined below) sufficiently addressed the comments we received and that our manuscript is now suitable for publication.

Please note that we have decided to change the title of the manuscript to fit its focus better: “A novel in vitro 3D model and computational pipeline to quantify the vasculogenic potential of iPSC-derived endothelial progenitors”.

Editorial Comments:

Comment 1: Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues

Response: We apologize for the presence of any spelling or grammar issues in the original document. These have now been corrected.

Comment 2: 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. You may use the generic term followed by “(see table of materials)” to draw the readers’ attention to specific commercial names. Examples of commercial sounding language in your manuscript are: Matrigel, Falcon, Accutase, Eppendorf, Triton-X, Tween-20, MathWorks, MATLAB, etc.

Response: These have been removed.

Comment 3: Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Response: These have been removed.

Comment 4: Please revise the protocol to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Please move the discussion about the protocol to the Discussion.

Response: These have been fixed.

Comment 5: Please revise the Protocol steps so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step. Use sub-steps as necessary.

Response: These have been fixed.

Comment 6: Approximate volumes for all buffers, gradients, and stock solutions to be set up should be given.

Response: Reviewer 3 has kindly pointed this out to us and we have revised the manuscript to include these numbers. Please refer to our response to comment 4 of Reviewer 3.

Comment 7: Step 4.6: Please specify gating strategies.

Response: This needed further detail and the following step has been added as outlined below:
4.6 On the appropriate fluorescent channel, sort 10,000 cells that have not been labeled with a fluorescent antibody. Gate a region at a high fluorescent intensity that does not contain any of these 10,000 cells (Figure 1D). This serves as a negative control.

Comment 8: 6.3: Please specify how the primary antibody is appropriately diluted.

Response: This has been revised as outlined below:
Incubate with the desired primary antibodies diluted in blocking buffer overnight at 4°C. For example, if immunostaining with phalloidin and VE-cadherin, dilute 1:40 and 1:200, respectively, in blocking buffer.

Comment 9: 6.4: Please specify the secondary antibody used here.

Response: This was unclear, so we have added an example in the text: (e.g., 1:200 Alexa Fluor 488 in PBS)

Comment 10: Figure 1: Please label and describe different panels in the figure legend.

Response: This has been addressed and the figure caption has been updated accordingly.

Comment 11: Please rename Figure 2 as Movie 1 and update figure numbers in the manuscript. Please include a scale bar in the movie if possible.

Response: This has been addressed and a scale bar has been added to the movie, as requested.

Comment 12: Table of Materials: Please sort the items in alphabetical order according to the name of material/equipment.

Response: This has been addressed. We have also added additional items as requested by Reviewer 3.

Reviewer 1

Comment 1: The manuscript does not include the percentage of ECs and mural cells (SMCs or pericytes) generated from the protocol. The authors would benefit from including FACS data of EC and SMC markers to characterize the efficiency of differentiation using this specific protocol.

Response: To address this comment we added **Figure 1D**, a flow cytometry distribution that shows the percentage of $CD34^+$ iPSC-EPs generated from this protocol (in our experience, this percentage varies from 10-25%). Regarding the generated number of ECs and SMCs, we have thoroughly characterized $CD34^+$ iPSC-EP bipotency in a manuscript recently accepted for publication in Tissue Engineering; we have reproduced the relevant data below.

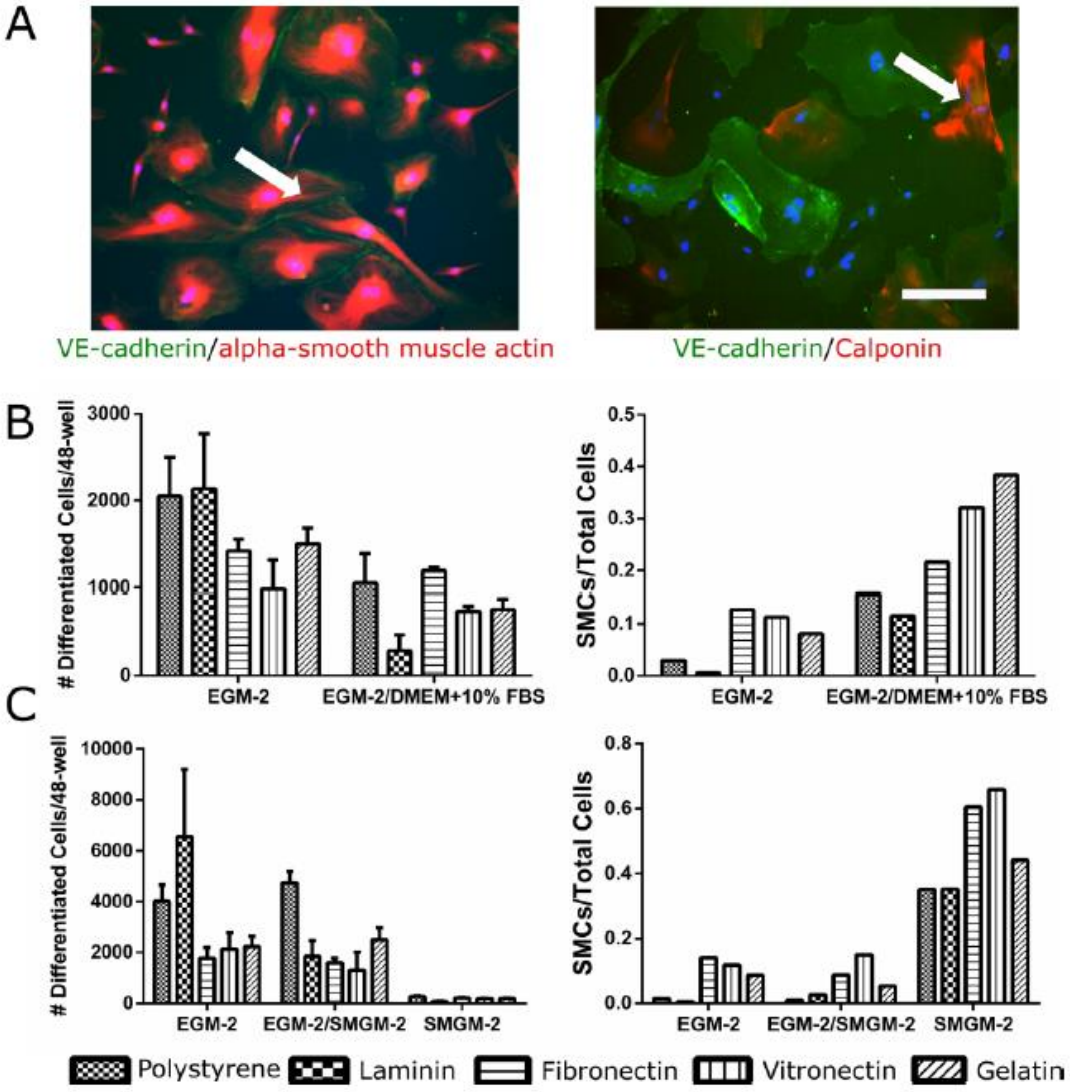


Figure S.2: FACS-isolated CD34$^+$ iPSC-EP are bipotent. (A) Following differentiation of iPSC-EPs into iPSC-ECs and iPSC-SMCs, cells were immunostained with endothelial marker (VE-cadherin) and smooth muscle cell markers (alpha-smooth muscle actin (α-SMA), and calponin). Most iPSC-ECs stained positively for both VE-cadherin and α-SMA (left image; arrow points to VE-cadherin positive junctions). We therefore used calponin to distinguish iPSC-SMCs from iPSC-ECs as shown in the right image (arrow points to calponin positive SMC). Scale bar for both images is 400 μm. (B) CD34$^+$ iPSC-EPs were cultured on ECM protein-coated plates for 7 days in EGM-2, DMEM supplemented with 10% FBS, or a combination of the two media (n=3). No data is shown for the latter medium because the cells underwent rapid apoptosis hours after seeding. (C) CD34$^+$ iPSC-EPs were cultured on ECM protein-coated plates for 7 days in EGM-2, SMGM-2, or a combination of the two media (n=3). All counts are reflective of the total cell populations 7 days after seeding.

This is now cited as **reference 20** in the manuscript; additionally, to aid the reader, we have added the following:
NOTE: These endothelial progenitors can be further differentiated along an endothelial lineage (35-99%) or a smooth muscle lineage (1-65%). Differentiation efficiencies vary depending on the type of ECM coating and the cell culture medium used during differentiation [20].

Comment 2: While there is inclusion of F-actin staining to determine the development of a primary capillary plexus from iPSC-EPs, it would be more convincing to stain the networks with an EC specific marker such as CD31.

Response: F-actin is indeed insufficient to claim that extended cells have formed an endothelial lumen. To image the network, we employed rhodamine-phalloidin because the fungal toxin exhibits very low off-target binding; in other words, there is very little fluorescence visible in the interstitial space between lumens. However, to better define the endothelial nature of the described capillary plexus, **we stained cell-laden collagen hydrogel containing fully formed lumen with VE-cadherin**, a canonical endothelial marker. We rendered this image in three dimensions and have added it as a supplemental movie (**Movie S.1**).

Comment 3: The authors developed a computational pipeline that uses ImageJ to pre-process acquired confocal images, however, they have not included examples of the data output. Furthermore, there is no mention of a comparison of the accuracy of their method to the standard methods commonly used and listed in the manuscript. It would be helpful to show a side-by-side comparison of their results, in comparison to those from more established approaches like Imaris or Metamorph.

Response: Please refer to our response to Reviewer 3, who asked for similar data. In short, we have included a sample data file, figures of this confocal network and node-based topology, and the associated statistics.

Comment 4: The authors state that "We have observed that the collagen hydrogels do not bind to the ultra-low attachment plates; the hydrogels tend to remain suspended in the media and will occasionally float to the top of the well." Are these acellular hydrogels or cell-seeded ones?

Response: We should clarify that this observation holds true for acellular and cell-laden hydrogels. The text has been amended as follows:
Acellular and cell-laden collagen hydrogels do not bind to the ultra-low attachment plates; the hydrogels tend to remain suspended in the media and will occasionally float to the top of the well.

Comment 5: The authors should cite the origin of their differentiation protocol (i.e., "Directed endothelial progenitor differentiation from human pluripotent stem cells via Wnt activation under defined conditions. Xiaoping Bao, Xiaojun Lian, and Sean P. Palecek.")

Response: We thank the reviewer for their valuable comment. In addition to the original paper, “Lian, X. et al. Efficient differentiation of human pluripotent stem cells to endothelial progenitors via small-molecule activation of WNT signaling. Stem Cell Reports” (citation 11), we added the suggested reference (citation 19) as follows:
Replace the medium daily with 2 mL of LaSR Basal for 2-3 additional days. At this time point (D5 of differentiation), a significant proportion of these cells will express CD34 and can be characterized as endothelial progenitors. A schematic of this protocol is outlined with representative results in Figure 1 and described in full by the investigators who first published this method [11,19].

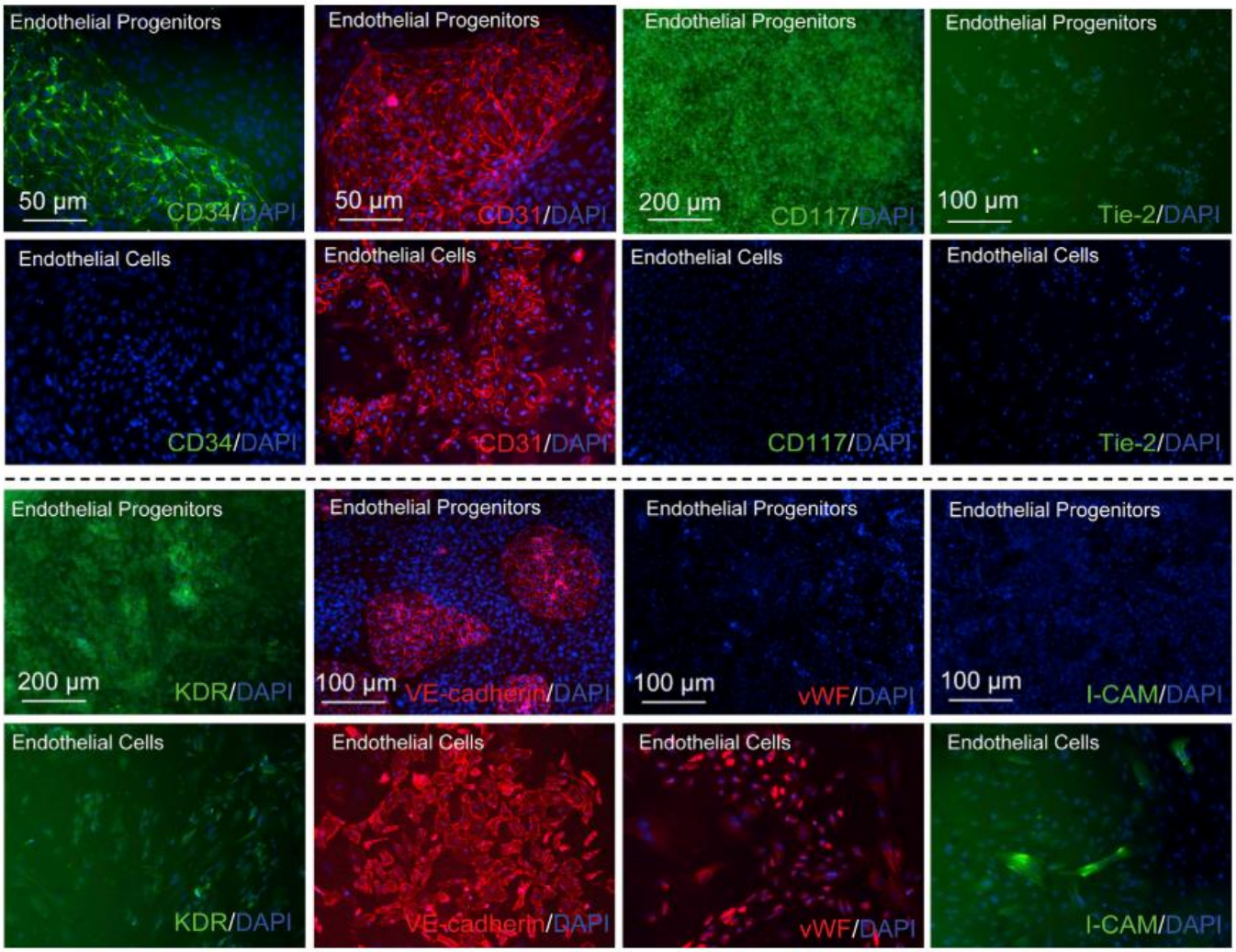
Comment 6: What is the concentration of collagen used? And what vendor? The volume of NaOH to be added is dependent on the molar concentration of acid in the collagen.

Response: The reviewer makes two excellent points, and we would like to address each individually.
First, to address the importance of the concentration of collagen, we have added the following note:
NOTE: the final concentration of collagen can have a significant impact on the formation of the capillary plexus. This protocol assumes that the collagen has been supplied at 10 mg/mL and that the hydrogels have a final concentration of 3.5 mg/mL. Adjust these volumes to achieve their final collagen concentration; it is recommended to restrict the final collagen concentration from 2 mg/mL to 4 mg/mL.
The supplier of the collagen used in this study, as noted in the Table of Materials, is VWR (Corning, Product number 354249).
Second, as noted by the reviewer, the purpose of the NaOH is to neutralize the acetic acid present in the collagen solution and to thereby encourage spontaneous fibrillation at elevated (e.g., incubator) temperatures. 5 μL of 1M NaOH has been shown to be sufficient to neutralize collagen that has been solubilized in 0.02 N acetic acid at a final concentration of 3.5 mg/mL[10].

Reviewer 2

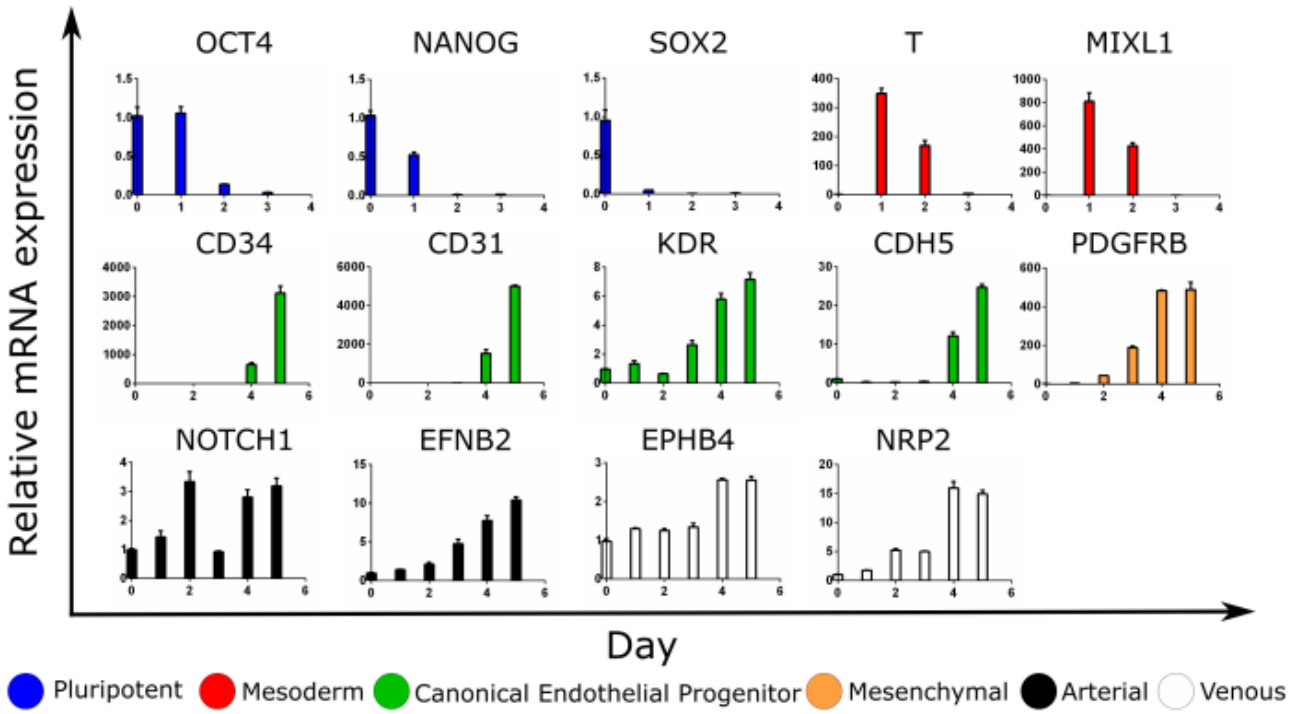
Comment 1: The "progenitor" character of endothelial cells is very often defined not just by CD34 expression but also the presence of CD133 and VEGFR2 - did the authors check for that or do they would like to include this in the characterization step?

Response: We agree that CD34 alone is insufficient to define a population of endothelial progenitors. The Palecek research group that first described this method to generate pluripotent stem cell-derived endothelial progenitors, performed a thorough characterization of these markers and found that these iPSC-EPs also displayed high levels of **CD31, CD117, Tie-2, KDR, and VE-cadherin (**reproduced below for Reviewer 2's convenience**)**[11]**.**



In our recently accepted manuscript in *Tissue Engineering: Part A*, we have also analyzed a set of canonical endothelial progenitor, pluripotency, arterial, venous, and mesenchymal markers. We characterized these markers for the following populations: D5 differentiated cells, sorted CD34 positive ($CD34^{+}$) cells, and their counterpart cell population expressing low or negative levels of CD34 ($CD34^{low/negative}$). We have reproduced this data below and cited this data in the manuscript as reference 20. We would like to direct the reviewer's attention to VEGFR2 (*KDR*) expression levels in these $CD34^{+}$ iPSC-EPs. Based on these data we anticipate that iPSC-EPs, expressing CD34 are highly likely to co-express the markers discussed above. We, therefore, do not believe a characterization step is necessary but added this as an optional step, as follows:

4.7.1 Optional: if further iPSC-EP characterization is desired, add other primary antibodies (e.g., CD31-APC) simultaneously with CD34-PE. Ensure that crosstalk between different fluorescent channels is minimized.



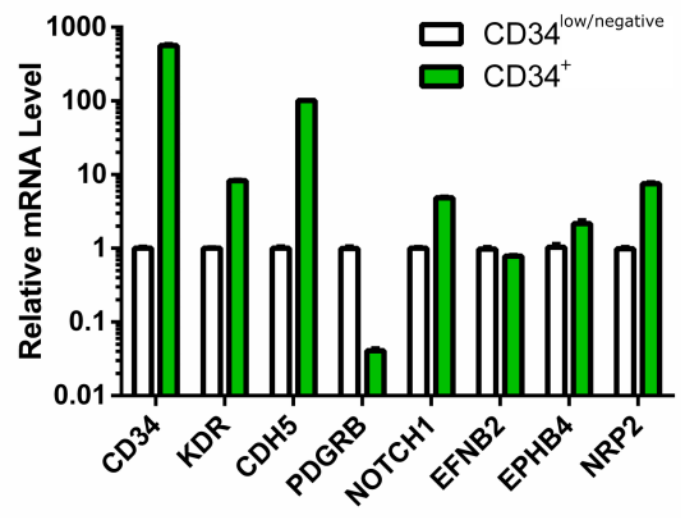


Figure 1: (Top) mRNA was isolated from the unsorted cells every 24 hours after CHIR99021 induction. qPCR was performed to evaluate temporal gene expression profiles during the five days of differentiation into iPSC-EPs. (Bottom) Expression levels of EP and EC markers of sorted $CD34^{+}$ iPSC-EPs were compared to the counterpart subpopulation which is low or negative for CD34 expression ($CD34^{low/negative}$).

Comment 2: I would suggest including one chapter on troubleshooting. Sometimes the networks in 3D are very difficult to analyze with the given software. Such a chapter would be very beneficial for the reader.

Response: We have expanded sections 6 and 7 significantly to address these concerns. Also, we believe that a considerable amount of detail regarding troubleshooting can be currently found in the discussion.

Comment 3: Please include also the following reference, where also detailed network analyses are described: https://www.ncbi.nlm.nih.gov/pubmed/25687101 (Charwat et al., 2015)

Response: We apologize for not including this reference in the first version of the manuscript. We have added the following sentences:
In the past decade, different open-source computational pipelines and skeletonization algorithms have been developed to quantify and compare vascular network length and connectivity. For example, Charwat et al. developed a Photoshop-based pipeline to extract a filtered, binarized image of vascular networks derived from a co-culture of adipose-derived stem cells and outgrowth ECs in a fibrin matrix [13,14]. Perhaps the most widely used topology comparison tool is AngioTool, a program published online by the National Cancer Institute [15]; despite the program's widespread adoption and well-documented fidelity, the program is limited to analyzing vessel-like structures in two dimensions; other programs, including AngioSys and Wimasis, share the same dimensionality limitation [16].

Reviewer 3

Comment 1: In line 49: it's not clear what does "to inform current models of cardiovascular disease" means.

Response: We thank the reviewer for pointing out the lack of clarity in this sentence; This sentence has been changed as follows:
Such vascular platforms promise to illuminate molecular and tissue-level mechanisms of cardiovascular disease and may present physiological insight into the development of primitive vascular networks [2,3].

Comment 2: In line 89: the acronym iPSC-EPs is used for the first time, but it is not explained. Add the meaning of the acronym.

Response: The acronym has now been defined as follows:
In particular, protocols have been published that outline the generation and isolation of iPSC-derived endothelial progenitors (iPSC-EPs) [10,11]; iPSC-EPs are bipotent and can, therefore, be further differentiated into endothelial cells and smooth muscle cells/pericytes, the building blocks of mature, functional vasculature.

Comment 3: In step 1.1: Can the vitronectin solution be stored for future uses?

Response: As for most proteins, we do not recommend storing vitronectin in a sparse solution. The text has been updated as follows:
Once diluted, it is not recommended to store this solution for future use.

Comment 4: In steps 1.4, 1., 3.3, and 5.6: It would be more convenient to add actual volume values instead of concentrations. How much should the reader prepare? Also, how should it be done? This means to elaborate on how much material should be weighted, and which volume should be used to dilute the material. Please, be thorough when explaining if the reader should dilute the entire vial in a specific volume or should previously weight a certain amount of material. This helps making protocols much clearer.

a. Step 1.4: 100 mg/ml ascorbic acid magnesium-2-phosphate in ultrapure water.
b. Step 1.5: 10 mM CHIR99021 in DMSO.
c. Step 3.3: 10 µM Y-27632 in E8 medium.
d. Step 5.6: 50 ng/ml VEGF in Y-27632-supplemented EGM-2 medium.

Response: We thank the reviewer for their valuable comment, We have updated the text as follows:

a. **Step 1.4**: Prepare a 100 mg/mL stock solution of ascorbic acid magnesium-2-phosphate (a white lyophilized powder) in ultrapure water by adding 500 mg of powder to 5 mL of water in a 10 mL glass scintillation vial. Agitate with a stir bar until the solution is completely transparent (this may take up to an hour). This solution can be stored at -20 °C for up to one year.
b. **Step 1.5**: Create a 10 mM stock of CHIR99021 (CHIR) by dissolving 10 mg of lyophilized powder in 1.9928 mL of dimethyl sulfoxide (DMSO) in a 15 mL conical centrifuge tube and warm in a 37 °C bead (or water) bath until the solution is transparent. Aliquot into 1.8 mL microcentrifuge tubes and store at -20 °C for up to one year.
c. **Step 3.3**: During the above one-hour incubation, remove the E8 medium and Y-27632 from the refrigerator and freezer, respectively. Once E8 medium and Y-27632 reach room temperature, prepare E8 + ROCKi by adding 13 µL of 10 µM Y-27632 to 13 mL of E8 in a 15 mL conical centrifuge tube.

d. **Step 5.6**: Prepare 1 mL of culture medium comprised of EGM-2 supplemented with 10 µM Y-27632 and 50 ng/mL vascular endothelial growth factor (VEGF) by adding 1 µL of each stock solution to a microcentrifuge tube. After mixing well, pipette 100 µL of this cell culture medium onto the cell-laden hydrogels. Transfer the plate to 37°C for long-term culture.

Comment 5: In step 2.2: Is there a recommended number of cells for thawing and seeding? This may arise from a cell amount that the authors usually freeze per vial, but it is not revealed to the reader.

Response: We have added the following note to Step 2.2:
Note: Even under optimal conditions, iPSCs tend to recover poorly from cryopreservation. It is recommended to cryopreserve a near-confluent 6-well for future seeding into a single 6-well. Significant debris may be normal for 2-3 days during cell recovery.

Comment 6: In step 2.6:

a. It would be helpful to add some kind of parameter that will help the reader determine when to stop the EDTA incubation. There is a range (between 5 and 7 minutes), but with no information on how to choose either incubation time.

b. The same goes for the cell suspension. How can the reader know if he/she should take 75 µl or 200 µl?

Response: We have added the following note to Step 2.6.2:
CAUTION: The incubation time for iPSC detachment is cell-line dependent; it is recommended that the user of this protocol tests a range of times upon sufficiently expanding a received/generated iPSC line. 75 µL of cell suspension generally results in 4 days between passages; 150 µL or more leads to 2-3 days between passages.

Comment 7: In step 3.7:

a. It is not clear when the E8 medium added in step 3.6 should be removed to start step 3.7.

b. The preparation of the LaSR Basal could be explained in section "1. Preparation of Culture Media and Coating Solutions".

Response: We apologize for lack of clarity; We have added the following to Step 3.6:
Incubate for another 24 hours under the same conditions.

As suggested by the reviewer, we have moved the preparation of LaSR Basal to “1. Preparation of Culture Media and Coating Solutions”.

Comment 8: In step 3.8: Even though it is shown in the timeline in figure 1, it should be explicitly stated in the text the incubation duration of the LaSR Basal supplemented with ascorbic acid.

Response: LaSR culture duration is now described in steps 3.9 and 3.10 as follows:
3. 9 After 48 hours of incubation with CHIR99021, replace with 2 mL of LaSR Basal.
3.10 Replace the medium daily with 2 mL of LaSR Basal for 2-3 additional days.
.

Comment 9: In step 4.4:

a. The line "that has been conjugated to a bright secondary fluorophore" gives the notion that the reader should do this step. Maybe re-writing the sentence could help understand that the authors use a commercially available fluorophore-conjugated secondary antibody.

b. How much of the CD34 antibody should the reader use? What did the authors do in this step?

Response: We apologize for lack of clarity, we have updated the sentence as follows:
Add 5 µL of the concentrated CD34-PE antibody to the cell-sorting buffer suspension and incubate for 10 minutes at 4°C.

Comment 10: In step 4.5:

a. "centrifuge at for 5 minutes". Remove "at".

b. In line 257, after the centrifugation, there shouldn't be a suspension. Please, clarify if the reader should remove supernatant and resuspend in sorting buffer.

Response: This has been correct as follows:
Re-suspend in 5 mL of ice-cold sorting buffer. Filter this suspension through a cell strainer with a 40 µm cap before placing on the sorter.

Comment 11: In step 4.6, line 261: Is the term "isolation" used instead of "sorting"?

Response: We have changed “isolation” to “sorting” throughout the manuscript to prevent future confusion.

Comment 12: In step 5.4: Why do you add the 1M NaOH solution when the cells are suspended into the material? Can this be done to the collagen separately to avoid harming the cells?

Response: In our experience, gelation occurs relatively quickly after the addition of NaOH. This makes the addition of cells after the addition of NaOH difficult and can result in cell aggregation in the hydrogel. Based on our experience, more than 80% of the cells are viable 24 hours after encapsulation.

Comment 13: In step 6.1:

a. It is convenient to remind the reader that the used volume should cover the entire hydrogel.

b. Frequently used staining protocols perform a quick PBS wash between removing the medium and adding the PFA. Is this missing?

Response: We thank the reviewer for their valuable comment, we have updated our protocol as follows:
After one week of culture, add 250 µL of 4% paraformaldehyde (PFA) solution to a 48-well plate. Fill as many wells as there are hydrogels. Remove the medium from the hydrogels (PFA) and use fine-tipped tweezers to transfer the hydrogels to PFA containing wells. Incubate for 10 minutes at room temperature and remove the PFA by washing rapidly with PBS.

With regards to point b, we have noted that the networks are sensitive to temperature fluctuations and changes in cell culture medium composition. Therefore, to maximally preserve the integrity of the 3D networks, we do not perform an additional washing step. We have not noticed any adverse effects during the immunostaining process.

Comment 14: In step 6.2 and 6.3: There is no blocking step, nor is the primary antibody suspended in blocking solution. This is not common for staining protocols. These steps might have been forgotten.

Response: We apologize for this omission. In the first section, we have added the following paragraph:
To prepare blocking buffer, add 500 mg of lyophilized bovine serum albumin (BSA) and 50 µL of Tween-20 to 48 mL of DPBS. Incubate at 37 °C for at least 15 minutes to ensure that the BSA does not clump and subsequently separate from the solution.

We have added the following to step 6.2:
Block by immersing the hydrogels in 250 µL of blocking buffer for 30 minutes.

Step 6.3 now reads:
Incubate with the desired primary antibodies diluted in blocking buffer overnight at 4°C.

Comment 15: In step 6.6, line 319: "of" instead of "if".

Response: Corrected.

Comment 16: In step 6.7, line 322: "using" instead "with". Makes it clearer.

Response: Corrected.

Comment 17: In step 6.8: It would be helpful to explain the image output format/extension (e.g., TIF, LSM, CZI).

Response: The following note has been added to step 6.8:
NOTE: For future processing, ensure that the z-stacks are saved with minimal compression (.czi is recommended on Zen instruments).

Comment 18: In step 7.1:

a. Is this step performed using ImageJ?

b. The authors should comment more about the chosen correction parameters. What should be the expected output after the contrast correction?

Response: We apologize for not explaining adequately the utility of ImageJ. We have updated step 7.1 as follows:
Step 7.1 Inspect each z-stack to ensure that slices only contain vessels. Open the z-stack in ImageJ and enhance the contrast by clicking “Image → Adjust → Brightness/Contrast.” The vessel borders are now clearly demarcated, and the background level is minimized.

CAUTION: ECs will migrate towards the edges of the gel and will form small cobblestone colonies. While these are useful to estimate the boundaries of the hydrogel, they will interfere with the final image analysis and should be deleted.

NOTE: ImageJ is a Java-based open-source image analysis software developed in concert by the National Institutes of Health and the Laboratory for Optical and Computational Instrumentation. It is recommended to download Fiji, which is simply ImageJ bundled with useful plugins (https://fiji.sc/)

Comment 19: In steps 7.3, 7.4, 7.5: These steps should be described more thoroughly.

a. How are the commands opened? Explain the exact steps as it was done in step 7.1 using the arrows.

b. Which parameter values do the author recommend for each command?

Response: These steps have been expanded as follows (please note that the step numbers have changed since the last version):

Step 7.2: In ImageJ, blur the image in 3-dimensions clicking Process→Filters→ Gaussian Blur 3D and then setting the sigma values in all 3 dimensions to 2.0 (this value might need to be adjusted by the end user).
Step 7.3: In ImageJ, click Process → Binary → Make Binary and then select an appropriate thresholding algorithm. The cross-entropy thresholding algorithm developed by Li et al. is effective in separating vessels from the background 21. Calculate the threshold for each image and set the background to "Default".
Step 7.4: In ImageJ, remove spurious noise and fill in "holes" in lumen by clicking Process → Noise → Remove Outliers.
Sub-step: Removing "bright" outliers will fill in small holes in connected vessels; removing "dark" outliers will remove dead cells. The size of the removal radius will vary based on the magnification and size of the vessels. For images acquired with a wide-field objective that are 512 x 512 pixels, the radii will typically range from 4-6 pixels.

Comment 20: In step 7.6: Please explain how to load a multi-paged TIFF file in MATLAB into a variable. A better option is to add a code that does this.

Response: We have added the code responsible for this upload and updated the description of these Step 7.6 as follows:
Save the processed .tif files in the same folder as the file "BatchProcessSkeleton.m", available for download in the manuscript. This script, developed by the authors, calls the functions published by Phillip Kollmannsberger22 and conducts some additional file manipulation.

Comment 21: In step 7.7 and 7.9: Add links to the corresponding functions for the paper's online version. Explain how to install/unzip the files for the user to work with them.

Response: We have added the links to download the necessary files from the MATLAB depository. We will assume that the reader can download and unzip these files, as this only requires to follow the instructions provided on-screen and will vary considerably based on the requirements of different OS's.

Comment 22: In step 7.8 and 7.9: There are many missing key points for a reader with little experience in MATLAB. The authors should explain thoroughly how to perform ALL the steps to run the functions. The best way to achieve this is adding an example code with comments for each line, or at least for the lines that involve actions that might not be known to the reader. Moreover, an example image to perform the analysis would be a great and helpful addition, especially if the authors add the results they obtained for said image.

Response: We have overhauled this section and attached the necessary scripts for application. Please refer to manuscript for all of the updated text.

Comment 23: In step 7.9: There is no explanation on how the parameters are obtained for use. How can the reader analyze and compare the data they obtained after doing the image analysis? Where is this data stored? Again, this would be better explained by adding a detailed code. Furthermore, it is very important that the authors will give full detail of the steps performed to obtain the images in figure 3B, since they seem to use the data from step 7.9.

Response: We have overhauled this section and attached the necessary scripts for application. We have also attached a sample z-stack, its skeleton, its analyzed network, and final data output.

Comment 24: In lines 409-410: This should be added into the protocol section.

Response: This has been moved as suggested.

Comment 25: The next materials are missing from the materials list:
*Ultrapure water
*Accutase

Response: Both have been added to the Table of Materials.

Sincerely,

Janet Zoldan

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Supplemental Coding Files

BatchProcessSkeleton.m