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Method Article

Generation and morphological quantification of large scale, three-dimensional, self-assembled vascular networks

Jasmine Shirazi, Joshua T. Morgan, Erica M. Comber, Jason P. Gleghorn*

Dept. of Biomedical Engineering, University of Delaware, Newark, DE, United States

A B S T R A C T

One of the largest issues facing the field of tissue engineering is scaling due to tissue necrosis as a result of a lack of vascularization. We have developed an accessible method for generating large scale vascular networks of arbitrary geometries through the self-assembly of endothelial cells in a collagen gel, similar to vasculogenesis that occurs in the developing embryo. This system can be applied to a wide range of collagen concentrations and seeding densities, resulting in networks of varying phenotypes, lending itself to the recapitulation of vascular networks that mimic those found across different tissues. Methods are thus described for the generation and imaging of these self-assembled three-dimensional networks in addition to image processing methods for rigorous quantitative measurement of various morphological parameters. There are several advantages to the system described herein.

- Varied molding procedures allow for irregular geometries, similar to those that would be required for tissue grafts.
- Robust network formation translates into centimeter scale constructs.
- Whereas similar processes suffer from a high degree of variability and inconsistent characterization, our method employs image analysis techniques to stringently characterize each network based on several objective characteristics.

* Corresponding author.
E-mail addresses: jshirazi@udel.edu (J. Shirazi), jmorgan@engr.ucr.edu (J.T. Morgan), ecomber@andrew.cmu.edu (E.M. Comber), gleghorn@udel.edu (J.P. Gleghorn).

Specifications Table

Subject Area:	Engineering
More specific subject area:	Biomedical engineering, regenerative medicine
Method name:	Generation and analysis of self-assembled vascular networks
Name and reference of original method:	Morgan JT*, Shirazi J*, Comber EM, Eschenburg C, Gleghorn JP "Fabrication of centimeter-scale and geometrically arbitrary vascular networks using in vitro self-assembly" (2019) Biomaterials 189:37-47 (*indicates equal contribution)
Resource availability:	NA

Method details

Introduction

Here we describe a protocol for the fabrication of millimeter scale vascular networks in collagen gels of arbitrary shape and size. By seeding human umbilical vein endothelial cells (HUVECs) in collagen gels, we can induce vasculogenesis, or the *de novo* formation of a self-assembled vascular network [1]. We have also developed a novel image processing method to characterize the resulting network architecture and quantify network connectivity, coverage, tortuosity, lumenization, and vessel diameter. The advantage of using this accessible method is that it would introduce consistency to a field that otherwise relies on various methods of quantifying network formation. By quantitatively characterizing multiple aspects of the resulting vascular network, we are able to compare conditions in a way that captures a large range of variability among culture conditions.

Procedure

A. Collagen isolation

Collagen isolation can be carried out as described by others [2]. Whereas commercial sources of collagen may be used, rat-tail extraction protocols allow for greater flexibility in terms of starting stock concentration. We recommend dissolving the collagen sponge in 0.1% acetic acid at 8 mg/mL to take advantage of a larger range of collagen densities.

B. Creating wells of varying shapes and sizes

Large scale collagen gels can be seeded in polydimethylsiloxane (PDMS) wells of arbitrary shape and size. Wells can be shaped by cutting the desired shape out of the PDMS with a biopsy punch, or the PDMS can be poured around a 3D printed shape.

Materials:

- 90 mm petri dish
- Sylgard 184 Elastomer Base
- Sylgard 184 Curing Agent
- Biopsy punch (7 mm or other size, can go up to 19 mm), Leather punches of various shapes and sizes, 3D printed molds
- Scalpel or razor blade

Protocol (for punched wells):

1 Pour 18 g of Sylgard 184 elastomer base and 2 g of Sylgard 184 curing agent into a 90 mm petri dish. Mix well and allow to degas under vacuum for thirty minutes to remove air bubbles. Place the mixture into an oven (65 °C) overnight to cure. This will result in a 3 mm layer of PDMS. While the PDMS will cure in several hours, it is better to leave it overnight to minimize uncured oligomers, which are cytotoxic.
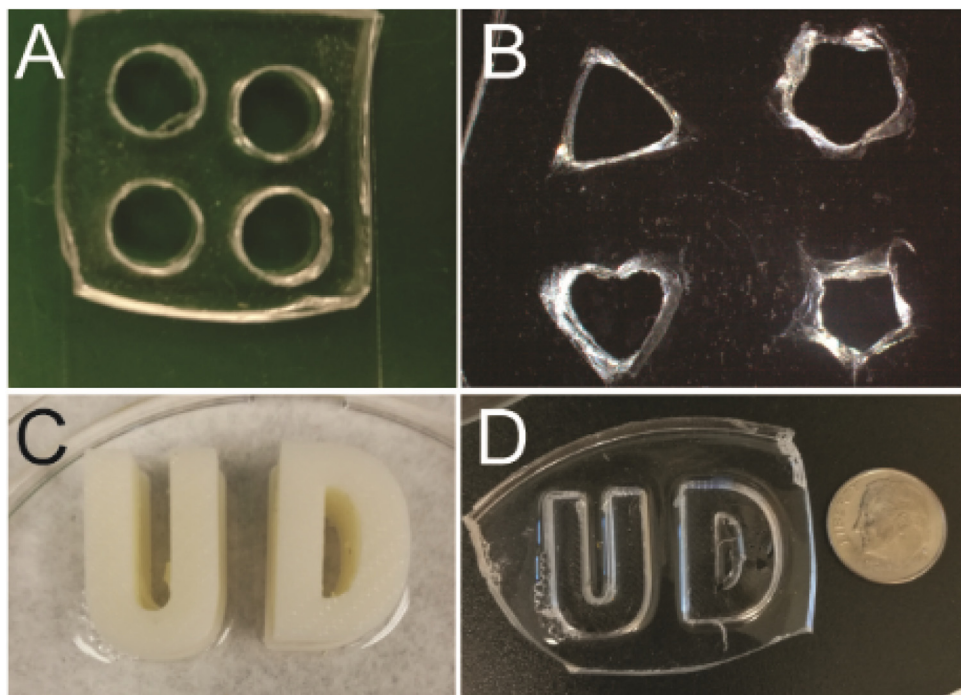
Fig. 1. Wells can be created using (A) a traditional biopsy punch (7 mm shown), (B) leather punches of arbitrary shape (flower, star, triangle, and heart shown), or (C) 3D printed constructs ["U" and "D" shown] that can be (D) de-molded.

2 Use a biopsy punch of the desired size to punch holes into the PDMS. Four wells with a diameter of 7 mm (100 μL of final gel mixture in each) are enough for a 500 μL final gel volume (Fig. 1A).
3 Alternatively, leather punches with irregular shapes can be used to form wells of varying shapes and sizes (Fig. 1B).
4 Use a scalpel or razor to cut the wells out. PDMS wells can be stored away from dust or functionalized as described on the following section.

Protocol (for 3D printed shapes):

1 Place the 3D printed shape in a 90 mm petri dish.
2 Pour 18 g of Sylgard 184 elastomer base and 2 g of Sylgard 184 curing agent into the dish, degas under vacuum for 30 min (Fig. 1C), and cure in an oven as described above.
3 De-mold (remove) 3D printed constructs from the cured PDMS.
4 Use a scalpel or razor to cut the away excess PDMS (Fig. 1D).
5 PDMS wells can be stored in a dust-free container until surface functionalization as described in the following section.

C. Surface functionalization of PDMS wells for collagen bonding

In order to avoid cell-induced contraction of the collagen gel, the edges of the PDMS well must be functionalized so that the collagen is bonded to the PDMS. This ensures that the boundary conditions of the gel are maintained.

Materials:

- 2% polyethylenimine (PEI) in deionized (DI) water
- 0.2% glutaraldehyde (GA) in DI water
- DI water (sterile)
- Harrick Plasma Cleaner
- Coverslips

Protocol:

1 Plasma clean the PDMS wells for 27 s at 800 mTorr on low using a Harrick Plasma Cleaner
2 Place the PDMS wells on a coverslip of appropriate size.
3 Cover the construct in 2% PEI and incubate for 30 min at room temperature
4 Wash three times with DI water
5 Incubate the construct in 0.2% GA for one hour at room temperature.
6 Wash three times with DI water and leave to air dry.
7 Place functionalized wells on coverslip of desired size.

D. Fabrication and culture of cellular collagen gels

Collagen gels of varying densities (2 mg/mL–6 mg/mL) can be fabricated using the values in Table 1. Cell density can be varied between 0.5×10^6 cells/mL to 2.0×10^6 cells/mL. To minimize gel contraction and maximize network formation, it is recommended that conditions along the axis shown in Fig. 2 are used.

Materials:

- Bucket of ice
- Biosafety cabinet
- Positive displacement pipette (P1000)
- 10X Hank's Buffered Salt Solution (HBSS)
- 1 M NaOH
- Human umbilical vein umbilical cells (HUVECs) (routinely cultured)
- Endothelial Growth Media-2 (EGM-2)
- Sodium ascorbate
- Phorbol 12-myristate 13-acetate (PMA)
- 0.05% Trypsin
- 1.5 mL microcentrifuge tubes
- Collagen stock solution in 0.1% acetic acid

Protocol:
NB: Unless stated otherwise, all steps should be performed aseptically on ice.

1 To make a 500 μL gel, pipette 50 μL of 10X HBSS into a 1.5 mL microcentrifuge tube (Table 1)

Table 1
Component ratios for collagen gelation mixture.

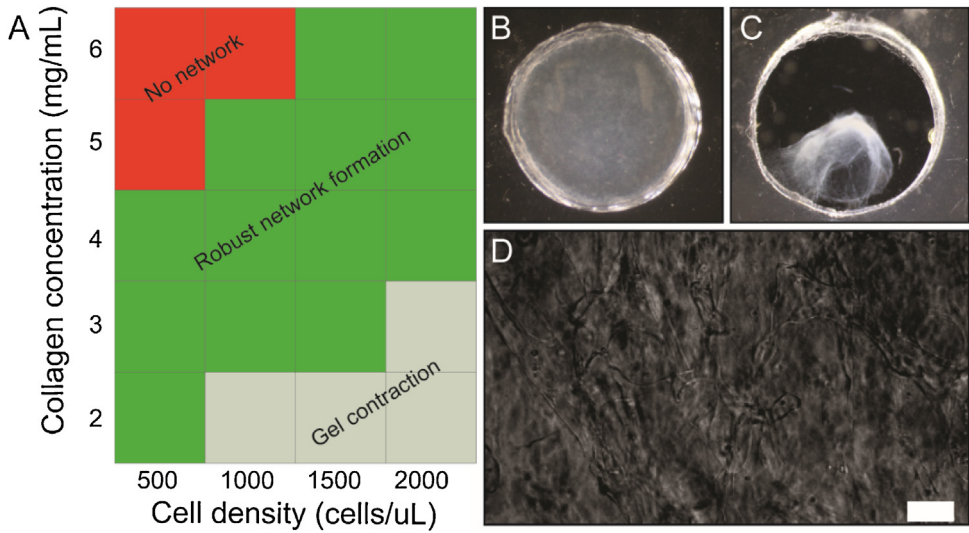|	Collagen stock conc: 8 mg/mL Final volume: 0.5 mL				
	2 mg/mL	3 mg/mL	4 mg/mL	5 mg/mL	6 mg/mL
10X HBSS (μL)	50	50	50	50	50
Collagen stock (μL)	125	188	250	313	375
1N NaOH (μL)	3	4	6	7	9
Cell suspension (μL)	322	258	194	130	66

Fig. 2. We have characterized a (A) large state space to determine which conditions result in optimal network formation. The areas highlighted in green will result in robust network formation, the conditions in red result in no network. (B) PDMS well edges are functionalized so that gels adhere; however parameters in the gray region of the state diagram result in significant cellular contraction and (C) gel contraction even with our surface functionalization protocol. (D) Brightfield image of robust network formation (scalebar = 150 μm).

2 Depending on the desired collagen concentration, add the appropriate amount of 1 M NaOH to neutralize the acidic collagen stock to a pH of 7. (Note: Each batch of collagen will require pH calibration. To do this, create a test sample with no cells using the recommended amount of 1 M NaOH and test with pH paper. Adjust NaOH as needed, and scale accordingly. For example, if half the recommended amount of NaOH is required for a 2 mg/mL gel, then also use half of the recommended amount for a 5 mg/mL gel.)

3 Trypsinize HUVECs, count, and aliquot the appropriate volume (based on desired seeding density) into a separate microcentrifuge tube.

4 Centrifuge the aliquoted HUVECs at 0.3 rcf for 5 min and resuspend in the appropriate volume of EGM-2 medium according to Table 1.

5 Pipette the appropriate volume of the stock collagen solution onto the side of the microcentrifuge tube containing the 10X HBSS and 1 M NaOH. A positive displacement pipette is recommended for the viscous collagen solution.

6 Quickly add the cell solution to the same tube while also washing the collagen off of the side and into the mixture. This neutralizes the solution quickly to prevent premature gelation (collagen rapidly gels under basic conditions) or cell death due to exposure to a base (NaOH) or acid (acetic acid from the collagen stock).

7 Gently mix everything by slowly pipetting. Aspirate from the bottom of the tube and deposit near the top. Pipetting too quickly will result in air bubbles.

8 Slowly pipette the appropriate volume into a PDMS well. One 7 mm well can hold 100 μL of gel. A 500 μL sample can fill four 7 mm wells, accounting for sample loss.

9 Leave the resulting gels at 37 °C for 30 min. to polymerize. Gels will look opaque when fully polymerized (Fig. 2B).

10 Following the incubation, add EGM-2 media supplemented with 50 ng/mL PMA and 50 μg/mL sodium ascorbate (vasculogenesis media; VM). Culture in an incubator at 37 °C and a humidified 5% CO_2 environment.
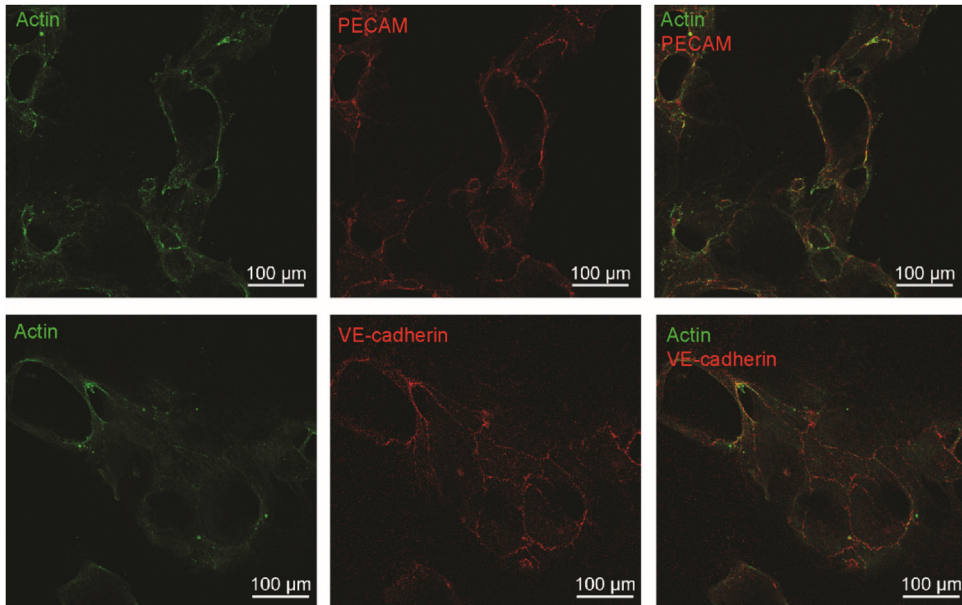
Fig. 3. Immunofluorescent staining for vascular markers produces comparable images to phalloidin.

11 Change the media daily. Network formation should occur within 1–3 days and be complete within 4–7 days.

E. Fixing and staining

Constructs must be fixed and fluorescently stained for subsequent imaging. A simple phalloidin stain is sufficient for the vascular networks described herein. However, this protocol can be used for immunostaining endothelial cell markers such as PECAM or VE-cadherin as well (Fig. 3). This ability and compatibility with imaging processing methods described herein allow for identification of endothelial cells and vascular networks from any additional stromal cells seeded in the bulk collagen gel.

Materials:

- 4% paraformaldehyde (PFA)
- Dulbecco's phosphate-buffered saline (DPBS)
- Triton-X
- Fluorescently conjugated phalloidin
- Relevant primary and secondary antibodies

Protocol:

1 Wash the cultured gels three times in DPBS.
2 Incubate the gels in 4% PFA with 0.1% Triton-X for 2 h at 4 °C.
3 Wash the gels three times in DPBS.
4 Block overnight at 4 °C with 1% bovine serum albumin/0.2% cold-fish gelatin/0.1% Tween-20 in PBS. *(optional)*
5 Incubate the gels overnight in mouse anti- PECAM1 (1:3200) (Cell Signaling), mouse anti-VE-cadherin (1:500), or any other relevant antibodies overnight at 4 °C and wash three times in DPBS.
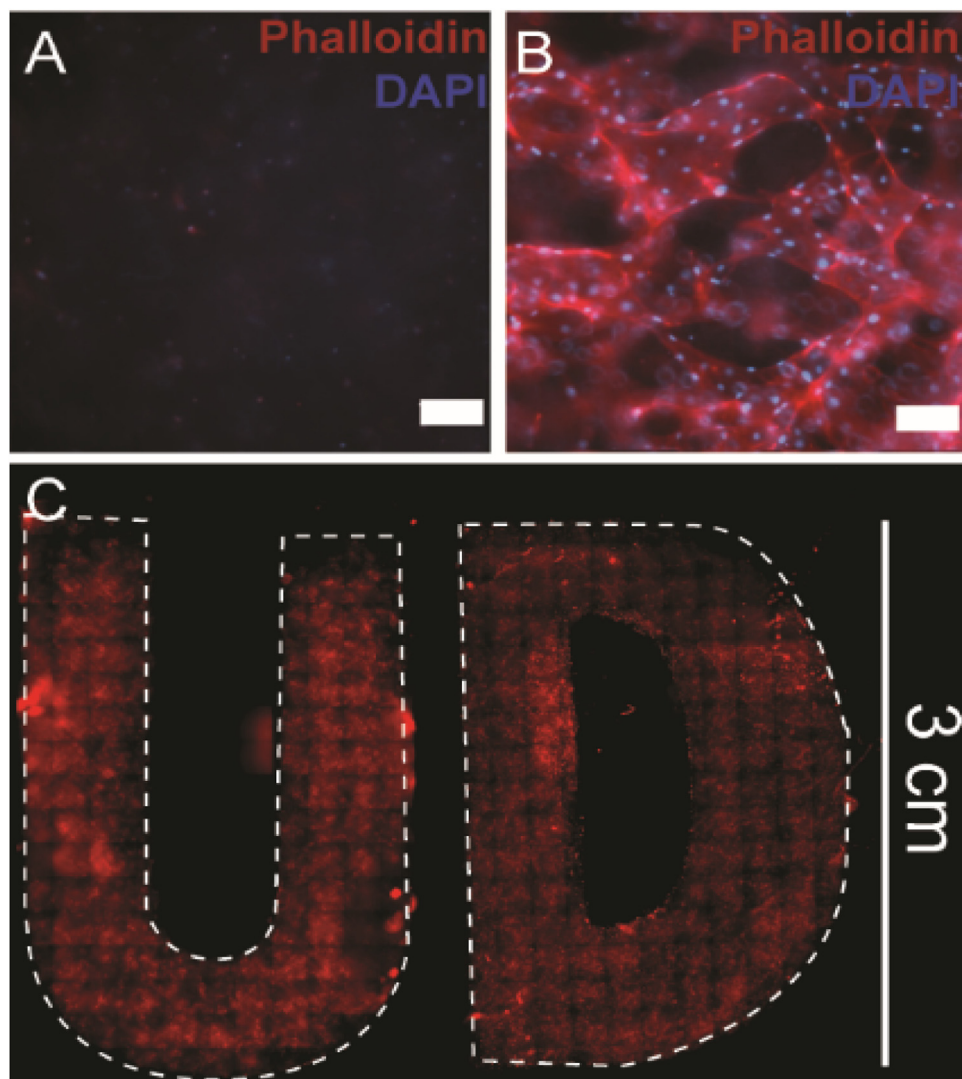
Fig. 4. Fixed and stained gels showing (A) no network formation and (B) robust network formation (scalebars = 100 μm), and (C) large-scale custom shape.

6 Incubate gels in the appropriate secondary antibodies (optional) along with fluorescently conjugated phalloidin (1:200) (Cell Signaling) overnight at 4 °C.

7 Wash gels three times in DPBS and store hydrated until imaging (Fig. 4).

F. Imaging

The image analysis and quantification methods in this protocol rely on confocal z-stacks that capture fluorescently labeled cells (to assess network phenotype) and reflectance microscopy to capture the collagen gel (to confirm lumenization).

Materials:

• Confocal microscope

Protocol:

1 Image collagen gel on a confocal microscope using the following settings:
   a 10X objective
   b 2 channels: (1) Fluorescence channel that corresponds with phalloidin stain; (2) Reflected light to capture collagen
   c Z-stack: Include as many slices as desired, preferably more than 200. 6 μm increments are recommended.
   d Include a minimum of 4 tiles stitched with 10% overlap

G. Image Analysis

We have developed custom algorithms to quantify various metrics pertaining to network phenotype. For clarity, the overall algorithm is broken into several individual sub-algorithms. Each sub-algorithm is detailed with steps and pseudocode provided for each step. For clarity, variables are indicated with *italics* and operations in ALL CAPS ().

Materials

• MATLAB (Release 2015a or later, Mathworks, Natick, MA)

Protocol (Enhancing and Segmenting Phalloidin Signal) (Fig. 5):

1 Compensate for decreasing laser illumination with depth using linear adjustment of the image to provide 5% low-end saturation and 0.2% high-end saturation
   a FOR each *plane*
      ADJUST *plane* mapping to saturate 5% and 0.2% of dark and bright image pixels, respectively
      ENDFOR
2 Smooth the image using 3D median filtering with a $5 \times 5 \times 5$ pixel ($6.9 \times 6.9 \times 30$ μm) median filter
   a MEDIANFILTER *volume* with $5 \times 5x5$ kernel
3 Correct for uneven illumination using reconstruction [3] based tophat filtering using a disk structuring element with a radius of 50 pixel (69 μm)
   a DEFINE *strel* as a 50 pixel (69 μm) disk
      FOR each *plane*
         DEFINE *mask* as *plane*
         DEFINE *marker* as morphological EROSION of *plane* using *strel*
         DEFINE *newplane* as the image RECONSTRUCTION of *mask* and *marker*
         SUBTRACT newplane from plane to form the tophat filtered plane
         ASSIGN tophat filtered plane to plane
      ENDFOR
4 Enhance contrast using the CLAHE algorithm [4] with 24×24 tiling and 256 output bins
   a DEFINE *strel* as 50 pixel (69 μm) disk
   b FOR each *plane*
      APPLY CLAHE to *plane* using 24×24 tiling and 256 output bins
      ENDFOR
5 Smooth and connect the enhanced image using morphological closing on a $5 \times 5 \times 5$ pixel ($6.9 \times 6.9 \times 30$ μm) neighborhood
   a CLOSE *volume* with $5 \times 5 \times 5$ kernel
6 Segment the image into a binary volume using hysteresis thresholding with thresholds of 5% and 19% of full-scale intensity
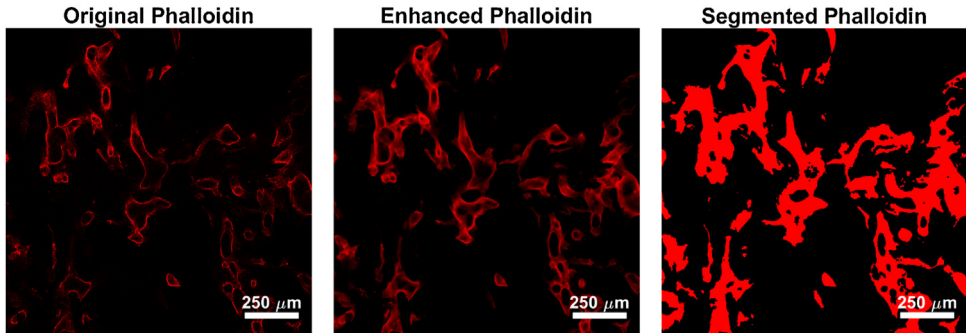   a THRESHOLD *volume* using hysteresis with a low threshold of 5% and high threshold of 19% full-scale

Fig. 5. Enhancement and segmentation of representative phalloidin stain.

Protocol (Enhancing and Segmenting Reflectance Signal) (Fig. 6):

1 Apply phase-preserving denoising [5] to smooth the reflection signal using the following parameters: 3 standard deviations of noise rejection, 7 filter scales with a factor of 3 multiplication between each, 12 filter orientations (15° apart), and no soft thresholding
   a FOR each *plane*
      DENOISE *plane* with 3 standard deviations of noise rejection, 7 filter scales with a factor of 3 multiplication between each, 12 filter orientations (15° apart), and no soft thresholding
      ENDFOR
2 Smooth the image using 3D median filtering with a $5 \times 5 \times 5$ pixel ($6.9 \times 6.9 \times 30 \mu$m) median filter
   a MEDIANFILTER *volume* with $5 \times 5 \times 5$ kernel
3 Compensate for decreasing laser illumination with depth using linear adjustment of the image to provide 1% low-end saturation and 0.5% high-end saturation
   a FOR each *plane*
      ADJUST *plane* mapping to saturate 1% and 0.5% of dark and bright image pixels, respectively
      ENDFOR
4 Enhance using reconstruction [3] based bottom-hat filtering using a sphere structuring element with a radius of 50 pixel (spatially ellipsoidal with radius of 69 μm in image plane and 300 μm)
   a DEFINE *strel* as a 50 pixel sphere (spatially ellipsoidal with radius of 69 μm in image plane and 300 μm)
      DEFINE *mask* as the COMPLEMENT of *volume*
      DEFINE *marker* as the COMPLEMENT of morphological DILATION of *volume* using *strel*
      DEFINE *newvolume* as the image RECONSTRUCTION of *mask* and *marker*
      SUBTRACT the COMPLEMENT of *newvolume* from *plane* to form the *bottom-hat filtered volume*
      ASSIGN the COMPLEMENT of bottom-hat filtered volume to volume
5 Smooth and connect the enhanced image using morphological closing using a sphere structuring element with a radius of 5 pixel (spatially ellipsoidal with radius of 6.9 μm in image plane and 30 μm)
   a CLOSE *volume* with 5 pixel radius sphere (spatially ellipsoidal with radius of 6.9 μm in image plane and 30 μm)
6 Correct planar (X–Y) variations in image intensity using reconstruction [3] based tophat filtering using a $5 \times 5 \times 61$ neighborhood ($6.9 \times 6.9 \times 366 \mu$m)
   a DEFINE *strel* as a $5 \times 5 \times 61$ neighborhood ($6.9 \times 6.9 \times 366 \mu$m).
      DEFINE *mask* as *volume*
      DEFINE *marker* as morphological EROSION of *volume* using *strel*
      DEFINE *newplane* as the image RECONSTRUCTION of *mask* and *marker*
      SUBTRACT new volume from volume to form the tophat filtered volume
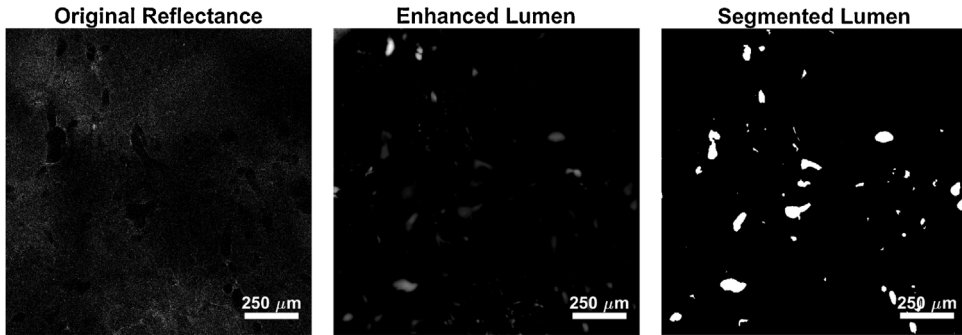   ASSIGN tophat filtered volume to volume

Fig. 6. Enhancement and segmentation of representative reflectance image.

7 Remove small defects in image using area opening to eliminate anything under 100 voxels (1140 μm^3)

  a AREAOPEN *volume* with 100 voxel threshold (1140 μm^3)

8 Perform an initial greedy segmentation on the image using hysteresis thresholding with thresholds of 2% and 5% of full-scale intensity

  a THRESHOLD *volume* using hysteresis with a low threshold of 2% and high threshold of 5% full-scale

9 Reference to phalloidin volume to ignore voids in collagen not lined with cells.

  a DEFINE *mask1* as the CLOSING of *binary phalloidin volume* with a 5-pixel radius spherical structuring element

    DEFINE *mask2* as the FILLING of *mask1* holes.

    ASSIGN logical AND of *volume* and *mask2* to *binary lumen volume*

  Protocol (Volume Based Metrics) (Fig. 6)

1 Define a Volume Fraction, V_F, as the number of network voxels compared to total number of voxels

  a DEFINE *network volume* as a logical OR of *binary lumen volume* and *binary phalloidin volume*

    DEFINE *volume fraction* as the COUNT of true voxels in *network volume* pDIVIDED by the size of *network volume*

2 Define tissue coverage through a representative Diffusion Length, L_D, a 90th percentile of tissue distance from the closest vessel.

  a DEFINE *distance map* as the EUCILDEAN DISTANCE TRANSFORM of *network volume*

    DEFINE *cdf* as the empirical cumulative distribution function of all voxel values in *distance map*

    DEFINE *diffusion length* as the 90% percentile distance of *cdf*

3 Define a Contiguous Fraction, Φ_C, as the volume of the largest connected component normalized to the network volume

  a DEFINE *cc* as a list of the CONNECTED COMPONENTS of *network volume*

    DEFINE *cc volumes* as the number of voxels within each entry of *cc*

    DEFINE *total volume* as the number of voxels in *network volume*

    DEFINE normalized cc volumes as cc volumes DIVIDED by total volume

    DEFINE contiguous fraction as the MAXIMUM of normalized cc volumes

  Protocol (Skeletonization) (Fig. 7):

1 Perform a skeletonization using a modified form of the fast marching method described by van Uitert and Bitter [6].

  a DEFINE *distance map* as the INVERSE EUCILDEAN DISTANCE TRANSFORM of *network volume*

    DEFINE *max distance* as the MAXIMUM value of *distance map*
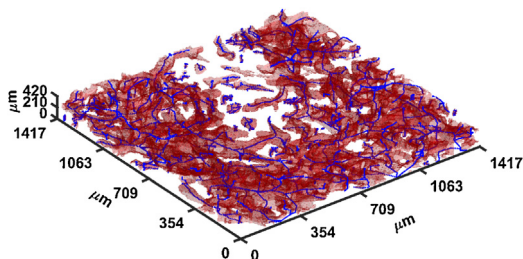
Fig. 7. Representative skeletonization (blue) of network volume (red). For clarity, only a partial volume is shown.

DEFINE speed volume as the distance map DIVIDED by max distance
DEFINE *cost volume* as RECIPROCAL of *speed volume*
DEFINE *source* as the location of the MAXIMUM of *distance map*
DEFINE *geodesic distance map* as the GEODESIC DISTANCE TRANSFORM of *network volume* from *source*
DEFINE *start* as the location of the MAXIMUM of *geodesic distance map*
DEFINE *skeleton branch* as the FAST MARCHING PATH between *source* and *start* across *cost volume*
DEFINE *branch length* as the GEODESIC DISTANCE along *skeleton branch*
DEFINE length threshold as max distance MULTIPLIED by 2
WHILE branch length is GREATER THAN length threshold
    DEFINE *source* as locations of all prior *skeleton branch*
    DEFINE *geodesic distance map* as the GEODESIC DISTANCE TRANSFORM of *network volume* from *source*
    DEFINE *start* as the location of the MAXIMUM of *geodesic distance map*
    DEFINE *skeleton branch* as the FAST MARCHING PATH between *source* and *start* across *cost volume*
    DEFINE branch length as the length of skeleton branch
ENDWHILE
DEFINE skeleton volume as all skeleton branches

Protocol (Skeleton Based Metrics)

1 Define a Lumen Fraction, Φ_L, as the fraction of skeleton voxels that overlap with the binary lumen volume
  a DEFINE *skeleton lumen volume* as a logical OR of *binary lumen volume* and *skeleton volume*
    DEFINE *lumen fraction* as the COUNT of true voxels in *skeleton lumen volume* pDIVIDED by the size of *skeleton volume*
2 Define average diameter based Euclidean distance between the skeleton and the edge of the network volume
  a DEFINE *distance map* as the INVERSE EUCILDEAN DISTANCE TRANSFORM of *network volume*
    DEFINE *average diameter* as the average of *distance map* values using a region defined by *skeleton volume*
3 Define average tortuosity, τ, as the average of branch length and branch chord overall all skeleton branches
  a DEFINE *branch length* as the GEODESIC DISTANCE along each *skeleton branch*
    DEFINE *branch chord* as the EUCILDEAN DISTANCE between ends of each *skeleton branch*
    DEFINE branch tortuosity as branch length DIVIDED by branch chord
    DEFINE average tortuosity as the MEAN over all branch tortuosity

Acknowledgements

References

[1] J.T. Morgan, J. Shirazi, E.M. Comber, C. Eschenburg, J.P. Gleghorn, Fabrication of centimeter-scale and geometrically arbitrary vascular networks using in vitro self-assembly, Biomaterials 189 (Jan 2019) 37–47.
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