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.

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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.
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 $0.5 \times 10^6$ cells/mL to $2.0 \times 10^6$ 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)

| 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% CO₂ environment.
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.
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) Reflecte 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
   b Smooth the image using 3D median filtering with a 5 × 5 × 5 pixel (6.9 × 6.9 × 30 μm) median filter
   a MEDIANFILTER volume with 5 × 5x5 kernel
   b 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
   c 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
   c Smooth and connect the enhanced image using morphological closing on a 5 × 5 × 5 pixel
   (6.9 × 6.9 × 30 μm) neighborhood
   a CLOSE volume with 5 × 5 × 5 kernel
   b 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
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
      - END FOR

2. Smooth the image using 3D median filtering with a $5 \times 5 \times 5$ pixel ($6.9 \times 6.9 \times 30 \, \mu \text{m}$) 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
      - END FOR

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 ($6.9 \times 6.9 \times 366 \, \mu \text{m}$)

   a. DEFINE strel as a $5 \times 5 \times 61$ neighborhood ($6.9 \times 6.9 \times 366 \, \mu \text{m}$)

      - 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

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**Fig. 5.** Enhancement and segmentation of representative phalloidin stain.
7 Remove small defects in image using area opening to eliminate anything under 100 voxels (1140 µm³)
   a AREAOPEN volume with 100 voxel threshold (1140 µm³)
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, VF, 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, LD, 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, FC, 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
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
ENDWHILE
DEFINE skeleton volume as all skeleton branches

Protocol (Skeleton Based Metrics)

1 Define a Lumen Fraction, \( \Phi_L \), 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 EUCLIDEAN 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, \( \tau \), 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

Fig. 7. Representative skeletonization (blue) of network volume (red). For clarity, only a partial volume is shown.
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