INTRODUCTION

VasQ Kit is a turn-key solution to create 3D perfusable vasculature models through vasculogenesis. It is designed to support high-throughput applications in research and drug development. Utilizing AIM's idenTx 40 plates, which feature 40 individual experimental sites on each plate, the VasQ Kit enables the co-culture of immortalized human umbilical vein endothelial cells (HUVEC) and immortalized lung fibroblasts (LF) within a fibrin gel to promote the formation of microvascular networks through self-assembly. This innovative platform facilitates the generation of microvascular structures that mimic *in vivo* microenvironments, making it ideal for assessing vascular geometry and permeability in various experimental settings. The accompanying protocol includes detailed instructions for cell seeding, maintenance, and quantification techniques to streamline the experimental workflow for consistent and reproducible results.

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VASQ KIT COMPONENTS

MATERIALS

Included in the Kit

- Thrombin from bovine plasma (1 X 50 µl)
- Fibrinogen from bovine plasma Type I-S (1 X 300 µl)
- G418 (Geneticin) (1 X 100 µl)
- Vascular Endothelial Growth Factor (VEGF, 1 X 35 µl)
- Human Umbilical Vein Endothelial Cells/TERT2 (HUVEC/TERT2, 1 X 0.5 M cells)
- Lung Fibroblasts/TERT309 (LF/TERT309 ,1 X 0.5 M cells)
- idenTx 40 plate

Non-included in the kit

- EGM Endothelial Cell Growth Medium BulletKit (Lonza, Cat. No. CC-3124)
- DMEM/F12 (1:1), w: stable Glutamine, w: 1.2 g/L NaHCO3 (Pan Biotech, Cat. No. P04-41150)
- Fetal bovine serum, certified, heat-inactivated (Sigma-Aldrich, Cat. No 10082147)
- Bovine gelatin (Sigma-Aldrich, Cat. No. G1393)
- 1X PBS (Life Technologies, Cat. No. 20012027)
- Trypsin (Life Technologies, Cat. No. 25300054)
- 70 kDa FITC-dextran (Sigma Aldrich, Cat. No. FD70S)
- 1.5 ml microcentrifuge tube
- 0.2 ml PCR tubes
- Ice bucket or styrofoam box
- Ice
- AIM Cooling Block

CELL CULTURE

MATERIALS

Included in the Kit

- G418 (Geneticin) (1 X 100 µl)
- Vascular Endothelial Growth Factor (VEGF, 1 X 35 µl)
- Human umbilical vein endothelial cells/TERT2 (HUVEC/TERT2, 1 X 0.5 M cells)
- Lung Fibroblasts/TERT309 (LF/TERT309, 1 X 0.5 M cells)

Non-included in the kit

- EGM Endothelial Cell Growth Medium BulletKit (Lonza, Cat. No. CC-3124)
- DMEM/F12 (1:1), w: stable Glutamine, w: 1.2 g/L NaHCO3 (Pan Biotech, Cat. No. P04-41150)
- Fetal bovine serum, certified, heat-inactivated (Sigma-Aldrich, Cat. No 10082147)
- 0.1% bovine gelatin (Sigma-Aldrich, Cat. No. G1393)
- 1X PBS (Life Technologies, Cat. No. 20012027)
- T175 flask
- T75 flask

Preparing cell culture medium ⏱**TIMING** ***20 min***

1. Supplement the EGM™ Endothelial Cell Growth Medium BulletKit™ with 10% FBS to make the complete EGM medium:
   a) Remove 50 ml of medium from a new bottle of EBM basal medium (500 ml).
   b) Add the EGM™ SingleQuot™ Supplements into the remaining 450 ml of EBM basal medium:
   c) Add an extra 40 ml of FBS to reach a final concentration of 10% FBS.
2. Supplement the DMEM/F12 (1:1) medium with 10% FBS:
   a) Remove 50 ml of medium from a new bottle of DMEM/F12 basal medium (500 ml).
   b) Add 50 ml of FBS into the remaining 450 ml of DMEM/F12 basal medium.
3. Prepare the media needed for 1 idenTx40 plate:
   a) 200 ml of EGM supplemented with 40 µl of G418: EGM complete medium.
   b) 50 ml of DMEM/F12 supplemented with 100 µl of G418: DMEM/F12 complete medium.
4. The complete media can be stored at 4°C for a maximum of 4 weeks.
5. Bring the media to room temperature (not 37°C) before use.

! Critical *Lonza's EGM only contains 10 ml of FBS, additional 40 ml of FBS are required to prepare the complete EGM medium.*

Thawing and culturing HUVEC/TERT2 **(3 Days before the Cell Seeding)**
⏱**TIMING 1h and 20 min**

6. Prepare the gelatin coating solution by adding 19 ml of 1X PBS (room temperature) to a 1 ml 2% gelatin stock solution that is warmed up to 37°C.

7. Coat a T175 flask by following the procedures below:
   a) Transfer 20 ml of the gelatin coating solution to a T175 flask.
   b) Make sure the culture surface of the flask is completely wetted.
   c) Incubate the culture flask at 37°C for 10-60 min.
   d) Remove the gelatin coating solution.
   e) Use the coated culture flask immediately for seeding cells.
   f) Add 20 ml of EGM complete medium into the culture flask and place the culture flask in the incubator for at least 30 min to allow the medium to reach 37°C and its normal pH.

! Critical *The gelatin coated culture flask must not be dry before the cell seeding step.*

8. Prepare 9 ml of EGM complete medium in a 15 ml centrifugation tube.

9. Thaw a vial of frozen HUVEC/TERT cells and immediately transfer the cells to the tube with EGM complete medium and centrifuge it for 5 min at 300 x*g*.

10. Discard the supernatant and resuspend the cell pellet in 1ml of pre-warmed media

11. Transfer the cells to the prepared culture flask and incubate them at 37°C in a CO_2 incubator.

12. If the cells are very sparse on day 2, perform a media change by removing the medium from the flask and replacing it with 20 ml of pre-warmed EGM complete medium.

Thawing and culturing LF/TERT2 **(3 Days before the Cell Seeding)**
⏱**TIMING 30 min**

13. Add 15 ml of DMEM/F12 complete medium into a T75 culture flask and place the culture flask in an incubator for at least 30 min to allow the medium to reach 37°C and its normal pH.

14. Prepare 9 ml of DMEM/F12 complete medium in a 15 ml centrifugation tube.

15. Thaw a vial of frozen LF/TERT cells and immediately transfer the cells to the tube with DMEM/F12 complete medium and centrifuge it for 5 min at 300 x*g*.

16. Discard the supernatant and resuspend the cell pellet in 1ml of pre-warmed DMEM/F12 complete media.

17. Transfer the cells to the prepared culture flask and incubate them at 37°C in a CO_2 incubator

18. If the cells are not confluent on day 3, perform a media change by removing the medium from the flask and replacing it with 15 ml of pre-warmed DMEM/F12 complete medium.

PREPARING & FILLING FIBRIN GEL WITH CELLS ⏱TIMING 50 min

MATERIALS

Reagents

- Thrombin from bovine plasma (Sigma Aldrich, Cat. No. T4648)
- Fibrinogen from bovine plasma Type I-S (Sigma Aldrich, Cat. No. F8630)
- G418 (Geneticin) (Invivogen, Cat. No. ant-gn-1)
- Vascular Endothelial Growth Factor (VEGF) (R&D Biosciences, Cat. No. 293-VE-500)
- idenTx 40 plate

Others

- Human umbilical vein endothelial cells/TERT2 (HUVEC/TERT2)
- Lung Fibroblasts/TERT309 (LF/TERT309)
- EGM complete medium
- DMEM/F12 complete medium
- Trypsin (Life Technologies, Cat. No. 25300054)
- 1.5 ml microcentrifuge tube
- 0.2 ml PCR tubes
- Ice bucket or styrofoam box
- Ice
- idenTx 40 plate
- AIM Cooling Block

Preparing working cell suspension in thrombin solution ⏱TIMING ***60 min***

19. Perform the following steps in a biosafety cabinet; every item should be sterilized before use.

20. Add 10 µl of thrombin stock solution (100 U/ml) into 90 µl of DMEM/F12 to yield **10 U/ml** of 4X concentrated thrombin solution. Keep this on ice.

21. The target seeding density in the gel for this kit is 6 M cells/ml for HUVEC/TERT and 1 M cells/ml for LF/TERT.

22. Do backward calculations to determine the cell concentrations of HUVEC/TERT and LF/TERT in the master cell suspension. As the cell suspension will be diluted with fibrinogen solution in a 1:1 ratio, a 4x more concentrated cell suspension as compared to the target seeding concentrations should be prepared. In this example, a cell suspension with a mixture of HUVEC/TERT and LF/TERT at 24 M cells/ml and 4 M cells/ml respectively is needed.

$$c_{HUVEC} = Seeding\ Concentration\ x\ Dilution\ Factor$$

$$c_{HUVEC} = 6\frac{Mcells}{ml} \times 4$$

$$c_{HUVEC} = 24\frac{Mcells}{ml}$$

Similarly,

$$c_{LF} = 4\frac{Mcells}{ml}$$

23. Trypsinize cells as per protocol. Briefly, remove medium from the cell culture flask/dish and wash twice with sterile 1x PBS. Use 0.25% trypsin with EDTA solution to dissociate cells.

Reminder *Both cell types are mixed to create the master stock. The master stock is then centrifuged and resuspended in DMEM/F12 first, also known as master cell suspension. This is mixed with a 4x concentrated thrombin solution in a 1:1 ratio to make the working cell suspension. The working cell suspension is then mixed with fibrinogen solution in a 1:1 ratio again to form fibrin gel. Therefore, the seeding concentrations are multiplied by a dilution factor of 4 to obtain the concentrations of the master cell suspension.*

24. Add EGM complete medium for HUVEC/TERT and DMEM/F12 complete medium for LF/TERT) that is at least the same volume as Trypsin, into the culture flasks/dishes to dilute/neutralize the Trypsin.

25. Transfer the trypsinized cell suspensions to 15 ml tubes and pellet the cells by centrifuging at 300 x *g* for 5 min at RT. After centrifugation, discard the supernatant. Resuspend the cells in 1 ml of DMEM/F12 media. Count the cells.

! Critical *Cells are resuspended in DMEM/F12 because this is the medium used to prepare the cell suspension with Thrombin.*

26. Table 1 is the respective cell numbers that are needed to prepare 90 µl of master cell suspension that is sufficient to fill at least 1 idenTx 40. Based on the number of counted cells obtained in Step 22, calculate the required volume and then draw the cells from the respective cell stock and mix these 2 cells together in a 1.5 ml microcentrifuge tube.

Reminder *The volume of master cell suspension is determined by dividing total volume of fibrin gel needed by the dilution factor as detailed in Step 22*

$$Volume\ of\ fibrin\ gel = (3\ \mu l \times No.\ of\ sites) \times 1.5\ Extra\ volume$$
$$= (3\ \mu l \times 40) \times 1.5$$
$$= 180\ \mu l$$
$$Volume\ of\ master\ cell\ suspension = \frac{180\ \mu l}{2} = 90\ \mu l$$

Table 1 Cell numbers for 90 µl of master cell suspension

Cell	Cell Numbers
HUVEC/TERT	2.16 M cells
LF/TERT	0.36 M cells

27. Centrifuge the cell mixture at 300 x *g* for 5 min at RT.

28. Remove the supernatant as much as possible. Resuspend the cell pellet in 60 µl of DMEM/F12 to get the master cell suspension. Measure the volume of master cell suspension using a micropipette and top it up with more media to get a final volume of 90 µl.

Reminder *The cell pellet will always have a few microliters of leftover media. Always add lesser volume of DMEM/F12 to resuspend the cells to avoid exceeding the final volume, in this example we use 60 µl of media to resuspend the pellet. Then measure the volume using a micropipette and add more media if needed to obtain the final volume of 90 µl.*

29. Keep the master cell suspension on ice.

! Critical *Only add the 4x concentrated thrombin solution to the master cell suspension right before the gel filling step.*

30. (optional) If the seeding of HUVEC/TERT cells in the media channels is needed, withdraw 0.5 M of HUVEC/TERT cells from the remaining stock solution and plate them in a gelatin-coated T-175 flask in EGM media.

Filling fibrin gel (with cells) ⏱ **TIMING 40 min + 15 min incubation**

31. Prepare 8 mg/ml of working fibrinogen solution (See REAGENT SETUP for fibrinogen measurement)

Table 2 Final concentrations of fibrinogen and thrombin in the gel

	Concentration
Fibrinogen	4 mg/ml
Thrombin	2.5 U/ml

32. Use these values to prepare 8 mg/ml of working fibrinogen solution if you don't have access to a Nanodrop.

$$Fibrinogen\ concentration\ (from\ Nanodrop) = 10.8\ \text{mg/ml}$$

$$Clottable\ Protein\ (92\%) = 10.8\ x\ 92\ \% = 9.971\ \text{mg/ml}$$

$$Volume\ of\ Fibrinogen = Volume\ x\ \frac{Working\ concentration}{Stock\ concentration}$$

$$= 180\ \mu\text{l}\ x\ \frac{8\ mg/ml}{9.971\ mg/ml} = 144.4\ \mu\text{l}$$

33. Mix 144.4 µl of Fibrinogen with 35.6 µl of sterile PBS.

34. Split the master cell suspension in 2 tubes. In this example, 45 µl of the master cell suspension was added in each tube.

! Critical *Prepare 2 aliquots of the master cell suspension. Only add the Thrombin to the second tube until the first half of the plate has been seeded. This will increase the reproducibility of the assay.*

35. Mix master cell suspension in a 1:1 ratio with 4X concentrated thrombin solution to obtain the working cell suspension. In this example, 45 µl of thrombin solution is added to the first tube.

36. Aliquot 3 µl of working cell suspension into 5 0.2 ml PCR tubes on ice.

! Critical *Prepare only 5 aliquots at a time. This will increase the reproducibility of the assay.*

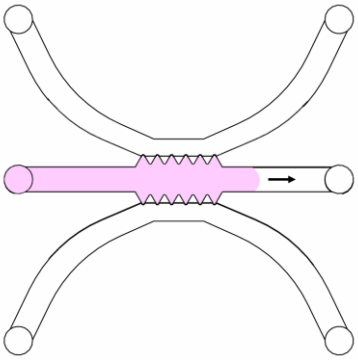
37. Make sure the working suspension and fibrinogen solution are always kept on ice. Mix 3 µl of working cell suspension with 3 µl of fibrinogen solution to make the fibrin gel solution in a microcentrifuge tube. This volume is sufficient to fill only 1 site.

! Critical *Fibrinogen polymerizes very quickly when it is mixed with thrombin. This mixing step should be done in less than 10 s. If unsure, pipette up and down for about 10 times.*

38. Draw 5 µl of fibrin gel solution with a 1- 10 µl micropipette.

! Critical *Limit the volume of fibrin gel to 5 µl to prevent the fibrin gel from overflowing into media channels.*

39. Insert the micropipette tip into either gel inlet. Push the plunger steadily to inject the fibrin gel until it reaches the other inlet.



! Critical *Hold the plunger firmly while removing the micropipette from the inlets, otherwise the negative pressure will suck the gel up.*

! Critical *Inject the fibrin gel solution deftly (but not abruptly) to complete the gel filling step before it polymerizes. If unsure, fill the gel solution within 5 s.*

40. Repeat Steps 38- 39 for the other 5 tubes.

41. Repeat Steps 36-40 until half of the plate is seeded.

! Critical *Mix the working cell suspension well before aliquoting it.*

42. Repeat Steps 35-40 until the whole plate is seeded.

43. Distribute 4 ml of sterile water or 1X PBS around the reservoir to ensure the entire reservoir is fully covered with water or 1X PBS.

44. Allow polymerization of hydrogel to take place for 15 min at room temperature.

! Critical *Chips with unpolymerized gel must be handled with care. Excessive agitation or impact may cause the unpolymerized gel to leak out of the gel channel.*

HYDRATING THE MEDIA CHANNELS ⏱TIMING 10 min

MATERIALS

Reagents

- Vasculogenesis medium (Lonza's EGM enriched with 50 ng/ml VEGF)

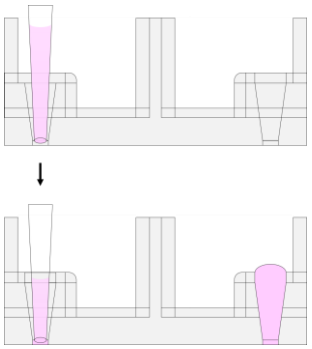
Others

- Fibrin-filled idenTx 40

45. Prepare the Vasculogenesis medium by adding 0.5 µl of VEGF (100 µg/ml) per ml of EGM complete medium to get a final concentration of 50 ng/ml VEGF.

Reminder *The total media needed for 1 idenTx40 plate for 7 days is 56 ml.*

46. After incubation, insert a pipette tip into either inlet of the media channel and push gently until the tip fits. Inject 10 µl of Vasculogenesis medium into the channel. Due to surface tension, the injected media will form a spherical cap at the opposite inlet. Repeat this step for the other channel.

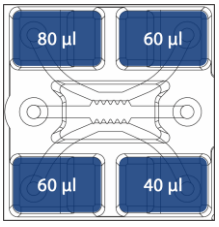


Insert a tip into a media inlet until it fits, then inject solution till it reaches the opposite inlet.

! Critical *Hold the plunger firmly while removing the micropipette from the inlets, otherwise the negative pressure will suck the medium up.*

! Critical *Do not inject more than 15 µl of medium at this step or the high injection pressure may disrupt the fibrin gel.*

47. Add Vasculogenesis medium with the indicated volumes on the right. Repeat this for all channels.



! Critical *There is a total volume difference of 40 µl between the top and bottom media channels. This difference allows for transient interstitial flow across the gel channel to facilitate vasculogenesis.*

48. Keep the plate in an incubator and change medium daily.

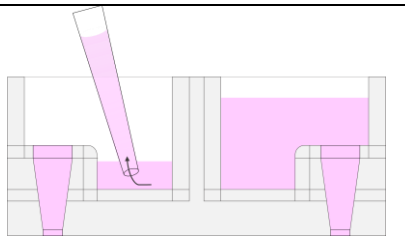
CHANGING MEDIUM ⏱TIMING 15 min

MATERIALS

Reagents

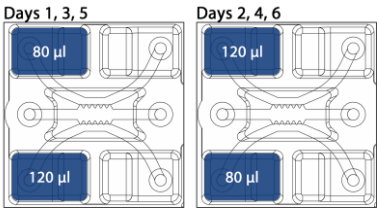
- Vasculogenesis medium (EGM complete media enriched with 50 ng/ml VEGF)

49. Remove medium from all 4 ports by carefully aspirating the medium from the troughs.



! Critical *Do NOT aspirate medium from inlets to avoid accidental removal of medium from the channels.*

50. Replace the medium with the indicated volumes on the right to re-establish the interstitial flow and to alternate its direction daily.



Reminder *Adding medium to 1 port of a media channel is sufficient to generate flow through the channel due to the previously wetted surfaces in all the media ports. If the medium did not flow through, refer to Step 47*

51. Keep the chips in an incubator. Microvasculature should start forming within 2 days and continue to mature in the chips.

SEEDING ENDOTHELIAL CELLS IN MEDIA CHANNELS (OPTIONAL) ⏱TIMING 1 h 30 min

MATERIALS

Reagents

- 1X PBS
- Trypsin
- Vasculogenesis medium (EGM complete media enriched with 50 ng/ml VEGF)

Others

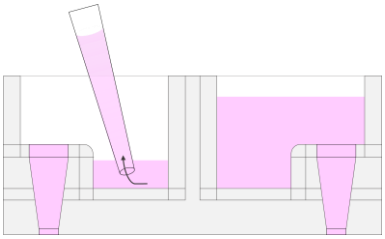
- idenTx 40 plate seeded with cells in the gel channel
- 1x T175 flask of HUVEC/TERT2

52. Seed endothelial cells in both media channels on day 4.

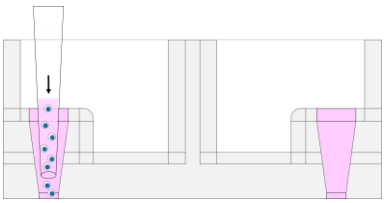
Reminder *Endothelial cells are seeded in the media channels to increase the cell coverage on the gel interface, to fill gaps at the gel-post borders and to improve the connections between the microvascular network and media channels.*

53. Trypsinize endothelial cells as per protocol and resuspend the cells at 2 M cells/ml.

54. Remove medium from all 4 ports by carefully aspirating the medium from the troughs. Doing so prevents backflow or overflow of cells into the media ports during the cell seeding Step 55.



55. Use a micropipette to withdraw 10 µl of the endothelial cell suspension (2 M cells/ml). Position the tip in the media channel inlet and inject the cell suspension slowly (e.g., over a duration of 3 s).



! Critical *Do not insert the tip completely into the inlets to avoid introducing cells into the media channels at a high flow rate. High flows will not allow cells to settle along the channel, resulting in uneven distribution.*

! Critical *Lay the idenTx 40 on a flat surface while seeding cells into the idenTx 40. Inclination of the plate affects the flow in the media channel, thus disturbing cell distribution.*

56. Visual inspection of the channel under a microscope is recommended. If the cell density or distribution is not optimal, add 120 µl of culture medium to 1 media port to flush out the cells. Adjust the cell concentration and repeat Step 55.

57. Keep the idenTx 40 in a 5 % CO_2 incubator at 37°C for at least 30 min to allow the endothelial cells to adhere.

58. Repeat Step 55 for the opposite media channel.

59. Incubate at 37°C for another 30 min to allow the endothelial cells to adhere.

60. Add respective medium in volumes stated in Step 50

61. Keep the idenTx 40 in a 5 % CO_2 incubator at 37°C. Continue changing media daily.

PERFUSING MICROVASCULATURE WITH FLUORESCENT DEXTRAN ⏱TIMING 1 hour

MATERIALS

Reagents

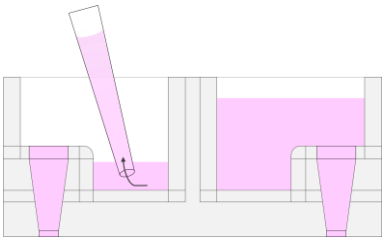
- 70 kDa FITC-dextran (Sigma Aldrich, Cat. No. FD70S)
- EGM complete medium

Others

- idenTx 40 with perfusable microvasculature (day 7 onwards)

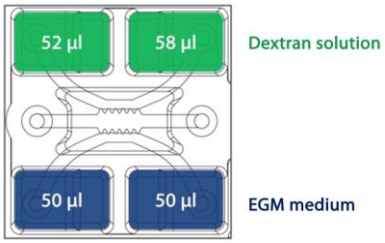
62. Prepare the FITC-dextran stock solution in PBS at 10 mg/ml and aliquot it accordingly. Store the aliquots at −20 °C. Dilute it with EGM in 1:100 to obtain a final concentration of 0.1 mg/ml for perfusion tests. 5 ml of dextran solution is sufficient for 1 plate of idenTx 40.

63. Remove medium from all 4 ports by carefully aspirating the medium out from the troughs before imaging.



64. Add 50 µl of EGM medium into each of the 2 ports of the bottom media channel.

    (Total volume = 100 µl)



65. Add 52 µl of dextran solution into the top left media port and 58 µl of dextran solution into the top right media port.

    (Total volume = 110 µl)

! Reminder *There is a total volume difference of 10 µl between the top and bottom media channels. This difference allows the perfusion of fluorescent dextran solution through the microvasculature.*

66. Image the dextran-perfused microvasculature with a confocal microscope or high content imaging system immediately. Ideally, the time-lapse images shall be taken every 2 min for a total of 10 minutes.

Table 3 Image acquisition settings

Objective	10x
Timepoints	6
Planes	25
Distance (between planes)	10 µm

QUANTIFICATION OF MICROVASCULAR GEOMETRY ⏱TIMING Variable

To quantify the microvascular geometry in idenTx 40, we recommend labelling the cells with appropriate fluorophores (such as VE-cadherin staining that is specific to endothelial cells). Bright field, phase contrast and epifluorescence microscopy are all compatible with idenTx 40 but 3D imaging techniques such as confocal microscopy are preferred due to the nature of this assay. The following quantification methods use images taken from confocal microscopy as illustrative examples.

TOTAL BRANCH LENGTH (L_{branch}), LATERAL VESSEL AREA ($A_{lateral}$) & LATERAL DIAMETER($D_{lateral}$)

The total branch length is an informative metric that can describe the complexity of the microvascular network. The lateral vessel area measures the projected 2D coverage of microvascular network in the hydrogel. As most of the vessels are parallel to the bottom laminate, the lateral diameter can then be derived based on the total branch length and lateral vessel area.

67. We recommend using confocal images of endothelial cells that are either fluorescently tagged or stained to quantify the sprout length and the number of branching points.

68. Project the stacks of confocal images into 2D images based on the maximum intensity projection method.

69. Pre-process the images if necessary. Depending on the image quality, you may reduce noise through despeckle and background reduction or apply a Gaussian filter to smoothen the edges and fill up the gaps between the bright signals around the cell membrane if the endothelial cells are stained for junction proteins (e.g., VE-cadherin).

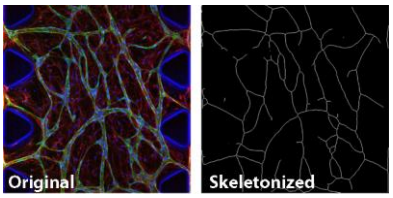
Reminder *You need to optimize the pre-processing steps based on the stained proteins/organelles and the image quality. You should always pre-process your images as a whole.*

70. Try all threshold methods on at least three individual projected images. Choose the method that segments your data best and produces the closest estimation to the original images. Threshold the pre-processed images to binarize the images.

71. Use Measure (a built-in function of ImageJ) on the binarized images to obtain the area of the images and the area fraction of the microvascular network. Multiply the total area by the area fraction to get lateral vessel area ($A_{lateral}$).

Reminder *You have to select area fraction as one of the measurements and set the scale according to your images.*

72. Use Skeletonize (2D/3D) plugin (http://imagej.net/Skeletonize3D) in ImageJ to find the centerlines (also known as skeleton) of objects in the input image.



A 3D projection of a stack of confocal images (left) and its corresponding skeletonized image (right).

73. Use AnalyzeSkeleton plugin (http://imagej.net/AnalyzeSkeleton) in ImageJ to analyze the skeletons you have generated. This yields information including the average branch length and the number of branches. Multiply the average branch length by the number of branches to get total branch length (L_{branch}).

74. You can then derive the lateral diameter ($D_{lateral}$) by dividing the lateral vessel area by the total branch length.

$$D_{lateral} = \frac{A_{lateral}}{L_{branch}}$$

3D VESSEL VOLUME (V), 3D SURFACE AREA ($A_{surface}$), TRANSVERSE DIAMETER ($D_{transverse}$) & CIRCULARITY

The 3D vessel volume and 3D surface area measure the extent of microvascular network in the 3D hydrogel. In combination, they can help derive the transverse diameter that is perpendicular to the bottom laminate. The circularity of the microvasculature is an indicative metric that describes if the vessels are mainly circular (more *in vivo*-like) or ellipsoidal.

75. We recommend using confocal images of endothelial cells that are either fluorescently tagged or stained in the cytoplasm to quantify the sprout area.

76. Pre-process the images if necessary. Depending on the image quality, you may apply pre-processing techniques such as despeckle and background subtraction.

Reminder *You should always pre-process your images as a whole.*

77. Try all threshold methods and choose the method that segments your data best and produces the closest estimation to the original images based on at least three individual stacks of images. Threshold the pre-processed images to binarize the images.

78. Use Trainable Weka Segmentation 3D plugin (https://imagej.net/Trainable_Weka_Segmentation) in ImageJ to segment the binarized images. Briefly, select representative region of interest (ROI) of the microvascular network and add that as first classifier. The region outside of microvasculature network is selected as the second classifier. Train the classifiers and create results afterwards.

79. Adjust the segmented image into 8-bit.

80. Use 3D geometrical measure in 3D ImageJ Suite (http://imagejdocu.tudor.lu/doku.php?id=plugin:stacks:3d_ij_suite:start) to measure the 3D vessel volume (V) and 3D surface area ($A_{surface}$).

81. You can then derive the transverse diameter ($D_{transverse}$) by using the following equation:

$$D_{transverse} = \sqrt{\frac{D_{lateral}^2}{\frac{A_{surface}^2 D_{lateral}^2}{8V^2} - 1}}$$

82. Compute circularity by dividing the transverse diameter by the lateral diameter.

Reminder The circularity of a circle is 1.

$$Circularity = \frac{D_{transverse}}{D_{lateral}}$$

QUANTIFICATION OF MICROVASCULAR PERMEABILITY ⏱TIMING Variable

To quantify the microvascular permeability in idenTx 40, we recommend using fluorescent-labelled dextran to perfuse the microvascular network. Once the dextran passes through the vascular barrier and enters tissue (3D hydrogel) region, the changes in fluorescent intensity of the region that correlate to the flux of dextran are measured over time. The following quantification method uses images taken from confocal microscopy as an illustrative example.

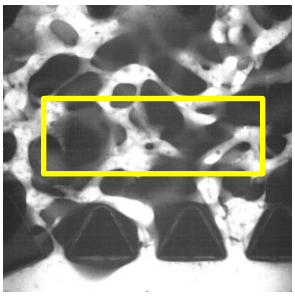
FLUORESCENT INTENSITIES OF VESSELS (I_V) & TISSUE (I_T)

The fluorescent intensities of vessels and tissue at two time points are used to determine the vascular permeability. The greater the difference in fluorescent intensities of tissue between the two time points, the leakier the microvasculature.

83. We recommend using confocal images of microvasculature that is perfused with fluorescent-labelled dextran to quantify the fluorescent intensities.

84. Set scale in the unit of µm based on the image resolution data.

85. Select region at the bottom section of the image and crop the image to avoid the posts.



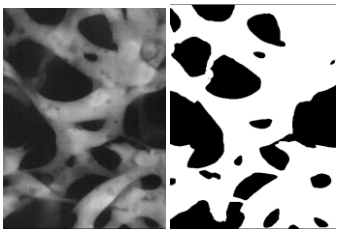
Reminder *To avoid the side diffusion affecting the analysis, crop the image nearer to the bottom posts as shown in the yellow box.*

86. Project a stack of confocal images at time point 2 (t_2) into 2D images based on the maximum intensity projection (MIP) method. Duplicate the projected image.

87. Pre-process the duplicated image if necessary. Depending on the image quality, you may apply pre-processing techniques such as Gaussian blur.

Reminder *You should always pre-process your images as a whole.*

88. Binarize the duplicated image. Choose the threshold method that gives the best outline of the vessels. Use the Auto Threshold: Select the preferred method and select white objects (vessels) on black background (tissue).



Cropped and MIP image (left) of a stack of confocal images and its corresponding binarized image (right).

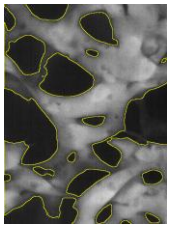
89. Use the built-in Selection: Create Selection function in ImageJ to select the binarized microvasculature as the first region of interest (ROI) and add that into the ROI manager.

The yellow outline represents the selected area of the binarized microvasculature.

90. Use the built-in Selection: Enlarge in ImageJ to include an expanded area outside of the microvasculature as second ROI and add that into the ROI manager. A value of 10 expands the selection to include a thin area outside of the vessels and that will be treated as the tissue region.

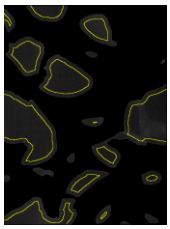
The yellow outline has been enlarged to include tissue area along with the microvasculature.

91. Select the original projected image so that it is the active window. Select the first selection in ROI manager which outlines the vessels. Use the Measure function to measure the fluorescent intensities of vessels (I_V), perimeter of the microvasculature (P_{vessel}) and area of vessel (A_V) at t_2 (typically $t_2 = 2$ minutes).



Outline of binarized microvasculature selected to measure the microvasculature on the original max projected image.

92. After completing the measurements for the vessels, clear the selected vessel area. Next, select the second selection in ROI manager which outlines the enlarged area to include the tissue. Use the Measure function to measure the fluorescent intensities of vessel plus tissue (I_{V+T}) and area of vessel plus tissue (A_{V+T}) at t_1 (typically $t_1 = 0$ minute).



Enlarged outline selected to measure the microvasculature plus tissue after clearing out the microvasculature on the original max projected image.

93. Repeat Steps 86 to determine the fluorescent intensities at time point 3, t_3 (typically $t_3 = 4$ minutes).

Reminder *A Macro can be created to analyze multiple images together.*

94. Calculate the lateral tissue area and true tissue intensity.

$$Lateral\ tissue\ area\ (A_{lateral_tissue}) = A_{V+T} - A_V$$

$$True\ tissue\ intensity\ (I_T) = \frac{(A_{V+T} \times I_{V+T})}{A_{lateral_tissue}}$$

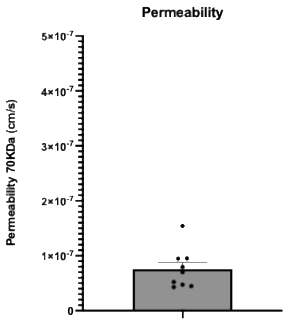
Reminder *The measurements that were done for tissue include the vessel area and intensity that was cleared. Therefore, they have to be adjusted accordingly to obtain the lateral tissue area and true tissue intensity.*

95. Calculate the apparent permeability P_{app} by using the following equation:

$$P_{app} = \frac{1}{(I_V^{t2} - I_T^{t2})} \frac{(I_T^{t3} - I_T^{t2})}{\Delta t} \frac{V_{tissue}}{A_{surface}}$$

V_{tissue} is the volume of the tissue space and $A_{surface}$ is the surface area of the microvasculature. Based on the assumption that the ratio $V_{tissue}/A_{surface}$ can be approximated as $A_{lateral_tissue}/P$, the P_{app} can be calculated as followed: Based on the publication by Campisi et al. [2].

$$P_{app} = \frac{1}{(I_V^{t2} - I_T^{t2})} \frac{(I_T^{t3} - I_T^{t2})}{\Delta t} \frac{A_{lateral_tissue}}{P_{vessel}}$$



The permeability of a microvasculature for 70 kDA FITC-dextran in idenTx 40 is typically in the range of 10^{-8} cm/s.

Reminder *$A_{lateral_tissue}/P$ is an estimation for the ratio between 3D vessel volume and 3D surface area, $V_{tissue}/A_{surface}$.*

STAINING CELLS ⏱TIMING 2 days

MATERIALS

Reagents

- 4% Formaldehyde (See REAGENT SET UP; Sigma Aldrich, Cat. No.158127)
- 0.1% Triton X-100 (See REAGENT SET UP; Sigma Aldrich, Cat. No. T8787)
- Blocking buffer (Life Technologies, Cat. No. B-10710)
- 1X PBS (Life Technologies, Cat. No. 20012027)
- Alexa Fluor® 488 Anti-CD31 antibody [JC/70A] (Abcam, Cat. No. ab215911)
- Rhodamine Phalloidin (Life Technologies, Cat. No. R415)
- Hoechst (Life Technologies, Cat. No. H1399)

Cell Fixation ⏱TIMING **30 min**

96. Remove medium from the reservoirs by carefully aspirating the medium away from the media inlets.

97. Add 120 µl of 1X PBS into one media reservoir and then add 80 µl into the other media reservoir. The differential volumes in the two reservoirs create flow through the gel to wash the sample.

98. Remove 1X PBS from the media reservoirs by carefully aspirating the PBS away from the media inlets.

99. Add 120 µl of 4% formaldehyde into one media reservoir and then add 80 µl into the other media reservoir.

Reminder *The 1X PBS within the media channels can be carefully removed using a micropipette if the dilution effect contributed by the 1X PBS is a concern. Replenish the media channels with 10 µl of 4% formaldehyde after the removal of PBS.*

100. Incubate for 20 min at RT.

101. Remove the 4% formaldehyde solution from the reservoirs with a micropipette. Wash the channels twice with 1X PBS as described in Steps 97-98.

! Critical *Dispose 4% formaldehyde and the 1st subsequent wash in a designated waste bottle.*

▪ **PAUSE POINT** *Fixed cells can be kept for up to 1 month (depending on target antigens as some antigens may degrade over time after fixation) at 4°C as long as the PBS in the ports does not dry up.*

Cell Permeabilization ⏱TIMING **30 min**

(Optional: Permeabilization is only necessary when cell-impermeable fluorescent probes are used, e.g., phalloidin)

102. Remove 1X PBS from the media reservoirs by carefully aspirating the PBS away from the media inlets. Add 120 µl of 0.1 % Triton X-100 into one media reservoir and 80 µl into the other media reservoir.

Reminder *The 1X PBS within the media channels can be carefully removed using a micropipette if the dilution effect contributed by the 1X PBS is a concern. Replenish the media channels with 10 µl of 0.1 % Triton X-100 after the removal of PBS.*

103. Incubate for at least 30 min at RT.

104. Wash once with 1X PBS as described in Steps 97-98.

Blocking ⏱**TIMING 2 h**

(Optional: Blocking is only necessary for immunofluorescent staining)

105. Remove 1X PBS from the media reservoirs by carefully aspirating the PBS away from the media inlets. Add 120 µl of blocking buffer into one media reservoir and 80 µl into the other media reservoir.

Reminder *The 1X PBS within the media channels can be carefully removed using a micropipette if the dilution effect contributed by the 1X PBS is a concern. Replenish the media channels with 10 µl of blocking buffer after the removal of PBS.*

106. Incubate for 2 h at RT.

Primary/Conjugated Antibody Staining ⏱**TIMING 18 h**

107. Prepare antibodies according to the manufacturer's recommendations. In this example, the antibody is conjugated with GFP. Dilute the anti-CD31 antibody with 1X PBS in a 1:250 ratio.

108. Remove the blocking buffer from the media reservoirs by carefully aspirating the blocking buffer away from the media inlets. Add 120 µl of the antibody solution into one media reservoir and 80 µl into the other media reservoir.

! Critical *Do not wash with 1X PBS after the blocking step.*

109. Incubate overnight at 4°C.

Secondary Antibody Staining ⏱**TIMING 1.5 h**

(**Note**: Secondary antibody staining is only necessary if the primary antibody is not conjugated)

110. Remove the primary antibodies from the media reservoirs by carefully aspirating the antibody solution away from the media inlets. You may reuse the antibodies depending on the antigen and antibody.

111. Wash with 1X PBS 5 times, with a 10 min incubation between each wash, as described in Steps 97-98.

112. Prepare the secondary antibodies according to the manufacturer's recommendations.

113. Add 120 µl of the secondary antibody solution into one media reservoir and 80 µl into the other media reservoir.

! Caution *Cover the plate with aluminium foil to minimize photobleaching.*

114. Incubate for 1 h at RT.

115. Remove the secondary antibodies from the media reservoirs by carefully aspirating the antibody solution away from the media inlets.

116. Wash with 1X PBS 5 times, with a 10 min incubation between each wash, as described in Steps 97-98.

Reminder *The staining protocol should be optimized for your specific application (with different cell types and different proteins of interest).*

▪ PAUSE POINT *Stained cells can be kept for up to 1 month (depending on your application) at 4°C in dark as long as the PBS in the reservoirs does not dry up.*

REAGENT SETUP

FIBRINOGEN MEASUREMENT

Reagents

- Fibrinogen from bovine plasma (Sigma-Aldrich, Cat. No. F8630-1G)
- 1X PBS (Life Technologies, Cat. No. 20012027)

Others

- Nanodrop 1000 Spectrophotometer (Thermo Scientific)

1. Measure the protein concentration of the fibrinogen solution using a spectrophotometer (Nanodrop 1000) before aliquoting. In this example, Protein A280 is selected to measure the protein concentration. Choose E1% as the sample type and enter the mass extinction coefficient as 15.1%.

2. Thaw the aliquot in fridge (4 °C) for a maximum of 1 hour until thawed and then transfer to ice for protein measurement before experimental use.

3. Use cold 1X PBS to make a blank measurement.

4. Keep the fibrinogen cold and measure it thrice to obtain an average.

5. Use the measured average protein concentration, (e.g. 10.9 mg/ml), and the clottable protein percentage (92%) to derive the final stock concentration.

$$Final\ stock\ concentration\text{: } 10.8\ \frac{mg}{ml}\ x\ 92\% = 9.971\ mg/ml$$

6. With the protein concentration now being 9.971 mg/ml, use this as the stock concentration to prepare the 8 mg/ml fibrinogen for experimental use.

VASCULOGENESIS MEDIUM (EGM complete media enriched with 50 ng/ml VEGF)

Reagents

- $VEGF_{165}$ (R&D Systems, Cat. No. 293-VE-500)
- Complete EGM™ Endothelial Cell Growth Medium BulletKit™ (Lonza, Cat. No. CC-3124)

1. Add 0.5 µl of VEGF (100 µg/ml) per ml of medium to get cell culture medium enriched with 50 ng/ml VEGF.

4% FORMALDEHYDE

Reagents

- Paraformaldehyde powder (Sigma-Aldrich, Cat. No. 158127)
- 1X PBS (Life Technologies, Cat. No. 20012027)

Others

- Hot plate with magnetic stirrer

1. Mix 40 g of paraformaldehyde (PFA) powder with 800 ml of 1X PBS and heat it up to 60 °C by using a hot plate.

! Caution *Wear protective clothing and gloves while working with PFA. Prepare this solution in a ventilated hood.*

2. Stir for approximately 6 h.

Reminder *pH can be adjusted by using 1 M NaOH to facilitate the dissolution of PFA. If so, neutralize the pH back to approximately pH 7.0 by using dilute HCL after the PFA is dissolved.*

3. Adjust the volume to 1L with 1X PBS and filter the solution before aliquoting.

0.1% TRITON-X

Reagents

- Triton X-100 (Sigma Aldrich, Cat. No. T8787)
- 1X PBS (Life Technologies, Cat. No. 20012027)

1. Dilute Triton X-100 with 1X PBS to yield 0.1 % (v/v) working concentration.

2. Aliquot the solution and store them at room temperature.