INTRODUCTION

AIM idenTx 40 plate is utilized to create 3D perfusable vasculature models through vasculogenesis for high-throughput assessments. The model, which consists of a co-culture of human umbilical vein endothelial cells (HUVEC) and normal human lung fibroblasts (NHLF), self-organize and form microvascular networks within a fibrin gel. The idenTx 40 holds 40 individual experimental sites on a single standard SBS-format plate. This protocol covers the calculations and techniques for seeding cells in idenTx 40 as well as the quantifications of microvascular geometry and permeability.

TABLE OF CONTENTS

INTRODUCTION....1
TABLE OF CONTENTS....1
PREPARING & FILLING FIBRIN GEL WITH CELLS....2
HYDRATING & COATING MEDIA CHANNELS....6
CHANGING MEDIUM....7
SEEDING ENDOTHELIAL CELLS IN MEDIA CHANNELS....8
PERFUSING MICROVASCULATURE WITH FLUORESCENT DEXTRAN....10
QUANTIFICATION OF MICROVASCULAR GEOMETRY....11
QUANTIFICATION OF MICROVASCULAR PERMEABILITY....13
STAINING CELLS....16
TROUBLESHOOTING....19
REAGENT SETUP....20

PREPARING & FILLING FIBRIN GEL WITH CELLS ⏱ TIMING 50 min

MATERIALS

Reagents

- Thrombin from bovine plasma (see REAGENT SETUP, Sigma-Aldrich, Cat. No. T4648)
- Fibrinogen from bovine plasma Type I-S (see REAGENT SETUP, Sigma-Aldrich, Cat. No. F8630-1G)
- EGM-2 MV Microvascular Endothelial Cell Growth Medium-2 (Lonza, Cat. No. CC-3202)
- DMEM high glucose with GlutaMAX Supplement, pyruvate cell culture medium (Life Technologies, Cat. No. 10569010)
- 1X PBS (Life Technologies, Cat. No. 10010023)
- Trypsin (Life Technologies, Cat. No. 25300054)
- TrypLE (Life Technologies, Cat. No. 12605010)

Others

- Human umbilical vein endothelial cells (HUVECs)
- Normal Human Lung Fibroblasts (NHLFs)
- 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

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

2. Add 10 µl of thrombin stock solution (100 U/ml) into 90 µl of EGM-2 MV to yield **10 U/ml** of 4X concentrated thrombin solution. Keep this on ice.

! Critical *Lonza's EGM-2 MV is heparin sulfate-free. Heparin sulfate interferes with the enzymatic activity of thrombin in converting fibrinogen to fibrin. If another medium is to be used, keep it free of heparin sulfate in this step.*

3. The target seeding density in the gel for this example is 5 M cells/ml for HUVECs and 1 M cells/ml for NHLFs.

4. Do backward calculations to determine the cell concentrations of HUVEC and NHLF 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 HUVECs and NHLFs at 20 M cells/ml and 4 M cells/ml respectively is needed.

Reminder *Both cell types are mixed to create the master stock. The master stock is then centrifuged and resuspended in EGM-2 MV 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.*

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

$$c_{HUVEC} = 5\frac{Mcells}{ml} \times 4$$

$$c_{HUVEC} = 20\frac{Mcells}{ml}$$

Similarly,

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

5. Trypsinize cells as per protocol. Briefly, remove medium from the cell culture flask/dish and wash twice with sterile 1x PBS. Use TrypLE (for HUVEC) and 0.25% Trypsin with EDTA solution (for NHLF) to dissociate cells.

6. Add respective medium with FBS (EGM-2 MV for HUVEC and DMEM for NHLF) that is at least the same volume as TrypLE/Trypsin, into the culture flasks/dishes to dilute/neutralize the TrypLE/Trypsin.

7. 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 EGM-2 MV media. Count the cells.

! Critical *Cells are resuspended in EGM-2 MV only to make sure the cell suspension is heparin sulfate-free.*

8. 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 4.. Calculate the required volume and then draw the cells from the respective cell stock and mix 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 4..*

Table 1 Cell numbers for 90 µl of master cell suspension

Cell	Cell Numbers
HUVECs	1.8 M cells
NHLFs	0.36 M cells

$$Volume\ of\ fibrin\ gel = (3\ \mu l \times No.of\ sites) \times 1.5\ Extra\ volume$$
$$= (3\ \mu l \times 40) \times 1.5$$
$$= 180\ \mu l$$

$$Volume\ of\ working\ cell\ suspension = \frac{180\ \mu l}{2} = 90\ \mu l$$

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

10. Remove the supernatant as much as possible. Resuspend the cell pellet in 60 µl of EGM-2 MV 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 EGM-2 MV 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.*

11. 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.*

12. Withdraw 0.5 M of HUVEC cells from the remaining stock solution and plate them back in a collagen-coated T-175 flask in EGM-2 MV media for side seeding on day 4.

Reminder *HUVEC must be sub-cultured at this stage or thawed for side channel seeding (see SEEDING ENDOTHELIAL CELLS IN MEDA CHANNELS). Subculturing 0.5 M of HUVEC in a T175 flask to achieve at least 2 M cells by day 4, so that they are sufficient to seed the side channels of 1 idenTx 40 plate. The seeding density is subject to the growth rate of the cells used.*

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

13. Prepare 5 mg/ml of working fibrinogen solution (See REAGENT SETUP for fibrinogen preparation)

Table 2 Final concentrations of fibrinogen and thrombin in the gel

	Concentration
Fibrinogen	2.5 mg/ml
Thrombin	1 U/ml

14. Prepare a 5 mg/mL working fibrinogen solution using these values if a Nanodrop is not available.

$$Fibrinogen\ concentration\ (from\ Nanodrop) = 10.9\ \text{mg/ml}$$

$$Clottable\ Protein\ (92\%) = 10.9\ x\ 92\ \% = 10.028\ \text{mg/ml}$$

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

$$= 180\ \mu\text{l}\ x\ \frac{5\ mg/ml}{10.028\ mg/ml} = 89.75\ \mu\text{l}$$

15. Mix 89.8 µl of Fibrinogen with 90.2µl of sterile PBS.

16. 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 4X thrombin solution to the second tube until the first half of the plate has been seeded. This will increase the reproducibility of the assay.*

17. 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.

18. 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.*

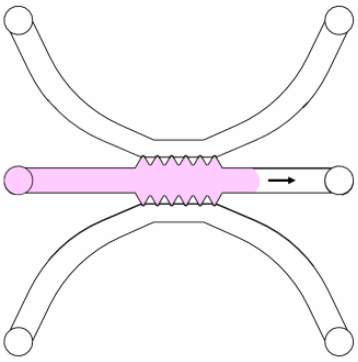
19. Make sure the working suspension and fibrinogen solution are always kept on ice. Mix 3 µl of working cell suspension with 3 µl of fibrinogen solution to make fibrin gel solution in a microcentrifuge tube. This volume is sufficient to fill only 1 site.

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

20. 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.*

21. 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 5s.*

22. Repeat Steps 19. - 21. for the other 4 tubes.

23. Repeat Steps 18. - 22. until half of the plate is seeded.

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

24. Repeat Steps 17. - 23. until the whole plate is seeded.

25. Distribute 4 ml of 1X PBS around the reservoir to ensure the entire reservoir is fully covered with 1X PBS.

26. 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.*

? Troubleshooting (see Table 3 for troubleshooting advice at page 20)

HYDRATING & COATING MEDIA CHANNELS ⏱TIMING 20 min + 60 min incubation

MATERIALS

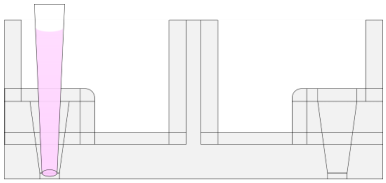
Reagents

- Human fibronectin (Sigma-Aldrich, Cat. No. F0895), 60 µg/ml in endothelial medium
- EGM-2 MV Microvascular Endothelial Cell Growth Medium (Lonza, Cat. No. CC-3202) enriched with 50 ng/ml Vascular Endothelial Growth Factor (VEGF, see REAGENT SETUP SETUP at page 23)

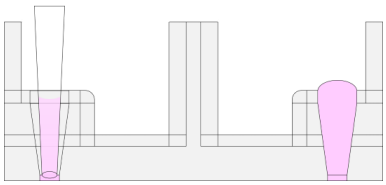
Others

- Fibrin-filled idenTx 40 plate

27. After incubation, insert a pipette tip into either inlet of the media channel and push gently until the tip fits. Inject 10 µl of coating solution (60 µg/ml fibronectin solution diluted in EGM-2 MV) into the channel. Due to surface tension, the injected coating solution will form a spherical cap at the opposite inlet. Repeat this step for the other channel. Use culture medium instead if coating is not required.



Insert a tip into a media inlet until it fits



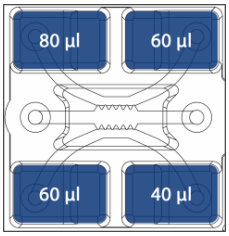
Inject the solution until it reaches the opposite inlet.

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

! Critical *Do not inject more than 10 µl of solution at this step or the high injection pressure may disrupt the fibrin gel.*Update volume for picture below*

28. Incubate the media-channel-hydrated idenTx 40 sites for 1 h in a 37°C incubator.

29. Add EGM-2 MV medium enriched with 50 ng/ml VEGF with the indicated volumes on the right to flush out the coating solution. 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.*

30. Keep the chips in an incubator and change medium daily.

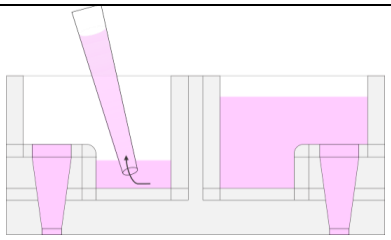
CHANGING MEDIUM ⏱TIMING 15 min

MATERIALS

Reagents

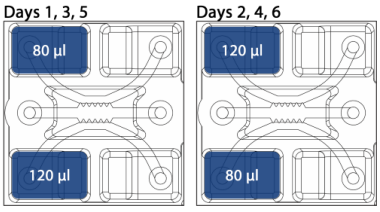
- Lonza's EGM-2 MV enriched with 50 ng/ml Vascular Endothelial Growth Factor (VEGF, see REAGENT SETUP)

31. 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.*

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



! Note *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.*

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

? Troubleshooting (see Table 3 for troubleshooting advice)

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

MATERIALS

Reagents

- 1X PBS (Life Technologies, Cat. No. 10010023)
- TrypLE (Life Technologies, Cat. No. 12605010)
- EGM-2 MV Microvascular Endothelial Cell Growth Medium-2 (Lonza, Cat. No. CC-3202) enriched with 50 ng/ml Vascular Endothelial Growth Factor (VEGF, see REAGENT SETUP at page 23)

Others

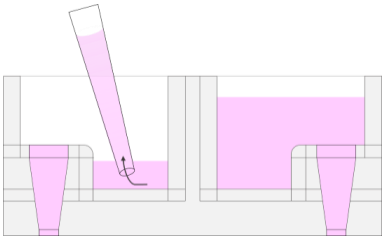
- idenTx 40 plate seeded with cells in the gel channel
- One T175 flask of HUVEC

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

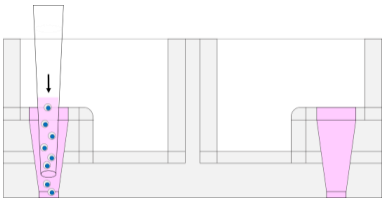
Reminder *Endothelial cells are seeded in the media channels to increase the cell coverage on the gel interface, to fill gaps at the gel-post borders and to improve the connections between the microvascular network and media channels.*

35. Trypsinize endothelial cells as per protocol and re-suspend the cells at 2 M cells/ml.

36. 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 cell seeding (Step 37.).



37. 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.*

38. 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 Steps 36. and 37. .

? Troubleshooting (see Table 3 for troubleshooting advice)

39. Repeat Step 37. for all the remaining TOP media channel.

40. 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.

41. Repeat Step 37. for the remaining BOTTOM media channel.
42. Incubate at 37°C for another 30 min to allow the endothelial cells to adhere.
43. Add respective medium in volumes stated in Step 32..
44. Keep the idenTx 40 in a 5 % CO_2 incubator at 37°C. Continue changing media daily.

PERFUSING MICROVASCULATURE WITH FLUORESCENT DEXTRAN ⏱TIMING 20 min

MATERIALS

Reagents

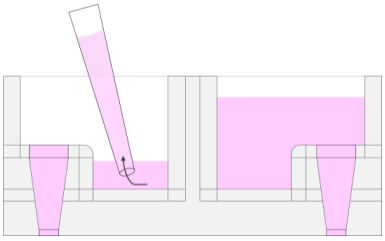
- 70 kDa FITC-dextran (Sigma Aldrich, Cat. No. FD70S)
- EGM-2 MV Microvascular Endothelial Cell Growth Medium-2 (Lonza, Cat. No. CC-3202)

Others

- idenTx 40 with perfusable microvasculature (day 7 onwards)

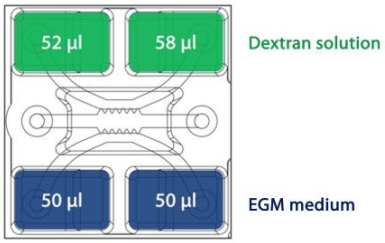
45. Prepare FITC-dextran stock solution in PBS at 10 mg/ml and aliquot it accordingly. Store the aliquotes at −20 °C. Dilute it with EGM-2 MV in 1:100 to obtain a final concentration of 0.1 mg/ml for perfusion tests. 5 ml of dextran solution is sufficient for 1 plate of idenTx 40.

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



47. Add 50 µl of EGM-2 MV only into each of the 2 ports of the bottom media channel.

    (Total volume = 100 µl)



48. Add 52 µl of dextran solution into the top left media ports and 58 ul 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.*

49. 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 min.

Table 3 Image acquisition settings

Objective	10x
Timepoints	6
Planes	35
Distance (between planes)	5 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.

50. 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.

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

52. Pre-process the images if necessary. Depending on the image quality, noise can be reduce 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).

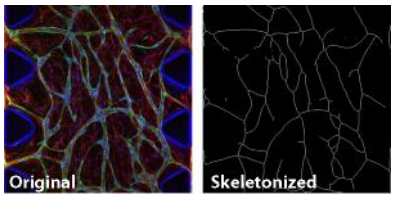
Reminder *The pre-processing steps should be optimized based on the stained proteins/organelles and the image quality. Image should always be pre-processed as a whole.*

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

54. 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 *Area fraction should be selected as one of the measurements, and the scale set according to the images.*

55. 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).

56. Use AnalyzeSkeleton plugin (http://imagej.net/AnalyzeSkeleton) in ImageJ to analyze the generated skeletons. 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}).

57. The lateral diameter ($D_{lateral}$) can then be derived 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.

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

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

Reminder *Images should always be pre-processed as a whole.*

61. Try all threshold methods and choose the method that segment the data most effectively 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.

62. 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.

63. Adjust the segmented image into 8-bit.

64. 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 ($A_{surface}$).

65. The transverse diameter ($D_{transverse}$) can then be derived by using the following equation:

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

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

Reminder *The circularity of a circle is 1.*

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

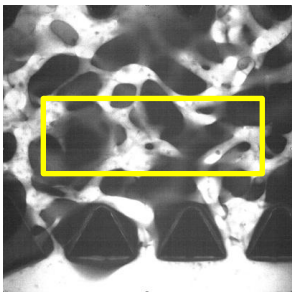
QUANTIFICATION OF MICROVASCULAR PERMEABILITY ⏱TIMING Variable

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.

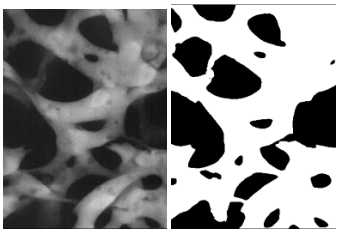
67. We recommend using confocal images of microvasculature that is perfused with fluorescent-labelled dextran to quantify the fluorescent intensities.
68. Set scale in the unit of µm based on the image resolution data.
69. Select region at the bottom section of the image and crop the image to avoid the posts.



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

70. Project a stack of confocal images at time point 1 (t_1) into 2D images based on the maximum intensity projection (MIP) method. Duplicate the projected image.
71. Pre-process the duplicated image if necessary. Depending on the image quality, pre-processing techniques such as Gaussian blur.
72. 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).

Reminder *Images should always be pre-processed as a whole.*



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

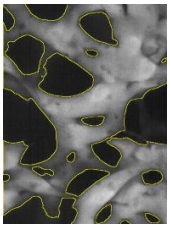
73. Use the built-in Selection: Create Selection function in ImageJ to select the binarized microvasculature as the first region of interest (ROI) and add that into the ROI manager.

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

74. 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.

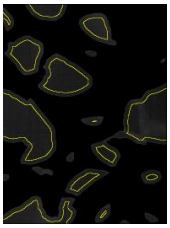
The yellow outline has been enlarged to include tissue area along with the microvasculature.

75. 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_1 (typically $t_1 = 0$ minutes).



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

76. After completing the measurements for the vessels, clear the selected vessel area. Next, select the second selection in ROI manager (ROI 2) which outlines the enlarged area to include the tissue. Use the Measure function to measure the fluorescent intensities of vessel plus tissue ($I_{V+T, cleared}$) 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.

77. Repeat step 70. 76. to determine the fluorescent intensities at time point 2 ,t_2 (typically $t_2 = 2$ minutes).

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

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

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

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

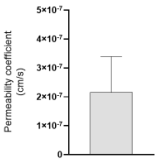
Reminder *The measurements that were done for tissue include the vessel area and intensity that was cleared. Therefore, they have to be adjusted accordingly to obtain the lateral tissue area and true tissue intensity.*

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

$$P_{app} = \frac{1}{(I_V^{t1} - I_T^{t1})} \frac{(I_T^{t2} - I_T^{t1})}{\Delta t} \frac{V_{tissue}}{A_{surface}}$$

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^{-7} 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. 10010023)
- 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**

80. Remove medium from the reservoirs by carefully aspirating the medium away from the media inlets.
81. 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.
82. Remove 1X PBS from the media reservoirs by carefully aspirating the PBS away from the media inlets.

83. 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.*

84. Incubate for 20 min at RT.

85. Remove the 4% formaldehyde solution from the reservoirs with a micropipette. Wash the channels twice with 1X PBS as described in Steps 80. and 81..

! 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)

86. 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.*

87. Incubate for at least 30 min at RT.

88. Wash once with 1X PBS as described in Steps 80. and 81..

Blocking ⏱**TIMING 2 h**

(Optional: Blocking is only necessary for immunofluorescent staining)

89. 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.*

90. Incubate for 2 h at RT.

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

91. 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.

92. 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.*

93. 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)

94. Remove the primary antibodies from the media reservoirs by carefully aspirating the antibody solution away from the media inlets. The antibodies may be reused depending on the antigen and antibody.

95. Wash with 1X PBS 5 times, with a 10 min incubation between each wash, as described in Steps 80. and 81..

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

97. Add 120 µl of the secondary antibody solution into one media reservoir (directly into the media channel inlet) and 80 µl into the other media reservoir (directly into the media channel inlet).

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

98. Incubate for 1 h at RT.

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

100. Wash with 1X PBS 5 times, with a 10 min incubation between each wash, as described in Steps 80. and 81..

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

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

TROUBLESHOOTING

Table 3 Troubleshooting advice

Step	Problem	Possible Reason	Solution
26.	Large fibrin fibers in the gel that affect EC cell alignment	Fibrin gel started to polymerize before the injection of gel into the chips	Avoid mixing the fibrin gel for too long before injecting it into the chips
		Fibrinogen stock solution is too old	Prepare a new batch of fibrinogen solution
33.	Microvasculature is too narrow	Seeding density of endothelial cells is too low	Increase the seeding density of endothelial cells
33.	Sheet like-microvasculature	Seeding density of endothelial cells is too high	Reduce the seeding density of endothelial cells
38.	Cells do not distribute evenly	The interval between the injections of cell suspension is short thus the flow of cells in the channel may be disrupted	Wait for at least 2 min before seeding cells into the opposite connected inlet
38.	Too many cells in a channel	Concentration of cell suspension is too high	Flush out unattached cells with culture medium immediately and repeat the seeding steps with cell suspension that is less concentrated
38.	Too few cells in a channel	Concentration of cell suspension is too low	Increase the concentration of cell suspension or repeat the seeding steps (without modifying the concentration of cell suspension) until the target cell density is obtained
38.	Cells do not adhere to the gel interface	The pressure head applied is insufficient	Increase the volume of cell suspension

REAGENT SETUP

THROMBIN STOCK SOLUTION

Reagents

- Thrombin from bovine plasma (Sigma-Aldrich, Cat. No. T4648)
- BSA (Sigma Aldrich, Cat. No. A9647)
- Sterile deionized water (Thermo Water Purifying System)

Others

- 0.2 µm 250 ml bottle top filter or 0.2 µm syringe filter

1. Prepare thrombin stock solution at a concentration of 100 units/ml in a 0.1 % (w/v) BSA solution (water).
2. Sterilize thrombin stock solution by passing through a 0.2 µm bottle top filter or syringe filter in a sterile laminar flow hood.
3. Aliquot the thrombin stock solution and store them at -80 °C.

Reminder *Water is used instead of PBS to keep the pH at 6.5 which is optimum to maintain the stability of thrombin.*

FIBRINOGEN STOCK SOLUTION

Reagents

- Fibrinogen from bovine plasma (Sigma-Aldrich, Cat. No. F8630-1G)
- 1X PBS (Life Technologies, Cat. No. 10010023)

Others

- 0.2 µm 250 ml bottle top filter or 0.2 µm syringe filter
- Nanodrop 1000 Spectrophotometer (Thermo Scientific)

4. Prepare aliquots of fibrinogen stock solution from one bottle of fibrinogen to prevent variability in experiments. Prepare the fibrinogen based on clottable protein (%). For this batch of fibrinogen, it has 73% protein measured by Biuret assay of which 92% is clottable where 67% is clottable protein per preparation.

   $$92\%\ clottable\ protein\ x\ 73\%\ protein = 67\%\ clottable\ protein$$

   Reminder *Use the lot number of the fibrinogen to view the certificate of analysis with the protein and clottable protein results.*

5. Account for the protein amount (73%) by dissolving 1000mg of bovine fibrinogen powder in 73ml of sterile 1X PBS.

   Reminder *The volume of PBS used can be higher or lower depending on the protein concentration needed at the end.*

6. Incubate the fibrinogen solution in a 37°C water bath for 1 hour to completely dissolve it. Do not vortex.

7. Sterilize the fibrinogen solution by passing it through a 0.2 µm syringe filter in a sterile laminar flow hood.

   Reminder *Some protein may be lost during the sterilization.*

8. 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%.

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

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

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

    $$Final\ stock\ concentration\text{: } 10.9mg/ml\ \ x\ \ 92\% = 10.028mg/ml$$

12. With the protein concentration now being 10.028mg/ml, use this as the stock concentration to prepare the 5mg/ml fibrinogen for experimental use.

13. Store the aliquots in -80 °C. 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.

GROWTH FACTOR-ENRICHED MEDIA

Reagents

- $VEGF_{165}$ (R&D Systems, Cat. No. 293-VE-500)
- BSA (Sigma Aldrich, Cat. No. A9418)
- 1X PBS (Life Technologies, Cat. No. 10010023)
- EGM-2 MV Microvascular Endothelial Cell Growth Medium-2 (Lonza, Cat. No. CC-3202)

14. Reconstitute recombinant human $VEGF_{165}$ at 100 µg/ml in sterile PBS containing at least 0.1 % BSA. Aliquot it into smaller volumes and store them at -20 °C.
15. Add 0.5 µl of VEGF (100 µg/ml) per ml of medium to get cell culture EGM-2 MV medium enriched with 50 ng/ml VEGF.

4% FORMALDEHYDE

Reagents

- Paraformaldehyde powder (Sigma-Aldrich, Cat. No. 158127)
- 1X PBS (Life Technologies, Cat. No. 10010023)

Others

- Hot plate with magnetic stirrer

16. 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.*

17. 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.*

18. 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. 10010023)

Others

- Hot plate with magnetic stirrer

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

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