INTRODUCTION

AIM idenTx 3 chips and idenTx 9 plates are utilized to create a 3D BBB model. The model is a tri-culture of human brain microvascular endothelial cells (HBMEC), brain pericytes (PC) and astrocytes (AC). These all-human cells self-organize and form microvascular networks within a fibrin gel through vasculogenesis. The idenTx 9 encompasses three individual idenTx 3 chips and an idenTx Holder, enabling 9 independent experiments on a single standard SBS-format plate. This protocol covers the calculations and techniques for seeding cells in idenTx 3 or idenTx 9 as well as the quantifications of microvascular geometry and permeability.

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THAWING CELLS ⏱ TIMING 70 min

MATERIALS

Reagents

- Collagen type I, rat tail (Corning Life Science, Cat. No. 354236)
- Acetic acid (Sigma-Aldrich, A6283)
- Poly-L-Lysine (ScienCell, Cat. No. 0413)
- Sterile deionized water (Thermo Water Purifying System)
- Attachment Factor (Cell-Systems, Cat. No. 4Z0-210)
- EGM-2MV Microvascular Endothelial Cell Growth Medium-2 (Lonza, Cat. No. CC-3202)
- Astrocyte medium (ScienCell, Cat. No. 1801)
- The System: Pericyte medium (Cell-Systems, Cat. No. CSS-A101)
- 1X PBS (Life Technologies, Cat. No. 10010023)

Others

- Human Brain Microvascular Endothelial Cells (Angio-Proteomie, Cat. No. cAP-0002)
- Astrocytes (ScienCell, Cat. No. 1800)
- Pericytes (Cell-Systems, Cat. No. ACBRI-498-V)
- Ice bucket or styrofoam box
- Dry Ice
- 0.22 uM syringe filter
- Syringe
- T75, T175 flasks

Coating flasks and thawing cells ⏱ **TIMING *60 min***

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

2. Prepare collagen coating solution for HBMECs. Prepare acetic acid stock by adding 575 µl of 99% acetic acid to 500 ml of dH20. Mix the solution and pass it through a sterile filter. Prepare 50 ml of 50 µg/ml collagen coating solution by diluting the collagen stock in acetic acid. Coating solution can be stored at 4°C.

3. For HBMECs, coat one T175 flask with 11 ml of collagen coating solution and incubate it at 37°C for at least 1 hour. Pre-warm 25 ml of EGM-2MV medium. After 1 hour, wash the flask with 1X PBS twice and add 25 ml of media into it.

4. Thaw the cryopreserved vial of HBMECs (0.5 M cells) quickly (approximately 1 min) by placing the lower half of the vial in a 37°C water bath. Monitor the vial closely during this process.

   ! Critical *Remove the vial from the water bath when only a small amount of ice remains. Do not allow the cells to thaw completely.*

5. Add 1 ml of warm EGM-2MV dropwise to the vial. Gently resuspend the cells in the vial by pipetting the cells 5 times. Transfer the cell suspension to the flask with media and keep them in a 37°C CO_2 incubator.

6. After 24 hours, remove the media from the flask, wash the flasks with 1X PBS twice, and replace it with fresh warm EGM-2MV to remove any leftover DMSO. Culture the cells for another 2 days before using them for experiment.

7. For astrocytes, coat one T75 flask with 15 ul of 10 mg/ml of poly-l-lysine in 10 ml of dH20 incubate it at 37°C for at least 1 hour. Pre-warm 15 ml of Astrocyte medium. After 1 hour, wash the flask with dH20 twice and add 25 ml of media into it.

8. Thaw the cryopreserved vial of Astrocytes (0.25 M cells) like step 4. Add 1 ml of warm astrocyte medium dropwise to the vial. Gently resuspend the cells in the vial by pipetting the cells 5 times. Transfer the cell suspension to the flask with media and keep them in a 37°C CO_2 incubator.

9. After 24 hours or the next day, remove the media from the flask, wash the flasks with 1X PBS twice, and replace it with fresh warm Astrocyte medium to remove any leftover DMSO. Culture the cells for another 2 days before using them for experiment.

10. For pericytes, coat one T75 flask with 5 ml of attachment factor for 5 seconds. Remove the attachment factor and add pre-warmed 25 ml of Pericyte medium into it.

11. Thaw the cryopreserved vial of Pericytes (0.5 M cells) like step 4. Add 1 ml of warm pericyte medium dropwise to the vial. Gently resuspend the cells in the vial by pipetting the cells 5 times. Transfer the cell suspension to the flask with media and keep them in a 37°C CO_2 incubator.

12. After 24 hours or the next day, remove the media from the flask, wash the flasks with 1X PBS twice, and replace it with fresh warm Pericyte medium to remove any leftover DMSO. Culture the cells for another 2 days before using them for experiment.

PREPARING & FILLING FIBRIN GEL WITH CELLS ⏱ TIMING 50 min

MATERIALS

Reagents

- Fibrinogen from bovine plasma (see REAGENT SETUP at the end of this protocol, Sigma Aldrich, Cat. No. F8630)
- Thrombin stock solution (see REAGENT SETUP at the end of this protocol, Sigma-Aldrich, Cat. No. T4648)
- Sterile deionized water (Thermo Water Purifying System)
- EGM-2MV Microvascular Endothelial Cell Growth Medium-2 (Lonza, Cat. No. CC-3202)
- Endothelial Growth Medium (Angio-Proteomie, Cat. No. cAP-02)
- Astrocyte medium (ScienCell, Cat. No. 1801)
- The System: Pericyte medium (Cell-Systems, Cat. No. CSS-A101)
- 1X PBS (Life Technologies, Cat. No. 10010023)
- Trypsin (Life Technologies, Cat. No. 25300054)
- TrypLE (Life Technologies, Cat. No. 12604013)
- Passage Reagent Group™ (Cell-Systems, Cat. No. 4Z0-800)
    - PRG-1 EDTA-dPBS Solution (4Z0-610)
    - PRG-2 Trypsin-EDTA Solution (4Z0-310)
    - PRG-3 Trypsin Inhibitor Solution (4Z0-410)

Others

- Human Brain Microvascular Endothelial Cells (Angio-Proteomie, Cat. No. cAP-0002)
- Astrocytes (ScienCell, Cat. No. 1800)
- Pericytes (Cell-Systems, Cat. No. ACBRI-498-V)
- 1.5 ml microcentrifuge tube
- Ice bucket or styrofoam box
- Ice
- idenTx 3 or idenTx 9
- idenTx holders
- 0.22 µm syringe filter

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

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

14. Determine the target seeding concentrations for endothelial cells (EC), astrocytes (AC) and pericytes (PC) in the fibrin gel. Table 1 is the recommended seeding concentrations based on the publication by Hajal *et al.* [1].

Table 1 Target seeding concentrations

Cell	Concentration
HBMECs	6 M cells/ml
Astrocytes	1 M cells/ml
Pericytes	0.5 M cells/ml

15. Do backward calculations to determine the cell concentrations of HBMEC, AC and PC in the master cell suspension.

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

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

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

Similarly,

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

$$c_{Pericyte} = 2\frac{Mcells}{ml}$$

Reminder *All 3 cell types are mixed to create the master stock. The master stock is then centrifuged and resuspended in EGM-2MV 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.*

16. Trypsinize cells as per protocol. Briefly, wash the culture flasks/dishes twice with sterile 1X PBS (for HBMEC and AC) and with PRG 1 (for PCs). Use TrypLE (for HBMEC), trypsin (for AC), and PRG 2 (for PC) to dissociate cells.

Reminder *HBMEC 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 HBMEC 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.*

17. Add respective medium with FBS (for HBMEC and AC) and PRG 3 (for PC), at least the same volume as TrypLE/trypsin/PRG 2, into the culture flasks/dishes to dilute/neutralize the TrypLE/trypsin/PRG 2.

18. Transfer the trypsinized cells to 15 ml tubes and pellet the cells by centrifuging at 300 x*g* for 5 min at RT (for EC and AC) and at 900 x*g* for 10 min at RT (for PC). After centrifugation, discard the supernatant. Resuspend the cells in 1ml of EGM-2MV media. Count the cells.

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

19. Table 2 is the respective cell numbers that are needed to prepare 35 µl of master cell suspension that is sufficient to fill at least 3 idenTx 3 or 1 idenTx 9. Based on the number of counted cells obtained in Step 18. calculate the required volume and then draw the cells from the respective cell stock and mix all 3 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 15.*

$$Volume\ of\ fibrin\ gel = (10\ \mu l\ x\ No\ of\ sites) + Extra\ volume$$
$$= (10\ \mu l\ x\ 9) + 50\ \mu l$$
$$= 140\ \mu l$$

$$Volume\ of\ working\ cell\ suspension = \frac{140\ \mu l}{2} = 70\ \mu l$$

$$Volume\ of\ master\ cell\ suspension = \frac{70\ \mu l}{2} = 35\ \mu l$$

Table 2 Cell numbers for 35 µl of master cell suspension

Cell	Cell Numbers
HBMECs	0.84 M cells
Astrocytes	0.14 M cells
Pericytes	0.07 M cells

20. Centrifuge the cells mixture at 300 x*g* for 5 min at RT.

21. Remove the supernatant as much as possible. Resuspend the cell pellet in 35 µl of EGM-2MV to get the master cell suspension. Keep this on ice.

Reminder *The cell pellet will always have a few microliters of leftover media. Always add lesser volume of EGM-2MV to resuspend the cells to avoid exceeding the final volume. Then measure the volume using a micropipette and add more media if needed to obtain the volume of 35 µl.*

22. Add 3.6 µl of thrombin stock solution (100 U/ml) into 56.4 µl of Lonza's EGM-2MV to yield **6 U/ml** of 4X concentrated thrombin solution. Keep this on ice.

! Critical *Lonza's EGM-2MV 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.*

23. Mix master cell suspension in a 1:1 ratio with 4X concentrated thrombin solution to obtain the working cell suspension. In this example, 35 µl of thrombin solution is added. Aliquot 5.5 µl of working cell suspension into 9 PCR tubes (0.2 ml) on ice.

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

24. Assemble idenTx 3 into an idenTx holder (see Instructions For Use for the idenTx holder included in the package). The idenTx 9 is ready-to-use.

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

Table 3 Final concentrations of fibrinogen and thrombin in the gel

	Concentration
Fibrinogen	2. 5 mg/ml
Thrombin	1.5 U/ml

26. Make sure the working suspension and fibrinogen solution are always kept on ice. Mix 5.5 µl of working cell suspension with 5.5 µl of fibrinogen solution to make fibrin gel solution in a microcentrifuge tube.

Reminder *Prepare fibrin gel that is only sufficient for one site at a time to avoid polymerization from taking place in the microcentrifuge tube.*

! 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 15 times.*

27. Draw 10 µl of fibrin gel solution with a 1- 10 µL micropipette.

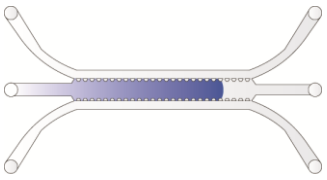
! Critical *Limit the volume of fibrin gel to 10 µl to prevent the fibrin gel from overflowing into media channels.*

28. Fill fibrin gel solution through either of the gel inlets at room temperature:

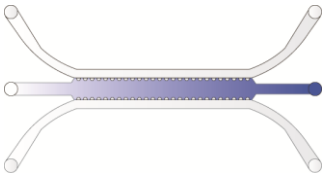
! Critical *Hold the plunger firmly while removing the micropipette from the inlets, otherwise the negative pressure will suck the gel up.*

! 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 10 s.*

a. **Option 1**: Fill fibrin gel solution from either of the inlets and stop near the end of posts. Fill from the other inlet until the gel fronts merge. This method is recommended for new users.



Inject from one end



Inject from the other end until the gel fronts merge

b. **Option 2**: Fill fibrin gel solution from one side all the way to the other side. Continue injecting the fibrin gel solution gently until it reaches the other inlet. This method ensures that the gel solution is being filled homogeneously but it requires greater control over pipetting pressure (especially when the fibrin gel solution reaches the opposite inlet) to prevent the fibrin gel solution from overflowing into the flanking media channels.

29. Repeat Steps 26 – 28 for all other sites.

30. Distribute 6 ml of 1X PBS into the reservoirs of the idenTx holder.

! Critical *idenTx 3 or idenTx 9 is laminated with a gas-permeable film that enables gas exchange to take place. The bottom of the chips should therefore be exposed to allow for air circulation.*

31. 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 4 for troubleshooting advice)

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

MATERIALS

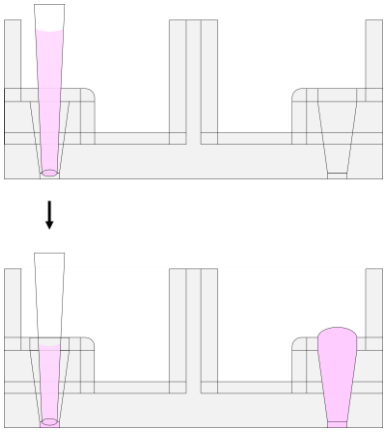
Reagents

- Human fibronectin (Sigma-Aldrich, Cat. No. F0895), 60 µg/ml in endothelial medium
- Medium A: Lonza's EGM-2MV enriched with 50 ng/ml Vascular Endothelial Growth Factor (VEGF) and 1% v/v Astrocyte Growth Supplement (See REAGENT SETUP)

Others

- Fibrin-filled idenTx 3 or idenTx 9

32. After incubation, insert a pipette tip into either inlet of the media channel and push gently until the tip fits. Inject 15 µl of coating solution (60 µg/ml fibronectin solution diluted in EGM-2MV) 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.



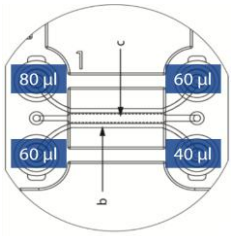
Insert a tip into a media inlet until it fits. Inject solution till it reaches the opposite

! 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 20 µl of solution at this step or the high injection pressure may disrupt the fibrin gel.*

33. Incubate the media-channel-hydrated chips for 1 hour in a 37°C incubator.

34. Add medium A 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.*

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

CHANGING MEDIUM ⏱ TIMING 15 min

MATERIALS

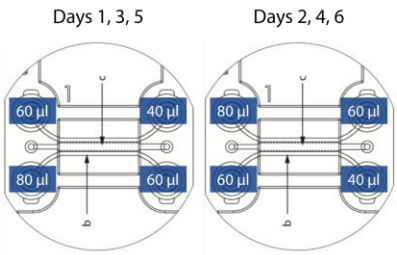
Reagents

- Medium A: EGM-2MV enriched with 50 ng/ml Vascular Endothelial Growth Factor (VEGF) and 1% v/v Astrocyte Growth Supplement (See REAGENT SETUP)
- Medium B: EGM-2MV enriched with 1% v/v Astrocyte Growth Supplement (See REAGENT SETUP)

36. 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.*

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



! Critical *The differential volumes in the two ports allow the replacement of medium to take place in the channel. The minimum volume of medium is 30 µl to ensure the inlets are covered and the troughs are wetted. If less than 30 µl of medium is used, the surface tension at the inlets will prevent the medium from flowing through the channel. We recommend using at least 40 µl of medium for easier handling.*

38. Use medium A from day 0 to day 3 and switch to medium B on day 4. Subsequently, add medium C on day 5 and 6. Keep the chips in an incubator. Microvasculature shall start forming within 2 days and continue to mature in the chips.

? Troubleshooting (see Table 4 for troubleshooting advice)

SEEDING ENDOTHELIAL CELLS IN MEDIA CHANNELS ⏱ TIMING 30 min

MATERIALS

Reagents

- 1X PBS (Life Technologies, Cat. No. 10010023)
- TrypLE (Life Technologies, Cat. No. 12604013)
- EGM-2MV Microvascular Endothelial Cell Growth Medium-2 (Lonza, Cat. No. CC-3202)
- Medium B: EGM-2MV enriched with 1% v/v Astrocyte Growth Supplement (See REAGENT SETUP)

Others

- Fibrin-filled and fibronectin-coated idenTx 3 or idenTx 9
- One T175 flask of EC

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

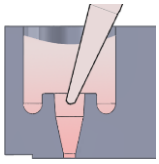
Reminder *Endothelial cells are seeded in the media channels to increase the cell coverage on the gel interface, to fill gaps at the gel-post borders and to improve the connections between the microvascular network and media channels.*

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

41. Add an additional 20 µl of EGM-2MV into one of the ports at the media channel that is to be seeded with cells.

Reminder *Ports must be filled with medium before seeding cells into the media channels.*

42. Use a micropipette to withdraw 20 µl of endothelial cell suspension. Position the tip near the inlet of a media channel and inject the cell suspension. The additional 40 µl of fluid (20 µl of cell suspension and 20 µl of medium) creates a height difference between the two media channels thus generating interstitial flow across the gel. This helps the attachment of endothelial cells on the gel interface.



Position the pipette tip at media inlets while injecting cell suspension

! 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 chips (on idenTx holders) on a flat surface while seeding cells into idenTx 3 or idenTx 9. Inclination of the chips affects the cell distribution.*

43. Visual inspection under a microscope is recommended. If the cell distribution is not optimal for your application, adjust the concentration of the cell suspension and repeat the seeding steps.

? Troubleshooting (see Table 4 for troubleshooting advice)

44. Keep the idenTx 9 in a 5% CO2 incubator at 37°C for at least 30 min to allow the endothelial cells to adhere.

45. Repeat steps 1. and 43. for the opposite media channel.

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

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

48. Add Medium B in volumes stated in Step 1. .

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

PREPARATION OF MEDIUM B WITH BBB^{Boost} ⏱ TIMING 30 min

MATERIALS

Reagents

- 100X BBB^{Boost}
- Medium B: EGM-2MV enriched with 1% v/v Astrocyte Growth Supplement (See REAGENT SETUP)

Others

- idenTx 9 plate seeded with cells in the gel and side channel
- 0.22 µm syringe filter
- Syringe

50. Switch to medium B with BBB^{Boost} from Day 5 onwards.

51. Add 10 µl of BBB^{Boost} (100X) per ml of medium B to achieve a final concentration of 1X BBB^{Boost}.

Reminder *Prepare extra to account for loss during sterile filtering.*

52. Sterile filter the medium using a 0.22 µm syringe filter.

53. Change the medium accordingly.

PERFUSING MICROVASCULATURE WITH FLUORESCENT DEXTRAN ⏱ TIMING 20 min

MATERIALS

Reagents

- 70 kDa FITC-dextran (Sigma Aldrich, Cat. No. FD70S)
- EGM-2MV Microvascular Endothelial Cell Growth Medium-2 (Lonza, Cat. No. CC-3202)
- 1X PBS (Life Technologies, Cat. No. 10010023)

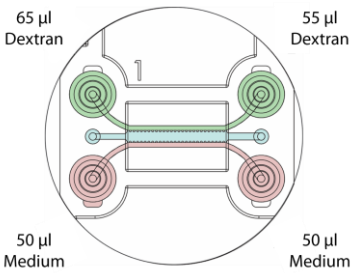
Others

- idenTx 3 or idenTx 9 with perfusable microvasculature (day 7 onwards)

54. Prepare FITC-dextran in PBS at 10 mg/ml and aliquot accordingly. Store at −20 °C. Dilute 1:40 in EGM-2MV to obtain final concentration of 0.25 mg/ml for perfusion.

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

56. Add 50 µl of EGM-2MV into the two ports in the bottom media channel.



Add the indicated volumes of dextran solution and medium into the respective ports as shown above.

57. Add 55 µl and 65 µl of dextran solution sequentially into the respective empty ports on the top media channel.

Rationale *The higher total volume of the dextran solution will drive its perfusion across any perfusable microvasculature within the gel channel, and the resulting volume difference between the left and right ports will generate shear flow across the top media channel.*

58. Image the dextran-perfused microvasculature with confocal microscopy or high content imaging system immediately with a 2 min interval for 12 min.

QUANTIFICATION OF MICROVASCULAR GEOMETRY ⏱ TIMING Variable

In order to quantify the microvascular geometry in idenTx 9, 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 9 but 3D imaging techniques such as confocal microscopy is 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.

59. 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.

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

61. 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).

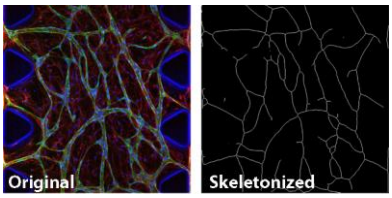
Reminder *You need to optimize the pre-processing steps based on the stained proteins/organelles and the image quality. You should always pre-process your images as a whole.*

62. 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.

63. 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.*

64. 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).

65. 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}).

66. 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.

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

68. 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.*

70. 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.

71. 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.

72. Adjust the segmented image into 8-bit.

73. 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 ($A_{surface}$).

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

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

75. 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 9, 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.

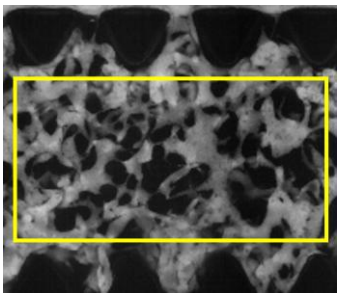
FLUORESCENT INTENSITIES OF VESSELS (I_V) & TISSUE (I_T)

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.

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

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

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



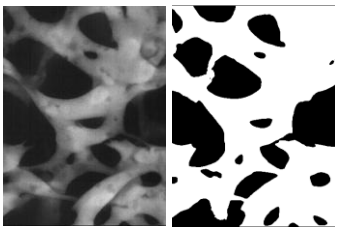
Reminder *To avoid the side diffusion affecting the analysis, crop the image nearer to the bottom posts as shown in the yellow box.*

79. 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.

80. 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.*

81. 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).

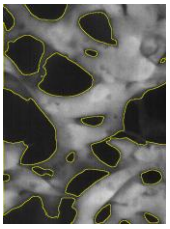
82. Use the built-in Selection: Create Selection function in ImageJ to select the binarized microvasculature as the first region of interest (ROI) and add that into the ROI manager.

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

83. Use the built-in Selection: Enlarge in Image J 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.

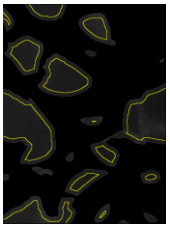
The yellow outline has been enlarged to include tissue area along with the microvasculature.

84. 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 $t_1 = 0$ minute).



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

85. 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.

86. Repeat step 79. – 85. 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.*

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

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

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

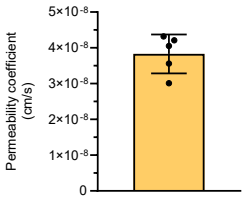
Reminder *The measurements that were done for tissue include the vessel area and intensity that was cleared. Therefore, they have to be adjusted accordingly to obtain the lateral tissue area and true tissue intensity.*

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

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

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].

$$P_{app} = \frac{1}{\left(I_V^{t1} - I_T^{t1}\right)} \frac{\left(I_T^{t2} - I_T^{t1}\right)}{\Delta t} \frac{A_{lateral_tissue}}{P_{vessel}}$$



The permeability of a microvasculature for 70 kDa FITC-dextran in idenTx 9 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, $V_{tissue}/A_{surafce}$.*

TROUBLESHOOTING

Table 4 Troubleshooting advice

Step	Problem	Possible Reason	Solution
31.	Large fibrin fibres 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
38.	Microvasculature is too narrow	Seeding density of endothelial cells is too low	Increase the seeding density of endothelial cells
38.	Sheet like-microvasculature	Seeding density of endothelial cells is too high	Reduce the seeding density of endothelial cells
43.	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
43.	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
43.	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
43.	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).

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

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.

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 1000 mg of bovine fibrinogen powder in 73 ml 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. 10.29 mg/ml), and the clottable protein percentage (92%) to derive the final stock concentration.

    $$Final\ stock\ concentration\text{: } 10.29\ mg/ml\ \ x\ \ 92\% = 9.47\ mg/ml$$

12. With the protein concentration now being 9.47 mg/ml, use this as the stock concentration to prepare the 5 mg/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)
- Astrocyte Growth Supplement (ScienCell, Cat. No. 1852)
- 1X PBS (Life Technologies, Cat. No. 10010023)
- EGM-2MV 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. **Medium A**: Add 0.5 µl of VEGF (100 µg/ml) and 10 µl of Astrocyte Growth Supplement (100X) per ml of medium to get cell culture medium enriched with 50 ng/ml VEGF and 1% v/v Astrocyte Growth Supplement.

16. **Medium B**: Add 10 µl of Astrocyte Growth Supplement (100X) per ml of medium to get cell culture medium enriched with 1% v/v Astrocyte Growth Supplement.

1. HAJAL, C., ET AL., *ENGINEERED HUMAN BLOOD-BRAIN BARRIER MICROFLUIDIC MODEL FOR VACSULAR PERMEABILITY ANALYSES.* NATURE PROTOCOLS, 2021. **17:** P. 95-128.

2. CAMPISI, M., ET AL., *3D SELF-ORGANIZED MICROVASCULAR MODEL OF THE HUMAN BLOOD-BRAIN BARRIER WITH ENDOTHELIAL CELLS, PERICYTES AND ASTROCYTES.* BIOMATERIALS, 2018. **180**: P. 117-129.