INTRODUCTION

AIM idenTx 3 chips and idenTx 9 plates are utilized to create a 3D BBB model through the co-culture of human induced pluripotent stem cell (iPSC)-derived endothelial cells (EC), brain pericytes (PC) and astrocytes (AC). These all-human cells rearrange themselves and form microvascular network within a fibrin gel through vasculogenesis. The idenTx 9 encompasses three individual idenTx 3 chips and a 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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PREPARING & FILLING FIBRIN GEL WITH CELLS ⏱ TIMING 50 min

MATERIALS

Reagents

- Fibrinogen from bovine plasma (Sigma Aldrich, Cat. No. F8630)
- Thrombin stock solution (see REAGENT SETUP at the end of this protocol, Sigma-Aldrich, Cat. No. T9549)
- Sterile deionized water (Thermo Water Purifying System)
- Vasculife® VEGF Endothelial Medium Complete Kit (see REAGENT SETUP at the end of this protocol, Lifeline Cell Technology, Cat. No. LL-0003)
- Astrocyte medium (ScienCell, Cat. No. 1801)
- Pericyte medium (ScienCell, Cat. No. 1201)
- 1X PBS (Life Technologies, Cat. No. 70011044)
- Trypsin (Life Technologies, Cat. No. 25300054)
- TrypLE (Life Technologies, Cat. No. 12604013)

Others

- Human iPSC-ECs (Cellular Dynamics International, Cat. No. R1112)
- Astrocytes (ScienCell, Cat. No. 1800)
- Pericytes (ScienCell, Cat. No. 1200)
- 1.5 ml microcentrifuge tube
- Ice bucket or styrofoam box
- Ice
- idenTx 3 or idenTx 9
- idenTx holders or humidified chambers
- 0.22 µm syringe filter

Preparing fibrin gel with dispersed cells ⏱ **TIMING 10 min**

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

2. Dissolve 12 mg of fibrinogen in 2 ml of 1X PBS to yield **6 mg/ml** of fibrinogen working solution. Incubate in a 37 °C water bath for > 1 h until the fibrinogen powder completely dissolves.

3. Filter the fibrinogen working solution with a 0.22 µm syringe filter. Keep the working solution on ice.

   Reminder *The working solution can be kept in 4°C for not more than 2 weeks.*

4. Add 40 µl of thrombin stock solution (100 U/ml) into 960 µl of endothelial medium to yield **4 U/ml** of suspension medium. Keep the suspension medium on ice.

   ! Critical *The heparin sulfate in the endothelium medium interferes with the conversion of fibrinogen to fibrin gel. Use heparin sulfate free-medium for this step.*

5. Determine the target seeding concentrations for iPSC-ECs, astrocytes and pericytes in the fibrin gel. Table 1 is the recommended seeding concentrations based on the publication by Campisi et al. [1].

Table 1 Target seeding concentrations

Cell	Concentration
iPSC-ECs	6 M cells/ml
Astrocytes	2 M cells/ml
Pericytes	2 M cells/ml

6. Do backward calculations to determine the concentrations of cell suspensions.

$$c_{iPSC-EC} = Seeding\ Concentration \times No.Cell\ Type \times Dilution\ Factor$$

$$c_{iPSC-EC} = 6\frac{Mcells}{ml} \times 3 \times 2$$

$$c_{iPSC-EC} = 36\frac{Mcells}{ml}$$

Similarly,

$$c_{Astrocye} = 12\frac{Mcells}{ml}$$

$$c_{Pericyte} = 12\frac{Mcells}{ml}$$

Reminder *Individual cell suspensions are mixed to obtain a master cell suspension stock. The master stock is then mixed with fibrinogen solution in a 1:1 ratio to form fibrin gel. Therefore the seeding concentrations are multiplied by the number of cell types and the dilution factor of 2 to obtain the concentrations of cell suspensions.*

7. Trypsinize cells as per protocol and re-suspend the cells in the suspension medium in respective concentrations. Briefly, wash the culture flasks/dishes with sterile 1x PBS twice. Use TrypLE (for iPSC-ECs) or trypsin (for astrocytes and pericytes) to dissociate cells.

8. Add medium with FBS, at least 5 times the volume of TrypLE/trypsin, into the culture flasks/dishes to dilute/neutralize the TrypLE/trypsin.

9. Transfer the cell suspensions to 15 ml tubes and pellet the cells by centrifuging at 250 *xg* for 5 min at RT. Re-suspend the cells in the suspension medium in respective concentrations. Keep the cell suspensions on ice.

10. Draw 30 µl of cell suspension from each tube, and mix them to make a 90 µl master cell suspension stock. This amount is sufficient for filling at least 4 idenTx 3 or 1 idenTx 9.

Filling fibrin gel (with cells) ⏱ TIMING 40 min

11. 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.

12. Mix 6 µl of master cell suspension with 6 µl of fibrinogen solution to make fibrin gel solution in a microcentrifuge tube. Make sure the fibrin gel solution is kept on ice at all times.

Reminder *Prepare fibrin gel that is only sufficient for a 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 than10 s. If unsure, pipette up and down for not more than 15 times.*

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

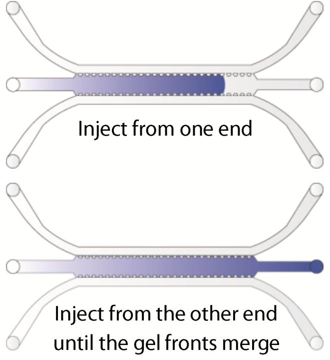
! Critical *Limit the volume of fibrin gel to 10 µl to prevent the fibrin gel from overflowing into media channels.*

14. 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.



    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.

15. Repeat steps 12 -14 for all other sites.

16. Add 6 ml of water into the reservoirs of the idenTx holder. Alternatively, prepare a humidified chamber to house the chips (e.g. by adding water into a pipette tip box until approximately 1/3 is filled; both water and pipette tip box should be sterile).

! 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.*

17. 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 2 for troubleshooting advice)

HYDRATING & COATING MEDIA CHANNELS ⏱ TIMING 5 min

MATERIALS

Reagents

- Human fibronectin (Sigma-Aldrich, Cat. No. F0895), 60 µg/ml in endothelial medium
- Medium A: Endothelial medium 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

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18. After incubation, insert a pipette tip into either inlet of the media channel and push gently until the tip fits. Inject 15 µl of fibronectin coating solution into the channel. Due to surface tension, the injected 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.*

19. Incubate the media-channel-hydrated chips (on idenTx holders or in humidified chambers) for 1 h in a 37°C incubator.

20. Add 70 µl of medium A into one port and then add 50 µl into the opposite port of the same media channel to flush out the coating solution. Repeat this for the other channels.

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

CHANGING MEDIUM ⏱ TIMING 10 min

MATERIALS

Reagents

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

22. Remove medium from all 4 ports by carefully aspirating the medium out from the troughs. To replace the medium in a media channel, add 70 µl of medium into one port and then add 50 µl into the opposite connected port. Repeat this for the other channel.

Always remove medium from troughs

! 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 50 µl of medium for easier handling.*

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

23. Use medium A from day 0 to day 4 and switch to medium B from day 4 onwards. Keep the chips in an incubator. Microvasculature shall start forming within 2 days and continue to mature in the chips.

? Troubleshooting (see Table 2 for troubleshooting advice)

SEEDING ENDOTHELIAL CELLS IN MEDIA CHANNELS ⏱ TIMING 20 min

MATERIALS

Reagents

- 1X PBS (Life Technologies, Cat. No. 70011044)
- 0.25% trypsin with EDTA (Lonza, Cat. No. CC5012)
- Medium A: Endothelial medium enriched with 50 ng/ml Vascular Endothelial Growth Factor (VEGF) and 1% v/v Astrocyte Growth Supplement (See REAGENT SETUP)

Others

- Fibrin-filled and fibronectin-coated idenTx 3 or idenTx 9

24. Seed endothelial cells in one of the media channels on day 2 after the daily medium change.

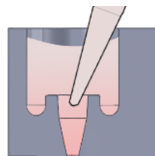
Reminder *Endothelial cells are seeded in the media channels to increase the cell coverage on the gel interface, to fill gaps at the gel-post borders and to improve the connections between the microvascular network and media channels.*

25. Trypsinize endothelial cells as per protocol and re-suspend the cells at 1.5 M cells/ml.

26. Add an additional 30 µl of medium A 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.*

27. Use a micropipette to withdraw 10 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 (10 µl of cell suspension and 30 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 or in humidified chambers) on a flat surface while seeding cells into idenTx 3 or idenTx 9. Inclination of the chips affects the cell distribution.*

28. 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 2 **for troubleshooting advice)**

29. Wait for 5 min and then remove medium from all the ports. Add 50 µl of medium A into each port.

30. Use a micropipette to withdraw 10 µl of endothelial cell suspension. Position the tip at the same inlet that has been injected with endothelial cell. Inject the cell suspension.

31. Remove water from the reservoirs of the idenTx holders. Flip the holder (with chips) upside down and incubate for 1.5 h in a 37°C incubator. This helps the attachment of endothelial cells on the top surafce of the media channel.

32. Flip the holder back to its upright position after the incubation. Fill the reservoirs with water.

33. (Optional) Change medium to remove unattached cells.

34. Keep the chips in an incubator. Allow the endothelial cells to grow for 24 h and repeat steps 25 – 33 on day 3 for the other media channel.

Reminder The *surface tension at the ports prevents the medium from dripping even though the chips are flipped.*

PERFUSING MICROVASCULATURE WITH FLUORESCENT DEXTRAN ⏱ TIMING 20 min

MATERIALS

Reagents

- 1X PBS (Life Technologies, Cat. No. 70011044)
- 10 kDa FITC-dextran (Sigma Aldrich, Cat. No. FD10S)
- Medium B: Endothelial medium enriched with 1% v/v Astrocyte Growth Supplement (See REAGENT SETUP)

Others

- idenTx 3 or idenTx 9 with perfusable microvasculature (day 7 onwards)
- 0.2 µm syringe filter

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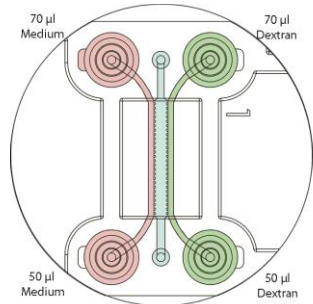
35. Prepare 2 µg/ml of 10 kDa FITC-dextran in PBS. Sterilize it using 0.2 µm syringe filter.

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

37. Add 70 µl of dextran solution into one port and then add 70 µl of medium B into the port of the other media channel.

Reminder *The surface tension at the media channel inlets prevents the dextran solution/medium from flowing through the channels at this step.*

38. Add 50 µl of dextran solution and medium B into the respective empty ports with a 20 s interval to allow the dextran solution to flow into the microvascular network.



Add 70 µl of dextran solution and medium into the top ports and then add 50 µl of dextran solution and medium into the bottom ports.

39. Image the dextran-perfused microvasculature with confocal microscopy or high content imaging system starting immediately after step 38. with a 5 min interval for 30 min.

QUANTIFICATION OF MICROVASCULAR GEOMETRY ⏱ TIMING Variable

In order to quantify the microvascular geometry in idenTx 3 or 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 3 or 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.

40. 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.

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

42. 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.*

43. 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.

44. 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.*

45. 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.

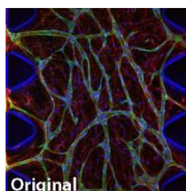


Figure 1 A 3D projection of a stack of confocal images (left) and its corresponding skeletonized image (right).

46. 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}).

47. 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.

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

49. 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.*

51. 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.

52. 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.

53. Adjust the segmented image into 8-bit.

54. 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 ($A_{surface}$).

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

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

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

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

Reminder *The circularity of a circle is 1.*

QUANTIFICATION OF MICROVASCULAR PERMEABILITY ⏱ TIMING Variable

In order to quantify the microvascular permeability in idenTx 3 or 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.

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

58. Project a stack of confocal images at time point 1 (t_1) into 2D images based on the maximum intensity projection method. Duplicate the projected image.

59. Pre-process the duplicated image if necessary. Depending on the image quality, you may apply pre-processing techniques such as enhance contrast.

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

60. Binarize the duplicated image.

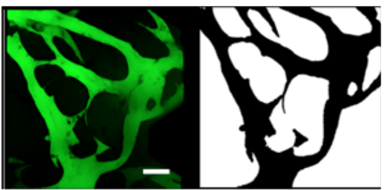


Figure 2 The projected image (left) of the fluorescent dextran perfused microvasculature and its binarized image (right). Scale bar is 50 µm. Adapted from Campisi et. al. 2018 [1]

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

62. Use the built-in Selection: Make Inverse function in Image J to select the area outside of the microvasculature as second ROI and add that into the ROI manager.

63. Select the original projected image so that it is the active window. Use the Measure function in the ROI manager to measure the fluorescent intensities of vessels (I_V), fluorescent intensities of tissue (I_T), the perimeter of the microvasculature (P) and lateral tissue area ($A_{lateral_tissue}$) at t_1 (typically $t_1 = 0$ minute).

64. Repeat step 58. – 63. to determine the fluorescent intensities at time point 2 ,t_2 (typically $t_2 = 30$ minute).

65. 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:

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

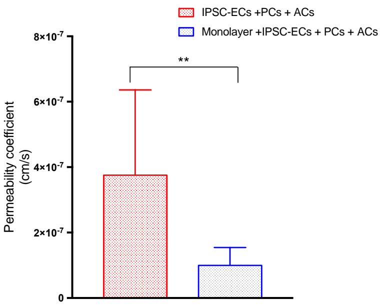


Figure 3 The permeability of microvasculature for 10 kDA FITC-dextran in idenTx 3 is typically in the range of 10^{-7} cm/s. Unpublished work by Marco Campisi.

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 2 Troubleshooting advice

Step	Problem	Possible Reason	Solution
17.	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
23.	Microvasculature is too narrow	Seeding density of endothelial cells is too low	Increase the seeding density of endothelial cells
23.	Sheet like-microvasculature	Seeding density of endothelial cells is too high	Reduce the seeding density of endothelial cells
28.	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
28.	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
28.	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
28.	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 (Sigma-Aldrich, Cat. No. T9549)
- 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 -20 °C.

VASCULIFE® VEGF ENDOTHELIAL MEDIUM

Reagents

- Vasculife® VEGF Endothelial Medium Complete Kit (Lifeline Cell Technology, Cat. No. LL-0003)
- iCell Endothelial Cells Medium Supplement (Cellular Dynamics International, Cat. No. M1019)

Others

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

4. Supplement the Vasculife Basal Medium with all the components in the kit except for FBS LifeFactor and L-glutamine LifeFactor.
5. Replace FBS LifeFactor with iCell Endothelial Cells Medium Supplement. Reduce the volume of L-glutamine LifeFactor to 10 ml.
6. Sterilize the enriched Vasculife Endothalial Medium by passing through a 0.2 µm bottle top filter or syringe filter in a sterile laminar flow hood.

GROWTH FACTOR-ENRICHED MEDIA

Reagents

- $VEGF_{165}$ (R&D Systems, Cat. No. 293-VE-010)
- BSA (Sigma Aldrich, Cat. No. A9647)
- Astrocyte Growth Supplement (ScienceCell, Cat. No. 1852)
- Vasculife® VEGF Endothelial Medium Complete Kit (Lifeline Cell Technology, Cat. No. LL-0003)
- 1X PBS (Life Technologies, Cat. No. 70011044)

Others

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

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7. 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. Dilute the aliquots to 10 µg/ml with PBS as secondary stock solutions before use.

8. **Medium A**: Add 5 µl of VEGF (10 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.

9. **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.

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1. 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.