INTRODUCTION

VasQ Kit is a turn-key solution to create 3D perfusable vasculature models through vasculogenesis. It is designed to support high-throughput applications in research and drug development. Utilizing AIM's organiX plates, which feature 24 individual experimental sites on each plate, the VasQ Kit enables the co-culture of immortalized human umbilical vein endothelial cells (HUVEC) and immortalized lung fibroblasts (LF) within a fibrin gel to promote the formation of microvascular networks through self-assembly. Researchers can also incorporate spheroids, organoids, or patient-derived biopsies into the model, enabling the vascularization of complex 3D tissues for more physiologically relevant studies. This innovative platform facilitates the generation of microvascular structures that mimic *in vivo* microenvironments, making it ideal for assessing vascular geometry and permeability in various experimental settings. The accompanying protocol includes detailed instructions for cell seeding, maintenance, and quantification techniques to streamline the experimental workflow for consistent and reproducible results.

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VASQ KIT COMPONENTS

MATERIALS

Included in the Kit

- Thrombin from bovine plasma (1 X 50 µl)
- Fibrinogen from bovine plasma Type I-S (2 X 300 µl)
- G418 (Geneticin) (2 X 100 µl)
- Vascular Endothelial Growth Factor (VEGF, 1 X 35 µl)
- Human Umbilical Vein Endothelial Cells/TERT2 (HUVEC/TERT2, 2 X 0.5 M cells)
- Lung Fibroblasts/TERT309 (LF/TERT309 ,1 X 0.5 M cells)
- orgainX plate

Non-included in the kit

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

CELL CULTURE

MATERIALS

Included in the Kit

- G418 (Geneticin) (2 X 100 µl)
- Vascular Endothelial Growth Factor (VEGF, 1 X 35 µl)
- Human umbilical vein endothelial cells/TERT2 (HUVEC/TERT2, 2 X 0.5 M cells)
- Lung Fibroblasts/TERT309 (LF/TERT309, 1 X 0.5 M cells)

Non-included in the kit

- EGM Endothelial Cell Growth Medium BulletKit (Lonza, Cat. No. CC-3124)
- DMEM/F12 (1:1), w: stable Glutamine, w: 1.2 g/L NaHCO3 (Pan Biotech, Cat. No. P04-41150)
- Fetal bovine serum, certified, heat-inactivated (Sigma-Aldrich, Cat. No 10082147)
- 0.1% bovine gelatin (Sigma-Aldrich, Cat. No. G1393)
- 1X PBS (Life Technologies, Cat. No. 20012027)
- 2x T175 flasks
- T75 flask

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

1. Supplement the EGM™ Endothelial Cell Growth Medium BulletKit™ with 10% FBS to make the complete EGM medium:
   a) Remove 50 ml of medium from a new bottle of EBM basal medium (500 ml).
   b) Add the EGM™ SingleQuot™ Supplements into the remaining 450 ml of EBM basal medium:
   c) Add an extra 40 ml of FBS to reach a final concentration of 10% FBS.
2. Supplement the DMEM/F12 (1:1) medium with 10% FBS:
   a) Remove 50 ml of medium from a new bottle of DMEM/F12 basal medium (500 ml).
   b) Add 50 ml of FBS into the remaining 450 ml of DMEM/F12 basal medium.
3. Prepare the media needed for 1 organiX plate:
   a) 250 ml of EGM supplemented with 50 µl of G418: EGM complete medium.
   b) 50 ml of DMEM/F12 supplemented with 100 µl of G418: DMEM/F12 complete medium.
4. The complete media can be stored at 4°C for a maximum of 4 weeks.
5. Bring the media to room temperature (not 37°C) before use.

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

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

6. Prepare the gelatin coating solution by adding 38 ml of 1X PBS (room temperature) to a 2 ml 2% gelatin stock solution that is warmed up to 37°C.

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

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

8. Prepare 9 ml of EGM complete medium in two 15 ml centrifugation tubes.

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

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

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

12. Perform a media change at day 2 by removing the medium from the flask and replacing it with 20 ml of pre-warmed EGM complete medium.

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

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

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

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

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

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

18. If the cells are not confluent on day 2, perform a media change by removing the medium from the flask and replacing it with 15 ml of pre-warmed DMEM/F12 complete medium.

PREPARING & FILLING FIBRIN GEL WITH CELLS ⏱TIMING 50 min

MATERIALS

Reagents

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

Others

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

Preparing working cell suspension in thrombin solution ⏱TIMING 60 min

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

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

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

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

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

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

Similarly,

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

Reminder *Both cell types are mixed to create the master stock. The master stock is then centrifuged and resuspended in DMEM/F12 first, also known as master cell suspension. This is mixed with the stock thrombin solution in a 96:4 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.*

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

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

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

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

25. Table 1 is the respective cell numbers that are needed to prepare 660 µl of master cell suspension that is sufficient to fill at least 1 organiX plate. Based on the number of counted cells obtained in step 21 , calculate the required volume and then draw the cells from the respective cell stock and mix these 2 cells together in a 1.5 ml microcentrifuge tube.

Reminder *The volume of master cell suspension is determined by dividing total volume of fibrin gel needed by the dilution factor as detailed in Step 21*

$$Volume\ of\ fibrin\ gel\ = (25\ \mu l\ \times\ No.of\ sites) \times 1.1\ Extra\ volume$$
$$= (25\ \mu l\ \times\ 24) \times\ 1.1$$
$$= 660\ \mu l$$
$$Volume\ of\ master\ cell\ suspension = 660\ \mu l$$
$$Volume\ of\ Thrombin\ required =$$
$$= Volumen\ x\ \frac{Thrombin\ working\ concentration}{Thrombin\ stock\ concentration}$$
$$= 660\ \mu l\ x\ \frac{5\ U\ working\ concentration}{100\ \ U\ stock\ concentration} = (660\ \mu l\ \times\ 0.05)$$
$$Volume\ of\ Thrombin = 33\ \mu l$$

Table 1 Cell numbers for 660 µl of master cell suspension

Cell	Cell Numbers
HUVEC/TERT	7.92 M cells
LF/TERT	1.32 M cells

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

27. Remove the supernatant as much as possible. Resuspend the cell pellet in 500 µl of DMEM/F12 to get the master cell suspension. Measure the volume of master cell suspension using a micropipette and top it up with more media to get a final volume of 627 µl.

Reminder *The cell pellet will always have a few microliters of leftover media. Always add lesser volume of DMEM/F12 to resuspend the cells to avoid exceeding the final volume, in this example we use 500 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 627 µl.*

28. Keep the master cell suspension on ice.

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

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

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

Table 2 Final concentrations of fibrinogen and thrombin in the gel

	Concentration
Fibrinogen	4 mg/ml
Thrombin	2.5 U/ml

30. Prepare 8 mg/ml of working fibrinogen solution using these values if Nanodrop is not available.

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

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

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

$$= 660\ \mu\text{l}\ x\ \frac{8\ mg/ml}{9.971\ mg/ml} = 529.5\ \mu\text{l}$$

31. Mix 529.5 µl of Fibrinogen with 130.5 µl of sterile PBS.

32. Mix master cell suspension with thrombin stock solution to obtain the working cell suspension. In this example, 33 µl of thrombin solution is added to the master cell suspension tube (627 µl).

33. Aliquot 25 µl of working cell suspension into 24 0.2 ml PCR tubes on ice.

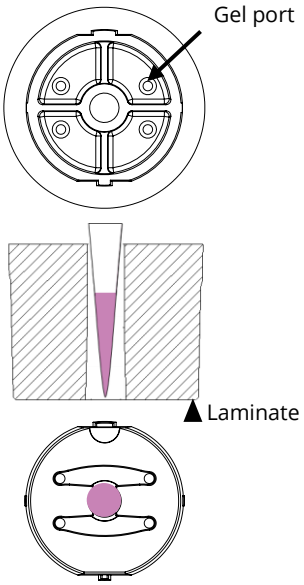
34. Make sure the working suspension and fibrinogen solution are always kept on ice. Mix 25 µl of working cell suspension with 25 µl of fibrinogen solution to make the fibrin gel solution in a microcentrifuge tube. This volume is sufficient to fill only 1 insert.

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

35. Withdraw 50 µl of fibrin gel solution with a 200 µl micropipette.

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

36. Insert the micropipette tip straight down into the gel port. Position it slightly above the bottom laminate. Then, dispense the fibrin gel gently. Repeat steps 34 and 35 for the rest of the sites.



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

37. Repeat steps 34 - 36 for the other 23 tubes.

38. Allow polymerization of hydrogel to take place for 20 min at room temperature.

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

HYDRATING THE MEDIA CHANNELS ⏱TIMING 10 min

MATERIALS

Reagents

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

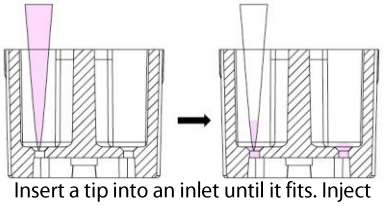
Others

- Fibrin-filled organiX plate

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

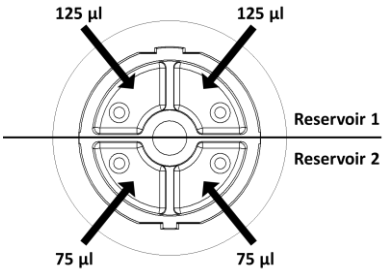
Reminder *The total media needed for 1 organiX plate for 7 days is 69 ml.*

40. After incubation, insert a pipette tip into either inlet of the media channel and push gently until the tip fits. Inject 35 µl of Vasculogenesis medium into the channel. Repeat this step for the other channel.



Insert a tip into an inlet until it fits. Inject medium till it reaches the opposite inlet.

41. Add Vasculogenesis medium with the indicated volumes on the right. Repeat this for all the sites.



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

CHANGING MEDIUM ⏱TIMING 15 min

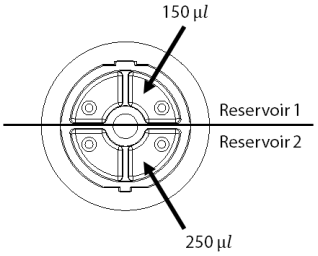
MATERIALS

Reagents

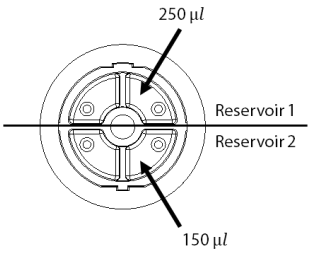
- Vasculogenesis medium (EGM complete media enriched with 50 ng/ml VEGF)

43. Remove medium from the 2 media reservoirs by carefully aspirating the medium away from the media inlets.

Days 1,3, 5



Days 2, 4, 6



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

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

Reminder *Adding medium to 1 port of a media channel is sufficient to generate flow through the channel due to the previously wetted surfaces in all the media ports. If the medium did not flow through, refer to Step 41*

45. Keep the plate in an incubator. Microvasculature should start forming within 2 days and continue to mature over time.

PERFUSING MICROVASCULATURE WITH FLUORESCENT DEXTRAN ⏱TIMING 2 hours

MATERIALS

Reagents

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

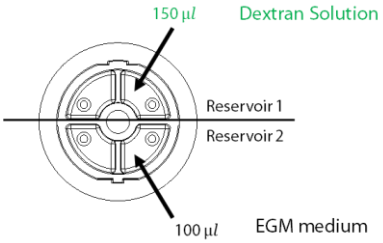
Others

- organiX plate with perfusable microvasculature (day 7 onwards)

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

47. Remove medium completely from the 2 media reservoirs by carefully aspirating the medium away from the media inlets.

48. Add 100 µl of EGM medium into one side of the reservoir 2.



49. Add 150 µl of dextran solution into the reservoir 1.

! Reminder *There is a total volume difference of 50 µl between both reservoirs. This difference allows the perfusion of fluorescent dextran solution through the microvasculature.*

50. Image the dextran-perfused microvasculature with a confocal microscope or high content imaging system immediately. Ideally, the time-lapse images shall be taken every 3 min for a total of 15 min.

Table 3 Image acquisition settings

Objective	10x
Timepoints	5
Planes	10
Distance (between planes)	10 µm

QUANTIFICATION OF MICROVASCULAR GEOMETRY ⏱TIMING Variable

To quantify microvascular geometry in organiX, image data obtained either from fluorescent dextran perfusion or from endothelial-specific labelling (for example, VE-cadherin immunostaining) can be used. The organiX platform is compatible with brightfield, phase-contrast, and epifluorescence microscopy; however, due to the three-dimensional nature of the assay, confocal microscopy is preferred for accurate structural characterization. Accordingly, the quantification approaches described below use confocal images acquired following fluorescent dextran perfusion as representative 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.

51. We recommend using confocal images of endothelial cells that are either fluorescently tagged, immunostained, or visualized via perfusion with fluorescent dextran to quantify sprout length and branching point number.

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

53. Pre-process the images if necessary. Depending on image quality, noise may be reduced using despeckle and background subtraction methods, or by applying a Gaussian filter to smooth edges and bridge gaps in the signal, particularly when endothelial junction proteins (e.g., VE-cadherin) are used for cell labeling.

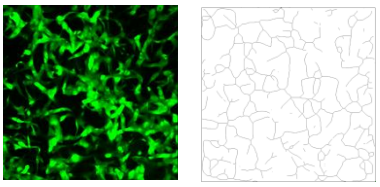
Reminder *The pre-processing steps should be optimized based on the stained proteins/organelles and the image quality. Image should always pre-process as a whole.*

54. Evaluate all thresholding methods on at least three individual projected images. Select the method that most effectively segments the structures and provides the closest representation of the original images. Apply the chosen threshold to the pre-processed images to generate binary masks.

55. 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 should be set according to the images.*

56. 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).

57. 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}).

58. 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.

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

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

Reminder *Images should always pre-process as a whole.*

61. Try all threshold methods and choose the method that segments the dataset 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.

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 organiX, 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.

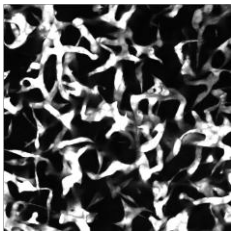
FLUORESCENT INTENSITIES OF VESSELS (I_V) & TISSUE (I_T)

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.

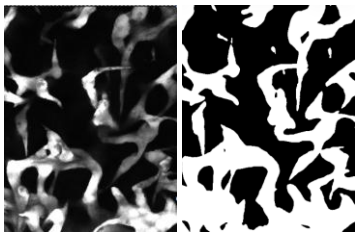
69. Project a stack of confocal images at timepoint 2 (t_2) into 2D images based on the maximum intensity projection (MIP) method. Duplicate the projected image.



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

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

71. 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).

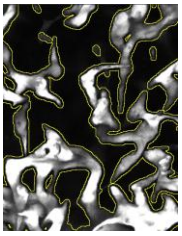
72. Use the built-in Selection: Create Selection function in ImageJ to select the binarized microvasculature as the first region of interest and add that into the ROI manager.

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

73. 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.

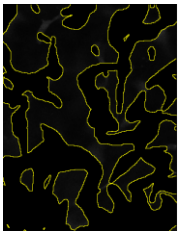
The yellow outline has been enlarged to include tissue area along with the microvasculature.

74. Select the original projected image so that it is the active window. Select the first selection in ROI manager which outlines the vessels. Use the Measure function to measure the fluorescent intensities of vessels (I_V), perimeter of the microvasculature (P_{vessel}) and area of vessel (A_V) at t_2 (typically $t_2 = 6$ mins).



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

75. 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 $t_1 = 0$ min).



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

76. Repeat Steps 69 to 75 to determine the fluorescent intensities at time point 3, t_3 (typically $t_3 = 9$ mins).

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

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

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

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

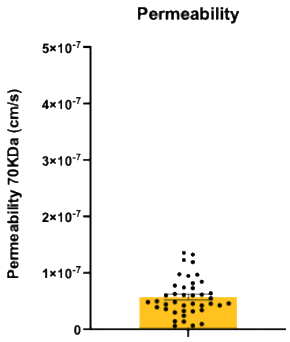
Reminder *The measurements that were done for tissue include the vessel area and intensity that was cleared. Therefore, they have to be adjusted accordingly to obtain the lateral tissue area and true tissue intensity.*

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

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

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

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



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

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

STAINING CELLS ⏱TIMING 2 days

MATERIALS

Reagents

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

Cell Fixation ⏱TIMING **30 min**

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

80. Add 250 µl of 1X PBS into one media reservoir and then add 100 µl into the other media reservoir. The differential volumes in the two reservoirs create flow through the gel to wash the sample.

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

82. Add 250 µl of 4% formaldehyde into one media reservoir and then add 100 µl into the other media reservoir.

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

83. Incubate for 20 min at RT.

84. 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)

85. Remove 1X PBS from the media reservoirs by carefully aspirating the PBS away from the media inlets. Add 250 µl of 0.1 % Triton X-100 into one media reservoir and 100 µl into the other media reservoir.

Reminder *The 1X PBS within the media channels can be carefully removed using a micropipette before the addition of Triton X-100, 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.*

86. Incubate for at least 30 min at RT.

87. Wash once with 1X PBS as described in steps 80 and 81.

Blocking ⏱**TIMING 2 h**

(Optional: Blocking is only necessary for immunofluorescent staining)

88. Remove 1X PBS from the media reservoirs by carefully aspirating the PBS away from the media inlets. Add 250 µl of blocking buffer into one media reservoir and 100 µl into the other media reservoir.

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

89. Incubate for 2 h at RT.

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

90. 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.

91. Remove the blocking buffer from the media reservoirs by carefully aspirating the blocking buffer away from the media inlets. Add 150 µl of the antibody solution into one media reservoir (directly into the media channel inlet) and 50 µl into the other media reservoir (directly into the media channel inlet).

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

92. Incubate overnight at 4°C.

93. Some antibodies will need longer incubation time. If needed, after overnight incubation, transfer 50 µl from the reservoir (where 150 µl of the antibody solution was added) to the opposite reservoir and 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 150 µl of the secondary antibody solution into one media reservoir (directly into the media channel inlet) and 50 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.*

HARVESTING SAMPLES ⏱ TIMING 20 min

MATERIALS

Others

- organiX Extractor

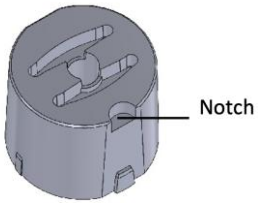
  Note: The provided organiX extractor is not sterile. ***Use 70% alcohol and/or UV-light to sterilize the organiX extractor, if needed.***

Sample harvesting ⏱ TIMING 20 min

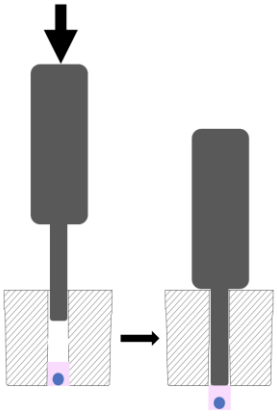
1. Remove the organiX insert from the organiX plate.

Reminder *The samples can be fixed or be frozen before the harvesting step. Greater force is needed to push frozen samples out from the organiX inserts as they expand in size.*

2. Grip the bottom laminate at the notch using a pair of tweezers and peel it off.



3. Use the organiX Extractor to push out the sample gently.



4. Collect the sample in a clean microcentrifuge tube or petri dish for further processing.

REAGENT SETUP

FIBRINOGEN MEASUREMENT

Reagents

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

Others

- Nanodrop 1000 Spectrophotometer (Thermo Scientific)

5. 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%.

6. 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.

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

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

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

   $$Final\ stock\ concentration: 10.9mg/ml\ x\ 92\% = 10.026mg/ml$$

10. With the protein concentration now being 10.026mg/ml, use this as the stock concentration to prepare the 8mg/ml fibrinogen for experimental use.

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

Reagents

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

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

4% FORMALDEHYDE

Reagents

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

Others

- Hot plate with magnetic stirrer

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

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

2. Stir for approximately 6 h.

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

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

0.1% TRITON X-100

Reagents

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

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

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