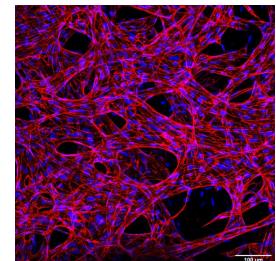


Apr 29, 2024

## Xeno-free microvasculature-on-a-chip model

DOI

[dx.doi.org/10.17504/protocols.io.261ge5obog47/v1](https://dx.doi.org/10.17504/protocols.io.261ge5obog47/v1)



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DOI: [dx.doi.org/10.17504/protocols.io.261ge5obog47/v1](https://dx.doi.org/10.17504/protocols.io.261ge5obog47/v1)

**Protocol Citation:** Robert Mertens, Jennifer Schwarzkopf, Katja Meier, André Rosa, Holger Gerhardt, PETER VAJKOCZY, Anja Nitzsche 2024. Xeno-free microvasculature-on-a-chip model. [protocols.io https://dx.doi.org/10.17504/protocols.io.261ge5obog47/v1](https://dx.doi.org/10.17504/protocols.io.261ge5obog47/v1)

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**Protocol status:** Working

**We use this protocol and it's working**

**Created:** April 18, 2024

**Last Modified:** April 29, 2024

Protocol Integer ID: 98378

**Keywords:** Microfluidic device, xeno-free, HUVEC, pericytes, endothelial

**Funders Acknowledgement:**

**Robert Mertens**

Grant ID:

Landestierschutzbeauftragte  
des Landes Berlin (CHE0011)

**Robert Mertens**

Grant ID: BIH - Charité Junior  
Clinician Scientist Program  
(Charité - Universitätsmedizin  
Berlin; Berlin Institute of  
Health)

## Abstract

Multicellular, complex human cell-derived models, such as organ-on-a-chip or microvasculature-on-a-chip models offer the opportunity of providing both, an alternative to animal experimentation and a more reliable bench-to-bedside translation. However, these models often rely on animal-derived products, for example fetal bovine serum or matrices like matrigel and collagen. Apart from inflicting animal suffering, the use of animal-derived products also prompt biological problems, as artificial phenomena could be a result of cross-species incompatibility. Here, we aimed to develop a completely xeno-free human microvasculature-on-a-chip model.

Three major steps of the protocol containing animal products had to be addressed: (1) routine cell culture of endothelial cells and pericytes, (2) culture of cells in the microfluidic chip and (3) immunofluorescence staining. We successfully replaced all animal-derived products and established a completely xeno-free human microvasculature-on-a-chip model that can be adapted to study vascular processes and specific neurovascular diseases.

## Image Attribution

Example image of human microvasculature-on-a-chip generated under xeno-free conditions.

## Materials

### **Consumables:**

A	B	C
Pipette tips (10 µl)	Sarstedt	70.3010.255
AIM Biotech idenTx 3 Chips	AIM Biotech/tebubio	DAX-1
AIM Biotech idenTx Holder	AIM Biotech/tebubio	HOL-2
Filtropur V25, Vacuum filtration unit, 250 ml, PES, 0.2 µm	Sarstedt	83.3940.001
Collection vessel 250 ml	Sarstedt	83.3940.505
Millex-GP Filter Unit, 0.22 µm pore size (Sterile)	Millipore	SLGP033R

### **Cell culture reagents:**

A	B	C
Fibronectin from human plasma	Sigma Aldrich	F0895
EndoGo XF Basal Medium	Sartorius	05-400-1A
EndoGo XF Supplement Mix	Sartorius	05-410-1-25
Human Serum off-the-clot, Type AB, male, sterile filtered	Pan Biotech	P40-2701
EBM-2 Endothelial Cell Growth Basal Medium-2	Lonza	CC-3156
EGM-2 Endothelial SingleQuots (only synthetic components)	Lonza	CC-4176
Recombinant Human VEGF-A165	Peprotech	100-20
Human serum albumin	Sigma Aldrich	A9731
Animal Component-Free Cell Dissociation Kit	StemCell	5426
D-PBS (1x) without CaCl <sub>2</sub> , MgCl <sub>2</sub>	Gibco	14190-094

### **Fibrin gel:**

A	B	C
Fibrinogen from human plasma	Sigma-Aldrich	F3879
Thrombin from human plasma	Sigma-Aldrich	T6884

### **Immunofluorescence staining:**

A	B	C
Synthetic Block (10X)	ThermoFisher	PA017
Triton X-100	Sigma-Aldrich	T8787

A	B	C
Recombinant Anti-VE Cadherin antibody	ThermoFisher	MA5-29141
ICAM-2 Recombinant Rabbit Monoclonal Antibody	ThermoFisher	MA5-29335
Collagen IV Recombinant Mouse Monoclonal Antibody (rCOL4, 4742)	ThermoFisher	1282-MSM2-P1ABX
Goat anti-Mouse IgG (H+L), Superclonal Recombinant Secondary Antibody, AlexaFluor Plus 647	ThermoFisher	A55060
Goat anti-Rabbit IgG (Heavy Chain), Superclonal Recombinant Secondary Antibody, Alexa Fluor 555	ThermoFisher	A27039
DAPI	Sigma-Aldrich	D9542
Paraformaldehyd	Sigma-Aldrich	16005
PBS tablets	ThermoFisher	18912014
Folded filter papers, MN 615, Qualitative, Medium, (22 s), Smooth	Macherey-Nagel	531024
Fluoromount-G mounting medium	ThermoFisher	00-4958-02

**Other lab equipment:**

- Sterile H<sub>2</sub>O (e.g. purified with Milli-Q Ultrapure Water Systems)
- Micropipettes
- Pipette tips (100µl, 200µl, 1000µl)
- T75 cell culture flasks (e.g. Sarstedt 83.3911.002)
- Sterile syringes for single use suitable for use with syringe filters (5 ml, 20 ml, 50 ml, e.g. BD Plastipak™ series)
- Table-top centrifuge
- Sterile 0.5-ml & 1-ml tubes
- Sterile 50-ml tubes
- Cell culture incubator
- Cell culture microscope (e.g. ZEISS Primovert)
- Water bath
- Biological safety cabinets for sterile cell culture work
- Ice

**Preparations of reagents:**

*A) Human VEGF-A<sub>165</sub> stock:*

Reconstitute human VEGF-A<sub>165</sub> at 100 µg/ml sterile D-PBS with 0.1% human serum albumin (HSA). HSA stabilizes VEGF-A<sub>165</sub>. Aliquots are stored at -80°C.

*B) Cell culture medium:*

1. *EndoGo XF Medium:* The EndoGo™ XF medium (05-400-1) needs to be supplemented with the EndoGo™ XF Supplement Mix (05-410-1) and 2% human AB serum (off the clot). After adding the supplements the medium is

sterile-filtered using the Filtropur system (Sarstedt) or single-use syringes and Millex-GP Filter Units. The medium is stored at 4°C, protected from light, and should be used within 1 month.

2. *EGM2-Mix*: The EBM<sup>TM</sup>-2 basal medium is supplemented with 2% human AB serum (off the clot), 5 ng/ml human VEGF-A<sub>165</sub> (see above) and three components from EGM<sup>TM</sup>-2 Endothelial SingleQuots<sup>TM</sup> supplemens: Hydrocortisone, ascorbic acid and gentamicin. After adding the supplements the medium is sterile-filtered using the Filtropur system (Sarstedt) or single-use syringes and Millex-GP Filter Units. The medium is stored at 4°C and should be used within 1 month.

### C) Immunofluorescence staining:

1. *Paraformaldehyd (PFA)*: *Caution! PFA is toxic! Work under a chemical hood!* To prepare 1 l of 4% PFA, add 40 g PFA to 800 ml of 1x PBS (add two PBS tablets, #18912014) in a glass beaker and heat to 60°C while stirring constantly. When PFA is dissolved completely, add H<sub>2</sub>O to reach a final volume of 1 l. The solution is filtered through a folded filter paper. Aliquots are stored at -20°C.

2. *DAPI*: Prepare a stock of 5 mg/ml in H<sub>2</sub>O and store aliquots at -20°C for long-term storage.

3. Antibody dilutions:

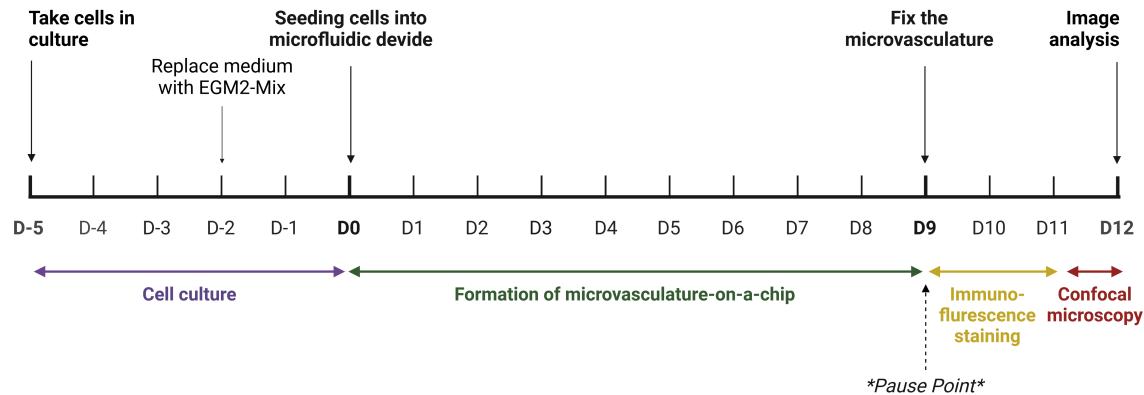
- Recombinant Anti-VE Cadherin antibody, rabbit (MA5-29141): 1:200
- Recombinant Anti-ICAM-2, rabbit (MA5-29335): 1:400
- Recombinant Anti-Collagen IV, mouse (1282-MSM2-P1ABX): 1:100
- Goat anti-Mouse IgG Alexa Fluor<sup>TM</sup> Plus 647 (A55060): 1:500
- Goat anti-Rabbit IgG Alexa Fluor<sup>TM</sup> 555 (A27039): 1:500

## Safety warnings

- ! All material of human origin should be treated as potentially infectious.  
PFA is hazardous and should be handled with appropriate safety equipment.

## Experimental Overview

1



This protocol describes all steps of the microvasculature-on-a-chip model, including cell culture, cell seeding into the microfluidic device, culture of cells in the device, fixation and immunofluorescence staining for subsequent image acquisition by confocal microscopy. Importantly, this protocol is completely xeno-free. Created with BioRender.com

## Cell culture

5d

2 *Critical: All steps need to be performed under sterile conditions!*

### 2.1 **Taking Human Umbilical Cord Endothelial Cells (HUVEC) and human Brain Vascular Pericytes (hBVP) in culture**

3d

1. Coat T75-flasks with human fibronectin ( $2 \mu\text{g}/\text{cm}^2$ ) for  $\geq 15$  min at  $37^\circ\text{C}$ .
2. Remove fibronectin (can be re-used twice) and add 10 ml pre-warmed EndoGo XF+2% human serum (HS).
3. Place cryovials with frozen HUVEC and hBVP in a  $37^\circ\text{C}$  water bath for  $\sim 2-5$  min until cells are thawed, take them up with 1 ml EndoGo XF+2% HS medium and add the cell suspension drop-wise to the coated T75-flask containing EndoGo XF+2% HS medium.
4. Place the cells in a cell culture incubator ( $37^\circ\text{C}$ , 5%  $\text{CO}_2$ , 90-95% rel. humidity). After the cells have settled and adhere to the cell culture flask ( $\sim 2-3$  h), replace the medium with fresh medium.

5. The cells are incubated as described for 2-3 days until they reach about 80-90% confluence.

Note: That protocol has been performed with HUVEC up to passage 5 and hBVP up to passage 8.

## 2.2 ***Pre-incubation with reduced-growth factor medium prior to seeding cells into the microfluidic chip***

1. After 2-3 days of culture in EndoGo XF+2% HS medium, remove the medium and wash once with ~10 ml pre-warmed D-PBS.

2. Add 10-12 ml of pre-warmed xeno-free, reduced-growth factor EGM2-Mix ("EGM2-Mix"; see materials for details) to the cells and culture under the described conditions for  $\geq 48\text{h}$ .

*(Comment: Critical step for successful formation of microvasculature.)*

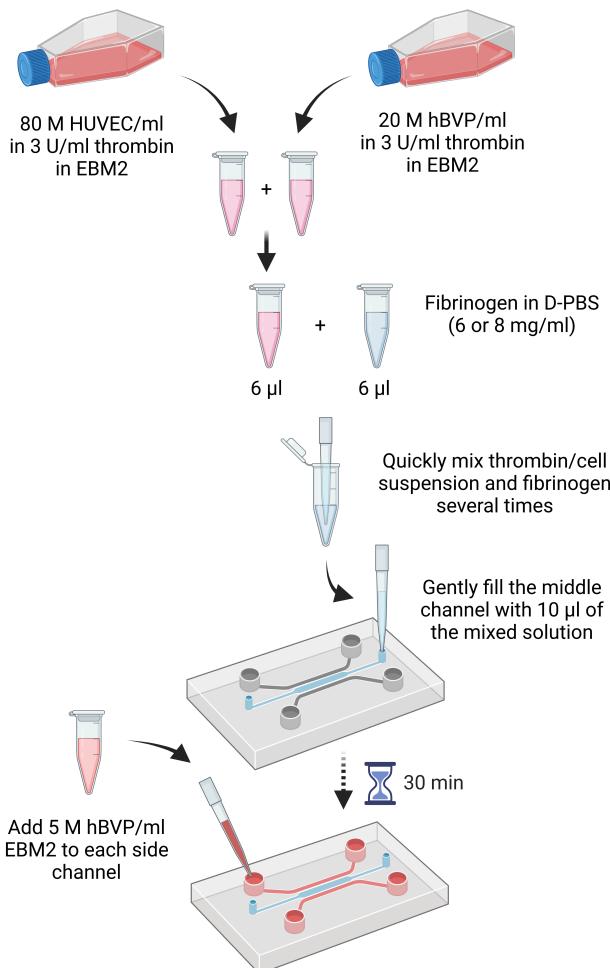
2d



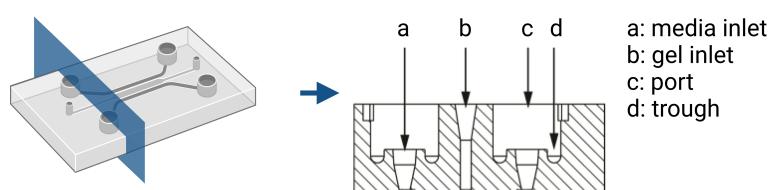
## Seeding cells into the microfluidic chip

3 *Critical: All steps need to be performed under sterile conditions!*

4h 30m



**Outline of cell seeding step:** HUVEC (80 M/ml) and hBVP (20 M/ml) are provided in a thrombin-EBM2 solution and pooled 1:1 to obtain a master cell suspension (= 40 M/ml HUVEC & 10 M/ml hBVP). This master cell suspension is then mixed in a 1:1 ratio with fibrinogen (= 20 M/ml HUVEC & 5 M/ml hBVP) and filled into the middle channel of the microfluidic device. Thrombin converts fibrinogen into a solid fibrin gel. After fibrin gel polymerization, hBVP (5 M/ml) are seeded into the side channels ("media channels"). Created with BioRender.com



**Terminology:** The cross-section through an empty microfluidic device illustrates the different terms used for the individual parts of the media and gel channel. Illustration is adapted from AIM Biotech (see "References") and created with BioRender.com.

### 3.1 Preparation

#### 1. Fibrinogen from human plasma:

- Dissolve 15-20 mg/ml in 1x D-PBS (without Ca<sup>2+</sup>/Mg<sup>2+</sup>) in a 37°C water bath for 1h. Mix gently in between.
- Filter with 0.22 µm syringe filter before measurement.
- Determine protein concentration with a spectrophotometer by measuring the absorbance at 280 nm and applying ε (molar extinction coefficient) or E1% (absorbance values for 1% [= 1g/100mL] solutions measured in a 1 cm cuvette). E1% of human fibrinogen is 15.1.
- Store aliquots at -80°C.
- Prepare 6 or 8 mg/ml solution by diluting the stock in sterile 1x D-PBS (A higher concentration of fibrinogen results in a stiffer gel.)

#### 2. Thrombin from human plasma:

- Reconstitute human thrombin in 0.1% human serum albumin/H<sub>2</sub>O to 100 U/ml stock.
- Sterile-filter with 0.22 µm syringe filters.
- Store aliquots at -80°C.
- Prepare 3 U/ml thrombin solution at a final concentration of 1.5 U/ml thrombin by diluting the stock in sterile EBM2 (endothelial basal medium without any supplements).

### 3.2 Preparation of master cell suspension (day 0)

HUVEC and hBVP are filled into the middle channel, and hBVP additionally into media channels.

*Critical: The fibrinogen, thrombin and final cell solutions should always be kept on ice!*

1. Warm up media and PBS in a water bath at 37°C and ACF Cell dissociation kit at room temperature (RT), respectively.

2. Perform a polymerization test of the fibrin gel: gently mix 6 µl diluted fibrinogen and 6 µl diluted thrombin in a 500-µl tube and allow polymerization for 15 minutes at RT. After 15 minutes, the gel should be solidified and it should no longer be possible to aspirate the gel, a small drop of gel should stick to the pipette tip.

3. Calculate required initial cell concentrations according to the following equation:

*Initial cell concentration = final seeding concentration \* number of cell types \* 2 (dilution factor)*

In this protocol, HUVEC and hBVP are seeded in the middle chamber of the chip at a final concentration of 20\*10<sup>6</sup> cells/ml HUVEC and 5\*10<sup>6</sup> cells/ml hBVP. Consequently, the initial concentration of HUVEC in thrombin solution is 20 x 2 x 2 = 80\*10<sup>6</sup> cells/ml. The initial concentration of hBVPs in thrombin solution is 5 x 2 x 2 = 20\*10<sup>6</sup> cells/ml.

4. Preparation of HUVEC suspension:

*Critical: Pipette gently during cell collection!*

- Wash cells 2x with warm D-PBS.
- For 1x T75 flask: add 3 ml of ACF Enzymatic Dissociation Solution and incubate at 37 °C for 2 - 6 minutes until cells are detached (visual control under a light microscope), add 3 ml of ACF Enzyme Inhibition Solution and collect cells in a polypropylene tube; wash the flask with 3 ml of EBM2 and add to polypropylene tube.
- Pellet the cells by centrifugation at 800 g for 5 minutes at RT.
- Remove medium and resuspend pellet in 5 ml of warm EBM2; determine cell concentration (e.g. with a automated cell-counter or counting chamber)
- Pellet the cells at 800 g for 5 minutes at RT.
- Take up the pellet in thrombin solution at the initial cell concentration of  $80 \times 10^6$  cells/ml and keep on ice.

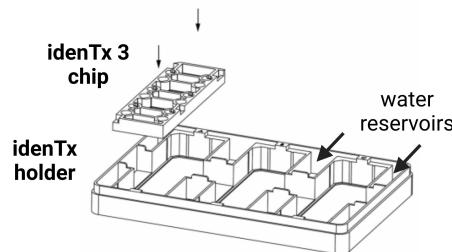
5. Preparation of hBVP: Follow the same procedure as for HUVEC, but re-suspend the final cell pellet in thrombin solution at a concentration of  $20 \times 10^6$  cells/ml and keep on ice.

6. Mix the HUVEC and hBVP cell solutions in a 1:1 ratio to create the master cell suspension and keep on ice. This step reduces the concentration of HUVEC in the master cell suspension to  $40 \times 10^6$  cells/ml and that of hBVP to  $10 \times 10^6$  cells/ml.

### 3.3 **Filling cell suspension into the middle gel channel of IdenTx 3 chips**

*Critical: Cell seeding steps should be performed on ice!*

7. Place IdenTx 3 chips into the holder.



Three idenTx 3 chips can be placed into one holder. Each idenTx 3 chip is composed of three individual sections (i.e. one gel channel surrounded by two media channels). The idenTx holder contains eight water reservoirs. Illustration adapted from AIM Biotech's holder manual (see "References").

8. Mix 6 µl of the master cell suspension (gently mix each time to avoid cell aggregates and varying cell concentrations) with 6 µl of the fibrinogen solution. This step reduces the final concentration of HUVEC in the chip to  $20 \times 10^6$  cells/ml and that of hBVP to  $5 \times 10^6$  cells/ml.

9. Take 10 µl of this mixed solution with a 10-µl micropipette and gently fill it into the gel inlet of the chip.

*Critical: Mixture must be filled into the chip in < 10 seconds: mix maximum 10 times with the pipette!*

*Critical: Fill in evenly within 5 seconds from either of the gel inlets all the way to the other side!*

*Critical: Keep the pipette straight and push the tip all the way into the gel inlet!*

*Critical: Always keep plunger pressed down while removing the pipette tip from the inlet!*

*Critical: Mix cells for only one channel at a time; master cell suspension and fibrinogen should be kept on ice to avoid early polymerization!*

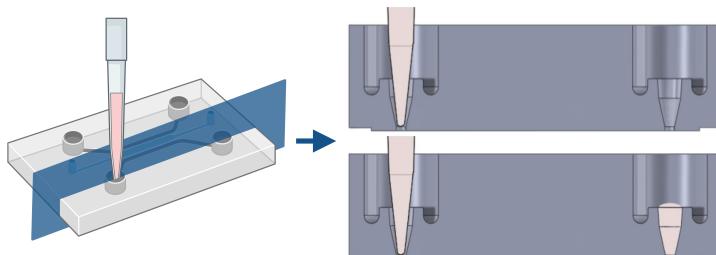
*Note: The pipette tip should have a big opening, the 10- $\mu$ l tips by Sarstedt (#70.3010.255) are a good choice. Choosing pipette tips with smaller openings leads to uneven filling of the middle channel!*

10. Optional: Flip holder with chips after each filled channel for 60 seconds each for better distribution of the cells in the gel.

11. Repeat step 8 - 10 for all other sides.

12. Close the lid of the holder and allow gels to polymerize for 15 minutes at RT while flipping the holder every 3 minutes.

13. Hydrate the media channels: Insert a 100- $\mu$ l pipette tip into either inlet of the media channel and push gently until the pipette tip fits ("Method A", see below). Then inject 10  $\mu$ l of EBM2 into the channel. Due to surface tension, the injected solution will create a spherical cap at the opposite inlet. Repeat this step for the remaining channels.



Method A: Insert the pipette tip into the media inlet and push the solution through the media channel until it is exiting through the opposite media channel. Illustration is adapted from AIM Biotech (see "References") and created with BioRender.com.

14. Place the holder upside down in 37°C incubator for 15 minutes.

15. Return holder to normal position. Then add 60  $\mu$ l of EGM2-Mix into one port and 50  $\mu$ l of EGM2-Mix into the opposite port of the same media channel. Repeat this step for the remaining channels.

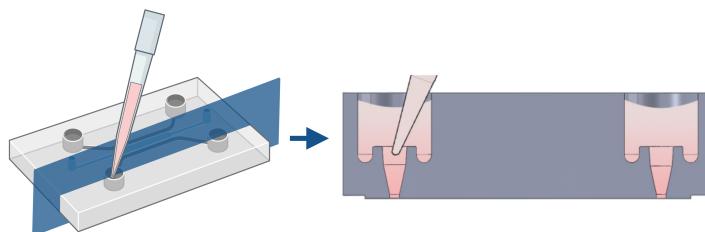
### 3.4 Preparation and filling of hBVP into the media channels of IdenTx 3 chips

16. Preparation of hBVP: Take a second flask of cultured hBVP and repeat step 5, take up hBVP pellet in EBM2 at the target concentration of  $5 \times 10^6$  cells/ml and keep on ice. Alternatively, use the remaining hBVP solution of  $20 \times 10^6$  cells/ml in thrombin and dilute to  $5 \times 10^6$  cells/ml using EBM2.

17. Add 15 µl of hBVP solution directly into the inlets of each media channel that contain 60 µl of EGM2-Mix (see step 15).

*Critical: When filling the hBVP into the media channels, do not push pipette tip completely into the media inlet but place the tip gently at the opening of the media channel (see "Method B" below)!*

*Critical: Visual control of hBVP in media channel is essential!*



Method B: Position the pipette tip at the opening of the media inlet, but do not insert the tip, and release the cell suspension. Due to the gradient in between the two ports, the cells will flow into the media channel. Illustration is adapted from AIM Biotech (see "References") and created with BioRender.com.

18. Fill the reservoirs of the holder with a total of 12 ml sterile water (1 ml into small and 2 ml into big reservoirs).

19. Incubate for 2 h in a cell culture incubator at 37 °C. Ensure attachment of hBVP in the media channels by visual control.

20. Change medium according to the protocol below.

### Culture microvasculature in the microfluidic chip

4 *Critical: All steps need to be performed under sterile conditions!*

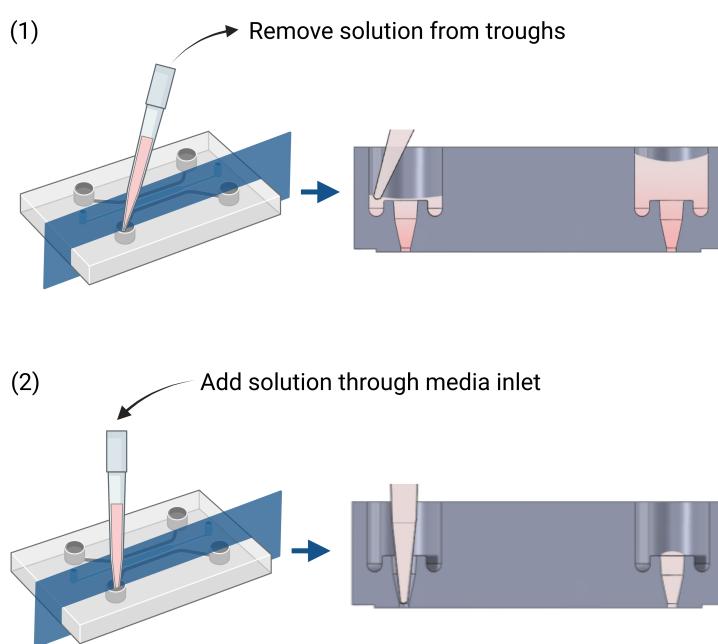
1w 2d

#### Medium change (day 0-9)

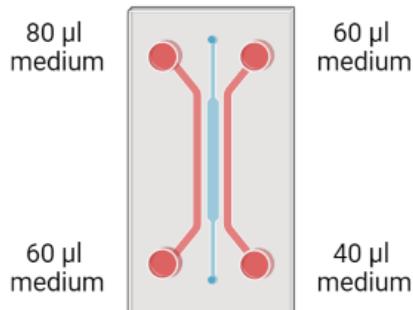
1. Remove medium from all four ports by carefully aspirating the medium from the troughs using a 200-µl pipette tip (see "Method C" below).

*Recommendation: Change pipette tips after each section to avoid contamination!*

2. Add EGM2-Mix medium into the ports according to the loading scheme shown below. For immediate and complete exchange of the medium, fully insert the pipette tip into the opposite media inlet and slowly push the medium through the channel until the target port is filled with the corresponding amount of medium (see "Method C" below). By following this loading scheme, flow is created between the upper and lower port and across the gel.  
*Recommendation: Use a new pipette tip for each port to avoid contamination!*  
*Recommendation: Start by adding 40 µl, continue with 60 µl and add the 80 µl at the end!*
3. Change medium daily.



Method C: Medium is removed from the troughs (1) and replaced by inserting the pipette tip all the way into the media inlet and pushing the new medium through the media channel (2). The same technique is used in the immunofluorescence protocol. Illustration is adapted from AIM Biotech (see "References") and created with BioRender.com.



Loading scheme for cell culture medium (80µl/60µl & 60µl/40µl). Created with BioRender.com

### Expected result

By day 5 a vascular network should have formed.

## Immunofluorescence staining

5

3d

### 5.1 Fixation

1h

*Caution: Fixative is hazardous!*



1. Remove the medium from all ports by aspirating the medium from the troughs (see "Method C").
2. Add 4% PFA by fully inserting the pipette tip into the media inlet and carefully pushing the PFA through the media channels (see "Method C"). Apply a gradient across the ports and the gel by using the 80µl/60µl & 60µl/40µl loading scheme. Incubate for 20 min at RT.
3. Wash three times with PBS ( $\geq 10$  min): Carefully push PBS through media channels using the 80µl/60µl & 60µl/40µl loading scheme (see "Method C").

Note: Fixed devices can be stored at 4°C (up to three days was tested). Ensure that the channels do not dry out during storage.

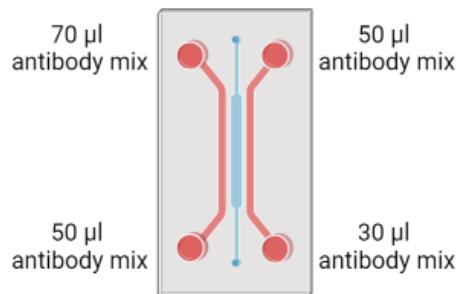
5.2 Staining

3d



1. Aspirate the PBS from all troughs (see "Method C").
2. Block and permeabilize in 1x Synthetic Block with 0.5% TritonX-100 for 60 min at RT. Use Method C, insert the pipette tip fully into the media inlet and push the buffer through the media channel. Apply a gradient across the ports and the gel by using the 80µl/60µl & 60µl/40µl loading scheme.

3. Add primary antibodies diluted in 0.5x block. Use the same technique as above and insert the pipette tip fully into the media inlet and push the antibody mix through the media channels, apply a 70 $\mu$ l/50 $\mu$ l & 50 $\mu$ l/30 $\mu$ l loading scheme (see illustration below). Note: Antibody mix is centrifuged at  $\geq$ 13,000g, 4°C for 20 min prior to application.
4. Incubate overnight at 4°C (Note: Shaking is not required, since the antibodies will enter the gel due to the applied gradient).
5. Remove antibodies by aspirating the solution from the troughs and carefully push wash buffer (0.5% TritonX-100 in 1x PBS) through media channels (Method C, apply the 80 $\mu$ l/60 $\mu$ l & 60 $\mu$ l/40 $\mu$ l loading scheme).
6. Wash 5 times for  $\geq$ 10 min at RT (Method C, apply the 80 $\mu$ l/60 $\mu$ l & 60 $\mu$ l/40 $\mu$ l loading scheme).
7. Add secondary antibodies diluted in 0.5x block as described above, apply the 70 $\mu$ l/50 $\mu$ l & 50 $\mu$ l/30 $\mu$ l loading scheme. Note: Antibody mix is centrifuged at  $\geq$ 13,000g, 4°C for 20 min prior to application. *Caution: Protect from light for all subsequent steps!*
8. Incubate overnight at 4°C.
9. Remove antibodies by aspirating the solution from the troughs and carefully push wash buffer through the media channels (Method C, apply the 80 $\mu$ l/60 $\mu$ l & 60 $\mu$ l/40 $\mu$ l loading scheme).
10. Wash 2x for  $\geq$ 10min at RT. (Optional: Check fluorescence under an appropriate epifluorescence microscope to verify successful staining before proceeding.)
11. Add DAPI diluted 1:100 in wash buffer, incubate for  $\geq$ 60 min at RT (Method C, apply the 80 $\mu$ l/60 $\mu$ l & 60 $\mu$ l/40 $\mu$ l loading scheme).
12. Wash channels with 1x PBS  $\geq$ 3 times for  $\geq$ 10 min (Method C, apply the 80 $\mu$ l/60 $\mu$ l & 60 $\mu$ l/40 $\mu$ l loading scheme)
13. Apply mounting media into the media channel, until media channels and troughs are filled (Note: The mounting media can be very viscous and caution must be taken not to introduce air bubbles). Depending on the chosen mounting media, it will solidify.



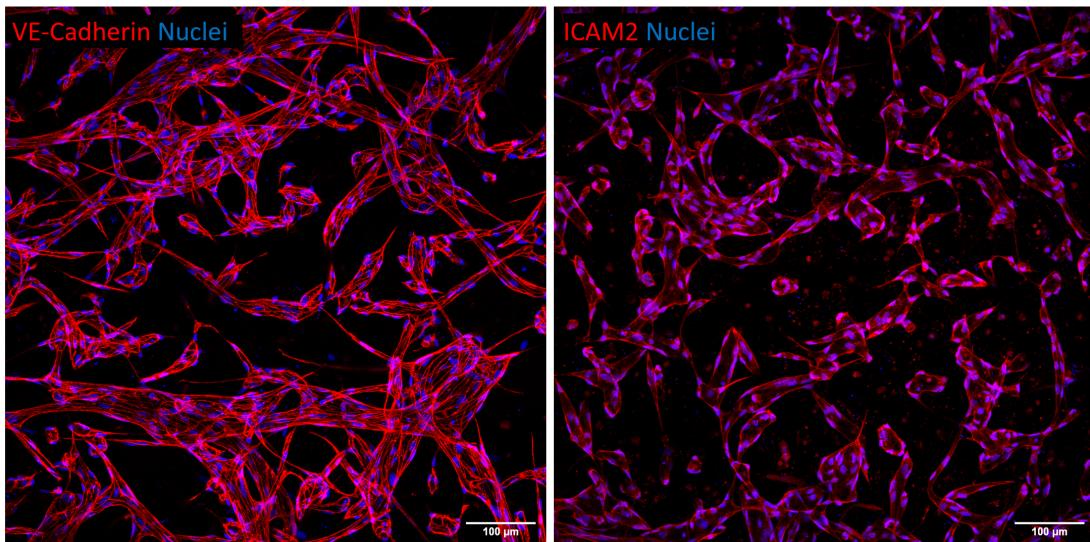
Loading scheme for antibody solutions (70 $\mu$ l/50 $\mu$ l & 50 $\mu$ l/30 $\mu$ l). Created with BioRender.com

## Image Acquisition

- 6 Images can be acquired with an epifluorescence or confocal microscope. Due to the three-dimensional composition of the vasculature, a confocal microscope is recommended.

Images were acquired with an inverse Leica SP8 microscope equipped with HC PL APO 40x/1.30 Oil and HC PL APO CS2 20x/0.75 Immersion objectives.

#### Expected result



Examples for immunofluorescence staining following the xeno-free staining protocol. Endothelial VE-Cadherin junctions (red, left image) and ICAM2 (red, right image) are shown. Scale bar: 100  $\mu\text{m}$

## Protocol references

The here presented protocol is an adaptation of AIM Biotech's "Blood-Brain-Barrier Model" and "General Protocol":  
<https://aimbiotech.com/wp-content/uploads/2022/01/Blood-Brain-Barrier-Model-v2.1.pdf>; [http://aimbiotech.com/wp-content/uploads/2022/01/General-Protocol\\_v6.1.pdf](http://aimbiotech.com/wp-content/uploads/2022/01/General-Protocol_v6.1.pdf)

Original research articles:

1. **3D self-organized microvascular model of the human blood-brain barrier with endothelial cells, pericytes and astrocytes.** Campisi M, Shin YJ, Osaki T, Hajal C, Chiono V, Kamm RD. *Biomaterials* 12 July 2018  
<https://doi.org/10.1016/j.biomaterials.2018.07.014>
2. **A 3D neurovascular microfluidic model consisting of neurons, astrocytes and cerebral endothelial cells as blood-brain barrier.** Adriani G, Ma DL, Pavesi A, Kamm R, Goh ELK. *Lab Chip*, 2016. 17 (3):448-459
3. **A Robust Method for Perfusionable Microvascular Network Formation In Vitro.** Wan Z, Zhong AX, Zhang S, Pavlou G, Coughlin MF, Shelton SE, Nguyen HT, Lorch JH, Barbie DA, Kamm RD. *Small Methods* 6, e2200143 (2022).

Others:

idenTx holder manual: [http://aimbiotech.com/wp-content/uploads/2021/06/HOL-2\\_Handling-Protocol\\_v1.0.pdf](http://aimbiotech.com/wp-content/uploads/2021/06/HOL-2_Handling-Protocol_v1.0.pdf)