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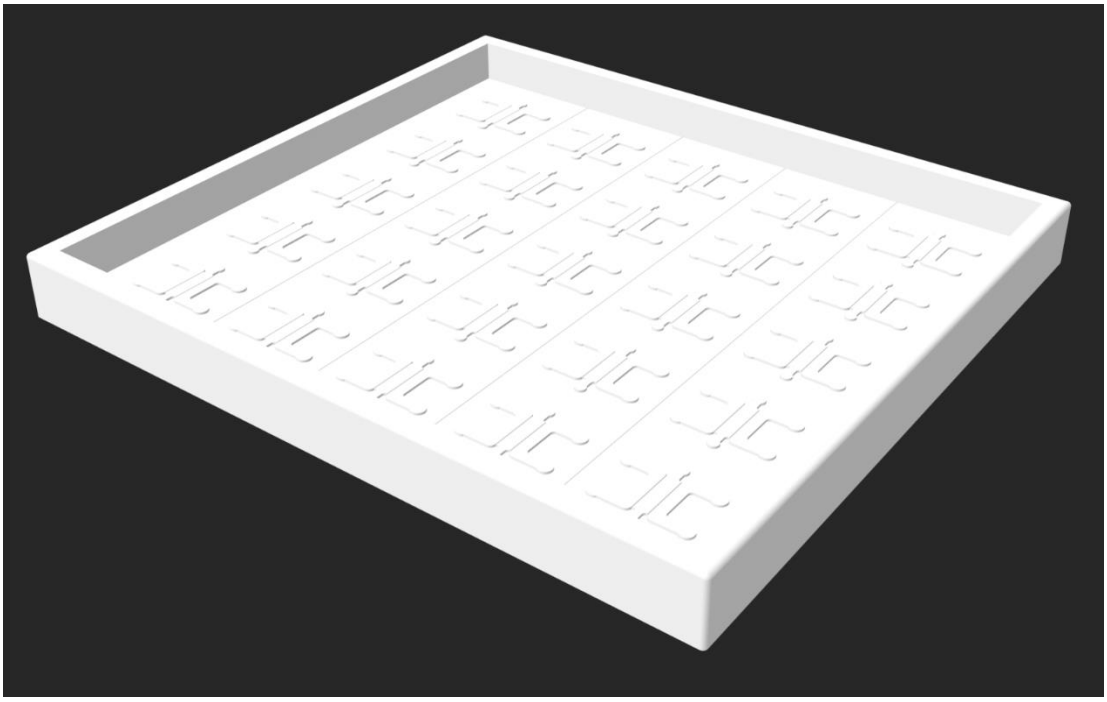
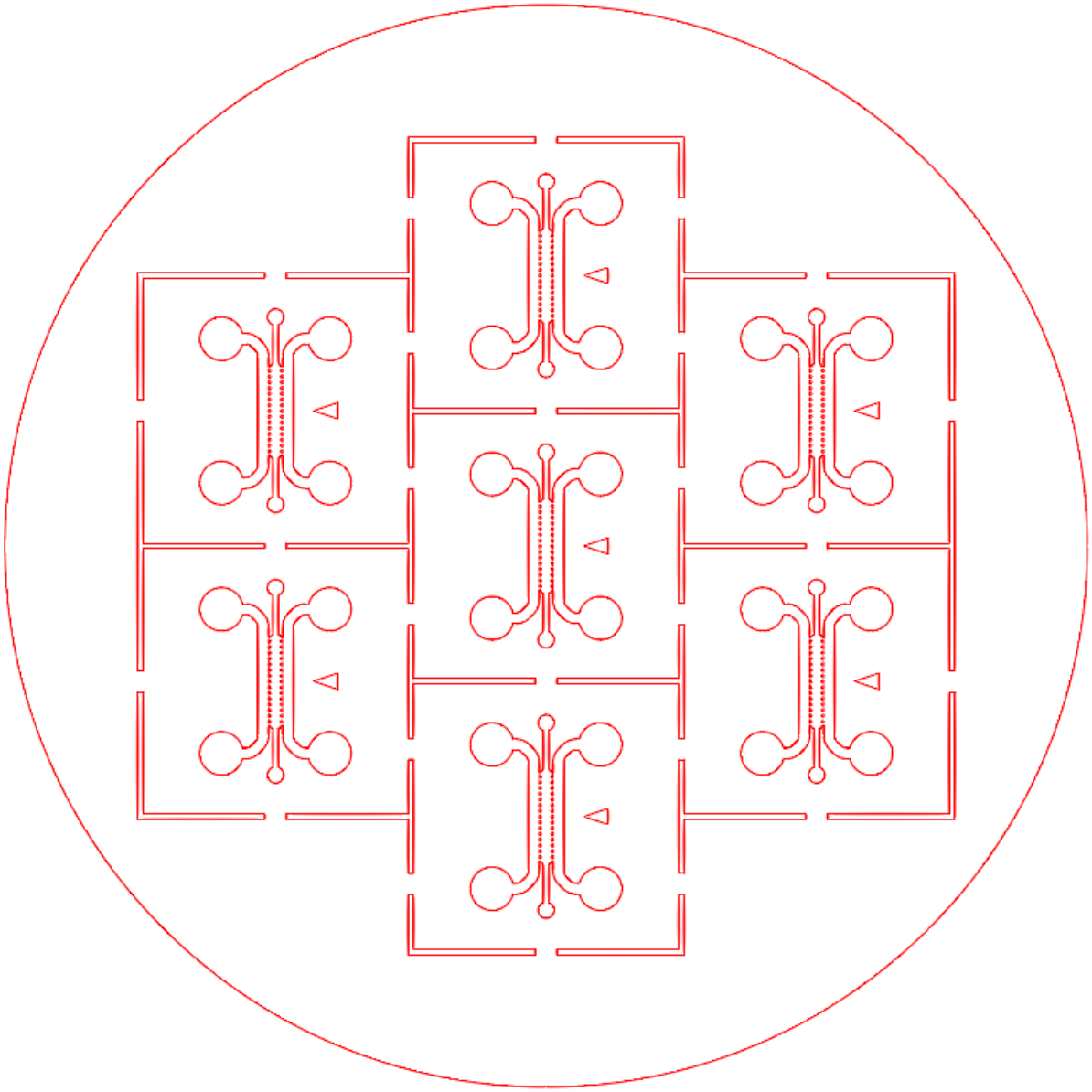
Supplementary information

Engineered human blood–brain barrier microfluidic model for vascular permeability analyses

In the format provided by the authors and unedited



Supplementary Figure 1. Image of the 3D printing template for the macro device. CAD file is included in Supplementary Data 1.



Supplementary Figure 2. Image of the CAD template for the micro device. CAD file is included in Supplementary Data 2.

	Micro device	**Macro device**
Height	150 µm	500 µm
Width	1,300 µm	3,000 µm
Length	8,150 µm	15,000 µm
Exposed gel surface to side channels	*Surface between posts:* Inter-post length x Height x # of openings x 2 = 200 µm x 150 µm x 17 x 2 = **1.02 mm^2**	*Uninterrupted surface between partial wall and glass slide:* Exposed length x Partial wall height x 2 = 10,000 µm x 300 µm x 2 = **6 mm^2**

Supplementary Table 1. Device dimensions and other parameters to transport measurements for the macro and micro devices.

Supplementary Method:

READ ME file for MVN permeability characterization using ImageJ/Fiji

The following steps describe the manual methodology to measure permeability from confocal microscopy images of MVNs perfused with fluorescent molecules. The methodology can be followed using the software FIJI (download details are below in step 1) on any Operating System.

A Macro for the software FIJI is provided with this READ ME document, which performs the analysis semi-automatically (initial confocal stack alignment and trimming by the user are required) and outputs separate Excel files for each time point containing both morphological and intensity parameters. These values can be copied and pasted into the "*Permeability template*" Excel file also provided with this document.

When using the Macro for the first time, ensure that the Macro file (macro_permeability_win.ijm) and classifier file (classifier.model) are in the same folder. The directory to this folder must be defined in the Macro: In FIJI, use *Plugins -> Macros -> Edit…* and select the Macro file. Edit line 6 to the folder directory (*e.g.*, "C:\\Users\\..."), then save the changes through *File -> Save*. The Macro can now be run using *Plugins -> Macros -> Run…* and selecting the Macro file.

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1. Download and install FIJI, available at: https://imagej.net/Fiji
2. Download and install the *3D Analysis* plugin, available at: https://imagejdocu.tudor.lu/plugin/analysis/3d_analysis/start
3. Open in FIJI confocal stacks representing two different time points for the same volume imaged.
4. Select volume of interest within the stacks by removing slices for which the signal may be faint, *i.e.* at larger depths within the tissue construct, or absent, *i.e.* below the glass cover slip. To do this, use *Image -> Stack -> Tools -> Slice Remover*. If needed, crop the stacks in the *xy*-plane by selecting an area of interest and using *Image -> Crop*. Repeat procedure for all time points, ensuring alignment in the *z*-direction in case of microscope drift.
5. Duplicate first time point stack: select stack -> *Image -> Duplicate…* -> select "*Duplicate stack*".
6. Select duplicated stack. Automatic thresholding: *Image -> Type -> 8 bit*, then *Image -> Adjust -> Auto Threshold -> Method: Otsu -> de-select "White objects…" -> select "Stack"*.
7. At this point, in order to account for the presence of the added thickness of the endothelium use: *Plugins -> Process -> Erode (3D)*.
8. Segmentation: *Plugins -> Segmentation -> Trainable Weka Segmentation 3D*. A classifier is required by the software to identify what constitutes vasculature and what matrix within the stack. Once created, this classifier can be saved using "*Save*

classifier" and used again without going through the process described below. In that case, simply use "*Load classifier*" and then "*Create result*". A classifier is provided together with this READ ME document, which can be used for solutes with little to no tendency for aggregation, *e.g.* dextran.

9. To create a new perfusate classifier for the first time, use the mouse to trace lines within the matrix and vasculature and add each to either class 1 or 2, respectively, using the buttons to the right of the thresholded image. Five lines per class will be enough. Press "*Train classifier*" and then "*Create result*" once done.
10. Select the classified image, and press *Image -> Type -> 8 bit*. The classified image color scheme will change from red/green to gray. At this point the morphology of the MVNs can be quantified using *Plugins -> 3D -> 3D Geometrical Measure*. Note that this option will only be available if the *3D Analysis* plugin is correctly installed. The values produced that are of interest for the analysis are the *Volume (unit)* and the *Surface (unit)*, where *(unit)* refers to those included in the metadata of the original file (as opposed to pixels), *e.g.* μm^3 for the volumes and μm^2 for the surfaces if the unit of the stack was microns. The two lines in the results represent the vasculature (first line) and matrix (second line). To check that this is indeed the case, a pixel intensity value is assigned to each line, *e.g.* 85 for the darker vasculature and 155 for the lighter matrix in the classified image.
11. Copy and paste the morphological results into the Excel file provided with this READ ME document ("*Permeability template*"). The template calculates 3D morphological parameters as:

    Vascular volume fraction: $\%V = \frac{V_v}{V_v+V_m}$

    Specific surface area: $SSA = \frac{SA_v}{V_v+V_m}$

    Average vessel diameter: $d = 4 * \frac{V_v}{SA_v}$

    where *V* and *SA* represent the volume and surface of the vasculature (subscript *v*) and matrix (subscript *m*).
12. Measure fluorescence intensity in the matrix and vasculature: select *Plugins -> 3D -> 3D Intensity Measure*, select the classified image for "Objects" and the raw data for "Signal".
13. Add intensity value results to the "Permeability template" file.
14. Repeat from step 5 for the second time point. The template outputs the permeability in cm s^{-1}.