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Interstitial flow regulates *in vitro* three-dimensional self-organized brain micro-vessels

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Abstract

Cell culture under medium flow has been shown to favor human brain microvascular endothelial cells function and maturation. Here a three-dimensional *in vitro* model of the

human brain microvasculature, comprising brain microvascular endothelial cells but also astrocytes, pericytes and a collagen type I microfiber – fibrin based matrix, was cultured under continuous medium flow in a pressure driven microphysiological system (10 kPa, in 60-30 s cycles). The cells self-organized in micro-vessels perpendicular to the shear flow. Comparison with static culture showed that the resulting interstitial flow enhanced a more defined micro-vasculature network, with slightly more numerous lumens, and a higher expression of transporters, carriers and tight junction genes and proteins, essential to the blood-brain barrier functions.

Keywords: Interstitial flow, 3D *in vitro* model, brain micro-vessels, blood-brain barrier

1 **Abbreviations**

- 2 ECM: extracellular matrix
- 3 HBEC: human brain microvascular endothelial cells
- 4 HA: human astrocytes
- 5 HP: human brain pericytes
- 6 CMF: collagen microfibers
- 7 Cld5: Claudin 5
- 8 ZO1: Zonula occludens-1
- 9 Glut1: Glucose transporter 1
- 10 TfR: Transferrin receptor
- 11 BRCP: Breast cancer resistant protein
- 12 Pgp: P-glycoprotein

1 **1. Introduction**

2 The structural organization of the human brain microvasculature enables functional
3 specificities and ensures the protection of the human nervous system thanks to the highly
4 selective blood-brain barrier. The brain microenvironment regulates the phenotypes of the
5 main cell types constituting the brain micro-vessels: the brain microvascular endothelial cells,
6 the astrocytes and the pericytes. The continual shear stress generated by the flow of blood
7 across the microvasculature especially affects the cell morphology and functions. It has
8 indeed been reported to upregulate some junctional proteins, transporters, ions channels and
9 limit the permeability of brain microvascular endothelial [1–4]. However, other studies
10 showed that none or only part of the key blood–brain barrier markers were significantly over-
11 expressed (Claudin 5, Zonula occludens-1, Occludin, CD31, Glucose transporter 1, P-
12 glycoprotein, and others) [5,6], although the mechanical stress from the medium did promote
13 differentiation or maturation of the endothelial cells specific to the brain microvasculature.
14 Differences in culture and flow parameters such as applied pressure and time of treatment
15 may partly explain those inconsistencies, especially as too high or disturb shear stress may
16 reverse its beneficial impacts [3,7]. The range of assessed biochemical signals are also
17 susceptible to be affected by the types of cells used in the model. The use of astrocyte
18 conditioned medium has been shown to be as important as the shear stress to upregulate
19 Zonula occludens-1 and decrease brain endothelial permeability [1]. Few studies introduced
20 astrocytes [2,4], pericytes [8] or both [9] to the flow cultured brain microvasculature models,
21 though they seemed to increase the differences observed compared to static conditions. Fewer
22 studies considered the importance of the extracellular matrix (ECM) and its combined
23 influence with flow on the cell culture. Systems of coculture and perfused vessels adjacent to
24 a collagen-I gel were engineered [10]. However, no direct contact were possible between the

1 endothelial cells seeded in the perfused channel and the astrocytes and pericytes seeded on the
2 other side of the gel ; and the vessels diameter had to remain over 150 μm to avoid for the
3 system to collapse [4,7]. Cells self-organization in a collagen or fibrin based gel is a state-of-
4 the-art technique to develop brain microvasculature like network. Two teams have proposed
5 such models with a coculture of brain microvascular endothelial cells, astrocytes and pericytes
6 in microfluidic chips [11,12]. However, if the micro-vessels were perfusable for a few hours
7 (< 20 h) after a maturation of 7 days, no data are yet available for a full culture under flow.

8 We have also previously developed such a self-organized three-dimensional (3D) *in vitro*
9 model of the brain microvasculature that was cultured in static condition [13]. Here we
10 focused on the impacts of the mechanical stimuli exerted during the 7 days of culture by
11 medium flow through the ECM, on the morphology, gene and protein expression of a scale-up
12 version of this human cerebro-vasculature model.

13 **2. Materials and Methods**

14 *2.1. Cell culture*

15 The cell lines have been developed in previous studies: human brain microvascular
16 endothelial cells/conditionally immortalized clone 18 (HBEC) [14], human
17 astrocyte/conditionally immortalized clone 35 (HA) [15], and human brain pericyte/
18 conditionally immortalized clone 37 (HP) [16]. All cells were cultured on collagen coated
19 dishes (100 mm, collagen type I, Iwaki, Shizuoka, Japan) and incubated at 33°C (to maintain
20 growth activity), 5% CO₂, in humidified environment. All culture media were supplemented
21 with 4 $\mu\text{g}\cdot\text{mL}^{-1}$ Blasticidin S HCl (Invitrogen, Waltham, USA) to maintain selective pressure
22 for routine culture. HBEC were cultured in Vasculife (VEGF-M_v, LifeLine) 500 mL
23 supplemented with 0.5 mL rh FGF-b, 0.5 mL ascorbic acid, 0.5 mL hydrocortisone

1 hemisuccinate, 25 mL L-glutamine, 0.5 mL rh IGF-1, 0.5 mL rh EGF, 0.5 mL rh VEGF, 0.5
2 mL heparin sulfate, 25 mL fetal bovine serum (kit, LifeFactor VEGF-Mv, LifeLine,
3 Frederick, USA), 25 mL supplementary fetal bovine serum (FBS, Gibco ThermoFisher,
4 Waltham, USA), and 1% penicillin-streptomycin (10,000 U.mL⁻¹ - 10,000 µg.mL⁻¹, Nacalai
5 tesque, Kyoto, Japan). HA were cultured in Dulbecco's Modified Eagle Medium (DMEM,
6 Nacalai tesque, Kyoto, Japan) 500 mL, complemented with 10% FBS, 1% penicillin-
7 streptomycin, 5 mL N2 supplement x100 (Gibco ThermoFisher, Waltham, USA). HP were
8 cultured in Pericyte Medium (ScienCell Research Laboratories, Carlsbad, USA) 500 mL,
9 supplemented by 5 mL Pericyte Growth Supplement 100x, 10 mL fetal bovine serum, and 5
10 mL penicillin-streptomycin. HA and HP were pre-differentiated at 37°C 3 days before
11 seeding of the 3D model.

12 *2.2. Collagen type I microfibers and fibrin based ECM model*

13 The ECM of our 3D *in vitro* model of brain vasculature is composed of collagen type I
14 microfibers (CMF) and fibrin. CMF were processed from freeze-dried porcine collagen type I
15 sponge, donated from Nippon Ham Foods Ltd. (Osaka, Japan). 50 mg of collagen sponge
16 were homogenized 6 min (Violamo VH-10 homogenizer, S10N-10G 10 mm diameter, 115
17 mm length probe) in 5 mL of ultrapure water (Milli-Q water purification system, Merck,
18 Kenilworth, USA), then centrifuged 10 min at ambient temperature at 12100 g. The pellet was
19 then sonicated (Ultrasonic processor VC50, 50W, 3.2 mm probe, Sonics, Newtown, USA) in
20 5 mL with fresh ultrapure water in an ice bath for 100 cycles of 20 s ultrasonication and 10 s
21 cooling. The sonicated collagen was filtrated (40 µm filter, microsyringe 25 mm filter holder,
22 Merck, Kenilworth, USA), and the filtrate freeze-dried for at least 40 h (Freeze dryer FDU-
23 2200, Eyela Co., Shanghai, China). The resulting CMF were stored in a desiccator at room
24 temperature prior use.

1 For 70 μ L hydrogels, 0.7 mg CMF and 0.4 mg Fibrinogen were dispersed in 40 μ L non-
2 complemented DMEM while 200,000 HBEC, 400,000 HA, 100,000 HP, and 0.3 U Thrombin
3 were dispersed in 30 μ L complemented Vasculife. Both solutions were mixed just before
4 dropping in cell culture inserts (3470 transwell, for 24 well plates, polyester membrane with
5 0.4 μ m pore, Corning Life Sciences, Tewksbury, USA) preliminarily coated with 100 μ L
6 Fibronectin at 0.04% (Fibronectin from human plasma, F2006-5G, Sigma-Aldrich, St Louis,
7 USA). 60 min incubation at 37°C allowed for the mixtures to gel. For static cultures, the
8 inserts were placed on top of 6 well culture plate thanks to a specifically design 24 to 6 well
9 plate adaptor (Figure 1), and 12 mL of 1:1:1 complemented mixed media were added (6 mL
10 of Vasculife, 6 mL DMEM-N2, and 6 mL of Pericyte Medium, all without Blasticidine). The
11 gels were incubated 7 days at 37°C, 5% CO₂, humidified atmosphere, with a medium change
12 at day 4.

13 *2.3.Flow system*

14 For flow culture, parts of the upper parts of the inserts were cut off, which allowed their
15 introduction after gelation in the Pressure driven microphysiological system (Figure 1 and
16 Figure S1) [system derived from 16,17]. The system was composed of 4 devices, each
17 accommodating 2 samples chambers and 2 medium chambers. A total of 8 samples could thus
18 be cultured in parallel. Each unit - sample and medium chamber - contained a total of 1.4 mL
19 medium (1:1:1 mixed media as for static culture). This medium continuously flowed from one
20 chamber to the other following cycles of 60 s (sample chamber to medium chamber) and 30 s
21 (medium chamber to sample chamber). A pressure of 10 kPa was applied to induce and
22 maintain this perfusion process. The system was optimized so that the samples were always
23 immersed, and perfused with a vertical flow. Medium changes were conducted every day
24 except at day 6.

2.4. *Fluorescence imaging and protein expression*

Fluorescent cell tracking dyes were used to identify HA and HP (CellTrackerTM CMFDA and red, ThermoFisher Scientific, Waltham, USA). After dilution of the dyes at 10 mM in dimethyl sulfoxide (DMSO, Fujifilm Wako, Osaka, Japan), they were further diluted in non-complemented medium at 1:1000 (v:v). 1 day prior to the experiment, the cells were rinsed with PBS, and exposed to the diluted dyes for 40 min at 37°C. After 3 additional washings with PBS, the cells were covered with their usual complemented medium and put back at 37°C for 24 h before seeding into the gel. Precautions were taken to minimize exposure to light of those gels.

After 7 days of culture, the gels (with stained or unstained HA and HP) were rinsed in PBS 3 times, and fixed in 4% paraformaldehyde (Fujifilm Wako, Osaka, Japan) at 4°C for at least 6 h. The gels were then detached from their inserts and a 15 min incubation in 0.05% Triton X-100 (Sigma-Aldrich, St Louis, USA) in PBS was carried out for permeabilization. After additional washing, a 1 h incubation with 1% bovine serum albumin (BSA, Sigma, St Louis, USA) in PBS was used to minimize non-specific staining. Samples were incubated at 4°C with primary antibodies diluted at 1% in BSA for at least 40 h: mouse anti-CD31 antibody (NCL-CD31-1A10, Leica, Wetzlar, Germany) alone; mouse anti-ZO-1 antibody (61-7300, Invitrogen, Waltham, USA) alone; or mouse anti-CD31 antibody and rabbit anti-tight junction proteins, carriers or transporters (anti-Aquaporine 4, A5971, Sigma-Aldrich, St Louis, USA ; anti-Claudin 5, ab131259, Abcam, Cambridge, UK; anti-PgP, ab170904, Abcam, Cambridge, UK; anti-Glut-1, ab115730, Abcam, Cambridge, UK; anti-BCRP, 4477, Cell Signaling, Danvers, USA). Samples were exposed to secondary antibodies diluted at 1% in BSA for 2 h at room temperature in the dark (goat anti-mouse, Alexa Fluor® 647, A21235, ThermoFisher Scientific, Waltham, USA, or goat anti-rabbit, Alexa Fluor® 488, ab150077, Abcam, Cambridge, UK). For TfR and ZO1, a second staining was then carried out with

1 conjugated antibodies (first mouse anti-CD31, then FITC-conjugated mouse anti-TfR,
2 NB500-493, NovusBio, Centennial, USA; or first mouse anti-ZO1, then Alexa Fluor 594
3 conjugated mouse anti-CD31, FAB3628T, R&D Systems, Minneapolis, USA). Nuclei were
4 counterstained with Hoechst (Thermo Fisher Scientific, Waltham, USA). For samples only
5 stained for CD31, a treatment with RapiClear (1.47, SUNJin Lab, Hsinchu, Taiwan) eased
6 deeper observations by making the ECM more transparent.
7 Samples were observed using epifluorescence microscopes (Confocal Laser Scanning
8 Microscope Fluoview FV3000, Olympus, Tokyo, Japan, for x20 and x60 magnifications). 3D
9 renderings were obtained with Imaris software (Oxford Instruments, Abingdon, UK). For
10 comparative protein expression assessment, the same exposition time and excitation power for
11 each correlated set of static and flow samples. Maximum and minimum z were chosen to
12 include all visible signals. Z-stack projections using maximum intensity projection were
13 analyzed using Fiji software [19]. The expression of tight junction proteins, transporters or
14 carriers were assessed by measuring the fluorescent intensity of the specific protein staining
15 divided by the fluorescent intensity of CD31 staining.

16 *2.5.Histology*

17 After 7 days culture, the hydrogels were rinsed and fixed in 4% Paraformaldehyde as
18 previously described, and sent to the Applied Medical Research Company for paraffin wax
19 embedding, sections mounting CD31 and Toluidine immuno-histostaining, or hematoxylin
20 eosin staining. The sections were then observed using the FL Evos Auto microscope (Thermo
21 Fisher, Waltham, USA). Lumen diameters, numbers, and sample thickness were measured on
22 the Fiji software.

1 2.6. Gene expression

2 After 7 days culture, the hydrogels were rinsed in PBS, and a RNA extraction with a DNase
3 step was carried out following the PureLink RNA Micro Kit instructions (Invitrogen,
4 Waltham, USA). RNA content was assessed with the NanodropTM spectrometer (N1000,
5 Thermo Fisher Scientific, Waltham, USA). A reverse transcription of the RNA samples into
6 cDNA using iSCRIPT cDNA synthesis kit (Bio-Rad, Hercules, USA) was undertaken. A real-
7 time quantitative polymerase chain reaction (RT-qPCR) was then conducted on 2 μ L of each
8 cDNA samples using Taqman Fast Advanced Mix (Taqman gene expression assays, Thermo
9 Fisher Scientific, Waltham, USA, see Supplementary data (Table S1) for references) in the
10 StepOnePlus Real-Time PCR System (Thermo Fisher Scientific, Waltham, USA). A
11 preliminary study determined PPIA as the most stable housekeeping gene. Results were
12 moreover standardized by CD31 expression.

13 2.7. Statistics

14 Three independent experiments were carried out for each outcome in duplicate. Error bars
15 represent standard deviations. Two by two comparisons of results (static and flow groups)
16 were performed. Student's t-test firstly assessed the variance similarity of the two groups. p
17 values were then calculated using two-tailed Fisher test. A difference was considered
18 significantly different if * $p < 0.05$, or ** $p < 0.01$.

20 3. Results and discussion

21 With optimized seeding parameters and after 7 days of culture, the cells self-organized into
22 capillary-like network whether the culture was carried out in static condition or under flow in
23 the pressure driven microphysiological system (measured flow rate of $379 \pm 37 \mu\text{L}\cdot\text{min}^{-1}$).

1 This was especially visible with CD31 immunostaining of the HBEC (Figure 2). Unlike
2 expected, the micro-vessels mainly grew horizontally in the gel, perpendicular to the flow
3 direction. It is thus not thought that the micro-vessels were perfused through the lumen, but
4 more likely that interstitial shear flow impacted the cells from the basal side. Such mechanical
5 stress affected however greatly the vessel formation and maturation. Flow culture seemed to
6 induce indeed a more defined network. Flow cultured cells self-organized in more tubular
7 shapes, less thick, with fewer connections, and slightly deeper in the hydrogels. It resembled
8 more to micro-vessels than in static culture. Our results were consistent with the often-
9 detected enhancement of the cells functions and maturation by flow although the experimental
10 set-up differed largely. Other studies on interstitial flow impact on non-brain micro-vessel
11 development have shown that the angiogenic activity was suppressed in the direction of the
12 flow, and favored in the opposite direction [20,21]. The interstitial flow could thus be used to
13 regulate and spatially guide angiogenesis.

14 Both static and flow conditions induced capillary-like tubules that displayed lumen, as seen in
15 histological sections (Figure 3, and Figure S2 for full gel picture). Interestingly, no significant
16 change in the lumen dimensions was found when comparing static and flow culture. However,
17 the microvasculature cultured under flow had a tendency to exhibit a higher lumen number
18 than when cultured in static condition. This is consistent with our observations of a more
19 defined network.

20 Gene expression of tight junction proteins, transporters and carriers essential to the blood-
21 brain barrier functions were analyzed by RT-qPCR. Flow culture seemed to boost such gene
22 expressions compared to static condition (Figure 4.A). All assessed genes: Claudin 5 (Cld5),
23 Zonula occludens-1 (ZO-1), Glucose transporter 1 (Glut1), Transferrin receptor (TfR), Breast
24 cancer resistant protein (BCRP), and P-glycoprotein (PgP), tended to be over expressed in
25 samples that undergone culture under flow compared to samples that were culture in static

1 condition. The increase was significant for Glut1, TfR and BCRP, not for the other genes
2 however. This may partly result from the high standard deviations, consequence from the
3 differences between collagen lots, and small differences in flow pressure (9% deviation in
4 average) that could results from limited shrinkage of the gels or bubbles, although those latter
5 have been widely reduced with method improvements. Despite some inconsistency in the
6 literature, similarly showing the importance of culture parameters, we have shown in the
7 introduction that the consensus was also tending to an improvement of tight junction proteins,
8 transporters and carriers expression by a shear stress.

9 Confirmation of this tendency was obtained by the assessment of the protein expression of
10 those same tight junction proteins, transporters and carriers. Comparison of
11 immunofluorescence staining intensity showed a similar tendency to an improvement of the
12 expression of Cld5, ZO1, Glut1, TfR, BCRP and PGP when the sample were cultured under
13 flow (Figure 4.B and C). This increase was significant for TfR and PgP. Differences between
14 gene and protein expressions are expected due to transcriptional and post-transcriptional
15 regulation processes [22]. This and the high standard deviations could explain that significant
16 increases in Glut1 and BCRP genes were not linked to higher protein expression, and that PgP
17 protein expression was on the other side much more enhanced by flow culture than its gene
18 expression lead to believe. The proteins seemed mostly expressed by the endothelial cells as
19 the colocalization between the red CD31 staining and the green staining prevailed. However,
20 some green staining could also be found in area not-specifically stained by CD31, which
21 might mean that astrocytes and pericytes could expressed those proteins too but at lower
22 levels It is known for example that Glut1 is overexpressed in endothelial cells but can also be
23 expressed in pericytes [23]. High shear stress can also modify the localization of those
24 proteins. Low shear stress supports ZO1 expression in the cell junctions for example, while
25 high shear stress induces ZO1 translocalization [3].

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4. Conclusion

Overall, this study showed that culture under continuous flow for 7 days lead to the self-organization of endothelial cells, astrocytes and pericytes in a brain micro-vessel network with increased definition compared to static culture. Although the lumen dimensions were not modified, a slight increase in lumen number was observed. Similarly, the gene and protein expression of tight junction proteins, transporters and carriers seemed to be increased by the interstitial flow. This model shows the significant impact of flow to regulate 3D *in vitro* brain micro-vessel models. Using the mechanical stress from the continuous flow to guide the micro-vessels orientation is a foreseen next step. The long-term goal is to aim for micro-vessels stable under long-term flow culture and perfusable to mimic even more closely the *in vivo* conditions.

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28 **Figure/Table Legends**

29 **Figure 1:** Schematic overview of the protocol for the 3D *in vitro* brain microvasculature
30 model, top: culture in static condition with a picture of a gel in a transwell and of this
31 transwell in a 6 well plate with an adaptor; bottom: culture in flow condition with a picture of
32 2 samples in the pressure driven microphysiological system.

33

34 **Figure 2:** Confocal images of human brain endothelial cells (in red, immunostained for
35 CD31) organized as a network after 7 days culture in static or flow culture. (A) z-stack
36 projections of x20 magnification, (B) samples after RapiClear treatment, xz and yz projection
37 on the sides, (C) 3D rendering of x20 and x60 magnification, (D) Sections of x60

1 magnification with astrocytes (in green, stained with CellTracker Green), and pericytes (in
2 blue, stained with CellTracker Red).

3

4 **Figure 3:** (A) Histological observation of a cross-sectioned gel, lumen surrounded by HBEC
5 (stained for CD31 in brown, Toluidine staining for nuclei) were easily visible. (B)

6 Distribution of lumen diameters. (C) Comparison of lumen number between static and flow
7 condition.

8

9 **Figure 4:** Gene and protein expression of tight junction proteins, transporters and carriers

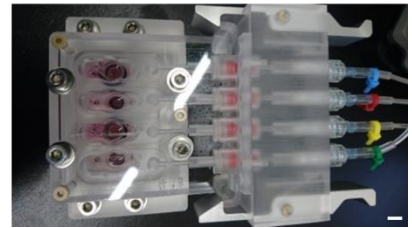
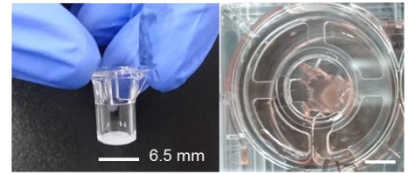
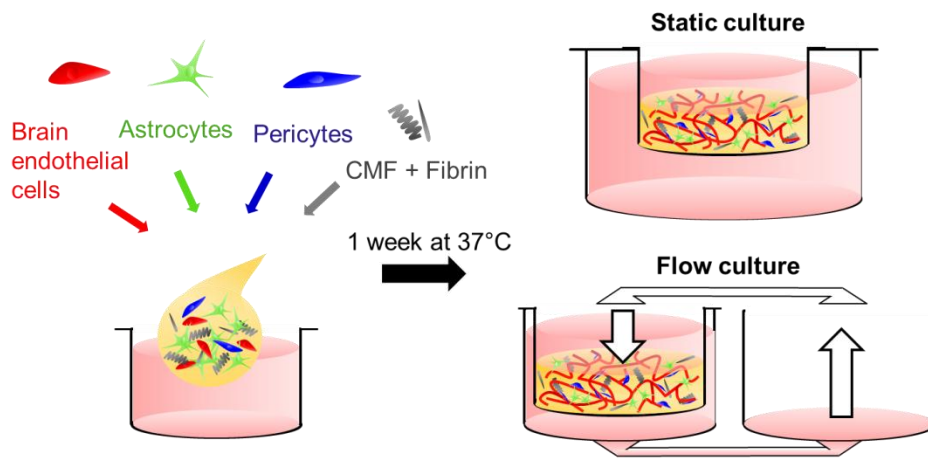
10 essential to the blood-brain barrier functions, assessed after 7 days culture under static or flow
11 conditions. (A) RT-qPCR results. (B) Immunostaining fluorescence intensity comparison. (C)

12 Immunostainings observed by confocal microscopy.

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14

1 **Figures/Tables**

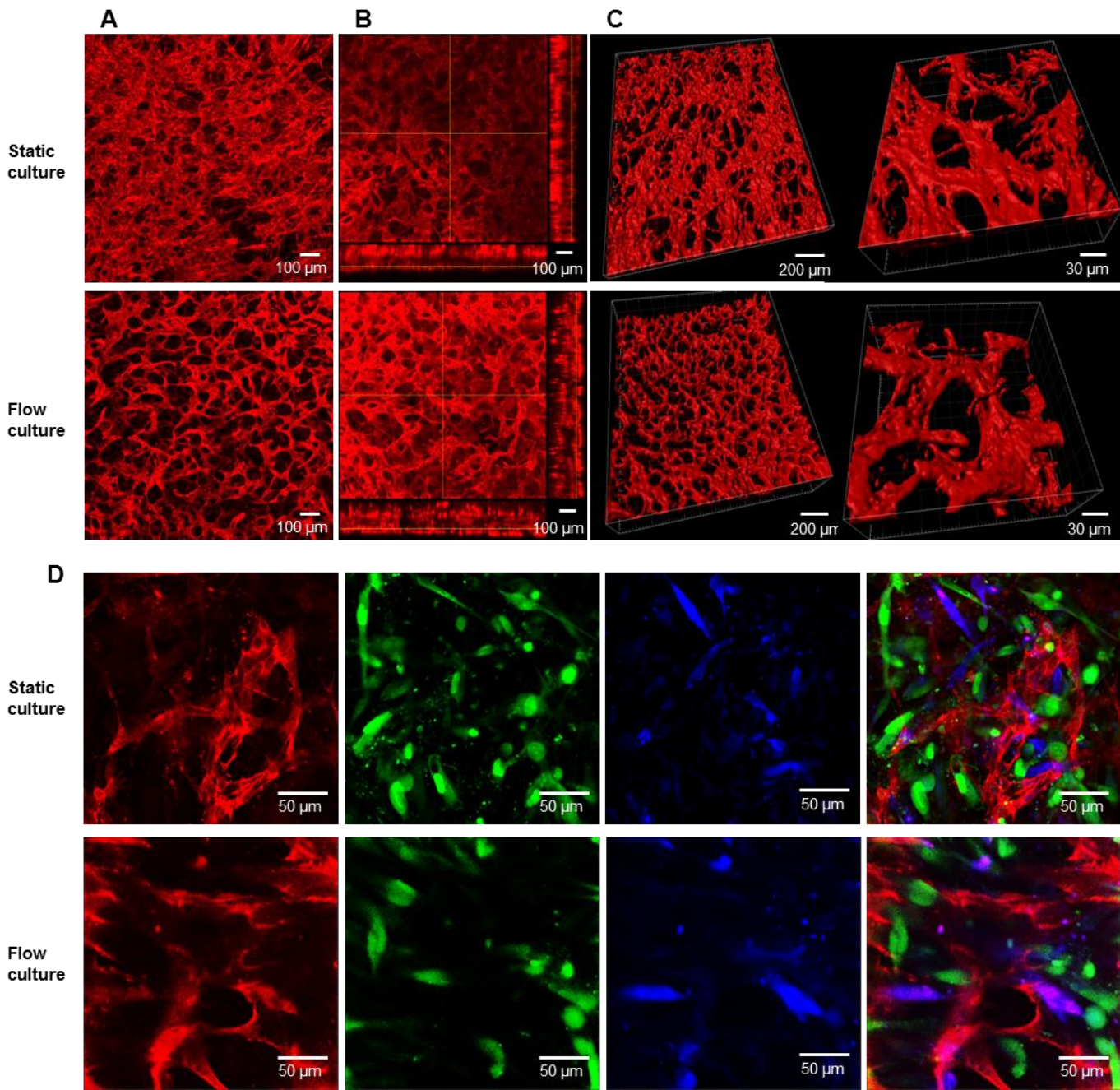


2

3 **Figure 1**

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2 Figure 2

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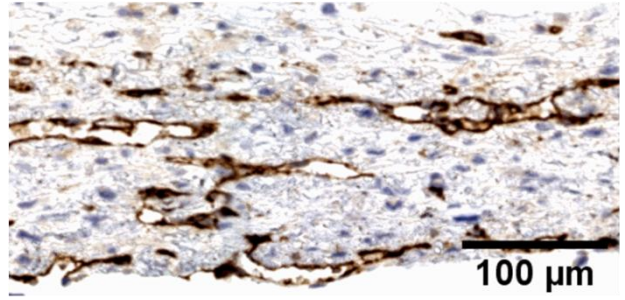
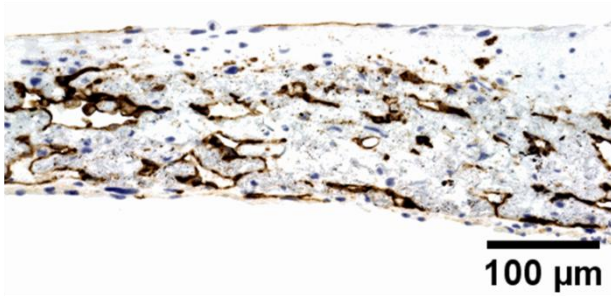
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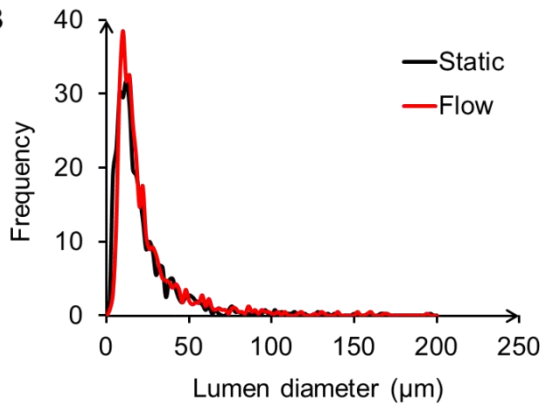
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Static culture

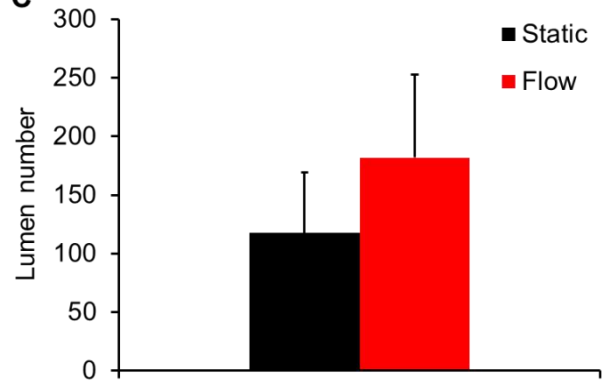
Flow culture



B



C

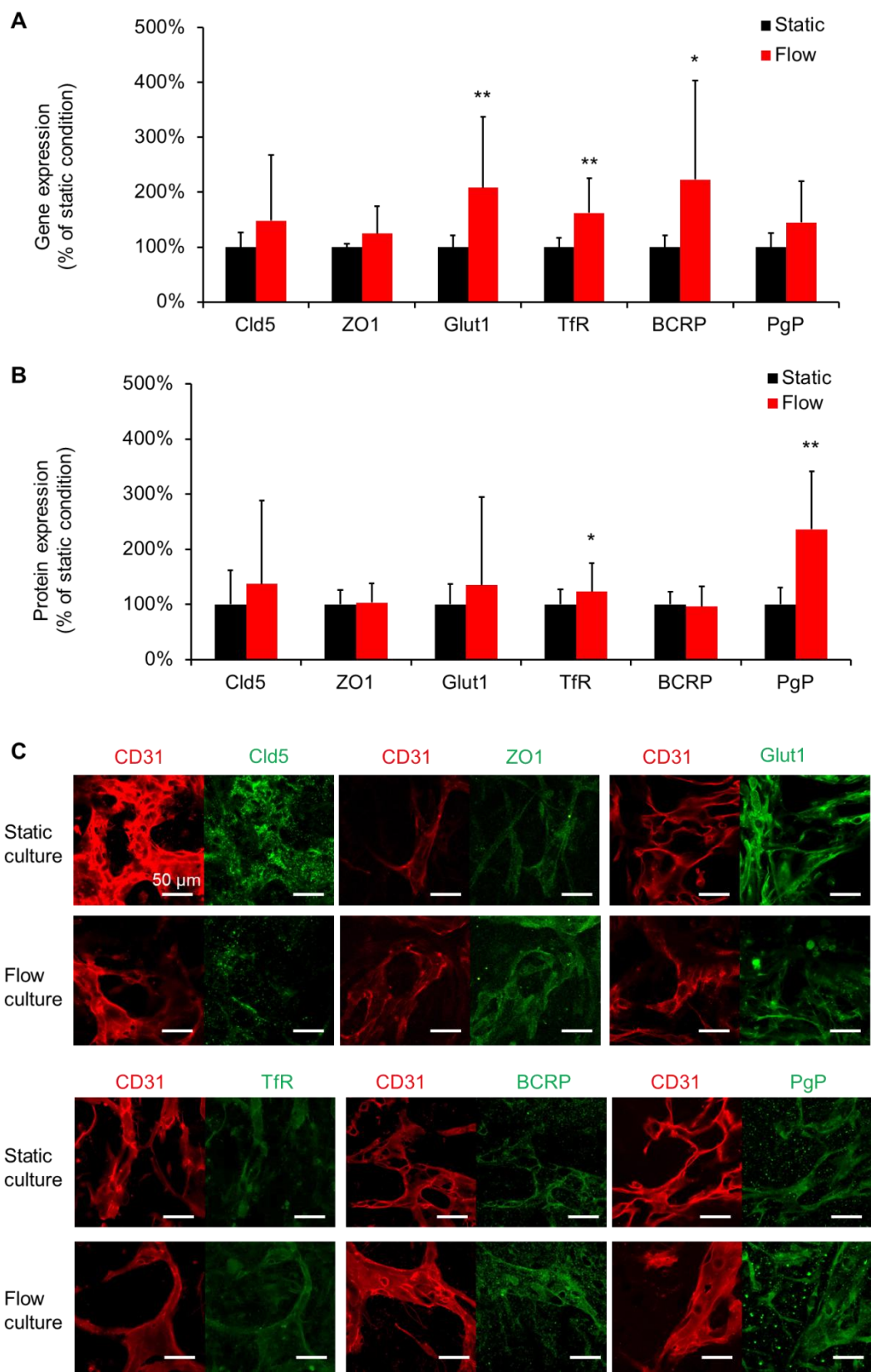


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2 Figure 3

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2 Figure 4

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