Interstitial flow regulates in vitro three-dimensional self-organized brain micro-vessels
Agathe Figarol, Marie Piantino, Tomomi Furihata, Taku Satoh, Shinji Sugiura, Toshiyuki Kanamori, Michiya Matsusaki

To cite this version:

HAL Id: hal-03740372
https://hal.science/hal-03740372
Submitted on 26 Aug 2022

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L’archive ouverte pluridisciplinaire HAL, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d’enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.
Interstitial flow regulates in vitro three-dimensional self-organized brain micro-vessels

Agathe Figarol¹ (agathe.figarol@gmail.com), Marie Piantino¹ (m-piantino@chem.eng.osaka-u.ac.jp), Tomomi Furihata² (tomomif@toyaku.ac.jp), Taku Satoh³ (taku.satoh@scetra.or.jp), Shinji Sugiura⁴ (shinji.sugiura@aist.go.jp), Toshiyuki Kanamori⁴ (t.kanamori@aist.go.jp), and Michiya Matsusaki¹* (m-matsu@chem.eng.osaka-u.ac.jp)

¹ Department of Applied Chemistry, Graduate School of Engineering, Osaka University, Osaka, Japan
² Laboratory of Clinical Pharmacy and Experimental Therapeutics, Tokyo University of Pharmacy and Life Sciences, Tokyo, Japan
³ Stem Cell Evaluation Technology Research Association, Tokyo, Japan
⁴ Cellular and Molecular Biotechnology Research Institute, Bio-Nanomaterials Team, Research Center of Advanced Bionics, National Institute of Advanced Industrial Science and Technology, Tsukuba, Japan

* Corresponding author:
m-matsus@chem.eng.osaka-u.ac.jp
2-1 Yamadaoka, Suita, Osaka 565-0871, Japan.
TEL: +81-6-6879-7356, FAX: +81-6-6879-7359

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. Introduction

The structural organization of the human brain microvasculature enables functional specificities and ensures the protection of the human nervous system thanks to the highly selective blood-brain barrier. The brain microenvironment regulates the phenotypes of the main cell types constituting the brain micro-vessels: the brain microvascular endothelial cells, the astrocytes and the pericytes. The continual shear stress generated by the flow of blood across the microvasculature especially affects the cell morphology and functions. It has indeed been reported to upregulate some junctional proteins, transporters, ions channels and limit the permeability of brain microvascular endothelial [1–4]. However, other studies showed that none or only part of the key blood–brain barrier markers were significantly over-expressed (Claudin 5, Zonula occludens-1, Occludin, CD31, Glucose transporter 1, P-glycoprotein, and others) [5,6], although the mechanical stress from the medium did promote differentiation or maturation of the endothelial cells specific to the brain microvasculature. Differences in culture and flow parameters such as applied pressure and time of treatment may partly explain those inconsistencies, especially as too high or disturb shear stress may reverse its beneficial impacts [3,7]. The range of assessed biochemical signals are also susceptible to be affected by the types of cells used in the model. The use of astrocyte conditioned medium has been shown to be as important as the shear stress to upregulate Zonula occludens-1 and decrease brain endothelial permeability [1]. Few studies introduced astrocytes [2,4], pericytes [8] or both [9] to the flow cultured brain microvasculature models, though they seemed to increase the differences observed compared to static conditions. Fewer studies considered the importance of the extracellular matrix (ECM) and its combined influence with flow on the cell culture. Systems of coculture and perfused vessels adjacent to a collagen-I gel were engineered [10]. However, no direct contact were possible between the
endothelial cells seeded in the perfused channel and the astrocytes and pericytes seeded on the other side of the gel; and the vessels diameter had to remain over 150 µm to avoid for the system to collapse [4,7]. Cells self-organization in a collagen or fibrin based gel is a state-of-the-art technique to develop brain microvasculature like network. Two teams have proposed such models with a coculture of brain microvascular endothelial cells, astrocytes and pericytes in microfluidic chips [11,12]. However, if the micro-vessels were perfusable for a few hours (< 20 h) after a maturation of 7 days, no data are yet available for a full culture under flow. We have also previously developed such a self-organized three-dimensional (3D) in vitro model of the brain microvasculature that was cultured in static condition [13]. Here we focused on the impacts of the mechanical stimuli exerted during the 7 days of culture by medium flow through the ECM, on the morphology, gene and protein expression of a scale-up version of this human cerebro-vasculature model.

2. Materials and Methods

2.1. Cell culture

The cell lines have been developed in previous studies: human brain microvascular endothelial cells/conditionally immortalized clone 18 (HBEC) [14], human astrocyte/conditionally immortalized clone 35 (HA) [15], and human brain pericyte/conditionally immortalized clone 37 (HP) [16]. All cells were cultured on collagen coated dishes (100 mm, collagen type I, Iwaki, Shizuoka, Japan) and incubated at 33°C (to maintain growth activity), 5% CO2, in humidified environment. All culture media were supplemented with 4 µg.mL-1 Blasticidin S HCl (Invitrogen, Waltham, USA) to maintain selective pressure for routine culture. HBEC were cultured in Vasculife (VEGF-Mv, LifeLine) 500 mL supplemented with 0.5 mL rh FGF-b, 0.5 mL ascorbic acid, 0.5 mL hydrocortisone.
hemisuccinate, 25 mL L-glutamine, 0.5 mL rh IGF-1, 0.5 mL rh EGF, 0.5 mL rh VEGF, 0.5 mL heparin sulfate, 25 mL fetal bovine serum (kit, LifeFactor VEGF-Mv, LifeLine, Frederick, USA), 25 mL supplementary fetal bovine serum (FBS, Gibco ThermoFisher, Waltham, USA), and 1% penicillin-streptomycin (10,000 U.mL-1 - 10,000 µg.mL-1, Nacalai tesque, Kyoto, Japan). HA were cultured in Dulbecco’s Modified Eagle Medium (DMEM, Nacalai tesque, Kyoto, Japan) 500 mL, complemented with 10% FBS, 1% penicillin-streptomycin, 5 mL N2 supplement x100 (Gibco ThermoFisher, Waltham, USA). HP were cultured in Pericyte Medium (ScienCell Research Laboratories, Carlsbad, USA) 500 mL, supplemented by 5 mL Pericyte Growth Supplement 100x, 10 mL fetal bovine serum, and 5 mL penicillin-streptomycin. HA and HP were pre-differentiated at 37°C 3 days before seeding of the 3D model.

2.2. Collagen type I microfibers and fibrin based ECM model

The ECM of our 3D in vitro model of brain vasculature is composed of collagen type I microfibers (CMF) and fibrin. CMF were processed from freeze-dried porcine collagen type I sponge, donated from Nippon Ham Foods Ltd. (Osaka, Japan). 50 mg of collagen sponge were homogenized 6 min (Violamo VH-10 homogenizer, S10N-10G 10 mm diameter, 115 mm length probe) in 5 mL of ultrapure water (Milli-Q water purification system, Merck, Kenilworth, USA), then centrifuged 10 min at ambient temperature at 12100 g. The pellet was then sonicated (Ultrasonic processor VC50, 50W, 3.2 mm probe, Sonics, Newtown, USA) in 5 mL with fresh ultrapure water in an ice bath for 100 cycles of 20 s ultrasonication and 10 s cooling. The sonicated collagen was filtrated (40 µm filter, microsyringe 25 mm filter holder, Merck, Kenilworth, USA), and the filtrate freeze-dried for at least 40 h (Freeze dryer FDU-2200, Eyela Co., Shanghai, China). The resulting CMF were stored in a desiccator at room temperature prior use.
For 70 µL hydrogels, 0.7 mg CMF and 0.4 mg Fibrinogen were dispersed in 40 µL non-complemented DMEM while 200,000 HBEC, 400,000 HA, 100,000 HP, and 0.3 U Thrombin were dispersed in 30 µL complemented Vasculife. Both solutions were mixed just before dropping in cell culture inserts (3470 transwell, for 24 well plates, polyester membrane with 0.4 µm pore, Corning Life Sciences, Tewksbury, USA) preliminarily coated with 100 µL Fibronectin at 0.04% (Fibronectin from human plasma, F2006-5G, Sigma-Aldrich, St Louis, USA). 60 min incubation at 37°C allowed for the mixtures to gel. For static cultures, the inserts were placed on top of 6 well culture plate thanks to a specifically design 24 to 6 well plate adaptor (Figure 1), and 12 mL of 1:1:1 complemented mixed media were added (6 mL of Vasculife, 6 mL DMEM-N2, and 6 mL of Pericyte Medium, all without Blasticidin). The gels were incubated 7 days at 37°C, 5% CO₂, humidified atmosphere, with a medium change at day 4.

2.3. Flow system

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

Fluorescent cell tracking dyes were used to identify HA and HP (CellTracker™ 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 there 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-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
conjugated antibodies (first mouse anti-CD31, then FITC-conjugated mouse anti-TfR, NB500-493, NovusBio, Centennial, USA; or first mouse anti-ZO1, then Alexa Fluor 594 conjugated mouse anti-CD31, FAB3628T, R&D Systems, Minneapolis, USA). Nuclei were counterstained with Hoechst (Thermo Fisher Scientific, Waltham, USA). For samples only stained for CD31, a treatment with RapiClear (1.47, SUNJin Lab, Hsinchu, Taiwan) eased deeper observations by making the ECM more transparent.

Samples were observed using epifluorescence microscopes (Confocal Laser Scanning Microscope Fluoview FV3000, Olympus, Tokyo, Japan, for x20 and x60 magnifications). 3D renderings were obtained with Imaris software (Oxford Instruments, Abingdon, UK). For comparative protein expression assessment, the same exposition time and excitation power for each correlated set of static and flow samples. Maximum and minimum z were chosen to include all visible signals. Z-stack projections using maximum intensity projection were analyzed using Fiji software [19]. The expression of tight junction proteins, transporters or carriers were assessed by measuring the fluorescent intensity of the specific protein staining divided by the fluorescent intensity of CD31 staining.

2.5. Histology

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

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

2.7. Statistics

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

3. Results and discussion

With optimized seeding parameters and after 7 days of culture, the cells self-organized into capillary-like network whether the culture was carried out in static condition or under flow in the pressure driven microphysiological system (measured flow rate of 379 ± 37 µL.min⁻¹).
This was especially visible with CD31 immunostaining of the HBEC (Figure 2). Unlike expected, the micro-vessels mainly grew horizontally in the gel, perpendicular to the flow direction. It is thus not thought that the micro-vessels were perfused through the lumen, but more likely that interstitial shear flow impacted the cells from the basal side. Such mechanical stress affected however greatly the vessel formation and maturation. Flow culture seemed to induce indeed a more defined network. Flow cultured cells self-organized in more tubular shapes, less thick, with fewer connections, and slightly deeper in the hydrogels. It resembled more to micro-vessels than in static culture. Our results were consistent with the often-detected enhancement of the cells functions and maturation by flow although the experimental set-up differed largely. Other studies on interstitial flow impact on non-brain micro-vessel development have shown that the angiogenic activity was suppressed in the direction of the flow, and favored in the opposite direction [20,21]. The interstitial flow could thus be used to regulate and spatially guide angiogenesis.

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

Gene expression of tight junction proteins, transporters and carriers essential to the blood-brain barrier functions were analyzed by RT-qPCR. Flow culture seemed to boost such gene expressions compared to static condition (Figure 4.A). All assessed genes: Claudin 5 (Cld5), Zonula occludens-1 (ZO-1), Glucose transporter 1 (Glut1), Transferrin receptor (TfR), Breast cancer resistant protein (BCRP), and P-glycoprotein (PgP), tended to be over expressed in samples that undergone culture under flow compared to samples that were culture in static
condition. The increase was significant for Glut1, TfR and BCRP, not for the other genes however. This may partly result from the high standard deviations, consequence from the differences between collagen lots, and small differences in flow pressure (9% deviation in average) that could results from limited shrinkage of the gels or bubbles, although those latter have been widely reduced with method improvements. Despite some inconsistency in the literature, similarly showing the importance of culture parameters, we have shown in the introduction that the consensus was also tending to an improvement of tight junction proteins, transporters and carriers expression by a shear stress.

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

Acknowledgements

This work was supported by the Japan Agency for Medical Research and Development (AMED-MPS 19be0304207h003), and Grant-in-Aid for Scientific Research (A) from the Japan Society for the Promotion of Science (20H00665).

References


https://doi.org/10.1016/j.jneumeth.2014.05.013.
Roquer, A. Ois, A. Principe, E.R. Edelman, M. Balcells, Pulsatility and high shear stress
deteriorate barrier phenotype in brain microvascular endothelium, J Cereb Blood Flow
Galie, Mechanical stress regulates transport in a compliant 3D model of the blood-brain
https://doi.org/10.1016/j.biomaterials.2016.11.012.
5 L. Cucullo, M. Hossain, V. Puvenna, N. Marchi, D. Janigro, The role of shear stress in
6 J.G. DeStefano, J.J. Jamieson, R.M. Linville, P.C. Searson, Benchmarking in vitro tissue-
engineered blood-brain barrier models, Fluids and Barriers of the CNS. 15 (2018) 32.
7 N. Bouhrira, B.J. DeOre, D.W. Sazer, Z. Chiaradia, J.S. Miller, P.A. Galie, Disturbed
flow disrupts the blood-brain barrier in a 3D bifurcation model, Biofabrication. 12 (2020)
capillaries-venules modular system: Cerebrovascular physiology in a box, BMC
9 P. Miranda-Azpiazu, S. Panagiotou, G. Jose, S. Saha, A novel dynamic multicellular co-
culture system for studying individual blood-brain barrier cell types in brain diseases and
cytotoxicity testing, Scientific Reports. 8 (2018). https://doi.org/10.1038/s41598-018-
26480-8.
10 N.R. Wevers, D.G. Kasi, T. Gray, K.J. Wilschut, B. Smith, R. van Vught, F. Shimizu,
Y. Sano, T. Kanda, G. Marsh, S.J. Trietsch, P. Vulto, H.L. Lanz, B. Obermeier, A
perfused human blood–brain barrier on-a-chip for high-throughput assessment of barrier
11 M. Campisi, Y. Shin, T. Osaki, C. Hajal, V. Chiono, R.D. Kamm, 3D self-organized
microvascular model of the human blood-brain barrier with endothelial cells, pericytes
https://doi.org/10.1016/j.biomaterials.2018.07.014.
12 S. Lee, M. Chung, S.-R. Lee, N.L. Jeon, 3D brain angiogenesis model to reconstitute
functional human blood–brain barrier in vitro, Biotechnology and Bioengineering. 117
13 A. Figarol, Y. Naka, Y. Shigemoto-Mogami, T. Furihata, K. Sato, M. Matsusaki, In
Vitro self-organized three-dimensional model of the blood-brain barrier microvasculature,
14 R. Ito, K. Umehara, S. Suzuki, K. Kitamura, K. Nunoya, Y. Yamaura, H. Imawaka, S.
Izumi, N. Wakayama, T. Komori, N. Anzai, H. Akita, T. Furihata, A human immortalized
cell-based blood-brain barrier tri-culture model: development and characterization as a
https://doi.org/10.1021/acs.molpharmaceut.9b00519.
15 T. Furihata, R. Ito, A. Kamichi, K. Saito, K. Chiba, Establishment and
characterization of a new conditionally immortalized human astrocyte cell line, J.


**Figure/Table Legends**

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

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

**Figure 3:** (A) Histological observation of a cross-sectioned gel, lumen surrounded by HBEC (stained for CD31 in brown, Toluidine staining for nuclei) were easily visible. (B) Distribution of lumen diameters. (C) Comparison of lumen number between static and flow condition.

**Figure 4:** Gene and protein expression of tight junction proteins, transporters and carriers essential to the blood-brain barrier functions, assessed after 7 days culture under static or flow conditions. (A) RT-qPCR results. (B) Immunostaining fluorescence intensity comparison. (C) Immunostainings observed by confocal microscopy.
1 **Figures/Tables**

2

3 **Figure 1**

4

5
Figure 2
Figure 3

A  Static culture  Flow culture

B  

C  

1
2  Figure 3
3
4
Figure 4

A

Gene expression (% of static condition)

B

Protein expression (% of static condition)

C

Static culture

Flow culture

1

2

3