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ARTICLE

A MULTIPLE-CHANNEL, MULTIPLE-ASSAY PLATFORM FOR CHARACTERIZATION OF FULL-RANGE SHEAR STRESS EFFECTS ON VASCULAR ENDOTHELIAL CELLS

R. Booth\textsuperscript{a}, S. Noh\textsuperscript{b} and H. Kim\textsuperscript{c}

Vascular endothelial cells (VECs), which line blood vessels and are key to understanding pathologies and treatments of various diseases, experience highly variable wall shear stress (WSS) \textit{in vivo} (1-60 dyn/cm\textsuperscript{2}), imposing numerous effects on physiological and morphological functions. Previous flow-based systems for studying these effects have been limited in range, and comprehensive information on VEC functions at the full spectrum of WSS has not been available yet. To allow rapid characterization of WSS effects, we developed the first multiple channel microfluidic platform that enables a wide range (~x15) of homogeneous WSS conditions while simultaneously allowing trans-monolayer assays, such as permeability and trans-endothelial electrical resistance (TEER), as well as cell morphometry and protein expression. Flow velocity/WSS distributions between channels were predicted with COMSOL simulation and verified by measurement with an integrated micro-flow sensor array. Biomechanical responses of the brain microvascular endothelial cell line bEnd.3 to the full natural spectrum of WSS were investigated with the platform. Under increasing WSS conditions ranging from 0-86 dyn/cm\textsuperscript{2}, (1) permeabilities of FITC-conjugated dextran and propidium iodide decreased respectively at rates of 4.06e0 \textsuperscript{-2} \text{cm/s per dyn/cm\textsuperscript{2}}; (2) TEER increased at a rate of 0.8 \text{Ωcm\textsuperscript{2}} per dyn/cm\textsuperscript{2}; (3) cells increased alignment along the flow direction under increasing WSS; and finally (4) increased protein expression of both tight junction component ZO-1 (~5x) and efflux transporter Pgp (~6x) were observed at 86 dyn/cm\textsuperscript{2} compared to static controls via western blot. We conclude that the presented microfluidic platform is a valid approach for comprehensively assaying cell responses to fluidic WSS.

Introduction

Vascular endothelial cells (VECs), which line all blood vessels and comprise the interface between blood and surrounding tissue, are a key to understanding pathologies and treatments of vascular systems, dictating numerous vascular functions critical to homeostasis and drug delivery throughout the body\textsuperscript{1-5}. Their governing functions\textsuperscript{3} include permeability\textsuperscript{4}, angiogenesis\textsuperscript{5}, cell migration, proliferation, and apoptosis\textsuperscript{6}, impacting processes involved in inflammation\textsuperscript{7}, thrombosis\textsuperscript{8}, metastasis\textsuperscript{9}, and drug pharmacokinetics, all of which play critical roles in pathology and treatment of the two leading causes of death in the US\textsuperscript{10}: cancer\textsuperscript{11} and cardiovascular disease\textsuperscript{12}, as an example.

VECs have been found to delicately regulate such functions in response to dynamic microenvironments, and one major environmental parameter is the shear stress experienced at the vessel wall, or wall shear stress (WSS), induced by the flow of blood through the vasculature. VECs are reported to experience \textit{mechotransductive effects on cell phenotype} when exposed to WSS via membrane-bound mechanosensors\textsuperscript{13-15} (Fig. 1A). Such effects include the induced modulation of a myriad of biomolecular pathways leading to various physiological responses\textsuperscript{16}, such as resistance to apoptosis\textsuperscript{19,20}, upregulation of tight junction proteins (ZO-1, occludin\textsuperscript{17}), extracellular matrix components (fibronectin, laminin\textsuperscript{18}), membrane-bound efflux transporters (P-gp\textsuperscript{19}) and integrins\textsuperscript{20}, as well as cytoskeletal restructuring and cell reorientation in relation to the flow direction\textsuperscript{21-23}. The effects caused by WSS impact VEC functions relevant to pathology. For example, atherosclerosis, the leading cause of heart attack and stroke\textsuperscript{24}, has been correlated with low stress regions\textsuperscript{25}, while atheroprotective responses have been observed in high-stress regions\textsuperscript{26}, and WSS as high as 300 dyn/cm\textsuperscript{2} has been measured in cases of vessel stenosis\textsuperscript{27}. This variability in cell microenvironment indicates the need for comprehensive understanding of the adaptive responses of VECs to the WSS applied by the highly dynamic, highly variable microenvironment of the \textit{in vivo} vasculature, and for delineating the practical limitations of dynamic \textit{in vitro} culture conditions.
The study of VECs’ responses to WSS has remained quite challenging both in vivo and in vitro, due to the difficulty in realizing the wide range of WSS conditions (1-60 dyn/cm²) in vivo, and utilizing it for comprehensive assays. Such a large WSS range is mainly caused by significant variations of vessel sizes (8µm-2.5cm) and their differently localized pressure (Fig. 1B). In vivo investigation does not provide reproducible and controllable testing conditions due to natural variations in tissue complexity and structural contiguity, forbidding precisely defined correlations between WSS and VEC response. In vitro methods, despite providing greater experimental control and repeatability of flow conditions, have failed thus far to effectively provide characterization spanning the physiologically relevant full spectrum of WSS. This is because all previous VEC cultures systems for assaying WSS effects (Tab. 1) are either limited to a single WSS condition per unit [13,21,24,34-37], or are limited in the types of feasible assays, due either to a lack of an integrated microfluidic chip (preventing assays of barrier properties), or to non-uniformity in applied WSS among a cell population under assay [45-50] (preventing reliable correlation of assay results with discrete WSS). Though recently a closed-loop braille-display device has applied distinctly different WSS to three parallel channels [52], it covered only a limited shear stress range of ~12 dyn/cm² due to the limited flow rates from the integrated micro pumps and did not allow trans-monolayer assay. In summary, no VEC culture system has simultaneously achieved (1) multiple on-chip WSS conditions for isolated cell populations, while (2) allowing trans-membrane assays yet.

To address such issues, we have developed a multi-channel and multi-assay platform (Fig. 1C) where a single fluidic input produces multiple distinct WSS magnitudes homogenously applied to isolated VEC populations cultured on membranes, allowing multiple types of assays in a reproducible and controllable manner. To allow rapid characterization spanning the full in vivo WSS range (1-60 dyn/cm²), the platform employed four parallel channels producing distinct WSS with ~15X range of magnitude. In practice, by compounding the on-chip WSS variance with varying input flow-rates, useful quantitative data spanning the full spectrum of WSS can be efficiently gathered from a single experimental pump setup, and seeded from a single standard plate (75cm²) of VECs (Fig. 2). For example, 8 chips in parallel in a standard 16-cartridge pump, compounded by varying input flow rates to each chip, will result in 32 distinct WSS. Furthermore, the designed ~15X magnitude range allows a single test to cover the full span of WSS experienced in brain capillaries (3-20 dyn/cm²) [53], which is the origin of the cell line used in this characterization study. Thus, the presented approach massively reduces required cells/reagents and turn-around time, while allowing unprecedented high-throughput, comprehensive WSS characterization assays. To allow trans-membrane assays, including trans-endothelial electrical resistance (TEER) and permeability, this platform employs the cross-junction structure used in our previously reported microfluidic BBB model [54].

<table>
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<tr>
<th>Dynamic in vitro platforms for characterization of wall shear stress effects on vascular endothelial cells</th>
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<tr>
<td><strong>System type</strong></td>
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<tr>
<td>Presented microfluidic system</td>
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<tr>
<td>Microfluidic (braille closed-loop)</td>
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<td>Rotational systems</td>
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<td>Hollow fiber / capillary systems</td>
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**Fig. 1** Studying the relationship between vascular wall shear stress (WSS) and endothelial cell (EC) physiology. (A) VECs respond through mechanotransduction via mechanosensors (integrins and kinases), ultimately leading to significant changes in protein expression, such as in membrane transport, tight junctions, and cytoskeletal re-organization. (B) The WSS experienced by vascular endothelial cells varies significantly (1-60 dyn/cm²) at different geometric locations in vivo. (C) An optimal, fully comprehensive approach to testing the relationship between WSS and VEC responses requires multiple well-defined, discrete WSS, and extraction of data via multiple assays, including trans-membrane testing, per replicate chip.
This paper reports the design, fabrication, and testing results of the developed multi-channel, multi-assay WSS platform. To evaluate the effectiveness of the platform to produce a wide range of WSS, quantitative testing results are discussed, including velocity discretization into parallel channels (1) by COMSOL simulation and (2) by experimental measurements with an integrated micro-flow sensor array. Next, the validity of the platform as a high-throughput tool to correlate full-spectrum WSS effects with VEC properties of a particular cell line was quantitatively verified on the brain microvascular endothelial cell line bEnd.3 by measuring (3) cell morphology (cell elongation and orientation), (4) trans-monolayer permeability, (5) monolayer TEER, and (6) protein expression of tight junctions (ZO-1) and membrane-bound efflux transporters (P-gp).

Structures and Fabrication

To produce high-variance WSS distributions, a parallel array microfluidic structure of four channels was fabricated utilizing standard PDMS processing techniques to have significant differences in fluidic resistance among the channels, causing variations in the flow velocities and thus the resultant WSS.

Microfluidic Parallel-Channel Structures

The parallel array platform consists of four top (luminal) channels and one bottom (abluminal) channel that cross perpendicularly, all of which were constructed from multiple stacked poly-dimethylsiloxane (PDMS) layers (Fig. 3A). The four luminal channels branch from one common inlet and have independent outlets to simplify fluidic control and enable high-throughput parallel permeability assays. The four luminal channels have discrete widths (e.g. 0.73, 1.53, 2.33, 3.13 mm) with varying geometries at the branching region, subsequently causing different fluidic resistances, flow velocities and uniform WSS in each channel. When these channels were constructed in multiple layers by stacking PDMS substrates, a porous polycarbonate (PC) membrane was inserted to be free-standing at the channel junction, providing a monolayer cell culture surface allowing tran-monolayer assays (permeability/TEER) at each junction, as in the previously described µBBB system. Flanking both sides of each PC culture surface, a set of thin film AgCl electrodes were located to monitor TEER in the standard four-point sensing configuration minimizing error from parasitic resistance from external wires and contacts. The channel heights were initially set at 200µm, ensuring laminar flow and maximizing aspect ratio of the channels for WSS uniformity.

The platform structure was fabricated (Fig. 3B) similarly to the previously described µBBB system with some modifications. First, embedded electrodes for TEER measurement were fabricated on a glass wafer by sputtering Cr/Au/Ag (20/80/800nm) and patterning layers using liftoff lithography (LOR-10B photoresist). The silver (Ag) surface was chlorinated with 30mM FeCl$_3$ for 50s to convert it into AgCl for corrosion resistant electrodes. Next, the channels were constructed among multiple PDMS layers. Channel molds were lithographically constructed from SU-8 2075 (200µm thick) on a silicon substrate, and silanized overnight with tridecafluoro-1,1,2,2-tetrahydrooctyl trichlorosilane in a vacuum chamber to minimize stiction and ease the separation during the molding process. On top of the SU-8 mold, PDMS layers were sequentially cast (10:1 elastomer:curing agent) and heat-cured (110°C, 30m). Following 30 minutes pre-treatment of the PC membrane with 5% 3-aminopropyltriethoxysilane (APTES) at 80°C, a reliable bond between the membrane and PDMS layers was generated with O$_2$ plasma (125W, 20s) at 25°C. Residual APTES on the membrane was dissolved in ethanol. This bond method significantly decreased occurrences of leaks under high flow compared with the standard method using spin-coated PDMS pre-polymer, with no observed losses of cell adhesion. To generate the minimum total protein required for western blot (>25 µg), a large 5mm-wide, 175mm-long microfluidic channel was separately constructed.
Cell Culture

The cell line tested in this study was the brain endothelial cell line bEnd.35. The cells were grown with DMEM/F12 (Lonza), and was supplemented with 10% fetal bovine serum (HyClone), 1% Penicillin/Streptomycin and amphotericin B (EMD). Media pH was buffered to ~7.35 for all experiments. All cells used for experiments were taken from confluent cultures only, within two days after confluence was reached. All cell cultivation and shear stress experiments were carried out in a humid incubator (Nu-Aire Autoflow 4750) with 5% CO2 kept at a constant 37°C. Cell suspensions were centrifuged in an Eppendorf 5810, and all sterile work was performed in a class II biosafety cabinet (Thermo Fisher). Sterilization of microfluidic devices and tubing was carried out with 70% ethanol and UV radiation prior to use. A single T-75 flask was sufficient for seeding a parallel array of microfluidic cell culture models for shear stress experiments. Antibodies used in this study were: Primary rabbit anti-ZO-1 (GeneTex GTX108592), primary rabbit anti-MDR-1 (Santa Cruz sc-8313), primary rabbit β-actin (Abcam ab8227), secondary HRP-conjugated goat anti-rabbit (Abcam ab6721), and secondary Alexa Fluor 488 goat anti-rabbit (Molecular Probes A-11008).

Testing methodology

First, the fabricated platform was evaluated on its capability of producing a wide range of WSS by (1) predictions based on analytical calculation and simulations and (2) direct flow velocity measurement from the integrated micro flow sensor array. Next, the validity of the platform as the high-throughput tool to correlate full-spectrum WSS effects withVEC properties was evaluated by monitoring the (3) cell morphology (shape and orientation angle), (4) trans-monolayer permeability, (5) TEER, and (6) protein expression of tight junctions and efflux transporters of the bEnd.3 cell line.

Prediction of the Wall Shear Stress by Simulation

In order to predict the resultant WSS levels that cells experience at the channel wall, (1) fine-mesh 3D COMSOL simulations were performed to obtain velocity profiles at different heights from the wall; (2) shear rate dU/dz at the wall was calculated by COMSOL based on the velocity gradient right above the wall; (3) shear rate was multiplied with the proportionality constant µ, or viscosity, as in the following equation (Fig. 5A) 56:

\[ \tau = \frac{du}{dz} \mu \]

COMSOL simulation utilized the laminar flow module that derives velocity fields from the Navier-Stokes Equations. Assumptions used in the model were Newtonian fluid (µ=1.2mPa’s for media with serum), no-slip condition, with equal pressure at all outlets. Note that the COMSOL simulations and analytical calculations also assumed that all flow in each of the described devices at all relevant flow-rates is completely laminar, with a Reynolds number several orders of magnitude lower than the turbulent threshold (2300), which is a reasonable assumption in microfluidics57. It was assumed that the effects of flow-induced deformation of the channel walls58 was negligible at all relevant flow-rates for the study, and that the channel walls remained rigid for the purpose of WSS calculation and measurement in this study.
Shear Stress Measurement with Integrated Micro Flow Sensors

In parallel to COMSOL simulation, the average WSS ($\tau_A$) at the wall was calculated from the velocity measurement by the fabricated micro flow sensor ($U_{FS}$) in each channel (Fig. 5B). Since the fabricated micro sensor was located at 70µm above the wall, the measurement does not directly represent the average velocity (located at 42µm height for our device), necessitating an adjustment process in order to utilize the well-established relationship between the average velocity ($U_A$) and $\tau_A$ in a rectangular channel\(^5\). For this adjustment, the ratio between $U_{FS}$ and $U_A$, as obtained from the Poiseuille flow velocity profile in the vertical direction of the channel, was found to be $\approx 1.36$, and was applied as a multiplication constant to the denominator of the standard equation for average WSS in a rectangular channel\(^5\), allowing calculation of $\tau_A$ from $U_{FS}$:

$$\tau_A = \frac{6\mu U_{FS}}{1.36 h}$$

where $h$ is channel height and $\mu$ is the dynamic viscosity.

To prepare for the induction of WSS into the parallel channels, fluidic interconnection was established by sealing marprene or silicone tubing to inlet ports in connection to a 16-cartridge peristaltic pump (Watson-Marlow 20SS). Then, measurement utilizing the fabricated micro flow sensor was performed, while certain flow rates were supplied through the platform. The terminals of the micro flow sensors were connected to a Wheatstone Bridge circuit to measure voltage offsets resulting from differential thermal dissipation. Defined flows of DI water from a steady-flow syringe pump (KDS210) were injected in reverse through the outlets to generate calibration curves for each sensor. Electrical measurements were made with a power supply (GW Instek PSP603) and an NI DAQ. After 10s continuous flow, thus when flow is stable, 5V was applied and output voltage was recorded (10s, 1kHz) through the DAQ. Following brief cooldown, the process was repeated at least three times. To measure the velocity distributions under forward flow, known flow rates were injected through the inlet, and the measured voltage outputs in each channel were fitted to their calibration curves to calculate uniform velocity.

For further comparison, volumetric flow measurements (at least 3 replicates) at the outlet of each chip were fit to the standard equation for average WSS:

$$\tau_A = \frac{60\mu U_{FS}}{h^2 w}$$

Application of Shear Stress to Cultured Endothelial Cells

To analyse physiological effects of WSS on confluent cultures, the platforms were prepared by sealing marprene or silicone tubing to inlet ports in connection to a 16-cartridge peristaltic pump. Permeability/TEER measurement required two dedicated cartridges, and imaging & western blot measurement required one dedicated cartridge. Depending on tubing volume, 8-well strips (300 µL) with poly-tetrafluoroethylene plugs or centrifuge tubes lined with parafilm were used as media reservoirs. The entire experimental setup (pump, platforms, and reservoirs) was placed in a CO\(_2\) incubator. Though flow-rates differed among the four parallel channels, they were appropriate for simultaneous cell seeding and channel flushing to be practical. To culture cells in the fabricated platform, the platform was sterilized first with 70% ethanol and coated overnight with fibronectin and collagen IV (100µg/mL each) to facilitate cell adhesion. After sterilization bEnd3 cells were seeded in the devices at a density of 6e4/cm\(^2\) and allowed to adhere in a static condition (no flows) for 2h. Then the platforms were flushed with sterile DMEM/F12 media and perfused at very low flows (uncharacterized minimum pump setting) for 3 days to allow cell confluence and optimal cell anchorage. Media reservoirs were changed daily. Experimental WSS was applied for 24h prior to quantitative assays to characterize the WSS effects on bEnd.3 physiology.

Morphometric analysis
In order to evaluate the shear stress effects on cell morphology, VECs were imaged on-chip and both shape and orientation angle of the cells were analyzed with CellProfiler software, while various WSS was applied to each channel. For cell...
imaging preparation, monolayers of b.End3 cells were fixed with 4% paraformaldehyde (Avantor) for 20 min at room temperature. Cell membranes were permeabilized with 0.1% Triton X-100 in PBS for 20 minutes. Cells were then blocked for 1 hour under gentle rotation with 5% bovine serum albumin in permeabilization buffer, and cells were incubated overnight at 4°C with anti-ZO1 primary antibody. Cells were then incubated with Alexa-fluor 488-conjugated secondary antibody for 1 hour under gentle rotation. To visualize the cells, they were imaged with a fluorescent Nikon microscope. To quantitatively assess changes in cell morphology under various WSS, images were processed with CellProfiler to measure cell dimensions and positions (Fig. 6A), modeling cells as ellipses.

To quantify cell elongation, we used shape index (SI)

\[ SI = \frac{4\pi A}{P^2} \]  

where \( A \) = area and \( P \) = perimeter. An object with SI of 1 is a circle, and SI of 0 is a straight line. To quantify cell alignment, the orientation angle (OA) is defined as the angle (0-90°) between the cell’s major axis and direction of flow. Negative orientation angles were inverted to their positive values.

Permeability assay

In order to evaluate the shear stress effects on the cross-membrane transfer of molecules, the permeability of two commonly used fluorescent tracers, fluorescein isothiocyanate (FITC)-dextran (4kD size) and propidium iodide, was monitored in each channel in reference to the corresponding WSS values. Fluorescent tracer concentrations were measured with a BioRad Synergy plate reader for FITC-Dextran 4k (490/525nm excitation/emission) and propidium iodide (536/617nm), and fitted to known standards to calculate the concentration values. Then, the concentration values were utilized to calculate the corresponding permeability (Fig. 6B).

The tracer flux \( J \) through the cell layer was measured with the following flux equation

\[ J = \frac{\Delta C_B}{\Delta t} \cdot V_S \]  

where \( \Delta C_B \) is bottom perfusate concentration change, \( \Delta t \) is assay time, and \( V_S \) is bottom perfusate sample volume. Permeability coefficients were calculated for each tracer with the conventional equation for permeability

\[ P = \frac{J}{\Delta C_T} \]  

\[ \frac{1}{P} = \frac{1}{P_c} - \frac{1}{P_b} \]  

where \( P \) is the permeability coefficient, \( A \) is culture area, and \( C_T \) is the concentration being flowed through the top channel. To normalize values for blank membrane flux, endothelial coefficients \( P_c \) were calculated by subtracting the inverse of the measured \( P \) value by the inverse of coefficient \( P_b \) through a blank membrane (no cells), as in the following equation.

TEER assay

TEER values were measured under various WSS levels to evaluate the changes in confluence and integrity of tight junctions. For measurement of TEER (Fig. 6C), voltage and current electrode pads were connected through 30-gauge wires with conductive silver epoxy via an electrode adapter (WPI) to an EVOM2 epithelial voltohmeter (WPI). The EVOM2 passes a constant 10µA AC current at 12.5Hz while measuring resistance changes. To calculate TEER, initial D0 Background resistances \( R_{blank} \) were subtracted from measured resistance following 24h WSS \( R \), and normalized for the cell culture area for that particular channel, giving TEER values in \( \Omega \text{cm}^2 \) from the following equation.

\[ \text{TEER} = (R - R_{blank})A \]
Results and discussion

Shear Stress Simulation and Measurement

COMSOL simulation results indicated that the horizontal profile of velocity and WSS within a channel is largely uniform except the high-drag regions near the side-walls within a distance of ~200 µm (Fig. 7A). This uniformity helps optimize the homogeneity of the discrete WSS experienced by the cells in the channel, as it is desired to achieve as uniform an environment for all cells in a particular population. Due to the high-drag region, which is independent of channel width and only related to channel height, the proportion of cells experiencing lower shear stress is smaller for the wider channels, so the wider channels have a more homogenous profile, indicating the need for a high aspect ratio to optimize accuracy of WSS measurements. Though the high-drag regions in the smallest channel make up a slightly larger surface area than the uniform region (~330), its lower aspect ratio was necessary to achieve high WSS variance between channels.

COMSOL simulation results also showed that uniform WSS distribution among four channels were repeatedly achieved with the span ratio of ~15x relative magnitude between the fastest and slowest channels regardless of input flow-rate (Fig. 7B). Thus, the simulation results indicated that the full in vivo shear stress spectrum of 1-60 dyn/cm² is achievable in as few as two parallel chips with two different input flow-rates of at least ~4x difference, allowing very rapid application and testing of the full physiological spectrum.

Figure 7C shows the COMSOL simulation results (eq. 1) in comparison to the micro-flow sensor measurements (eq. 2) and estimation from the volumetric measurements (eq. 3) at an example input flow-rate of 300 µL/min. The comparison revealed that all three values matched within 10% error for the two center channels, indicating the validity of both prediction and measurement methods. The WSS values from the micro flow sensors showed that the discrepancy becomes larger for the smallest (0.73mm) and largest (3.13mm) channel sizes by 22% and 66% of the simulated values, respectively.

Western blot

Protein expression assays were performed to affirm the causes of physiological responses under various shear stress levels. Particularly, two proteins were monitored, tight junction component ZO-1 and membrane efflux transporter p-glycoprotein (P-gp), at multiple WSS because these proteins correlate with monolayer tightness and membrane transport activity. Cells were scraped from the channel substrate surface, or 6-well static controls, with a cell scraper and lysed. Following 10s sonication, total protein was centrifuged (12000RPM, 15m) and separated from pellet. Protein was quantified with BCA total protein assay, and 25µg protein was loaded in 4-12% Bis-Tris gels (Novex) and run at 200V for ~1h, or until sufficiently separated. Following 1h transfer to nitrocellulose membrane at 30V, membranes were blocked with 5% skim milk (1h) in TBS-Tween-20. Rabbit primary antibodies for ZO-1, MDR-1 (P-gp), and β-actin as a loading control, were incubated overnight at 4°C. Goat anti-rabbit horseradish peroxidase secondary antibody was incubated for 1h, and chemiGlow (AlphaInnotech) was applied to the membrane, and imaged for band analysis with a FluorChem FC2 imaging system.

Morphometric Analysis

Image analysis data from optical measurements showed that the bEnd.3 cells did not exhibit any notable change in shape index, a measure of cell elongation, with increases in WSS (Fig.8A). It is known that the tested cell line in this study (bEnd.3) holds a characteristic highly-elongated morphology under static conditions, and we hypothesize that this trait makes the cell line less susceptible to changes in SI than other cell types with a rounder, more “cobble-stone” morphology under static conditions. For example, human aortic endothelial cells with a static SI of 0.7 have exhibited a decrease to 0.4 at 12 dyn/cm² WSS, and bovine aortic endothelial cells with a static SI of 0.76 have also shown a decrease to 0.31 at 20 dyn/cm² WSS, while the utilized bEnd.3 cell line has initially low SI of 0.13 or 0.1 (mean) or 0.1 (median) at static condition.

Optical measurement data also showed that the cell lines adjusted their orientation with the flow direction under increasing WSS (Fig. 8B). The mean orientation angles respectively decreased from 45.3° to 18.1° under the WSS range from 0 (static) to the highest tested at 60 dyn/cm², while the overall trend of the mean values formed a linear correlation (R² of 0.61), suggesting an increase in cell alignment with increasing WSS. Residual analysis of the linear regression of the orientation angle data (R² of 0.05) indicated a right
(positive) skew as indicated by the distribution of the residuals (Fig. 8C) and the normal probability plot (Fig. 8D). In congruence, the median values were consistently higher than the mean values, resulting in discrete mean and median values in Fig. 8. Median values also showed a linear decrease along with WSS, ranging from 47.6 to 14.6 for static control and 86 dyn/cm$^2$, respectively.

Permeability

Experimental measurement results demonstrated that the permeability of chemical compounds decreased with increasing WSS. Fig. 9 shows the resultant permeability coefficients of fluorescent tracers FITC-Dextran 4kD (Fig. 9A) and propidium iodide (Fig. 9B). The permeabilities of FITC-dextran and propidium iodide decreased from averages of 7.4e$^{-26}$ cm/s and 2.3e$^{-25}$ cm/s to 4.0e$^{-26}$ cm/s and 1.9e$^{-25}$ cm/s, respectively with increasing WSS from 0.35 to 86 dyn/cm$^2$. The decreasing rates for the permeability of the fluorescent tracers were 4.06e$^{-28}$ cm/s and 6.04e$^{-28}$ cm/s per dyn/cm$^2$, respectively. Minimum and maximum average values ranged from 7.4e$^{-2}$ cm/s and 2.3e$^{-2}$ cm/s (0.35dyn/cm$^2$) to 4.0e$^{-2}$ cm/s and 1.9e$^{-2}$ cm/s (86dyn/cm$^2$) for FITC-Dextran 4kD and PI, respectively. A reduction in standard deviation was observed at WSS above 20 dyn/cm$^2$. Though FITC-Dextran’s mean value increased slightly from 64 to 86 dyn/cm$^2$, the mean values fall within a standard error of each other. For all conditions, the tests were repeated at least 8 times (n>8). Permeability was consistently higher for propidium iodide than for FITC-Dextran. This agrees to the expectation based on the lower molecular weight (668D) than FITC-Dextran (~4kD), making diffusion more rapid. Note that for FITC-Dextran at higher WSS (near 86dyn/cm$^2$) the increased mean permeability may indicate a slight loss of cell adhesion, but the increase is not significant, and was not observed in the propidium iodide permeability data, nor was a decrease in TEER observed. Potential issues with cell adhesion are cell line-specific, so testing of other cell types with reduced anchorage strength may potentially indicate losses in anchorage with the presence of “pinholes”, or missing cells in the monolayer, increasing permeability at higher WSS. The bEnd.3 cell line was selected for the testing due to their characteristic high surface adherence.

TEER

TEER was measured with the independent electrode sets to evaluate monolayer integrity under varying flow conditions. In correlation with the permeability results in Fig. 10, the measurement data showed that there was an increase in TEER (Fig. 10) with increasing WSS at a rate of 0.8Ωcm$^2$ per dyn/cm$^2$, ranging from 183Ωcm$^2$ at near-static 1.4dyn/cm$^2$ to 230Ωcm$^2$ at 86dyn/cm$^2$. As with the previously discussed permeability results, apparent anchorage losses resulting in reduced TEER was not observed at high WSS.

It is noteworthy to mention that a consensus exists for BBB models that TEER levels must exceed 150Ωcm$^2$ for reasonably
TEER measured following high shear stress was increased at about 0.8 unit resistance/unit WSS. These data indicate increased barrier tightness with higher WSS, in correlation with permeability results. All replicate n>3. Representative permeability data to be obtained in comparison to typical in vivo TEER levels (>1000Ωcm$^2$). The measured TEER values in this paper exceeded 150Ωcm$^2$ at all tested values of WSS. This also supports the use of the bEnd.3 cell line under the described culture conditions for use in BBB testing studies at high WSS of 60 dyn/cm$^2$, at the high end of the shear stress seen in vivo.

**Western blot analysis**

The protein expression analysis data provided by western blot analysis (Fig. 11), showed significant increases in expression of both tight junction component ZO-1 and efflux transporter P-gp under three distinct WSS, relative to static control derived from 6-well plates. The protein expression relatively increased ∼5x for ZO-1 and ∼6x for P-gp at 58 dyn/cm$^2$ compared to the static condition (0 dyn/cm$^2$). Notably, a larger relative increase in ZO-1 was observed at 14 dyn/cm$^2$ (∼5x), while P-gp expression increased significantly (∼4x) at 4.7dyn/cm$^2$.

Both proteins are known to influence trans-monolayer properties, such as permeability and TEER; thus the increase in the measured value in protein expression under increasing WSS matches well to the results obtained in aforementioned methods: reduction in permeability and increase in TEER with increases in WSS conditions.

**Conclusions**

This paper reported the design, fabrication, and testing results of the microfluidic platform that enables application of the WSS range (1-60dyn/cm$^2$) of the full physiologically relevant spectrum on vascular endothelial cells (VECs), while allowing multiple physiological, biochemical, and trans-membrane assays in a high throughput manner on a chip. To allow rapid full-spectrum characterization of WSS effects, we developed the four channel microfluidic platform that simultaneously produces shear stresses spanning ∼15x in magnitude. Flow distributions were predicted with COMSOL simulation and verified by the direct measurement with a micro flow sensor array and volume measurement. Multiple assays were performed, including cell morphometry, protein expression, permeability and TEER, on the brain microvascular endothelial cell line bEnd.3.

Morphometric image analysis showed increased alignment with flow direction with increases in WSS. Permeability measurement exhibited decreasing permeability with increasing WSS at rates of 4.06e$^3$ and 6.04e$^2$cm/s per dyn/cm$^2$ for FITC-

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**Notes and references**

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