THE ROLE OF LAMINAR FLUID SHEAR STRESS ON THE MORPHOLOGY, MOTILITY, AND BIOCHEMICAL EXPRESSION OF BRAIN MICROVASCULAR ENDOTHELIAL CELLS

By

Adam Reinitz

A thesis submitted to Johns Hopkins University in conformity with the requirements for the degree of Master of Science in Engineering

Baltimore, Maryland

December, 2013

© 2013 Adam Reinitz
All Rights Reserved
Abstract

To determine the effect of laminar fluid shear stress on the morphology, motility, and biochemical expression of human brain microvascular endothelial cells (HBMEC), we designed a microfluidic flow system to allow live-cell, time lapse imaging of a monolayer of endothelial cells being exposed to physiological levels of shear stress (τ). Two cell lines were tested; HBMECs and human umbilical vein endothelial cells (HUVEC). We quantified the morphological response based on inverse aspect ratio (IAR), the ratio of the minor and major axes of a cell, and orientation, the angle (0-90°) between the major axis of the cell and the direction of flow. Time-lapse imaging allowed for determination of time-dependent changes in morphological parameters and cell motility. After 36h of flow, the HBMECs had orientations of 46° ± .02, 47°± .4, and 47° ± .6 and IAR values of 0.654 ± .007, 0.650 ± .006, and 0.658 ± .005 at 8, 12, and 16 dyn cm⁻², respectively. After 36h of flow, the HUVECs had orientations of 43° ± 3°, 36° ± 3°, and 31° ± 2° and IAR values of 0.60 ± .03, 0.58 ± .02, and 0.54 ± .03 at 8, 12, and 16 dyn cm⁻², respectively. Time-lapse videos showed significant HBMEC proliferation, as well as delamination at high shear stress. Both cell lines showed a transient motility response, experiencing a rapid increase in motility at the onset of flow, followed by a gradual decline to a steady state condition that was lower than the starting motility. Fluorescence staining showed actin stress fibers in HBMECs not restructuring significantly as compared to the HUVECs. Quantification of F-actin orientation indicated that the HBMEC actin network was randomly oriented, while the HUVECs showed preferential orientation with the
direction of flow. Biochemical expression was evaluated using polymerase chain reaction (PCR). Preliminary results indicate upregulation of the junctional proteins claudin-5 and β-catenin. The results suggest that the HBMECs do not have the same mechanisms for mechanical transduction as is seen in other parts of the vasculature. Additionally, the results indicate that in the absence of other physiological interactions, the HBMECs do not adhere tightly to the basement membrane and show minimal contact inhibition of cell growth.
Preface

This thesis is motivated by the work in the Searson Research Group to develop an improved model of the blood brain barrier (BBB). The research was conducted in the labs of the Institute for NanoBioTechnology (INBT) at Johns Hopkins University. In completing this project, I was responsible for designing, fabricating, and conducting experiments in a microfluidic, laminar flow cell culture system. I would like to thank Dr. Peter Searson for giving me the opportunity to take on this project and allowing me to work in the state of the art facilities that are a part of INBT. Additionally, I am particularly thankful for the help provided by Andrew Wong, who was vital to my development as a researcher and personally helped me learn a variety of techniques that were necessary for completing this project. Furthermore, I would like to thank Jackson Destefano for assistance in completing the necessary replicate experiments to make this work possible. Finally, I would like to thank the entire Searson Group and specifically Mao Ye, Yuja Huang, and Amanda Levy, each of whom provided invaluable help at points throughout this research.
# Table of Contents

Abstract .................................................................................................................. ii

Preface .................................................................................................................. iv

List of Figures ........................................................................................................ vi

Introduction .......................................................................................................... 1

Materials and Methods .......................................................................................... 20

  Cell Culture .......................................................................................................... 20
  Flow System .......................................................................................................... 21
  Live-Cell and Immunofluorescence Imaging ....................................................... 23
  Image Analysis ..................................................................................................... 24
  Polymerase Chain Reaction .................................................................................. 27

Results and Discussion .......................................................................................... 29

  Morphology .......................................................................................................... 29
  Cytoskeleton Structure .......................................................................................... 37
  Motility .................................................................................................................. 39
  Biochemical Expression ......................................................................................... 40

Supplementary Information ...................................................................................... 42

Bibliography ............................................................................................................ 43

Curriculum Vita ....................................................................................................... 46
List of Figures

Figure 1: Model of the Blood Brain Barrier (BBB) ................................................................. 3
Figure 2: Pathways for BBB Transport .................................................................................. 4
Figure 3: Mechano sensing Components .............................................................................. 5
Figure 4: Endothelial Alignment Under Laminar Flow ...................................................... 8
Figure 5: Endothelial Response to Laminar and Arterial Flow .................................... 10
Figure 6: Quantitative Analysis of Laminar and Arterial Flow Response ................. 11
Figure 7: Actin Alignment Under Laminar and Arterial Flow ....................................... 12
Figure 8: Diagram of Disturbed Flow Device ................................................................. 12
Figure 9: Endothelial Response to Laminar and Disturbed Flow ......................... 13
Figure 10: HBMEC Permeability and Protein Expression Under Laminar Flow .... 15
Figure 11: HBMEC Morphological Response Under Laminar Flow ..................... 16
Figure 12: HBMEC Barrier Function in the DIV-BBB ................................................... 18
Figure 13: Experimental Setup ......................................................................................... 20
Figure 14: Experimental Flow Rate versus Time ............................................................ 21
Figure 15: Microfluidic Device Design .......................................................................... 22
Figure 16: Fabrication of PDMS Channels ..................................................................... 23
Figure 17: Morphology Analysis Flow Chart ................................................................. 25
Figure 18: ZO-1 Border Tracing ....................................................................................... 26
Figure 19: Phase Images of HUVEC and HBMEC Under Laminar Flow ............ 30
Figure 20: HUVEC and HBMEC Morphology Parameters versus Shear Stress. 31
Figure 21: HUVEC and HBMEC Morphology Parameters versus Time .......... 32
Figure 22: F-actin Staining and Orientation Quantification .............................. 36
Figure 23: ZO-1 Staining of HUVEC and HBMEC After Flow ..................... 38
Figure 24: HUVEC and HBMEC Motility versus Time ................................ 39
Figure 25: HBMEC PCR Results .................................................................. 41
Introduction-

The blood brain barrier (BBB) is the interface between the blood and the brain that selectively allows certain molecules necessary for function to enter into the brain, while blocking the transport of virtually anything else that tries to cross it. Although the BBB is necessary for the body's homeostasis, as a number of diseases are linked to increased BBB permeability (Cucullo et al. 2012), its effectiveness at keeping unwanted molecules and pathogens out presents a unique challenge to research developing drugs targeted for the brain. In order for a drug to passively cross the BBB it must be smaller than 500 Da, highly lipophilic, and cannot be a substrate for the efflux pumps that prevent unwanted molecules from crossing into the brain. The only central nervous system (CNS) disorders with successful small molecule treatments are depression, schizophrenia, chronic pain, and epilepsy (Pardridge 2005). As a result, there is currently no effective way to treat a variety of CNS disorders, including neurodegenerative diseases, such as Parkinson's disease and Alzheimer's disease, brain cancer, CNS trauma, and brain infections (Pardridge 2005). Recently, Moreno et al. reported a small molecule treatment for the neurodegenerative prion disease by targeting its unfolded protein response (UPR) that is also associated with Alzheimer's disease and Parkinson's disease, but this work is still in the early development stages (Moreno 2013).

Current attempts at developing systems to deliver large drugs to the CNS are focused on using adsorptive or receptor mediated transcytosis (AMT and RMT), as these are known to transport large molecules across the BBB, but,
since the mechanisms behind this transport are not well understood, it is difficult to predict the success of these systems. To help streamline the testing of drugs intended to target the CNS and to improve understanding of the transport mechanisms across the BBB, a number of in vitro models have been developed, trying to replicate its barrier function (Abbot 2013). These models attempt to mimic the in vivo brain microenvironment, while also allowing for easy observation and quantification of transport across the artificial BBB. The most common models use a porous transwell dish coated with a monolayer of brain microvascular endothelial cells (BMECs) grown in co-culture with other cell types found near the brain capillaries (e.g astrocytes, pericytes) (Abbot 2013). Although these systems allow for high throughput testing of barrier transport properties, the transendothelial electrical resistance (TEER) for these systems, a measure of monolayer permeability, do not match that of the in vivo BBB (Naik and Cucullo 2011). In contrast, a dynamic in vitro BBB model (DIV-BBB), developed to apply physiologically relevant fluid shear stress to the endothelial cell monolayer, was successful in obtaining a physiological TEER value (Cucullo et al. 2007), but lacked the high throughput capability to replace the static models (Abbot 2013).

Up until now, there has not been a model that completely replicates the environment of the BBB, while still allowing for high throughput testing. To this end, our group is developing a high throughput model that accounts for all of the stimuli associated with BBB function. In order to understand the considerations that must be made in creating such a model and to demonstrate the important
role that fluid shear stress plays in BBB function I will provide a brief overview of BBB structure and a more thorough literature review regarding the vascular endothelial response to fluid shear stress.

The BBB broadly refers to three locations where capillaries provide exchange and transport with reservoirs of fluid in the brain, specifically with the brain interstitial fluid (ISF), cerebrospinal fluid (CSF), and subarachnoid CSF. For the purpose of this paper, we will only be concerned with the BBB at the brain ISF, since transport at this interface exhibits the largest total surface area between the blood and brain and represents the most direct exchange with the CNS. The blood brain barrier that regulates the brain ISF (Fig. 1) is made up of brain capillary endothelial cells surrounding the luminal side of the vasculature (i.e. the blood/plasma side) and forming tight junctions with neighboring endothelial cells, effectively separating the blood from the brain parenchyma.

Figure 1- Model of the blood brain barrier (BBB). Reprinted from Abbot et al. 2013.
These tight junctions are formed by proteins such as occludin and claudin, which make up a physical barrier that prevents polar molecules from slipping between the endothelial cells and into the brain ISF (Abbot 2006).

As a result of tight junction formation, the only remaining pathways into the brain are by transport through the cell (Fig. 2). The major mechanisms of transport via the transcellular route are: (1) passive lipophilic diffusion, (2) carrier mediated influx by transport proteins, (3) RMT, and (4) AMT. Passive diffusion through the lipid membranes and cytoplasm of the endothelial cells is the primary mechanism by which most commercial drugs cross the BBB. Transport proteins and receptor-mediated transport proteins are very selective, only allowing specific molecules and nutrients recognized as supporting brain function to cross. Furthermore, endothelial cells safeguard against the entry of foreign molecules.

Figure 2- Pathways for transport of biomolecules across the BBB. The only method currently employed by commercial drugs is passive diffusion. Reprinted from Abbot et al. 2013.
by producing enzymes responsible for metabolizing toxins (Abbot 2006). Surrounding the capillary cell is the basal lamina, along with pericytes and astrocytes, both of which have been shown to interact with the capillary to support BBB function (Abbot 2006, Siddharthan 2007).

In addition to interacting with components of the brain parenchyma, the brain capillary cells are also subject to fluid shear stress from the blood on the luminal side of the BBB. *In vivo*, fluid shear stress can range from 0-30 dyn cm$^{-2}$ and in small vessels between 5-25 dyn cm$^{-2}$ (Davies 1989). The goal of this work is to understand the effect of this shear stress component on the physical properties of the brain capillary cells and, therefore, it will be important to both understand the mechanisms of transducing this mechanical force into a biochemical function and review the current literature regarding vascular endothelial shear stress response.

A thorough review on the subject of mechanical transduction in vascular endothelial cells was recently performed by Ando and Yamamoto (Ando and Yamamoto 2013). In this work they make note of eight important shear stress...
sensing mechanisms (Fig. 3):

1) Ion channel activation is both directly and indirectly linked to the onset of shear stress. To be specific, shear stress directly causes the K\(^+\)-ion channels to stretch open, leading to membrane polarization, while ATP upregulation indirectly leads to the activation of Ca\(^{2+}\) channels.

2) Surface receptors, including vascular endothelial growth factor receptor (VEGFR), become activated under shear stress in the absence of their target ligand.

3) Adhesion molecules, including vascular endothelial cell cadherin (VE-cadherin) and platelet endothelial cell adhesion molecule-1 (PECAM-1), are involved in a transduction pathway resulting in cytoskeletal rearrangement and orientation of actin filaments.

4) The glycocalyx, a layer of glycoaminoglycans coating the luminal endothelial surface, is associated with the production of the vasodilator nitric oxide (NO) when exposed to fluid shear stress.

5) The presence of primary cilia, rod shaped organelles protruding from the cell surface, are linked to upregulation of Ca\(^{2+}\) signaling and NO production, but they are not required for generating a shear response.

6) Down regulation of surface caveolae, small folds in cell membranes that are dense with ion channels, receptors, and signaling factors, inhibits vascular remodeling with shear stress changes. Additionally, the caveolae have been shown to play a key role in the rapid ATP release, Ca\(^{2+}\)
channel activation, and NO production characteristic of the endothelial shear response.

7) While cytoskeletal rearrangement is a known shear stress response, there is not experimental evidence suggesting this is directly caused by stress on the cytoskeletal network. With that said, there are models suggesting that this network is responsible for maintaining uniform tension on the cell membrane and that it rearranges under shear stress in order to maintain this uniform condition.

8) Fluidity of the plasma membrane increases proportionally with shear stress intensity, a potential mechanism to facilitate rearrangement of surface proteins and allow for changes in cell morphology.

Although it is known that all of these responses occur under shear stress, it is not clear which responses are directly the result of shear stress, rather than a side effect from other biochemical changes (Ando and Yamamoto 2013). Regardless, the importance of fluid shear stress in maintaining the integrity of the endothelium is widely understood, as regions of disturbed flow in vivo are associated with the formation of atherosclerotic lesions (Ando and Yamamoto 2013, Chiu and Chien 2011, Tarbell 2010, Li et al. 2005).

Now that we have a fundamental understanding of the mechanisms by which the endothelium may sense fluid shear stress, we can now discuss the experimental results regarding detectable changes in endothelial cell function. Specifically, we will discuss the effect of shear stress on endothelial cell morphology, biochemical expression, and barrier function as this is the interest of
this work. Additionally, we will focus on outlining the current research on the human brain microvascular endothelial cell (HBMEC) shear stress response, as these cells compose the endothelial layer of the BBB in vivo.

Much of the pioneering work to understand the endothelial shear stress response was performed by Davies in the early 1980’s (Dewey et al. 1981). In 1981, he published the first work to suggest a morphological response of endothelial cells to shear stress (Dewey et al. 1981). Prior to this work, it was known that a number of biochemical responses are associated with exposure to fluid shear stress and that regions of the vasculature that experience disturbed flow show a greater tendency of damage to the endothelial layer. In contrast, little

**Figure 4**- Bovine aortic endothelial cell (BAEC) morphology in static conditions and 8 dyn cm$^{-2}$, 24 and 48 h after the start of flow. Reprinted from Dewey et al. 1981.
was known regarding the physical state of endothelial monolayers exposed to shear stress. In this work, they used a cone plate viscometer to expose bovine aortic endothelial cells (BAECs) to a physiological 8 dyn cm\(^{-2}\) of laminar fluid shear stress. Phase-contrast images of the BAECs in both static conditions and when exposed to shear stress for 24 or 48 h (Fig. 4) demonstrate a time-dependent, elongation and orientation with the flow direction. More specifically, while there is not a significant morphology change after 24h of 8 dyn cm\(^{-2}\) fluid shear stress, after 48h the morphology differences, as compared to the static condition, are striking. Additional experiments studying endothelial growth rate under laminar flow found that within the shear stress range of 1-5 dyn cm\(^{-2}\) there is no change in cell growth rate, as compared to the static condition (Dewey et al. 1981). These results set the stage for comprehensive studies regarding the morphological response of a number of endothelial cell lines exposed to various shear flow conditions, all of which display a similar morphological response to laminar flow (Masuda et al. 1993, Girard 1995, Chiu et al. 1998, Blackman et al. 2002, Cockroft et al. 2009, Walsh et al. 2011).

Although laminar flow is useful for in vitro models, as it is easy to generate artificially, this condition is not equivalent to the shear stress experienced by endothelial cells in vivo. To be specific, endothelial cells in vivo are not under a constant shear stress, but instead experience an arterial pulsatile flow associated with the pumping of the heart. Therefore, in order for the laminar shear stress condition to be used in models, it must first be confirmed that the endothelial response to laminar flow is comparable to that of the arterial flow condition. In
2002, Blackman et al. performed this experiment utilizing a cone plate viscometer system, similar to that used in Davies' original work, to apply both laminar flow and an arterial flow pattern, modeled after the human aorta, to a monolayer of human umbilical vein endothelial cells (HUVECs) (Blackman 2002). To appropriately compare these two flow conditions, the arterial flow pattern was applied such that the time averaged fluid shear stress was the same as the laminar flow condition. More specifically, the time averaged shear stress of both conditions was 7.5 dyn cm\(^{-2}\).

Images taken after 24h of flow (Fig. 5) indicate that both laminar and arterial flow profiles will generate an endothelial elongation response. Additionally, they used time-lapse imaging to assess the morphological changes.

![HUVEC morphology images](image)

**Figure 5**- HUVEC morphology prior to flow (a), after 7.5 dyn cm\(^{-2}\) laminar flow(b), and 7.5 dyn cm\(^{-2}\) time average arterial flow (c). Reprinted from Blackman et al. 2002.
as a function of time. Quantitative morphological analysis allowed comparison of the cell aspect ratio (AR), the ratio of the major and minor cell axes, and motility analysis provided information regarding speed and directional persistence of individual cells within the monolayer. The results of the morphological analysis

![Figure 6](image)

**Figure 6**- Quantitative analysis of HUVEC flow response. (a) Cell aspect ratio as a function of time, (b) Cell speed under static (ST), laminar (LSS), and arterial (ART) flow conditions, (c) Directional persistence under static (ST), laminar (LSS), and arterial (ART) flow conditions. Reprinted from Blackman et al. 2002.

(Fig. 6a) indicate that both the laminar shear stress (LSS) and arterial shear stress (ART) caused an increase in the AR with time, with the cells exposed to the laminar flow becoming slightly more elongated. The motility analysis indicates that the cell speed (Fig. 6b) for both flow profiles was reduced compared to the static condition, with no statistically significant difference in cell speed between the two flow patterns. In contrast, the directional persistence, an evaluation of the time-scale over which the cells will move without changing course, was significantly higher under laminar shear stress than either the static condition or the arterial flow condition. These results suggest that, while the
response of endothelial cells under these two conditions is not identical, the changes in morphology and cell speed are similar enough that laminar flow model is reasonable for in vitro use. Staining of the actin filaments (Fig. 7) further confirm that both flow conditions show comparable cytoskeletal reorganization (Blackman 2002).

Up to this point we have briefly discussed the link between disturbed flow regions in the vasculature and atherosclerotic lesion development. While this link

Figure 7- Actin fluorescence staining of HUVECs under static (ST), laminar (LSS), and arterial (ART) flow conditions. Reprinted from Blackman et al. 2002.

Figure 8- Parallel plate flow chamber with a vertical step to generate non-laminar flow. The locations labeled correspond the flow conditions: (a) stagnant flow, (b) center of the recirculation eddy, (c) disturbed flow region, and (d) fully developed laminar flow. Reprinted from Chiu et al. 1998.
is well documented *in vivo*, it is also important for determining the viability of our system to confirm that the disturbed flow response *in vitro* is discernible from the laminar response. This verification was performed by Chiu et al. in 1998 using a parallel plate flow chamber with a vertical step (Fig. 8) to generate a non-laminar flow region (Chiu et al. 1998). There are four flow profile regions within this channel (a-d), but, for the purpose of observing the difference between a disturbed flow and laminar flow response, we are most interested in comparing positions c and d. At position c the flow is disturbed, meaning there is a very small magnitude of wall shear stress (0.4 dyn cm$^{-2}$), but a very large wall shear stress gradient (740 dyn cm$^{-3}$). In contrast, at point d, there is no wall shear stress gradient (0 dyn cm$^{-3}$), but a significant, physiologically relevant wall shear stress (21.0 dyn cm$^{-2}$).

Phase-contrast images before and after flow (Fig. 9i) indicate that there is

![Phase images of HUVECs taken before flow and after 24h of flow at the points in the flow channel a-d.](image1)

**Figure 9**- i) Phase images of HUVECs taken before flow and after 24h of flow at the points in the flow channel a-d. ii) F-actin staining of HUVECs after 24h of flow at the points in the flow channel a-d. Reprinted from Chiu et al. 1998.
a difference in morphology between disturbed and laminar flow regions, while staining of the F-actin filaments (Fig. 9ii) confirm that cytoskeletal orientation is far more prevalent in the laminar flow region. Quantitative morphological analysis of these cells confirms qualitative observations. Evaluation of the average shape index, a scale from 0 to 1 with 1 referring to a completely circular cell and 0 being a line, resulted in values of .56, .59, and .34 for the pre-flow, disturbed flow, and laminar flow conditions, indicating the disturbed flow condition is even less elongated than cells under static culture. Additionally, they evaluated the amount of DNA replication occurring within the cell, a means for evaluating the amount of cell proliferation occurring, and found that, while the laminar flow and static conditions were comparable, there was significantly more proliferation occurring in the disturbed flow region (Chiu et al. 1998). These results confirm both that the disturbed flow response \textit{in vitro} is noticeably different from the laminar flow response and that a parallel plate system, similar to the system we use, is effective for generating a laminar flow response \textit{in vitro}.

As noted above, the \textit{in vitro} morphology of the HUVECs is very well characterized, which makes it a useful control group in our shear stress experiments. In comparison, HBMECs are far less researched and the extent of \textit{in vitro} studies in literature mostly provides information on barrier function and biochemical expression. Here we will review HBMEC responses seen in literature to determine what is known regarding this cell line and to clarify what we intend to learn about these cells.
The first notable paper on the barrier properties of HBMECs exposed to fluid shear stress was published in the 2007 by Siddharthan et al. In this work, they concurrently looked at the influence of astrocyte conditioned media and laminar fluid shear stress on HBMEC permeability and expression of proteins associated with barrier function. Their system was essentially a transwell plate that was inserted into a flow system, allowing for application of flow and subsequent testing of permeability. The HBMECs were grown to confluence under very low flow (0.5 dyn cm\(^{-2}\)) and subsequently exposed to 1-2 dyn cm\(^{-2}\) for 5 days. The membrane that the HBMECs are seeded on does not allow for easy phase-contrast imaging, but monolayer integrity after flow was confirmed by cell border staining.

Results from their permeability experiment (Fig. 10a) indicate that the HBMEC monolayer displayed improved barrier properties after flow, with a greater than 2-fold reduction in dextran permeability as compared to the static

![Figure 10](image)

**Figure 10** - (a) Percent change in HBMEC monolayer permeability to dextran; (b) Relative ratio of ZO-1 expression for HBMEC monolayer exposed to flow as compared to the static condition; c) Relative ratio of \(\beta\)-catenin expression for HBMEC monolayer exposed to flow as compared to the static condition; Reprinted from Siddharthan et al. 2007.
condition. In their analysis of protein expression, they found that the tight junctional protein zonula 16eflon16ng-1 (ZO-1) was upregulated under flow (Fig. 10b), while another cell-cell junction protein, β-catenin, did not show any significant change (Fig. 10c). This result suggests that the improved barrier function is due to increased tight junction formation (Siddharthan et al. 2007).

While this research was important for setting a groundwork regarding the HBMEC flow response, the applied shear stress they use is smaller than is seen in vivo and, therefore, does not give insight regarding the HBMEC response to physiologically relevant shear stress.

A more physiological experiment was published in 2011 by Walsh et al. using a commercially sold parallel plate flow system. In this experiment, they

![Image](image_url)

**Figure 11**—Phase contrast images and fluorescence staining of HBMvECs after static culture and 24h at 10 dyn cm^−2_. Staining was performed for F-actin filaments and ZO-1. Reprinted from Walsh et al. 2011.
exposed both bovine and human brain microvascular endothelial cells (BBMvEC and HBMvEC) to 10 dyn cm$^{-2}$ laminar flow and compared the permeability to the static condition. They found a 20% reduction in HBMvEC permeability after 24 h. Phase-contrast images and immunofluorescence staining (Fig. 11) taken after 24h indicate orientation and alignment of the HBMvECs comparable to the HUVECs (Walsh et al. 2011); although no morphology analysis was performed to quantify this difference. Regardless, this result suggests that we would expect a similar elongation response from our HBMECs.

Some of the most extensive work studying the HBMEC response to shear stress was performed by the creators of the DIV-BBB system at the Lerner Research Institute, a part of the Cleveland Clinic. Using their DIV-BBB, they cultured HBMECs for a month under a laminar shear stress of 6.2 dyn cm$^{-2}$. They took TEER measurements every 3 days and performed an extensive analysis of RNA concentration after completion of the experiment. Additionally, gel electrophoresis was used to evaluate the concentration of various proteins. As indicated earlier, since the DIV-BBB does not allow for imaging of the endothelial layer, we do not have information regarding the morphology of these cells. Nonetheless, this work provided a wealth of information regarding the HBMEC gene expression under flow.

Specifically, they found upregulation of genes coding for the junctional proteins ZO-1, claudin-5, and β-catenin. Interestingly, they observed a 3-fold increase in the expression of β-catenin, a result in conflict with the protein expression reported by Siddharthan et al. (Fig. 10c). They also assayed genes
associated with adhesion molecules, ion channels, and enzymes, though perhaps the most interesting result in regards to barrier function is the upregulation of the efflux protein p-glycoprotein (P-gp), as this protein has broad multidrug resistance effects that reduce endothelial permeability. Their protein assay determined that the HBMECs exposed to shear stress produced a much greater percentage of cytoskeleton proteins as compared to the static condition, suggesting the cells are stiffening in response to flow. TEER measurements over the course of 30 days (Fig. 12) indicate that the endothelial permeability was constantly increasing throughout this experiment, never reaching a steady peak (Cucullo 2011). While this work was very important for characterizing the biochemical expression of the HBMECs, without the ability to visualize the cell monolayer, it is difficult to attribute the decrease in permeability directly to the increase in TEER.

**Figure 12**- Phase contrast images and fluorescence staining of HBMvECs after static culture and 24h at 10 dyn cm⁻². Staining was performed for F-actin filaments and ZO-1. Reprinted from Walsh et al. 2011.
increased production of junctional or efflux proteins. More specifically, one would expect the TEER of a single monolayer to reach a plateau, but the steady rise observed in this case could be indicative of additional affects, such as cell overgrowth.

It is the goal of this work to more fully characterize the morphology, motility, and biochemical response of the HBMECs. Specifically, literature results suggest HBMECs will display orientation under laminar flow (Walsh 2011), but there has yet to be a quantitative morphological analysis to confirm this. In addition, there is no current information on the time dependent morphological or motility response of HBMECs under laminar flow. Furthermore, there is no example in literature evaluating the HBMEC morphological and biochemical response at a range of physiological shear stresses. Here we show that by designing a microfluidic flow system for live-cell, time-lapse imaging of cells under laminar flow, we are able to both qualitatively and quantitatively compare the response of HBMEC cells to the more thoroughly researched human umbilical vein endothelial cells (HUVEC). The microfluidic device design permits for the testing of multiple shear stresses within a single experiment, allowing for high throughput determination of time and shear stress dependent changes in morphology, motility, and biochemical expression.
Materials and Methods

Cell Culture - Immortalized human brain microvascular endothelial cells (HBMEC)\(^6\) were cultured in M199 (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA) and 1% penicillin streptomycin (Invitrogen). The HBMECs were isolated from an adult epilepsy patient and were immortalized by transfection with the simian virus 40 large T antigen (Nizet et al. 1997). Human umbilical vein endothelial cells (HUVEC) (Promocell, Heidelberg, Germany) were grown in endothelial cell growth medium containing 2% fetal calf serum (FCS) (Promocell) and 1% penicillin streptomycin. Both cell lines were cultured under physiological conditions on uncoated tissue culture polystyrene flasks. Upon introducing the cells into a microfluidic device, cells were thoroughly washed twice with PBS without Ca\(^{2+}\) or Mg\(^{2+}\) and removed from

![Figure 13. Device and flow setup on microscope.](image)

- (a) Media reservoir.
- (b) Humidified 5% CO\(_2\) chamber. (c) Microfluidic device. (d) Peristaltic pump.

System is incased in a heated chamber set to 37 °C.
their culture surface using 0.5% EDTA/trypsin for 3 min at 37 °C. Prior to seeding the cells into the device, the interior of the microfluidic channels were coated with fibronectin (BD Biosciences, San Jose, CA) at 62.5 μg ml\(^{-1}\) for 1 h at room temperature. HBMECs and HUVECs were introduced at concentrations of 50,000 cells cm\(^{-2}\) and 100,000 cells cm\(^{-2}\), respectively and grown to confluence in their respective culture media. Both cell lines were then exposed to shear stress in a common endothelial cell basal medium supplemented with 2% FCS (Promocell).

Flow System- The flow setup is composed of a custom machined Teflon media reservoir connected to 1/8" ID silicon tubing (Fig. 13). Media from the 21eflon reservoir passes through a 1.5 m coil of silicon tubing contained in a gas exchange chamber of humidified 5% CO\(_2\). The flow is then directed through the microfluidic device and returns to the media reservoir via the programmable peristaltic pump (New Era Pumps, NE-9000). Teflon tubing was used for the return flow from the peristaltic pump to the media reservoir. The peristaltic pump

![Figure 24](image-url)

**Figure 24.** (a) Flow rate versus time programmed into the peristaltic pump (b) Shear stress versus time for each of the 4 channels.
was programmed (Fig. 14) to increase flow stepwise from 1.25-7.5 ml min\(^{-1}\) over the first 6 h, increasing 1.25 ml/min/h, and then increase to 10 mL min\(^{-1}\), which is equivalent to 4, 8, 12, and 16 dyn cm\(^{-2}\) within the four channels over 36 h.

The microfluidic device (Fig. 15) was fabricated using a machined aluminum mold designed with four rectangular channels of heights 390 μm, 450 μm, 550 μm, and 770 μm connected in series with an areal dimensions of 2.22 mm by 50 mm. Flow rates were calculated using the following equation to approximate shear stresses in rectangular channels where \(\tau\) is shear stress, \(Q\) is:

**Figure 15.** Illustration of microfluidic device. Bubble traps prevent nucleated air bubbles from entering the rectangular channels. Channels are connected in series to maintain equal flow rate in all channels.
volumetric flow rate, \( \mu \) is fluid viscosity, \( h \) is channel height, and \( w \) is channel width:

\[
\tau = \frac{6Qu}{h^2w}
\]

The heights were chosen such that the shear stress in the four channels would proportionally scale in a ratio of 1:2:3:4. Polydimethylsiloxane (PDMS) is poured to fill half of the mold and partially cured at 100 °C for 15 min (Fig. 16a). Nylon spacers (5 mm ID) forming the bubble traps in Fig. 2 are placed on top of this PDMS layer (Fig. 16b) and sealed to the bottom with a second layer of PDMS cured at 100 °C for 45 min (Fig. 16c). The PDMS blocking the bubble traps is removed using a 5 mm inner diameter hole punch and the inlets and outlets are made using a 1.50 mm hole punch. The PDMS channels are then plasma bonded to a 50 mm x 75 mm glass microscope slide. 6.35 mm ID silicon tubing is used to connect the nylon inserts to the caps of the bubble traps, which is a male Luer to hosebarb connector with a female Luer cap.

Figure 16. Fabrication of polydimethylsiloxane (PDMS) channels. (a) A layer of PDMS is partially cured, (b) Nylon inserts are added as bubble traps and coated with a second layer of PDMS, and (c) The device is completely cured.
**Live-Cell and Immunofluorescence Imaging** - Imaging was performed using a Nikon TE-2000U inverted microscope controlled by NIS Elements software (Nikon, Japan). The live-cell imaging was performed in a temperature controlled chamber that is set at 37 °C (In ViVo Scientific). Phase-contrast images were captured every 20 min using a 10x Nikon Plan Fluor objective lens. Autofocus adjustment through NIS-Elements is performed before each image capture to account for any z-drift.

Monolayers of endothelial cells within the device were prepared for immunofluorescence staining immediately following the flow experiment by washing with warm PBS with Ca²⁺ and Mg²⁺ and fixing in 4% formaldehyde in PBS. Cells were washed with PBS and permeabilized with 0.1% Triton-X 100. Sample was blocked using 10% goat serum in PBS. Samples were then incubated with anti-zonula occluden-1 antibody (rabbit monoclonal 1:200, Invitrogen) for 1 hr at room temperature, washed, and followed by a secondary antibody incubation of goat anti-rabbit (1:200, Alexa Fluor 568, Invitrogen). Samples were subsequently stained for actin using Alexa Fluor 488 phalloidin (Invitrogen) and for nuclei using DAPI (1:2500, Roche Applied Science).

**Image Analysis** - Quantitative analysis of the time-lapse experiments (**Fig. 17**) was performed using ImageJ (NIH, Bethesda, MD). TIFF images of the cell monolayers were imported into ImageJ (**Fig. 17a**) and a custom macro was applied to the time-lapse sequence. Images were subjected to contrast enhancements and smoothing functions to optimize cell border contrast (**Fig. 17b**). Binary traces of the cell borders were obtained by auto-thresholding the
resulting images and converting them to a mask (Fig. 17c). An ImageJ algorithm is applied to expand each particle in this mask to fill the image frame (Fig. 17d). Holes in the mask as well as aggregates of indiscernible cellular borders were automatically excluded (Fig. 17e) and this mask can be overlayed on the original phase image to verify tracing (Fig. 17f). From the mask, the morphology of single cells in the monolayer could be calculated. This process outputs morphological parameters such as orientation, area, and inverse aspect ratio (IAR). Orientation refers to the angle (0-90°) between the major axis of the cell and the direction of flow, with 45° indicating a random orientation. IAR is defined
as the ratio of the minor and major cell axes. Data is presented if more than 65% of the monolayer could be traced by this method. To verify accurate cell border identification by the cell tracing program, images of subsequently fixed and fluorescently immunostained monolayers were manually traced and compared (Fig. 18).

Cell motility was evaluated using the MATLAB based, open source, particle image velocimetry software, OpenPIV. (Taylor et al., 2010) Time-lapse phase-contrast images at 20 min intervals were imported as greyscale TIFFs. The software divides the imaging field into a matrix of interrogation windows. Each interrogation window is compared to itself between successive images for the movement of particles. With an image resolution of 0.64 μm/px, we used a 32x32 px interrogation window, which is approximately one-fourth the area of a typical endothelial cell. The magnitude of vectors describing the motion of particles within each interrogation window were averaged across all the windows. The magnitudes were converted to units of speed by dividing by the

Figure 18. (a) ZO-1 staining image used to verify cell tracing program. (b) Hand tracing performed in ImageJ to verify morphology results.
imaging interval (20 min). These values encapsulate the global speed and direction of particles within the 2D endothelial monolayer.

F-actin orientation was quantified using a method described by Lee and Chen in 2002 for quantifying the directionality of a textured image.(Lee and Chen 2002) Starting with an RGB, TIFF image of an F-actin stain, this image is converted to grayscale and a Fourier transform is performed. A smoothing function is performed on the Fourier spectrum and a threshold is applied to binarize this smoothed spectrum. Principal components analysis is performed on this binarized Fourier spectrum, allowing for calculation of the directionality in the original image (Lee and Chen 2002). This analysis produces a percentage of actin fibers at a given angle to the flow direction. These orientation angles are binned into nine sections each containing a 20° range in orientation, meaning a random value for a given range would be 11.1%. For the purpose of this analysis, we used two ranges corresponding to the most perpendicular and the most parallel fibers.

Polymerase Chain Reaction (PCR)- Samples were prepared for PCR following the protocol for the Taqman® Gene Expression Cells-to-C_T Kit (Life Technologies). Briefly, cells were removed from the microfluidic channels with trypsin and suspended in cold PBS at concentration of about 2,000 cells μL⁻¹. 5 μL of the cell suspension is mixed with 50 μL of Cells-to-C_T Lysis Solution and incubated for 5 min. Triplicates were prepared from each shear stress channel. 5 μL of the Cells-to-C_T Stop Solution is added to the lysed cell suspension and incubated for 2 min. 5 μL of the cell lysate is mixed with 25 μL of the Cells-to-
Ct™ 2X RT Buffer, 2.5 μL of the Cells-to-Ct™ 20X RT Enzyme Mix, and 12.5 μL of nuclease-free water. A reverse transcription (RT) thermal cycle program is applied to the samples using the Applied Biosystems Step One Plus real-time PCR (Applied Biosystems), where the samples are held at 37 °C for 60 min, heated to 95 °C for 5 min, and maintained at 4 °C indefinitely. Triplicate samples are then prepared containing 4 μL of this sample mixed with 10 μL of Taqman® Gene Expression Master Mix (2X), 1 μL of the appropriate Taqman® Gene Expression Assay (Life Technologies) for the gene of interest (ZO-1, Claudin-5, or β-catenin), and 5 μL of nuclease-free water. These samples undergo real-time PCR consisting of 2 min at 50 °C, 10 min at 95 °C, and 40 repetitions of 15 sec at 95 °C and 1 min at 60 °C. Amplification data of fluorescent Taqman probes is collected by the Step One Software (Applied Biosystems). Cell monolayers cultured under no flow in the fibronectin coated microfluidic device were used as controls. The media in control samples was changed twice per day. The genes B2M, GapDH, and 18S were used as endogenous controls.
Results and Discussion

To study the influence of shear stress on the morphology and function of HBMECs, cells were exposed to a physiological range of shear stresses, between 4-16 dyn cm\(^{-2}\). Importantly, this flow was introduced gradually over the first 6h of the experiment to prevent the generation of a high wall shear stress gradient that could disturb the endothelial monolayer (Dolan et al. 2013). By imaging these cells before, during, and after the application of flow, observations could be made regarding changes that occur throughout the shear response process. Additionally, quantitative analysis of these images allowed for the explicit determination of trends in the HBMEC response at varying shear stresses. As will be shown, the information obtained from these experiments reveal response characteristics of the HBMECs that are noticeably different from cells found in other parts of the vasculature.

In order to clearly present this data, the results were grouped into four aspects of the shear response: 1) the morphological response, 2) the cytoskeletal response, 3) cell motility under flow, and 4) changes in biochemical expression. The response of the HUVECs will serve as a control to both verify the operation of the flow system and to provide a direct comparison for the HBMEC responses that are observed.

Morphological response to shear stress

As was discussed earlier, endothelial cells from throughout the vasculature, including those found in arteries (Dewey et al. 1981), veins (Blackman et al. 2002), and capillaries (Walsh et al. 2011) display a
characteristic morphological response described by significant elongation and orientation with the flow direction. In the case of the HBMECs, there has been one report indicating a similar response at 10 dyn cm\(^{-2}\) (Walsh et al. 2013), but there is no literature quantifying the HBMEC morphological changes or evaluating the response at a range of physiological stress intensities. This section will cover the findings regarding the morphological response of both the HUVECs and the HBMECs at varying shear stresses.

**Figure 39.** (a) HUVECs before and after 36 h of flow at 8, 12, and 16 dyn cm\(^{-2}\). (b) HBMECs before and after 36 h of flow at 8, 12, and 16 dyn cm\(^{-2}\).
HUVECs show shear stress dependent morphological response- HUVECs are a useful control because they have been shown in literature to elongate under laminar flow (Blackman et al. 2002, Chiu et al. 1998), while also showing a distinctly different response under disturbed flow (Chiu et al. 1998). Based on this property, a HUVEC elongation response can be used to verify the efficacy of our system.

HUVECs within our device respond to shear stress by elongating and orienting with the direction of flow (Fig. 19a), with the extent of elongation and orientation being proportional to the intensity of flow. Quantitative analysis of the HUVEC morphological response after 36h reveals the HUVECs show a noticeable decrease in IAR (Fig. 20a) under increasing shear stress, with respective values of 0.60 ± .03, 0.58 ± .02, and 0.54 ± .03 at 8, 12, and 16 dyn cm⁻². Similarly, the plot of orientation versus shear stress (Fig. 20b) shows the HUVECs become more aligned with the direction of flow with increasing shear stress, with orientation angles of 43° ± 3°, 36° ± 3°, and 31° ± 2° at 8, 12, and 16
There was minimal change in HUVEC area when exposed to shear stress (Fig. 20c).

**HUVECs show time-dependent shear stress response**—Analyzing time-lapse images of the shear response provides a basis for evaluating the temporal shear stress response and allows characterization of stages in the morphological response. The HUVECs appear to have a transient morphological response with time (Supplementary Video 1). In order to fully capture this process, the HUVEC experiment ran for 60h. Prior to flow they are slightly elongated and randomly oriented, with an IAR (Fig. 21a) of .576 ± .003 and an orientation angle (Fig. 21b) of 47° ± 2°. The initial flow response is the formation of a “cobblestone” monolayer after 6h, whereby the cells become more rounded, with IAR at all shear stresses increasing to ~0.62. At this point the HUVECs show no orientation with the flow direction. After 12-24h of flow, following the formation of this cobblestone morphology, the cells begin elongating, and the IAR decreases to respective values of 0.578 ± 0.004, 0.51 ± 0.004, and 0.45 ± 0.004 at 8, 12, and
The orientation angle of these elongated cells is dependent on the shear stress applied, with respective values of $31^\circ \pm 0.5$, $22^\circ \pm 0.4$, and $17^\circ \pm 0.4$ at 8, 12, and 16 dyn cm$^{-2}$. The HUVECs show no significant areal change (Fig. 21c) at 8 and 12 dyn cm$^{-2}$, with initial areas of $1800 \pm 20 \, \mu m^2$ and $1790 \pm 20 \, \mu m^2$ and final areas of $1770 \pm 20 \, \mu m^2$ and $1760 \pm 20 \, \mu m^2$. There is an increase in area at 16 dyn cm$^{-2}$, increasing from $1500 \pm 10 \, \mu m^2$ to $1700 \pm 20 \, \mu m^2$, that is a result of a high seeding density in that channel at the start of the experiment.

HBMECs do not show shear stress or time dependent shape change. The HBMECs did not elongate or orient with the direction of flow (Fig. 19b), instead they maintained a polygonal shape. The HBMECs did not show good contact inhibition at 8 dyn cm$^{-2}$, with overgrowth occurring that prevented detailed analysis in certain regions. The degree of overgrowth decreased at 12 dyn cm$^{-2}$. At 16 dyn cm$^{-2}$, prominent cell shearing resulted in the HBMECs spreading on the fibronectin surface in order to maintain monolayer integrity. Comparing IAR to the applied shear stress (Fig. 20a) shows that the HBMECs do not elongate under shear stress and show no significant shape change, maintaining a value of IAR between 0.65-0.66. Similarly, the plot of orientation versus shear stress in (Fig. 20b) supports the observation that the HBMECs did not show any preferential orientation, maintaining a near random $46^\circ$ orientation at all shear stresses. The plot of area versus shear stress (Fig. 20c) confirms qualitative observations regarding the HBMEC response, with the HBMECs exposed to 8 and 12 dyn cm$^{-2}$ being more compact than those under 16 dyn cm$^{-2}$, with respective areas of $1240 \pm 20$, $1360 \pm 75$, and $1680 \pm 180 \, \mu m^2$. 

33
The HBMECs do not show any significant change in IAR (Fig. 21a) or orientation (Fig. 21b) with time, maintaining a value near 0.65 and 45°, respectively, further supporting the claim that they do not undergo shear stress dependent shape change. The HBMEC areal response with time (Fig. 21c) further supports the shear stress dependence of the HBMEC size. More specifically, at 8 dyn cm\(^{-2}\) the cells quickly form a tightly packed monolayer with area decreasing from 1860 ± 20 μm\(^2\) to 1260 ± 20 μm\(^2\), at which point a steady state condition is reached where additional cell growth over this monolayer is torn from the surface. A similar area decrease is observed at 12 dyn cm\(^{-2}\), but this change occurs at a slower rate than at 8 dyn cm\(^{-2}\). At 16 dyn cm\(^{-2}\), delamination is the dominant factor, causing an increase in cell area from 1560 ± 10 μm\(^2\) to 1760 ± 20 μm\(^2\).

**HBMECs show shear stress dependent overgrowth:** The shear stress dependence of HBMEC area is thought to be a balance between cell proliferation, adhesion, and delamination. To be specific, at low shear stresses the cells are proliferating without sufficient stress to cause delamination. As a result, the cell monolayer becomes very densely packed. At higher shear stress, the non-elongated cells are not sufficiently adhered to the surface, so delamination becomes more prevalent and overgrowth less significant. The lack of orientation will cause more shear stress to be transduced through the cell (Davies 1995); potentially causing this cell shearing observed at high shear stresses. It is also possible that the HBMEC overgrowth is inhibiting elongation by compacting the monolayer, but the lack of elongation at 16 dyn cm\(^{-2}\), where
cell shearing has made room for areal expansion (Supplementary Video 2), suggests that even when given the opportunity to freely elongate the HBMECs do not.

**Comparison to morphology changes in literature:** The HUVEC elongation response is in agreement with reports in literature for both HUVECs and BAECs, both of which have been shown to elongate and orient with applied shear stresses between 7.5 and 21 dyn cm\(^{-2}\) after 24h of flow (Dewey et al. 1981, Blackman et al. 2002, Chiu et al. 1998). This reorientation is thought to minimize stress perpendicular to the cell cytoskeleton, effectively reducing tension on the cell membrane, while increasing cell stiffness (Davies 1995). Additionally, the observed time-dependent shape response is in agreement with the response from BAECs exposed to 10 dyn cm\(^{-2}\) (Masuda and Fujiwara 2003).

In contrast, the HBMEC morphological response does not match the elongation response of endothelial cells found in other parts of the vasculature. While there is literature evidence to suggest the HBMECs should elongate when exposed to laminar flow (Walsh et al. 2011), this morphological response has not been characterized, making it difficult to make a direct comparison to the lack of elongation observed in this system. It is important to note that, unlike our system, HBMECs *in vivo* are interacting with pericytes and astrocytes, which may play a role in stabilizing the monolayer to allow a more controlled response. With that said, proliferation observed at low shear stresses could explain the large increase in TEER observed from HBMECs grown in the DIV-BBB system for 30 days at 6.2 dyn cm\(^{-2}\) (Cucullo et al. 2011). In addition, endothelial cells that are
not oriented with the flow direction have been shown to respond with an

![Image](image1.png)

**Figure 22.** F-actin staining and orientation quantification: (a) HUVEC at 8 (*left*), 12 (*middle*), and 16 (*right*) dyn cm\(^{-2}\), (b) Percent of actin in HUVECs parallel and perpendicular to the flow direction at 8, 12, and 16 dyn cm\(^{-2}\), (c) HBMEC at 8 (*left*), 12 (*middle*), and 16 (*right*) dyn cm\(^{-2}\), (d) Percent of HUVEC actin parallel and perpendicular to the flow direction at 8, 12, and 16 dyn cm\(^{-2}\).
inflammatory response (Wang et al. 2013), which may be the cause of the proliferation observed from the HBMECs.

**Cytoskeletal response to shear stress**

As part of the elongation response, characteristic of most endothelial cells exposed to laminar flow, the cytoskeletal actin network reorganizes to orient itself with the direction of flow (Girard et al. 1995, Chiu et al. 1998, Blackman et al. 2002). To characterize the orientation of actin fibers in the cell, we used a Fourier transform method described by Lee and Chen (Lee and Chen 2002) that determines the orientation of lines on a textured, grayscale image. From this analysis, we are then able to compare the cytoskeletal rearrangement of the HUVECs and HBMECs based on the fraction of actin fibers that are parallel and perpendicular to the direction of flow.

**HUVECs show clear cytoskeletal orientation**-HUVECs respond to shear stress by aligning their actin filaments in the direction of flow (Fig. 22a). Quantitative analysis of F-actin orientation after 36h of flow (Fig. 22b) reveals an increase in the fraction of fibers parallel to flow and a decrease in fibers perpendicular to flow, as compared to a random value of 11.1%. To be specific, the percentage of parallel fibers was 12.8, 12.4, and 15.1 at 8, 12, and 16 dyn cm\(^{-2}\), respectively, and the percentage of perpendicular fibers was 9.1, 9.7, and 8.2 at 8, 12, and 16 dyn cm\(^{-2}\), respectively. For this experiment, the 8 and 12 dyn cm\(^{-2}\) channels had comparable orientation and IAR, accounting for the similar values for actin orientation. HUVECs have been shown to reorganize f-actin filaments at shear stresses as low as 7.5 dyn cm\(^{-2}\) (Blackman et al. 2002); stress
fiber reorientation along the direction of shear is energetically favorable for cells because it minimizes transduced stresses across the cell body perpendicular to flow and reinforces mechanical stability by directly resisting strain and elongation due to shear stress applied on the apical side (Davies 1995).

HBMEC cytoskeletal network is randomly oriented-HBMECs did not respond to shear stress by aligning actin filaments in the direction of flow (Fig. 22c). Although a dense actin network forms within the cell body, most noticeably at 8 and 12 dyn cm⁻², filament orientation analysis reveals that actin fibers do not organize preferentially in the direction of flow (Fig. 22d); instead, the fraction of fibers parallel and perpendicular to the direction of flow remain near a random value around 11%. The percentage of parallel fibers was 11.6, 11.7, and 12.1 at 8, 12, and 16 dyn cm⁻², respectively, and the percentage of perpendicular fibers was 11.3, 11.3, and 11.4 at 8, 12, and 16 dyn cm⁻², respectively. The high

![Figure 23. ZO-1 staining of HUVEC and HBMEC at 8 (left), 12 (middle), and 16 (right) dyn cm⁻²](image-url)
filament density suggests that the HBMEC cell body is stiffening under flow, but the lack of cell orientation means that it is not properly reorganizing to transduce the shear stress. This failure to reorganize could explain the increased delamination at shear stresses greater than 12 dyn cm\(^{-2}\) (Supplementary Video 2) and may account for the activation of a proliferative cell response (Wang et al. 2013). Similarly, the lack of HBMEC structural reorganization may contribute to global instability of the monolayer, shown by less continuous ZO-1 junctions as compared to the HUVEC (Fig. 23).

HUVECs and HBMECs show a transient motility response

PIV analysis was used to quantify the motility of monolayers of cells exposed to shear stress. This analysis revealed a transient motility response from both the HUVECs (Fig. 24a) and HBMECs (Fig. 24b). In both cases, there is an initial increase in motility during the 6h ramp up flow, followed by a gradual reduction in motility that levels off after 30h of high flow. The final motility

![Figure 24. Results from the PIV analysis: (a) HUVEC and (b) HBMEC. The time begins from -6h to account for the 6h ramp up period.](image)
showed shear stress dependence for both HUVECs and HBMECs, with a reduced motility at higher shear stress. With the exception of 4 dyn cm$^{-2}$, the motility of the HUVEC cells was lower than that of the HBMECs, in agreement with qualitative observation of the time-lapse videos (Supplementary Video 1 and 2).

This response is in agreement with the response of BAECs exposed to 6 dyn cm$^{-2}$, which show an initial increase in velocity, followed by a reduction in velocity to values lower than the pre-flow condition (Masuda and Fujiwara 2003).

HBMECs upregulate tight junction gene expression under shear stress

Although the morphological response of HBMECs has not been well characterized prior to this work, there has been significantly more work analyzing HBMEC gene and protein expression (Siddharthan et al. 2007, Cucullo et al. 2011). In particular, analysis of HBMEC biochemical expression under flow has revealed an upregulation of a number of tight junctional proteins associated with BBB function and the upregulation of other cell junction proteins. With this in mind, PCR was performed to assay the expression of genes coding for ZO-1, Claudin-5, and β-catenin, as these were all shown to be upregulated in previous work and are relevant to a BBB model.

It is important to emphasize that these are preliminary results (n=1), and, therefore, they must be repeated before it can be determined if any of the observed trends are representative.

Preliminary results from PCR (Fig. 25) suggest a significant upregulation of the gene coding for the tight junction protein claudin-5 at all shear stresses
and a slight up regulation for the junctional protein β-catenin at 4 and 8 dyn cm$^{-2}$, with a slight down regulation at 12 and 16 dyn cm$^{-2}$. These results are in agreement with results from HBMECs cultured under 6.2 dyn cm$^{-2}$ of laminar shear stress in the DIV-BBB (Cucullo et al. 2011). The relative expression of claudin-5, as compared to the static condition, is 38.8, 2.5, 2.4, and 1.4 at 4, 8, 12, and 16 dyn cm$^{-2}$, respectively. The relative expression of β-catenin, as compared to the static condition, is 1.2, 1.4, .8, and .5 at 4, 8, 12, and 16 dyn cm$^{-2}$, respectively. In contrast to previous results with HBMECs (Siddharthan et al. 2007, Cucullo et al. 2011), the HBMECs show a slight drop in expression of ZO-1. The relative expression of ZO-1, as compared to the static condition, is 0.5, 0.8, 0.6, and 0.2 at 4, 8, 12, and 16 dyn cm$^{-2}$, respectively. Without repeat experiments, it is difficult to explain the down regulation of ZO-1, but it could be associated with the instability of the HBMEC monolayer. With that said, the upregulation of certain junctional proteins further supports the idea that biochemical expression associated with barrier function is being upregulated under laminar flow, but further testing will be needed to confirm that result.

**Figure 25.** Preliminary PCR results for HBMEC expression of the genes coding for claudin-5 (*left*), β-catenin(*middle*), and ZO-1 (*right*). Results are reported as relative expression, as compared to the static condition.
Supplementary Information

Supplementary Video 1_16 dyn HUVEC

Supplementary Video 2_16 dyn HBMEC


Curriculum Vita

Adam Reinitz is a Master’s student at Johns Hopkins University pursuing his M.S.E in Materials Science and Engineering. As a member of the Searson Research Group, part of the Institute for NanoBioTechnology, he is aiding in creating an *in vitro* model of the blood brain barrier. In May 2013, he completed his Bachelor’s degree in Materials Science and Engineering at Johns Hopkins University. In addition to his work on this project, he has a background in nanoparticle synthesis, characterization of electrospun nanofibers, and fabrication of liquids to match the dielectric properties of the human body. After completing his Master’s he will be pursuing an industry position in the field of Materials Science.