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<td>TITLE: CHARACTERIZATION OF TIGHT JUNCTION FORMATION IN AN IN-VITRO MODEL OF THE BLOOD-BRAIN BARRIER</td>
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<td>AUTHOR: Michael Robert Machado</td>
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<td>DATE SUBMITTED: July, 2012</td>
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<td>COMMITTEE CHAIR: Kristen O’Halloran Cardinal, PhD</td>
</tr>
<tr>
<td>COMMITTEE MEMBER: Trevor Cardinal, PhD</td>
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<td>COMMITTEE MEMBER: Lily Laiho, PhD</td>
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ABSTRACT

CHARACTERIZATION OF TIGHT JUNCTION FORMATION IN AN IN-VITRO MODEL OF THE BLOOD-BRAIN BARRIER

Michael Robert Machado

Active and passive transport of substances between the microcirculation in the brain and the central nervous system is regulated by the Blood-Brain Barrier (BBB). This barrier allows for chronic and acute modulation of the CNS microenvironment, and protects the brain from potentially noxious compounds carried in the circulatory system. In-vitro modeling of the BBB has become the target of much research over the past decade, as there are many unanswered questions regarding modulations in the permeability of this barrier. Additionally, the development of a practical and inexpensive model of the BBB would facilitate a much more efficient drug development process. The goal of this project is to investigate the formation of the BBB through assessment of tight junction formation and endothelial cell monolayer permeability. Accomplishment of this goal will include completion of the two primary aims of this thesis, which are 1) development of an immunohistochemical staining protocol for the labeling of tight junctional proteins, and 2) characterization of permeability across a porous membrane co-cultured with bovine aortic endothelial cells (BAECs) and C6 glioma cells. Both of these aims were met, as a reliable IF protocol for tight junctional staining was developed, and permeability values across a permeable membrane seeded with BAECs and C6s were collected. The completion of these aims has helped to accomplish the goal of investigating the formation of tight junctions in an in-vitro model of the BBB. The IF protocol that has been developed, along with the collected permeability data will aid the development of a more dynamic in-vitro model
of the BBB to aid in research surrounding acute modulation of the BBB, along with facilitating a timelier drug development process.

Keywords: Blood-Brain Barrier, Tight Junctions, Immunofluorescence, Permeability Coefficient
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TABLE OF CONTENTS

List of Tables ........................................................................................................................................... x
List of Figures ........................................................................................................................................... xi

Chapter 1 – Introduction to the Blood-Brain Barrier .................................................................................... 1
  Section 1.0 General Overview .................................................................................................................. 1
  Section 1.1 Components of the Blood-Brain Barrier ................................................................................. 1
  Section 1.2 Blood-Brain Barrier-specific cell types .................................................................................. 2
    Section 1.2.1 Pericytes .......................................................................................................................... 3
    Section 1.2.2 Astrocytes ......................................................................................................................... 4
    Section 1.2.3 Endothelial Cells .............................................................................................................. 6
  Section 1.3 BBB Formation In-Vivo ......................................................................................................... 7
    Section 1.3.1 Transport Across the BBB Endothelium ......................................................................... 10
  Section 1.4 Pathologies Associated with Failure of the Blood-Brain Barrier ............................................. 11
  Section 1.5 The Shift to In-Vitro- Models .................................................................................................... 13
    Section 1.5.1 Methods for Assessing BBB formation in Bench top Model .............................................. 14
  Section 1.6 Modeling the Blood-Brain Barrier .......................................................................................... 16
    Section 1.6.1 Custom DIV-BBB Models ................................................................................................ 19
    Section 1.6.2 Current State of BBB Research in the Tissue Engineering Lab ....................................... 20
  Section 1.7 Summary and Aims of the Thesis ............................................................................................ 22

Chapter 2 – Immunofluorescence Protocol Development for Tight Junctional Proteins ............................... 25
  Section 2.0 Introduction ............................................................................................................................ 25
  Section 2.1 Methods .................................................................................................................................. 26
    Section 2.1.1 Study 1 - Modification to IF Protocol Version 1 ................................................................. 27
    Section 2.1.2 Study 2 - ZO-1 Expression as a function of Confluency .................................................. 30
      Section 2.1.2.1 Confluency Experiment 1 ............................................................................................. 31
      Section 2.1.2.2 Confluency Experiment 2 ............................................................................................. 33
Section 2.1.3 Study 3 - ZO-1 Expression as a Function of
BAEC and C6 Co-Culture ................................................................. 35
  Section 2.1.3.1 Co-Culture Study 1 - BAECs/C6s .................................. 35
  Section 2.1.3.2 Co-Culture Study 2 - C6s/BAECs .................................. 37
Section 2.2 Study 4 - IF stain for ZO-1, Claudin-5, and PECAM-1 on
EC and C6 mono- and co-culture groups ........................................ 41
Section 2.3 Study 5 - Blocking Buffer Experimentation .......................... 43
  Section 2.3.1 Claudin-5 stain on C6s alone ........................................ 43
  Section 2.3.2 Final Proof of Concept IF Stain ...................................... 45
Section 2.4 Results and Discussion .................................................. 47

Chapter 3 – Assessment of the Permeability across Monolayers of Endothelial
and Glial Cells .................................................................................. 52
Section 3.0 Introduction .................................................................... 52
Section 3.1 Methods ......................................................................... 53
  Section 3.1.1 Study 1 - Permeability Assessment of Transwell Inserts without Cells ... 54
  Section 3.1.2 Study 2 - Permeability Assessment of Transwell Inserts with Cells ....... 58
    Section 3.1.2.1 Permeability of 4 kDa FITC Dextran ................................. 59
    Section 3.1.2.2 Permeability of 250 kDa FITC Dextran ............................... 63
    Section 3.1.2.3 Calculation of Permeability Coefficients for
      Transwell Insert Studies .................................................................. 65
  Section 3.2 Results and Discussion .................................................. 66

Chapter 4 – Discussion and Conclusion .............................................. 69
Section 4.0 Overview ...................................................................... 69
Section 4.1 IF Protocol Development ................................................ 69
Section 4.2 Permeability Studies ...................................................... 74
Section 4.3 Future Work .................................................................. 76
Section 4.4 Conclusion .................................................................... 79
References ................................................................. 81

Appendix 2.0 – IF Protocol Version 1 ................................................................. 88
Appendix 2.1 – IF Protocol for PECAM-1 stain on hUVECs ........................................... 89
Appendix 2.2 – IF Protocol Version 2 ........................................................................ 90
Appendix 2.3 – Cell Culture and Passage Protocol .................................................... 91
Appendix 2.4 – DAKO Autostainer IF stain for ZO-1, Claudin-5, and PECAM-1
    using IF Protocol Version 2 .............................................................................. 92
Appendix 2.5 – Autostainer Protocol Adapted from Senior Project by Nicholas Hanne .. 97
Appendix 2.6 – Protocol for IF Stain for ZO-1, Claudin-5, and PECAM-1 on C6 only,
    BAEC only, C6/BAEC, BAEC/C6, and Mix Experimental Groups .................... 105
Appendix 2.7 – Results from IF Stain for ZO-1, Claudin-5, and PECAM-1 on C6 only,
    BAEC only, C6/BAEC, BAEC/C6, and Mix Experimental Groups .................... 106
Appendix 2.8 – IF Protocol Version 3 ........................................................................ 124
Appendix 2.9 – Permeability Testing Protocol ........................................................... 125
LIST OF TABLES

Table 1 – Filter Wheel Settings ................................................................. Page 28
Table 2 – Cell Counts .............................................................................. Page 94
Table 3 – Permeability Coefficients .......................................................... Page 66
LIST OF FIGURES

Figure 1 – Proteins involved in Tight Junction Formation .............................................Page 2
Figure 2 – Orientation of Cells involved in BBB ..........................................................Page 3
Figure 3 – Rosette-like Structures formed by Astrocytes .............................................Page 5
Figure 4 – Occlusion of Intercellular Clefts by Tight Junctions ................................Page 9
Figure 5 – Methods of Transport ..................................................................................Page 10
Figure 6 – CellMax Duo Hollow Fiber Bioreactor .........................................................Page 21
Figure 7 – Orientation of Experimental groups for IF Stain .......................................Page 28
Figure 8 – Widefield Fluorescence Microscope ..........................................................Page 28
Figure 9 – BAEC and HUVEC IF Stain ......................................................................Page 30
Figure 10 – Distribution of BAECs in 6-well Plates .....................................................Page 31
Figure 11 – ZO-1 Expression as a Function of Confluency Part 1 ...............................Page 32
Figure 12 – ZO-1 Expression as a Function of Confluency Part 2 ...............................Page 34
Figure 13 – Orientation of Cells in Co-culture, BAECs over C6 .................................Page 36
Figure 14 – Location of Cells for IF Stain .................................................................Page 36
Figure 15 – White Light Images ..................................................................................Page 37
Figure 16 – Orientation of Cells in Co-culture, C6 over BAECs .................................Page 38
Figure 17 – Location of Cells for IF Stain .................................................................Page 38
Figure 18 – Results of IF Stain ....................................................................................Page 40
Figure 19 – Location of Cells for Manual Co-Culture Stain ......................................Page 42
Figure 20 – Location of Cells for Blocking Buffer Experiment ...............................Page 44
Figure 21 – IF Results for Blocking Buffer Experiment ............................................Page 44
Figure 45 – Positive Controls for BAEC Over C6 ..................................................Page 112
Figure 46 – Negative Controls for BAEC Over C6.................................................Page 113
Figure 47 – Positive Controls for C6 Over BAEC ....................................................Page 114
Figure 48 – Negative Controls for C6 Over BAEC ....................................................Page 115
Figure 49 – Positive Controls for Mix .................................................................Page 116
Figure 50 – Negative Controls for Mix .................................................................Page 117
Chapter 1 – Introduction to the Blood-Brain Barrier

Section 1.0 General Overview

The homeostasis and regulated molecular exchange established between the central nervous system and the supporting microcirculation is a delicate relationship that is maintained via the Blood-Brain Barrier (BBB)\(^1,2\). This barrier serves as both a physical and metabolic barrier to modulations in plasma as well as circulating neurotransmitters and toxicants capable of disrupting neural signaling\(^3-5\). The acute regulation of the microenvironment in the brain facilitated by the BBB is critical to typical bodily function and behavior. As such, changes in the ability of the BBB to maintain this balance can result in pathologies including stroke, multiple sclerosis, Alzheimer’s disease, Parkinson’s disease, and epilepsy\(^4,6-17\). Research, therefore, has been largely focused on the development of BBB models to aid in the understanding of these diseases. While the anatomy and the physiology of the BBB have been well-characterized in-vivo, the development of an adequate in-vitro BBB model is ongoing.

Section 1.1 Components of the Blood-Brain Barrier

The BBB is a discriminatory barrier formed by the enhanced interaction between specialized endothelial cells located in the cerebral microcirculation\(^2\). The integral membrane proteins and transport channels involved include members of the occludin and the claudin families (See Figure 1). Specifically, these proteins are Zona Occludens 1, 2, and 3 (ZO-1-3), Claudin 1, 3, 5, 12, junctional adhesion molecules (JAMs), endothelial selective adhesion molecule (ESAM), glucose transporter (GLUT), vascular endothelial cadherin (VE-cadherin), and platelet endothelial cell adhesion molecule (PECAM-1)\(^18,19\).
Section 1.2 Blood-Brain Barrier-specific cell types

The formation and subsequent modulation of the tight junctions formed in the cerebral microcirculation is the result of the intricate interplay between three cell types: the pericyte, the astrocyte, and the endothelial cell (See Figure 2). Both up- and downregulation of BBB permeability are influenced through paracrine interactions between the endothelium and these other two cell types.

**Figure 1** Intramembranous endothelial cell proteins associated with tight junction formation.
**Figure 2** Three cell model of the capillaries composing the Blood Brain Barrier. This figure shows the location and interaction between the specialized endothelial cell, the astrocyte foot processes, and the perivascular cell.\(^2\)

**Section 1.2.1 Pericytes**

Pericytes, which typically form around endothelial tubes, share a basement membrane with the endothelial cells, facilitating the delivery of factors to encourage endothelial survival, cell proliferation, migration, and differentiation\(^{20,21}\). These cells are involved in the regulation of endothelial cell proliferation and differentiation in addition to synthesizing and secreting certain vasoactive agonists. In addition to modulation in vascular tone, pericytes are contractile in nature and contribute to changes in endothelial cell junctional leakage as a function of contraction and relaxation\(^{21}\). The ratio of pericytes to endothelial cells is considered to be highly variable, especially between tissue types (e.g. 1:1 in the retina, 1:10 in the lungs, 1:100 in striated muscle)\(^{21}\). However, this data has been suggested based upon single studies, and has yet to be verified with any statistical significance\(^{21}\). Overall, there is a positive correlation between pericyte density and endothelial cell junctional tightness, along with the level of microvascular blood pressure\(^{21}\). Taking this into account, the ratio in cerebral
capillaries has been experimentally determined to be 1 pericyte for every 3 ECs, which allows room for enhanced interaction between the endothelium and the astrocyte foot process\textsuperscript{21}. These podocyte-like projections permeate 99% of the basement membrane in brain capillaries, suggesting a significant interaction between astrocytes and endothelial cells\textsuperscript{20,22,22}.

Section 1.2.2 Astrocytes

Astrocytes specifically target individual ECs, sending out foot processes to single endothelial cells and forming rosette-like structures that wrap around the capillary tube (See Figure 3). The connection between the endothelium and the astrocyte is similar to that of the pericyte, forming its interface only 20nm away from the capillary wall (the effective thickness of the basement membrane) through podocyte-like projections referred to as astrocytic endfeet. These endfeet that attach to the circumference of cerebral capillaries are known to aid in regulation of brain ion volume and concentration, and are necessary for the formation of the tube-like capillary structures in vitro\textsuperscript{2}. This close relationship between the endfeet of the astrocytic glia and the endothelial cells strongly suggest maintenance and even induction of BBB-specific characteristics in the cerebral microvasculature due to the interaction between these two cell types. Indeed, in vitro BBB setups involving research surrounding co-culture of astrocytes and brain capillary endothelial cells has shown the necessity for the presence and interaction between these two cell types for the proper formation of tight junctions\textsuperscript{1,23-26}. 
Studies surrounding endothelial cell and astrocyte interaction have supported the hypothesis that co-culture of these two cells types will increase barrier “tightness”\textsuperscript{27}, showing that the removal of astrocytes from a co-culture model results in increased permeability across the endothelium\textsuperscript{18}. However, this research has also supported that the removal of astrocytes from a co-culture model does not reduce the expression of tight junction-specific cell surface proteins. This suggests that while the presence of astrocytes has no bearing on the incidence of BBB-exclusive junctional proteins, the commissioned interaction between the endothelial cells and the astrocytes plays a vital role barrier manipulation and changes in permeability.

The origin of BBB permeability modulating agents has been shown to stem from astrocytic cells\textsuperscript{28}. These cell types have been recognized to release several humoral compounds and modulating factors (including glutamate, aspartate, taurine, ATP, ET-1, NO, TNF\textsubscript{α}, MIP2, IL-1β, and Bradykinin) that open the barrier, effectively increasing paracellular transport\textsuperscript{28}. These observations support the assumption that astrocytes are able to modulate
BBB permeability through ligand-receptor communication over short time scales$^{28}$.

Additionally, the relationship between the glia and the ECs has been shown to influence gene expression through the upregulation of specific transport pathways, including GLUT-1, and L-system and A-system amino acid carriers$^{29}$. Supplementing the modifications in extracellular transport, astrocytes express their own membrane bound proteins (e.g. p-glycoprotein) that aid in transcellular transport of drugs. These efflux channels such as Pgp allow for changes in drug concentration within the brain microenvironment without modifications to endothelial cell permeability$^{20}$.

**Section 1.2.3 Endothelial Cells**

While signals from the pericytes and the astrocytes play a role in influencing the function of the barrier, the key cell type involved in tight junction formation is the brain microvessel endothelial cell. Despite the vital function in cerebral microenvironment homeostasis, there is only a small fraction of these cell types present in the brain, with a total EC volume per gram of brain tissue of 1µL (2% of the total brain volume)$^{20}$ compared to the almost 50% of the brain volume taken up by astrocytes$^{30}$. However, it is not the amount of cells present in the brain capillaries, but the membrane-bound proteins expressed that set apart these specialized endothelial cells. As previously mentioned, the combination of the expression of unique receptors, transporters, and effusive pumps provide protection for the CNS from a wide array of potentially cytotoxic hydrophobic substances$^{31,31,32}$. While retaining the ability to target specific receptors on adjacent cells, these ECs are able to both release cytokines and respond to them$^{33}$. This suggests that despite external stimuli, the specialized ECs in the brain are able to modulate their own permeability in response to changes in the neural microenvironment.
A change in the permeability of these endothelial cells is accomplished not only by the cells themselves, but is often a result of interaction between the ECs and astrocytes. Specific transcellular transport channels located on the astrocytic endfeet such as aquaporin-4 are observed to be up-regulated when introduced to endothelial cells in co-culture\textsuperscript{34}. In addition to changes in protein expression, release of leukemia inhibitory factor (LIF) by the endothelium has been shown to encourage astrocyte differentiation\textsuperscript{35}.

This interaction between ECs and astrocytes has been a point of great interest during the characterization of BBB-specific cell types. Once it became clear that the interaction of these two cell types was favorable (and in most cases, necessary) for developing tight junctions, endothelial cells and astrocytes have been included in most attempts to recreate the characteristics of the BBB. Typical induction of BBB properties has been shown achieved through physical contact between the endothelial cells and the astrocytes (astrocyte foot processes)\textsuperscript{36}. However, researchers have also demonstrated the upregulation of BBB properties in endothelial cells introduced by using astrocyte media alone\textsuperscript{37}.

Section 1.3   BBB Formation \textit{In-Vivo}

To understand this complex interaction between adjacent endothelial cells (ECs), it is important to discuss the anatomy of the three classes of capillaries (continuous, fenestrated, and discontinuous) and their relationship to the establishment of regulated trans-endothelium transport. In tissues such as the liver and spleen where filtration of macromolecules suspended in the blood is necessary, capillaries exhibit sinusoids and intercellular clefts on the order of microns, allowing the egress of compounds from the circulation\textsuperscript{38}. In other tissue types where filtration of smaller substrates and ions is conducted, fenestrated capillaries (which express pores close to 60nm in size and tighter intercellular gaps) are responsible for regulation of
molecular transport out of the lumen\textsuperscript{38}. However, because of the narrow band of appropriate ion and macromolecular concentrations in the CNS to support proper neuron function, transport of any molecule across the endothelium is highly regulated by continuous capillaries\textsuperscript{1}. These capillaries exhibit extremely tight intercellular junctions, ranging from 0-4nm. This physical barrier severely restricts paracellular transport of large molecules\textsuperscript{39}. Additionally, these specialized endothelial cells express a unique combination of receptors and transport channels, effectively limiting the volume and type transport of across the lumen (e.g. endocytosis, pinocytosis, directed diffusion, and channel-mediated transport of ions and molecules)\textsuperscript{40}.

The characteristics of these capillary types responsible for this high degree of regulated paracellular diffusion are in the form of tight junctions (TJs). These junctions can be visualized as a complex web of intramembranous particles that effectively occlude intercellular junctions and modulate transport across each endothelial cell (See Figure 4)\textsuperscript{1}. These tight junctions are comprised of three elements, the integral proteins and lipids, which help to form the permeability barrier, ancillary proteins forming the intracellular element, and the membrane-bound proteins.
Figure 4| Structure of tight junctions between endothelial cells; junctional proteins form webbed network between adjacent cells, effectively limiting efflux of molecules across the lumen based upon size and charge density\textsuperscript{41}. 
Section 1.3.1  Transport Across the BBB Endothelium

The barrier established in the cerebral microvessels through the interactions of the previously described cell types forms not only a physical barrier, limiting paracellular exchange, but also constitutes a transcellular transport and metabolic barrier (See Figure 5)\(^{27,42}\). While the physical barrier to transport is clear through the formation of tight intracellular junctions, the transport and metabolic barriers formed by the specialized ECs merits discussion. Compounds unable to traverse through the junctional proteins are obliged to either interact with membrane bound receptors or molecule-specific influx/efflux channels to stimulate transportation across the membrane. Smaller gaseous molecules such as O\(_2\) and (sometimes) CO\(_2\) are free to diffuse through the cytoplasm along their concentration gradients. However, other hydrophilic molecules such as glucose, amino acids, purine bases, nucleosides, and cholines must activate transport proteins to traverse the endothelial cells\(^{31}\).

**Figure 5** Depiction of the highly regulated transport mechanisms across blood-brain barrier. A shows the inhibition of hydrophobic molecules through the tight junctions. Drug delivery is typically achieved through transport mechanisms shown in B-E.
Likewise, specific proteins such as insulin and transferrin migrate out of the lumen through ligand-receptor interaction, known as receptor mediated transcytosis (RMT). This type of transport begins with receptor activation on the luminal side of the EC and endocytosis of the compound. Once through the lipid bilayer, the vesicle containing the agent diffuses through the 200 nm of cytoplasm until it is exocytosed on the abluminal side into the cerebral interstitial space. Alternatively, certain lipophilic compounds such as caffeine and alcohol are able to move through an EC in small vesicles via pinocytosis, or adorptive transcytosis (Figure 5). While there is no determinable cutoff size for this type of transport, it is possible to speculate on this size based upon the average size of the molecules that typically undergo pinocytosis. Specifically, caffeine molecules, which undergo pinocytosis to cross through the brain endothelium, can be close to 65 kDa, comparable to other similarly lipophilic compounds. It is has been determined that there are roughly 5 of these pinocytotic vesicles per square micron of brain endothelium, suggesting that this form of transport is minimally involved in transport across the endothelium. Finally, cerebral endothelial cells can also secrete extracellular and intracellular enzymes that interact both with luminal toxicants and neuroactive compounds that might be transcytosed, effectively metabolizing and degrading the agents before transport occurs.

Section 1.4 Pathologies Associated with Failure of the Blood-Brain Barrier

Ongoing research in the area of neurological disorders has supported that a wide array of cerebral pathologies are associated with alterations in tight junctional protein expression and endothelial cell permeability. Likewise, spontaneous changes in the structure and function of the endothelium can result in specific neuroinflammatory diseases including meningitis and encephalitis through interference with particular bacterial proteins. Similarly,
other pathological states can be attributed to breakdown of the BBB or even a deregulation of astrocyte-endothelial coordination, including stroke, multiple sclerosis, Alzheimer’s disease, Parkinson’s disease, and epilepsy\textsuperscript{4,6-17}. In some of these cases there is a change in the typical expression of characteristic ATP-binding cassette (ABC) transporters, such as P-glycoprotein (Pgp), and therefore the ability of the BBB to maintain homeostatic permeability traits is compromised\textsuperscript{16,17}. Modulation of the expression of some of these ABC transporters has been investigated as a therapeutic target\textsuperscript{49}. Acute modulation of BBB permeability would allow enhanced delivery of therapeutic agents designed to treat these disease states\textsuperscript{50}. Progress in this arena has shown significant promise in animals models, but has not resulted in reproducible safety and efficacy in clinical trials\textsuperscript{51,52}. Other therapeutic treatments for these inflammatory conditions often include administration of glucocorticoids (GCs), which bind to the GC receptor present on most cell types and reduce inflammation\textsuperscript{53,54}. Managing inflammation of the BBB is critical to return the permeability of the membrane back to normal.

Chronic and acute modulations in BBB permeability have been characterized to the greatest extent in traumatic brain injury. Research supports that an injured brain will exhibit ruptured blood vessels in the microcirculation localized to the site of injury, and will allow the seepage of blood contents into the brain tissue\textsuperscript{55}. While this initial damage can be quite devastating to brain microenvironment, the resulting secondary damage due to changes in barrier permeability, which can last up to a few days following the injury, can leave lasting effects on the CNS\textsuperscript{56}. This initial damage includes loss of control over the cerebral microenvironment and the subsequent passage of potentially toxic compounds across the endothelial layer, further magnifying the initial damage. The timeframe during which this
secondary damage occurs is typically within 4-6 hours, after which conventional barrier function is restored\textsuperscript{57,58}. It is important to note that while the majority of barrier function is reestablished, effectively limiting the passage of larger (10 KDa) molecules through the endothelium, seepage of smaller compounds can persist up to 4 days post-injury\textsuperscript{55}.

Section 1.5 The Shift to In-Vitro Models

While characterization of the BBB \textit{in-vivo} is vital to the expansion of our knowledge base surrounding both typical and atypical neurological function, it is important to realize the gradual shift in research models towards \textit{in-vitro} systems tailored to mimic the natural environment. Research and testing conducted regarding the barrier has been focused towards the development of pharmaceuticals. While many of these studies strive to develop strategies for clinical treatments involving BBB modulation, a large fraction of the testing is regulatory in nature. Currently, the US Food and Drug Administration (FDA) mandates the screening of potentially noxious compounds using animals models to determine permeability coefficients and assess the effect of drugs-in-development on the CNS\textsuperscript{59}. Typical drug validation studies in animal models will look for sensory and/or behavioral changes in addition to histological examinations of excised brain tissue. This type of substantiation requires large amounts of investment dollars for the housing, care of, and subsequent testing of animals in addition to the time demand to obtain usable data. To alleviate this pain in the drug development market along with the development of more applicable research tools, there has been a push in the BBB community to design novel \textit{in vitro} testing procedures\textsuperscript{60}. This type of model could provide high throughput screening (HTS) of new drug compounds through a large-scale and automated tissue engineered BBB mimic test platform.
Standards mandating the change to in vitro test systems have already been enacted in areas of Europe. In 2003, a gathering of the ICCVAM/ECVAM (International Coordinating Committee on the Validation of Alternative Methods and the European Center for Validation of Alternative Methods) developed research strategies for the ACuteTox consortium (“Optimization and pre-validation of an in vitro test strategy for predicting human acute toxicity”). These research strategies, which are guidelines for testing toxicity in humans, direct researchers toward in vitro models in an effort to limit the volume of animals studies conducted^61. While there is a desire to shift research and testing protocols from widespread animal trials to large-scale in vitro testing programs, the complexity of modeling the natural microenvironment to reproduce the BBB characteristics observed in vivo presents a significant hurdle. Researchers have observed significant changes to barrier characteristics when cultured in vitro, including down regulation in the activity of glucose and neutral amino acid carriers, RMTs, and both luminal and intercellular antitoxic enzymes[^22]^62. Additionally, changes in tight junction permeability have been observed in response to extended (>60 minutes) periods of perfusion, regardless of a change in tight junctional protein expression[^26]. Overlooking changes in phenotype and functional characteristics, non-immortalized primary cultures of human brain endothelial cells will rapidly dedifferentiate and begin to senesce even in response to a moderate passaging schedule[^39], thus adding to the difficulties surrounding such *in-vitro* modeling.

**Section 1.5.1 Methods for Assessing BBB formation in Bench top Models**

Due to the difficulties surrounding the development of an *in-vitro* BBB model, it is essential to develop robust methods for determining tight junction formation.

[^22]: https://doi.org/10.1016/S0014-4894(88)80316-4
[^26]: https://doi.org/10.1016/S0006-2952(87)80040-2
[^39]: https://doi.org/10.1016/S0021-9539(87)80389-7
straightforward method for determining formation of tight junctions is to measure levels of adherence of the specialized ECs. This can be quantified by testing the electrical resistance across a monolayer, known as transendothelial electrical resistance (TEER). An array of TEER values for cerebral endothelial cells cultured in-vitro have been offered, ranging from 1MΩ to 8MΩ. It is important to realize the difficulty in achieving in-vivo TEER values and appropriate endothelial cell junction tightness in an in-vitro system. It has been shown that an in-vitro co-culture setup can take longer than 2 weeks to develop a transcellular electrical resistance equal to ¼ that observed in vivo. This can be enhanced through the use of special substrata used to condition the culture flasks, such as type IV collagen and fibronectin. It has also been observed that fluctuations in pH and increases in buffer concentrations (through the addition of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 3-(N-morpholino)propanesulfonic acid (MOPS), or N-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic Acid (TES)) can have significant impacts on TEER values, potentially through upregulation of tight junctional proteins such as claudin-5. From these results, it is apparent that the in-vitro testing procedures currently explored by the majority of BBB researchers are lacking fundamental intercellular communication and expression of BBB-specific membrane bound tight junctional proteins.

In order to observe the extent to which tight junctions are forming between adjacent ECs, it is possible to tag these tight junctional proteins with a fluorescently labeled antibody. This allows for fluorescent imaging of a monolayer of cells to reveal the presence of intercellular proteins. Changes in endothelial cell protein expression in response to external stimuli can therefore be quantified and optimized. An additional method for determining tight junction formation involves the use of porous (~3µm) transwell inserts to separate the
endothelial cells from the astrocytes while still allowing for the foot processes to reach out and attach to the EC surface. These inserts serve as a scaffold for the cells to culture, and establish a permeable membrane. The membrane can then be used to determine transport rate of known compounds from one compartment to the other. As in any tissue-engineered construct, it is also necessary to understand the cross-sectional thickness of the construct to determine an appropriate diffusion distance for any compound. This distance will inherently be involved in the transport rates of any compound across a cultured membrane. In such a BBB model, which tests for permeability, it would be necessary to take into account the thicknesses of the ECs, the astrocytes, and the thickness of the transwell membrane. SEM imaging has shown cell diameter ranging from 5-13 µm for Endothelial cells and 2.3 µm for C6 cells. After taking into account the 10 µm thickness of the transwell insert membrane, it is possible to include the 17-23 µm distance through which a compound must diffuse into any calculations regarding transport rates.

Section 1.6 Modeling the Blood-Brain Barrier

Typical methods for modeling the BBB include in-silico (or computer-aided) models, static mono- and co-cultures, and dynamic or flow-associated mono- and co-cultures. As of now, the most cost-effective method of modeling the BBB properties is the use of computer-aided design (CAD) modeling. This type of modeling employs solid models that mimic the physiologic properties of the blood vessels, which help form the BBB. Through the incorporation of permeability values typical in-vivo, it is possible to simulate a compounds interaction with a biologic system. These models allow for pre-screening of a larger volume of potential drug candidates though the prediction of their permeability coefficients. It is necessary, however, to incorporate standard physiological parameters (e.g. solubility,
lipophilicity, molecular size, capacity for hydrogen bonding, and charge distribution) into the simulation to obtain usable data\textsuperscript{70}. These criterion are established through animal testing data, wherein small animals (typically rodents) are injected with a compound, euthanized, and their brains histologically examined for drug concentration values\textsuperscript{30}. This standardized measurement, referred to as the “LogBB” value, equates the concentration of the compound within the brain to the concentration found in the blood plasma\textsuperscript{71,72}. While this type of experimentation provides useful and necessary toxicological data, much research on the BBB is focused on the development of an \textit{in-vitro} system that would bridge this gap between the computer modeling and preclinical animal screening.

The most common technique for establishing an experimental BBB model \textit{in-vitro} includes the use of monocultures of brain microvessel endothelial cells (human or animal, typically bovine) or co-cultures of endothelial cells and astrocytes\textsuperscript{73}. The monoculture approach provides data indicating a downregulation of complete tight junction characteristics in the absence of astrocytic influence. By including a single cell type (ECs) onto a porous transwell insert, it is possible to test membrane permeability with multiple drug compounds in the same well or varying concentrations of the same drug across different wells\textsuperscript{69}. While solely providing a cursory examination of the limited expression of BBB properties in a monoculture of ECs, this type of model can be induced to express slightly enhanced BBB characteristics through the introduction of astroocyte or glia media (or alternatively, in a serum-free medium with the introduction of hydrocortisone)\textsuperscript{74-77}. The absence of natural stimuli such as astrocyte-EC interaction and physiologic flow conditions results in down-regulation of tight-junction associated proteins, and necessitates the use of a more relevant model\textsuperscript{18,78}. 
The preferred co-culture method demonstrates enhanced tight junctional protein expression and increased Transendothelial Electrical Resistance (TEER) values by taking into account the necessary interaction between the endothelium and the astrocyte endfeet processes. Co-culture of these two cell types often utilizes porous transwell inserts that simulate the separation of the astrocytes and the endothelial cells observed in vivo by the basement membrane, while allowing for the astrocytic endfeet to extend through the micropores and interact with the ECs. This separation and interaction facilitate enhanced development of BBB characteristics. While this model introduces the intercellular communication between the endothelial cells and astrocytes that is necessary for proper barrier formation, the lack of shear stress on the apical membrane of the ECs results in diminished TEER values, effectively increasing the membrane permeability coefficient. Therefore, it is desirable to develop a dynamic in-vitro model.

These tri-dimensional models involving the co-culture of astrocytes and ECs along with exposure to flow, referred to as dynamic in-vitro blood-brain barrier (DIV-BBB) models, result in enhanced tight junctional protein expression along with physiologically relevant TEER values. The majority of these models utilize a tubular hollow fiber scaffold with intraluminally seeded endothelial cells and extraluminally seeded astrocytes. With the incorporation of pulsatile changes in flow and shear stresses through the lumen of the hollow fibers, and a physiologically relevant pressure drop from inlet to outlet, the endothelium takes on a morphology that more closely resembles the in-vivo situation. However, the use of these (typically polypropylene) hollow fibers limits the ability for intraluminal observation, restricting the examination of changes in morphometry and endothelial cell phenotype.
Section 1.6.1 Custom DIV-BBB Models

There are few commercial perfusion bioreactor systems for BBB research. However, all of the options available incorporate the use of hollow fibers into their bioreactor. These hollow fiber setups are widely used in BBB experimentation\textsuperscript{23,83-85}, as they mimic the capillary bed where the barrier is formed \textit{in-vivo}. These fibers allow for intraluminal seeding of endothelial cells and extraluminal seeding of astrocytes. The permeable polypropylene fibers then allow for nutrient exchange between the lumen and extracellular media.

Experimentation that includes these hollow fibers involve typically involves the use of endothelial cells and glial cells, often BAECs\textsuperscript{23,26} and C6s\textsuperscript{23,26,83,86}. Flow conditions through each of the fibers is calculated to fall within typical BBB ranges (1-2 dynes/cm\textsuperscript{2})\textsuperscript{82}, and systems are cultured according to typical cell culture protocol (37°C, 5% CO\textsubscript{2}, etc.). Most hollow fiber bioreactor modules will disperse fluid flow through around 50 of these fibers, allowing for a pressure and shear stress drop typical of a capillary bed. Across this artificial vascular bed, these hollow fiber models of the BBB are also able to provide information on the permeability of certain compounds through the barrier, as well as indications of transport protein function.

However, there are some significant drawbacks to the use of hollow fiber cartridges. IF staining and histological sectioning is typically performed on any scaffold containing cells to determine presence, viability, location, and confluency of the cells. In the case of a hollow fiber bioreactor assembly, this means that the one time use housing must be cut open with a band saw and the individual fibers placed in fixative. Once fixed, the polypropylene fibers become brittle, and exposure of the lumen becomes difficult. This results in issues
surrounding whole mount imaging on a fluorescence microscope, or even histological sectioning with typical paraffin embedding.

These commercial systems represent the first attempt to satisfy the demand for a practical, easy to use DIV-BBB model. Further investigations into the characterization of a BBB model that could facilitate drug development and toxicological screening will require incorporation of reusable bioreactor components. Additionally, the use of a robust scaffolding material that is easy to stain and section in addition to providing dynamic permeability data will provide a simple and inexpensive method of examining the BBB in a bench top model.

**Section 1.6.2 Current State of BBB Research in the Tissue Engineering Lab**

BBB research in the Tissue Engineering Lab at Cal Poly, initially started by Bryan Brandon, and subsequently followed by several others\(^{87-89}\), has set the initial foundation for exploring the formation of tight junctions and proper barrier functionality both in static and dynamic culture conditions\(^{87}\). BAECs and C6s were carefully selected for the BBB model, and initial studies involved the development of a protocol for culturing both of these cell types. The CellMax DUO hollow fiber perfusion bioreactor system was initially chosen as the system to model the BBB (Figure 6). This hollow fiber system was then characterized in terms of the flow rates and shear stresses inside the fibers as they are related to the channel settings on the system. A protocol for sodding the hollow fiber system with BAECs only was then developed.

This previous experimentation established the necessary protocols for culturing both of the cell types crucial to formation of the BBB. These protocols, along with initial experimentation into seeding the BAECs into a dynamic model of the BBB, have helped to establish a working knowledge of how to manage these cell types and how to manipulate them
in a culture setting (both static and dynamic) to change the expression of the elements forming the barrier.

In addition, the previous research in this lab has shown the difficulties associated with the use of a commercial hollow fiber bioreactor as a model for the BBB. Issues arising from opening the bioreactor and subsequent imaging of the fibers indicate the need for the development of an in-house custom bioreactor system. This system should account for the shortcomings of these hollow fiber systems, in that it needs to be reusable and incorporate a scaffolding material that is easy to section and stain, while allowing co-culture of the two key cell types in a dynamic environment.

Figure 6| CellMax hollow fiber bioreactor system previously used in the Tissue Engineering Lab at Cal Poly for BBB Research.
Section 1.7 Summary and Aims of the Thesis

The BBB protects our CNS from potentially toxic compounds by selectively screening which molecules can diffuse from the circulatory system into the microenvironment in the brain. In the design of any drug compound, it is therefore necessary to both understand the physiology and functional characteristics of the BBB and to be able to model the barrier in an in-vitro setting. This research surrounding the BBB plays a critical role in both the development of novel methods to model the barrier for toxicological screening and in the development of methods for acute modulation of BBB permeability for treatment of CNS disorders.

The expression of tight junctional proteins and formation of the BBB has been established in static culture conditions\textsuperscript{26,90}, and the use of hollow fiber bioreactors has enabled the modeling of the BBB in a dynamic environment\textsuperscript{23,83,91}. While the majority of academia uses these hollow fiber systems, the issues regarding opening the bioreactor and imaging the fibers in addition to the high costs associated with repeated purchase of hollow fiber modules necessitates the development of a reusable and easy to use perfusion bioreactor system for ongoing work at Cal Poly.

To account for shortcomings related to ease of culture and ease of data collection of most commercially available perfusion bioreactor systems, including the one in the Cal Poly Tissue Engineering lab, current in-vitro BBB research in academia is focused on the construction of new dynamic BBB models that allow for assessment of cellular architecture at different time points, histological examination, and the induction of native blood vessel pressures (1-2 dynes/cm\textsuperscript{2} for BBB capillaries) in addition to the potential for permeability screening of new drug compounds.
The experiments conducted in this thesis examine the interaction between BAECs and C6 cells in mono- and co-cultures along with permeability data using transwell inserts. This data will lay the groundwork for full perfusion bioreactor setups, involving the sodding of the ECs and glial cells into a poly(lactic-co-glycolic acid) (PLGA) tubular scaffold in a novel perfusion bioreactor system. Such a system would allow for the cultivation of ECs and glial cells on a tubular scaffold inside of a custom designed bioreactor, and would facilitate the simultaneous cultivation of four vascular constructs, while administering native hemodynamic conditions. Additionally, the use of a PLGA scaffold will support post-experimental analysis through cryosectioning and immunohistochemical (IF) staining.

The overall goal of current work at Cal Poly is to develop an in-house dynamic model of the BBB that would facilitate the investigation of transport across a cellular monolayer. One aspect of this overall goal will be to successfully culture and assess the proper cell types. Therefore, the focus of this project is to develop a protocol to determine expression of BBB tight junctional proteins in culture, and to assess permeability of the cell monolayer. This project will therefore have two aims. The first aim is to develop a protocol to determine the presence of tight junctional proteins. An IF stain will be developed to label each cell surface protein with a fluorescent antibody. In imaging these groups, it will be possible to obtain data regarding baseline expression of tight junctional proteins, and also data regarding the change in expression of these proteins in response to external stimuli (including introduction to astrocytes). This protocol will provide the tools necessary to determine the location of protein expression as well as information regarding the extent of BBB formation.

The second aim of this project is to assess the permeability values associated with a co-culture model of astrocytes and ECs. This will be performed using transwell inserts, on
which glial cells will be cultured in the lower compartment, and ECs in the upper compartment. This will establish a dual-layer of cells, through which the permeability of known compounds can be tested. This aim will provide a functional assessment of the BBB formation, and provide the platform for scaling up the model to a perfusion-based system.

The completion of Aims 1 and 2 will provide the foundation for dual-sod setups of BAECs and C6s in an in-house custom perfusion bioreactor system. This perfusion system will not only mimic the functional characteristics of the BBB found in-vivo, but will also provide a BBB model that is easy to use and reuse, simple to analyze and perform histological analysis on, and provide a practical alternative to in-vivo permeability testing.
Chapter 2 – Immunofluorescence Protocol Development for Tight Junctional Proteins

Section 2.0 Introduction

The development of an Immunofluorescence (IF) protocol for tight junctional protein staining was necessary to determine the extent to which the BBB formed in an in-vitro model. The key junctional proteins that were investigated using this protocol included ZO-1 and Claudin-5, which are tight junction specific proteins, and PECAM-1, which is a standard endothelial cell marker. This protocol included the use of primary antibodies (specific to each of the tight junctional proteins listed above) and fluorescent secondary antibodies. The use of a non-fluorescent primary and a fluorescent secondary antibody enabled the labeling and imaging of the ZO-1, Claudin-5, and PECAM-1 proteins. Evaluation of the expression of these proteins provided insight into the extent of formation of tight junctions in our in-vitro model.

The previous protocol for immunohistological staining, henceforth referred to as IF Protocol version 1, was developed by T.J Eames and used for initial staining of tight junction-specific cell surface proteins (Appendix 2.0). Due to issues arising from the use of this protocol, the first few attempts at staining endothelial cells resulted in non-usable images. Therefore, the majority of this protocol was replaced with steps from a separate IF protocol, hereby referred to as IF Protocol version 2. This protocol allowed for staining of PECAM-1 on endothelial cells, and had previously been used by Marcus Foley and Elizabeth Curiel in the Tissue Engineering Lab at Cal Poly (Appendix 2.1). While this protocol resulted in usable fluorescent images, it had been tailored to stain for PECAM-1 on endothelial cells obtained
from human umbilical veins (hUVECs), and therefore demanded further investigation into its potential use in tight junctional protein staining.

**Section 2.1 Methods**

The endothelial cells used in this research were sourced from the bovine aorta (Lonza, Cat. No. BW-6002). To determine formation of BBB specific tight junctional proteins, the Zona Occludens-1 (ZO-1) protein was chosen as the principle target for IF staining, and the appropriate primary antibody (Rabbit Anti-ZO-1) was purchased (Invitrogen, Cat. No. 61-7300), along with a fluorescently labeled secondary antibody (Alexa Fluor 488 Donkey Anti-Rabbit, Invitrogen, Cat. No. A21206). This chapter outlines the use of both the primary and secondary ZO-1 antibodies to evaluate and modify the IF protocol version 1. Once the initial modifications to version 1 of the protocol had been made, primary and secondary antibodies for Claudin-5 (Invitrogen, Cat. No. 341600) and PECAM-1 (Invitrogen, Cat. No. 37-0700) were incorporated to obtain a more broad understanding of tight junctional protein expression and endothelial cell phenotype retention.

Experimentation with the IF protocol began with the initial modification of protocol version 1 (used by T.J. Eames) to incorporate the steps outlined in the protocol used by Marcus Foley and Elizabeth Curiel, termed IF protocol version 2. IF protocol version 2 was then used to determine changes in ZO-1 expression as a function of changes in monolayer confluency, and as a function of endothelial cell and glial cell co-culture. When the use of IF protocol version 2 resulted in the nonspecific binding of the ZO-1, Claudin-5, and PECAM-1 antibodies to the glial cells, the protocol was again modified to IF protocol version 3.
Section 2.1.1 Study 1 - Modification to IF Protocol Version 1

In this experiment, it was necessary to modify IF protocol version 1, and to validate the use of IF protocol version 2 as a means for labeling the ZO-1 protein. The initial changes to IF protocol version 1 included the use of Formalin as opposed to Paraformaldehyde as a fixative, a less concentrated solution of Triton X-100 for permeabilization, the use of Bovine Serum Albumin as a blocking buffer, and changes to the primary and secondary antibody dilution. These changes were in accordance with the protocol established for PECAM-1 staining of hUVECs (Appendix 2.1). Once the IF protocol version 1 had been modified to the IF protocol version 2 (Appendix 2.2), an IF stain was performed to ensure proper labeling of the ZO-1 protein. Because this protocol was modeled after the protocol listed in Appendix 2.1, hUVECs were stained and used as a comparison group, in addition to the BAECs of interest.

To validate the use of IF protocol 2 as a means of labeling the ZO-1 protein, P12 BAECs and P10 hUVECs were cultured to confluency according to the protocol in (Appendix 2.3) on coverslips (Fischer Science, Cat. No. 22CIR-1D) inside of 6-well plates (VWR International, Inc., Cat. No. 3506) and stained using the IF protocol (Appendix 2.2) as shown in Figure 7. Once ready, these coverslips were transferred to a glass slide, and placed on an Olympus BX41 Widefield Fluorescence Microscope (Figure 8). The filter wheel settings and turret positions for the microscope are listed in Table 1. The capture settings used were 100ms exposure for Bisbenzimide (BBI) and 1s 999ms exposure for the secondary antibody.
Figure 7| 6-well plate setups showing cell type and treatment group for IF stain for ZO-1.

Table 1| Filter Wheel Setting for the Widefield Fluorescent Microscope. Filter Wheel and Turret Positions are listed for BBI and IF staining.

<table>
<thead>
<tr>
<th>Filter Wheel 1</th>
<th>BBI</th>
<th>Filter Wheel 2</th>
<th>BBI</th>
<th>Turret Position</th>
<th>BBI</th>
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<tr>
<td></td>
<td>1</td>
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<td>1</td>
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Figure 8| Olympus BX41 Widefield Fluorescence Microscope. Turret is located directly above the stage and below the eyepieces. Filter wheel positions are controlled using the pad in the lower right corner.
The fluorescent images obtained from this experiment are shown below in Figure 9. Experimental groups for this experiment include administration of both the primary and secondary antibodies, while the negative control groups consisted of administration of only the secondary antibody. This ensured that the secondary antibody is only binding to primary antibody. The images obtained show comparable antibody staining in both the BAECs and the hUVECs, which suggested that it was appropriate to use IF protocol version 2 for tight junctional protein labeling. Having determined that IF protocol version 2 was practical for tagging the ZO-1 protein on BAECs, it was then possible to investigate the changes in ZO-1 expression as function of monolayer confluency and as a function of EC and astrocyte co-culture.
Figure 9] Images from IF stain performed on BAECs and HUVECs. Primary+Secondary Antibody groups (A and E) are positive for ZO-1 expression. The negative control groups (C and G) are negative for ZO-1 expression, indicating a lack of nonspecific binding of the antibody. Composite images (D and H) indicate ZO-1 expression localized to the nuclei, seen in the BBI images B and F.

Section 2.1.2 Study 2 - ZO-1 Expression as a function of Confluency

Once the IF protocol version 2 had been verified for primary and secondary antibody labeling of the ZO-1 protein on BAECs, it was necessary to determine if there was upregulation in the expression of this protein when the endothelial cells were more confluent. This increase in ZO-1 protein expression was to be determined through monoculture of BAECs in 6-well plates. Each of the wells in these plates contained a different concentration of cells, therefore allowing imaging and qualitative analysis of the extent to which ZO-1 was being expressed as a function of monolayer confluency.
Section 2.1.2.1  Confluency Experiment 1

In this experiment, P5 BAECs were cultured in 2 T225 flasks according to the protocol in (Appendix 2.3). These cells were then trypsinized, and resuspended in 20 ml of BAEC media. This media was then pipetted into 2 6-well plates in the configuration shown in Figure 10. By pipetting varying volumes of cell suspended media, increasing concentrations of BAECs were plated into the 6-well plates. These differences in concentration would facilitate the culture of monolayers varying in confluency.

White light analysis of the cells preceding the IF stain confirmed increasing confluency of the BAECs (Figure 11). The cells were then stained according to IF protocol version 2 and imaged using the Widefield Fluorescent Microscope. However, issues arising from a faulty pipette aid resulted in non-usable fluorescent images for ZO-1. These images, shown in Figure 11, show not only a lack of primary and secondary antibody staining, but also a lack of stained nuclei from the BBI. This indicated that the stains used weren’t necessarily at fault, but the instrumentation used to administer the stains was not functioning correctly. Therefore, this same experiment was repeated, taking care to ensure that the primary and secondary ZO-1 antibodies were appropriately introduced to each experimental group.

![Distribution of BAECs in two 6-well plates. BAECs were suspended in 20 ml of media and distributed to the positive and negative control groups at a concentration of 5.46E4 cells/cm².](image)

**Figure 10** Distribution of BAECs in two 6-well plates. BAECs were suspended in 20 ml of media and distributed to the positive and negative control groups at a concentration of 5.46E4 cells/cm².
**Figure 11** Results from ZO-1 expression as a function of Confluency experiment. The white light images (A, D, G, J) show increasing confluency as the concentration of cells increases from 1ml to 4ml. IF and BBI staining (middle and right columns) did not work.
Section 2.1.2.2 Confluency Experiment 2

In this study, a second experiment was setup similar to Confluency Experiment 1. P4 BAECs were cultured according to the protocol in (Appendix 2.3) in 1 T225 flask, trypsinized, suspended in 20 ml of media and pipetted into 6-well plates as shown in (Figure 10). Following a 24-hour time period, the coverslips were imaged on a glass slide under the Widefield Fluorescence Microscope using the settings shown in Table 1.

The white light images shown in Figure 12 indicate increasing monolayer confluency as the concentration of BAECs in the 6-well plate increased. Fluorescent imaging also shown in Figure 12 revealed a qualitative increase in ZO-1 expression in the BAECs as the cells became more confluent. The negative control groups consisting of the secondary antibody only, showed no fluorescent staining indicating a lack of nonspecific binding of the primary antibody. Once these changes in ZO-1 expression as a function of monolayer confluency had been verified using IF protocol version 2, it was necessary to evaluate similar changes in ZO-1 expression as a function of EC and astrocyte co-culture.
Figure 12 | Results from second ZO-1 expression as a function of Confluency experiment. The white light images (A, D, G, J) and BBI images (D, H, L, P) show increasing confluency as the concentration of cells increases from 5.32E4 to 2.14E5 cells/cm². IF staining (B, F, J, N) was positive in all groups, and control groups (C, G, K, O) were negative for ZO-1 expression.
Section 2.1.3 Study 3 - ZO-1 Expression as a Function of BAEC and C6 Co-Culture

This experiment investigated the change in ZO-1 expression due to astrocytic influences on endothelial cells in a co-culture setup. The upregulation of tight junction associated cell surface proteins located on endothelial cells has been supported in co-culture situations involving endothelial cells and astrocytes\textsuperscript{27}. At this point in the protocol development, IF stains using IF protocol version 2 have shown positive ZO-1 stains on endothelial cells. The upregulation in the expression of this protein as a function of monolayer confluency has also been established utilizing the current protocol. Because changes in the expression of ZO-1 have been established in endothelial cell-only culture conditions, it is necessary to examine the expression of this protein when the endothelial cells are exposed to astrocytic influences.

The astrocytic cell lines used were rat glioma cells (ATCC, Cat. No. CCL-107). To understand the way with which these cells interact with endothelial cells, it was necessary to investigate each possible orientation during culture. This includes BAECs grown on top of C6s, C6s grown on top of BAECs, and a mixture of the two cell types. Tracking of the location and possible migration each cell type was assessed using Cell Tracker (CT) Dye (Invitrogen, Cat. No. C34552 and Cat. No. C10094). This is a fluorescent nuclear stain, which is incorporated into daughter cells, and enables labeling of an entire colony.

Section 2.1.3.1 Co-Culture Study 1 - BAECs/C6s

This first co-culture experiment consisted of BAECs grown on top of a monolayer of C6 cells, shown in Figure 13. Three vials of P7 BAECs and P12 C6s were cultured according
to the protocol in (Appendix 2.3). Once confluent, BAECs were stained using Cell Tracker Violet, while the C6s were stained using Cell Tracker Red.

Once stained, the C6s were trypsinized and pipetted into their corresponding wells (Figure 14) at a concentration of 3.34E5 cells/cm², and allowed to culture for 24 hours. The BAECs were then trypsinized and pipetted in their corresponding wells at a concentration of 8.89E6 cells/ml and 1 ml per well. The concentrations used in this setup were to mimic the ratio of BAECs to C6s (1:3) that is typically found in vivo²¹.

**Figure 13** Orientation of both cell types in co-culture. BAECs were grown on top of C6s, which were cultured on coverslips inside a well of a 6-well plate. Eye indicates direction from which each sample was imaged using the Widefield Fluorescent Microscope.

**Figure 14** Location of each experimental group for IF stain for ZO-1. This experiment investigated the expression of ZO-1 when BAECs and C6s are co-cultured (BAECs grown on top of C6 cells).
Initial white light analysis (Figure 15) showed a change in endothelial cell morphology in the co-culture groups. Additionally, formation of large clusters of cells in the co-culture and C6 only groups indicated a lack of monolayer formation. Because of this, further IF staining was not possible. Both the formation of these cell clusters and the lack of cellular adherence in a confluent monolayer to the 6-well plates suggest issues regarding the plating of BAECs on top of C6s. Therefore, the following co-culture experiment investigated changes in ZO-1 expression in response to astrocytic influences, when C6s were plated on top of BAECs.

![Figure 15](image)

**Figure 15**| White light images of (A) BAECs only, (B) C6s only, and (C) BAECs cultured on top of C6s. B and C show the low level of adherence to the cell culture flask, and the congregations of cell into groups.

**Section 2.1.3.2 Co-Culture Study 2 - C6s/BAECs**

This experiment investigated the changes in ZO-1 expression as a function of C6 cells cultured on top of BAECs. Initial experimentation growing BAECs on top of C6 cells revealed issues relating to the surface tenacity of either the BAECs to the C6s, or of the C6s to the cell culture flask. Specifically, the C6 cells were unable to adequately adhere to the coverslips when BAECs were cultured on top of the astrocytes. Therefore, subsequent
experimentation investigated a different co-culture orientation to hopefully account for issues with cell adherence.

Similar to the first experiment, this study investigated upregulation of tight junctional proteins in co-culture of C6 cells grown on top of BAECs (Figure 16). P6 BAECs and P9 C6s were cultured according to the protocol in (Appendix 2.3). P6 BAECs were passed 1:3 into a T225 flask according to the protocol in (Appendix 2.3). Once Confluent, Cell Tracker dye was again used to label each cell type, BAECs with CT Red and C6s with CT Violet.

Once labeled with Cell Tracker, BAECs were trypsinized and pipetted into their corresponding wells (Figure 17) and allowed to culture for 24 hours. C6 cells were then trypsinized and added to the remaining wells. The positive control group was labeled with the

Figure 16| Orientation of both cell types in co-culture. C6s were grown on top of BAECs, which were cultured on coverslips inside a well of a 6-well plate. Eye indicates direction from which each samples was imaged using the Widefield Fluorescent Microscope.

Figure 17| Location of each experimental group for IF stain for ZO-1. This experiment investigated the expression of ZO-1 when BAECs and C6s are co-cultured (C6s grown on top of BAECs).
ZO-1 primary and secondary antibody, while the negative control group was only labeled with the secondary antibody (to test for nonspecific binding of the fluorescent secondary).

All experimental groups, including the C6 only group, stained positive for tight junction specific ZO-1 protein (Figure 18). The negative control showed no antibody staining, suggesting a lack of nonspecific binding of the secondary antibody. Location of BAEC and C6 nuclei can be seen in the BBI images, but issues with the CT dye (labeling C6 cells) made it difficult to distinguish between both cell types. The problems with imaging the CT dye were due to light reflecting off of the ceiling and down through the eyepiece, and were accounted for in future stains.

Due to the positive staining observed in the C6 group, it was necessary to determine whether this observed fluorescence was due to nonspecific binding of the ZO-1 primary antibody on the astrocytes, or if this labeling was due to the ZO-1 protein being expressed on the astrocytes. Therefore, the following experiment involved staining for ZO-1, Claudin-5, and PECAM-1 on the ECs and astrocytes both in mono- and co-culture groups to determine if the target proteins for the Claudin-5 and PECAM-1 antibodies were present on the C6 cells.
Figure 18 | IF stain for co-culture experiment. Primary+Secondary staining (A, E, I) is positive for ZO-1 expression. Negative control groups (D, H, L) are negative for ZO-1 expression, indicating a lack of nonspecific antibody binding. CT Violet images (C, G, K) indicate C6 cells, while BBI images (B, F, J) indicate location of both cell types.
Section 2.2 Study 4 - IF stain for ZO-1, Claudin-5, and PECAM1 on EC and C6 mono- and co-culture groups

In this experiment, it was necessary to determine if the positive ZO-1 staining observed in the C6 only group was due to nonspecific binding of the ZO-1 antibody, or if there is ZO-1 protein expression on the astrocytes. Therefore, this study involved the simultaneous staining for ZO-1, Claudin-5, and PECAM-1 on ECs and Astrocytes in mono- and co-culture groups to evaluate if there was also positive Claudin-5 and PECAM-1 staining in the C6 groups. Initially, this experiment was performed using the IF protocol version 2, which was programmed into a DAKO Autostainer. As the use of the autostainer proved unsuccessful as shown in Appendix 2.4, resulting in unusable slides, the same experimental setup was established, and each experimental group was stained by hand according to IF protocol version 2.

The following co-culture test included the same experimental setup as established for the autostainer experiment as listed in Appendix 2.4, however the IF stain was performed by hand. One vial of P6 and P8 BAECs along with 3 vials of P9 C6s were cultured according to the protocol in the (Appendix 2.3). The BAECs were trypsinized and plated onto their respective glass slides inside of 6-well plates for the BAEC only and C6/BAEC groups. Similarly, the C6s were trypsinized and placed into their respective wells for the C6 only and BAEC/C6 groups. After allowing each group of cells to adhere to the coverslips inside of the well for 24 hours, half of the remaining BAECs and C6s were trypsinized and plated onto the BAEC/C6 and C6/BAEC groups, respectively. The second half of the remaining BAECs and C6s were mixed together and placed into their respective wells. The protocol for establishing these experimental groups is available in Appendix 2.6, and the location of each of these
groups is shown in Figure 19. All 48 slides were stained using IF protocol version 2 and imaged on the Multiphoton Laser Scanning Microscope using the 40x oil objective.

**Figure 19** Location of each experimental group for IF stain for ZO-1, PECAM-1, and Claudin-5. This experiment investigated the expression of these three cell surface proteins when BAECs and C6s are co-cultured (BAECs grown on top of C6 cells, C6s grown on top of BAECs, and a mixture of the two cell types).
Graphs of the brightness values for each of these experimental groups can be found in (Appendix 2.7). Again, the C6 only groups were positive not only for ZO-1, but also exhibited fluorescence for the Claudin-5 and PECAM-1 antibodies, suggesting that there were most likely issues with the primary antibody binding to areas of the cell other than its intended protein target. Therefore, investigations into alternative blocking buffers were necessary to ensure that the IF protocol is allowing for accurate and specific protein labeling.

Section 2.3 Study 5 - Blocking Buffer Experimentation

To account for the issues associated with nonspecific binding resulting in labeling of tight junctional proteins on C6 cells, three different blocking buffers were compared. This version of the IF protocol, IF protocol version 3 shown in Appendix 2.8, included the use of Bovine Serum Albumin (BSA) (Invitrogen, Cat. No. 15561-020), 10% Normal Goat Serum (NGS) (Invitrogen, Cat. No. 50-197Z), or Normal Donkey Serum (NDS) (Calbiochem, Cat. No. 655460) as substitutions for the previous blocking buffer used in IF protocol version 2. IF protocol version 3 was then applied to the experimental groups as shown in Figure 19.

Section 2.3.1 Claudin-5 stain on C6s alone

To determine the correct blocking buffer to incorporate into the IF staining protocol, BSA, NGS, and NDS were used to block for nonspecific binding on C6 cells. P12 C6s were cultured according to the protocol in (Appendix 2.3) and stained according to Figure 20. The BSA groups were positive for Claudin-5 expression on C6s, while both the NGS and NDS groups did not show any fluorescent labeling (Figure 21). As there was some observable fluorescence in the NGS group, NDS replaced BSA as the blocking buffer in the IF protocol version 3.
Figure 20 | Location of each of the positive and negative control groups in the blocking buffer experiment using C6 cells.

Figure 21 | IF stain on C6 cells using three different blocking buffers: BSA, NGS, and NDS. Primary+Secondary antibody images (A, E, I) indicate positive ZO-1 expression in BSA group, and negative expression in NGS and NDS groups. Negative control groups (C, G, K) indicate a lack of nonspecific binding of the antibody. Cell nuclei locations are seen in BBI images (B, F, J), and composite images (D, H, L) indicate protein expression localized to cell membranes.
Section 2.3.2 Final Proof of Concept IF Stain

Finally, it was necessary to test the final IF protocol version 3 (Appendix 2.8) on a set of experimental groups including BAECs alone, C6s alone, and a group of BAECs mixed with C6s. This ensured that the protocol would stain for tight junctional-specific proteins only, while disallowing for nonspecific binding of the primary antibody. P9 BAECs and P12 C6s were cultured according to the protocol in Appendix 2.3. Once reaching confluency, the cells were placed in their respective wells (Figure 22) and stained using the protocol in Appendix 2.6 and imaged on the Multiphoton Laser Scanning Microscope.

The BAEC only group stained positive for Claudin-5 expression, while the C6 only group did not express Claudin-5, suggesting that NDS was sufficient to block for nonspecific binding of the primary antibody (Figure 23). Additionally, the Mix group qualitatively showed an increase in brightness for the Claudin-5 antibody. The negative control group supported the lack of nonspecific binding of the secondary antibody.

![Figure 22](image)

Figure 22| Location of each of the positive and negative control groups in the final IF staining experiment using monocultures and co-cultures of BAECs and C6s.
**Figure 23** IF stain showing Claudin-5 stain of BAECs only (A-D), C6s only (E-H), and Mix groups (I-L). Primary+Secondary antibody groups (A, E, I) show positive Claudin-5 expression in BAEC and Mix groups, and negative expression in the C6 group. The negative control groups (C, G, K) indicate a lack of nonspecific binding of the antibody. Cell nuclei location can be seen in the BBI images (B, F, J), and the composite images (D, H, L) indicate localization of protein expression to each cell membrane.

This data suggests that the following IF protocol for tight junctional protein staining is appropriate to provide specific protein labeling while inhibiting nonspecific binding of either the primary or the secondary antibody.
Final IF Protocol for Tight Junctional Protein Staining

1. Remove cell culture media and fix cells in 10% Formalin for 15 minutes.
2. 3x PBS washes for 5 minutes each.
3. Permeabilize cells in 0.2% Triton X-100
4. 3x PBS washes for 5 minutes each.
5. Block for non-specific binding using 3% Normal Donkey Serum (NDS)
6. Incubate in Primary Antibody and 3% NDS for 24 hours.
7. 3x PBS washes for 5 minutes each.
   ** Steps 8-12 are LIGHT SENSITIVE**
8. Incubate in secondary antibody and 3% NDS for 50 minutes.
9. 3x PBS washes for 5 minutes each.
10. Counter stain using Bisbenzimide for 15 minutes.
11. 1x PBS wash for 5 minutes.
12. Remove coverslips from each well, mount to glass slides using mounting medium, and allow to dry for 24 hours.

Section 2.4 Results and Discussion

This chapter focused on the development of a protocol for immunohistochemical staining of tight junctional proteins on endothelial cells. This included investigations into the expression of these proteins as a function of confluency, and also as a function co-culture with glial cells. Completion of this first aim has resulted in establishment of a robust protocol for IF staining of endothelial cells for the ZO-1, Claudin-5, and PECAM-1 proteins. Use of this protocol in our lab is critical to the development of our working knowledge of static and dynamic BBB modeling.
IF protocol version 1 was modified with elements from a similar protocol for primary and secondary antibody labeling of PECAM-1 on hUVECs. This hybrid staining protocol, IF protocol version 2, was then tested by staining for ZO-1 on BAECs and hUVECs. Positive ZO-1 expression and usable images on the fluorescent microscope supported the further use of this protocol in staining for tight junctional proteins.

The next set of experiments involved the use of IF protocol version 2 to determine the expression of ZO-1 as a function of endothelial cell confluency. Evaluations of the results from this study revealed an increase in the relative brightness of each image (positively correlated with protein expression). Because IF protocol version 2 was able to stain for these proteins and support the increase in protein expression as a function of confluency, it was then necessary to determine changes in ZO-1 expression in the presence of glial cells. It is also important to note, that the use of IF protocol version 2 in this experiment did result in fluorescence observed in the C6 only group, suggesting either nonspecific binding, or the expression of ZO-1 on the C6 cells. Because the presence of the ZO-1 protein has been described in astrocytes\textsuperscript{92}, it was necessary to determine if the observed fluorescence was in fact due to nonspecific binding. Therefore, the following set of experiments involved the use of Claudin-5 and PECAM-1 primary antibodies in addition to the ZO-1 antibody, to determine if Claudin-5 and PECAM-1 were also being labeled on the C6 cells.

The next set of experiments involved IF staining of co-cultures of BAECs and C6 cells again using IF protocol version 2. These investigations were to determine how glial cells interact with endothelial cells to change the expression of certain tight junctional proteins, and to determine if Claudin-5 and PECAM-1 were also being labeled on the C6 cells. Specifically, these experimental groups were stained for ZO-1, PECAM-1, and Claudin-5 proteins. IF
protocol version 2 was used in these tests to determine changes in the relative brightness (or expression of these tight junctional proteins) when glial cells interact with the endothelial cells.

Each group was manually stained with either primary+secondary or secondary only for ZO-1, PECAM-1, and Claudin-5. These groups were then mounted onto glass slides and imaged using the multiphoton laser scanning microscope. All of the groups stained for ZO-1 showed statistically significant increases in brightness over the negative control groups, indicating successful inhibition of nonspecific binding of the secondary antibody. Additionally, the BAEC on top of C6 group showed a statistically significant increase in brightness over the BAEC only group. Although not statistically significant, there was an overall trend toward increases in brightness in the co-culture groups.

Groups stained for Claudin-5 exhibited similar characteristics. Statistically significant differences were observed between the positive and negative control groups, indicating a lack of nonspecific binding of the secondary antibody. There was a general trend toward the co-culture groups showing an increase in brightness. However, the only statistical differences were between the BAEC on top of C6 and the C6 on top of BAEC groups and the BAEC only group.

The final treatment group, stained for PECAM-1, also showed a statistically significant difference between the positive and negative control groups. However, there was no statistical significance in brightness between any of the experimental groups. The data did not even reveal a general trend of changes in protein expression. These results are to be expected, as PECAM-1 is solely an endothelial cell marker. As it is not a tight junctional
protein specific to BBB formation, we should expect to see no changes in brightness as a function of co-culture with glial cells.

The Cell Tracker data for each of the experimental groups revealed little to no migration of the cell types between monolayers. This suggests that tight junction formation is not dependent upon the orientation of the cell types in co-culture. However, in each experimental group and in each antibody stain, it was observed that there was a trend toward a decreased brightness in the Mix group. This could suggest that while the orientation of the two cell types is not important, it is important to keep the two cell types in their distinct monolayers during co-culture.

Overall, the confocal images collected from this experiment indicate an upregulation in tight junctional protein fluorescence when BAECs and C6 cells are co-cultured. Quantitative data regarding differences between the experimental and control groups from this study can be found in Appendix 2.7. This study also revealed that IF protocol version 2 was allowing for nonspecific binding of the primary antibody, effectively invalidating all of the previous results.

Due to the failure of IF protocol version 2 to provide accurate and reliable tight junctional protein staining, it is important to reevaluate the interpretations of the previous studies. While the confluency study suggested that there is an increase in ZO-1 expression as the cell monolayer becomes more confluent, this observed increase in fluorescence could also be the result of increased nonspecific binding. Likewise, the trends observed in the final co-culture experiment describing an increase in tight junctional protein expression when ECs and astrocytes are co-cultured could also be a direct result of increased opportunity for nonspecific binding. Due to the presence of two cell types in culture, there is potentially twice the
opportunity for the primary antibody to nonspecifically bind to areas of both the BAECs and C6 cells. To hopefully rectify this issue, additional experimentation with different blocking buffers to include in the staining protocol was investigated

Experimentation with blocking buffers for the IF protocol involved the use of BSA, NGS, and NDS experimental groups. Confocal imaging revealed that NDS was the most appropriate buffer to use, and was successful at blocking for nonspecific binding of the primary antibody on C6 cells. To further support this conclusion, a final stain using IF protocol version 3 modified with NDS was performed on mono- and co-cultures of BAECs and C6s. This study supported the use of NDS as a blocking buffer in the staining protocol, and is sufficient to inhibit nonspecific binding.

Through these studies, a final protocol was developed for IF staining of tight junctional proteins on mono- and co-cultures of endothelial and glial cells (Appendix 2.8). This protocol can be used to verify the formation of tight junctional proteins in bench top modeling of the BBB.
Chapter 3 –Assessment of the Permeability across Monolayers of Endothelial and Glial Cells

Section 3.0 Introduction

Following the establishment of a protocol to effectively stain for and image the formation of tight junctions, the second aim of this thesis was to perform a functional assessment of the in-vitro BBB cell cultures. These functional assessment experiments evaluated the permeability of two distinct molecular weight FITC Dextrans. These static studies incorporated BAECs and C6 cells cultured on 0.4 µm microporous transwell inserts (Corning, Cat. No. 3401), allowing the partition of two separate chambers to simulate the luminal and abluminal sides of the capillaries in the brain (Figure 24).

Figure 24| - Diagram of transwell insert showing the upper and lower compartment along with the permeable membrane. C6 cells are cultured on the underside of the membrane while the BAECs are cultured on the top of the membrane.93
Transwell inserts are typically used to assess permeability of various known compounds across a monolayer of cells\textsuperscript{25,39,73,90,94-100}. This cell layer can be established in a monoculture, co-culture, and tri-culture configuration\textsuperscript{25}, such that the endothelial cells are cultured on the top portion of the permeable membrane, astrocytes or pericytes cultured on the underside of the membrane and/or on the bottom of the well. These studies will typically involve the assessment of concentration of a known molecular weight molecule (such as Dextran) as it passes through the membrane. Radiolabeled isotopes can also be employed to determine permeability across the transwell membrane, but are less common. Regardless of the compound used, it is possible to determine the change in concentration of the compound in the media in the lower compartment in relation to the initial concentration in the media in the upper compartment to determine a permeability coefficient for the established cell monolayer. Therefore, the work in this chapter aimed to use the transwell insert model to assess permeability across a dual layer of BAECs and C6 cells, to assess the formation of tight junctions.

Section 3.1 Methods

Initial permeability assessments of the transwell inserts were performed to obtain a baseline permeability coefficient. This coefficient describes the transport of both molecular weight Dextrans across a microporous membrane not seeded with either cell type. This information can then be compared to experimental data regarding inserts seeded with cells as a method of accounting for the obstruction in diffusion due to the membrane itself.

Diffusion of each Dextran across the insert was assessed as concentration (in µl) as a function of time. These changes in concentration over a given time period allow for the calculation of the permeability coefficient (in cm/s) of the cultured monolayer in response to
different sized compounds. The variables shown in Eq. 3.0, include \( \frac{dQ}{dt} \), which refers to the amount of compound transported per minute (\( \mu \text{g/min} \)), \( A \) is the surface area of the microporous membrane (\( \text{cm}^2 \)), \( C_o \) is the initial concentration of the compound (\( \mu \text{g/ml} \)), and 60 is a conversion factor from minutes to seconds.

\[
P_{\text{app}} = \frac{\frac{dQ}{dt} \cdot \frac{1}{A \cdot C_o \cdot 60}}{\text{(3.0)}}
\]

Section 3.1.1 Study 1 - Permeability Assessment of Transwell Inserts without Cells

This preliminary experiment involved the use of 12 inserts, arranged according to the time points and Dextrans as shown in Figure 25. The transwell inserts (Figure 26) were first precoated in the incubator overnight with a 1:1 mixture of BAEC and C6 cell culture media (1.5 ml in basal chamber and 0.5 ml in the apical chamber). Following this incubation period, the cell culture media was aspirated from each upper compartment and replaced with 500 \( \mu \text{l} \) of Dextran.
**Figure 25**| Location of time points in a 12-well plate for the permeability study of the 4 and 250 kDa Dextrans through transwell inserts not seeded with cells.

At each time point shown in Figure 25, a 500 µl sample was pipetted from the abluminal compartment, placed in a quartz cuvette, and imaged using a Fluorometer (Jasco FP-6500 Spectrofluorometer). A maximum intensity value (in arbitrary brightness units) was then generated for each sample.

**Figure 26**| Transwell Permeable Supports used for functional permeability assessment of cultured BBB. This insert is placed inside a well in a 6-well plate, effectively creating distinct upper and lower compartments representing the luminal and abluminal surfaces of the capillaries in the brain, respectively.
Brightness data describing the working solutions and maximum basal chamber fluorescence of the 4 and 250 kDa Dextran were also collected (See Figure 27). As each Dextran has a distinct maximum fluorescence, it was necessary to calculate a brightness coefficient (brightness per unit volume) for each Dextran (Eq. 3.1), where $B_n$ is the normalized brightness in arbitrary brightness units (au), $B_m$ is the maximum brightness (au), and $V$ is the volume analyzed in the fluorometer ($\mu l$). The collected maximum intensity values were then multiplied by this brightness coefficient to normalize the data.

$$B_n = \frac{B_m}{V}$$ (3.1)

**Figure 27** Brightness values for 4 and 250 kDa FITC Dextran in the quartz cuvette Spectrofluorometer. The undiluted Dextran shows the maximum fluorescence of the working solution, while the diluted groups show the maximum fluorescence that can be expected following each permeability study. (Maximum possible fluorescence in basal chamber is a 1:3 dilution of Dextran in media).
Figure 28] Fluorescence values for the 4 and 250 kDa FITC Dextran. These numbers are not normalized to the brightness per unit volume value assigned to each Dextran.

The raw brightness values are displayed in Figure 28, showing the difference in maximum fluorescence between 4 and 250 kDa Dextran. The normalized data, which provides a more accurate interpretation of the changes in abluminal Dextran concentration over time, are shown in Figure 29. This data shows an increase in the concentration of both compounds over a 25-minute time period. More importantly, this data reveals increased diffusion of the smaller molecular weight Dextran through the porous membrane.
Figure 29: Fluorescence values for the 4 and 250 kDa FITC Dextran. These numbers are normalized to the brightness per unit volume value assigned to each Dextran.

Section 3.1.2 Study 2 - Permeability Assessment of Transwell Inserts with Cells

Once the control data describing the diffusion of both the 4 and 250 kDa Dextran through the unseeded transwell inserts was collected, experimentation regarding hindered diffusion through monolayers of BAECs and C6 cells was assessed. This set of experimentation involved the use of control groups, including BAECs only and C6s only, to show changes in permeability across the established monolayer when both cell types are cultured separately. The orientation of the three experimental groups is shown in Figure 30.
Figure 30| Cross-section of the permeable inserts showing the orientation of the three experimental groups for the 4 kDa Dextran permeability assessment on seeded transwell inserts.

Section 3.1.2.1 Permeability of 4 kDa FITC Dextran

This set of experimentation evaluated the diffusion of 4 kDa Dextran across the cell culture layers. Three replicates of the experimental setup shown in Figure 31 were used in this experiment. All 36 inserts were precoated in the incubator overnight with a 1:1 mixture of BAEC and C6 cell culture media. To facilitate adhesion of the C6 cells to underside of the membrane, 24 inserts were inverted and placed into individual wells in a 6-well plate. P13 C6 cells were cultured according to the protocol in Appendix 2.3, and passed onto the bottom of the inserts (See Figure 32). Uncovered C6 cells were allowed to settle onto the inserts for 60 minutes in the laminar flow hood. The lid for the 6-well plate was then replaced and the cells cultured in the incubator for an additional 60 minutes. Once sufficiently adhered to the bottom of the membrane, the inserts were inverted again and placed back into their respective 12-well plates, and allowed to culture for 24 hours.
Figure 31| Experimental setup for the 4 kDa permeability study of seeded transwell inserts, showing each control group and each time point.

Figure 32| Inverted orientation of transwell inserts in 6-well plate following passage of C6 cells onto the bottom of the membrane.

P5 BAECs were cultured according to the protocol in Appendix 2.3, and then passed onto the upper compartment of 24 of the 36 inserts. All 3 sets of inserts were then allowed to culture in the incubator for an additional 48 hours to ensure adequate monolayer confluency.
and sufficient interaction between the ECs and the glial cells in the Mix groups. Following this culture period, the cell culture media in the luminal compartment of the inserts was aspirated and replaced with 500 µl of 4 kDa Dextran. A 250 µl sample was taken from the abluminal compartment of each well at the time points designated in Figure 31.

Due to the unavailability of the fluorometer used in the first permeability experiment, each sample was pipetted into a foil-wrapped conical and stored in the refrigerator at the designated time point. Fluorescence analysis for this set of experiments was performed on a Microplate Spectrofluorometer (SPECTRAmax GEMINI XS Microplate Spectrofluorometer). This machine required the use of an ELISA plate, and allowed simultaneous analysis of the maximum brightness for each of the 36 samples. The use of a different fluorometer in this set of experiments necessitated the reevaluation of the intensity values for the working solutions of Dextran along with the maximum projected intensity of the diluted Dextran (1:3 dilution of Dextran in cell culture media). The results of this analysis, shown in Figure 33, revealed a 5 and 3 fold increase in the intensity of the 4 and 250 kDa Dextrans, respectively. To make legitimate comparisons between the first set of experimentation and this set, all calculated brightness values shown in Figure 34 were divided by their respective fold increase in maximum intensity. Additionally, it was necessary to account for the 3 week time period that passed between collecting the samples from lower compartment and when these samples were analyzed on the Spectrofluorometer. As per the manufacturer’s suggested storage requirements, each sample was stored in a separate foil-wrapped conical inside the refrigerator. Following these guidelines for extended storage, the supplier of the Dextran said that there should be no issues with photobleaching of the fluorophores if the samples were stored for less than 1 month.
Figure 33: Brightness values for 4 and 250 kDa FITC Dextran in the ELISA plate Spectrofluorometer. The undiluted Dextran shows the maximum fluorescence of the working solution, while the diluted groups show the maximum fluorescence that can be expected following each permeability study. (Maximum possible fluorescence in basal chamber is a 1:3 dilution of Dextran in media).

Figure 34: Change in concentration of 4 kDa Dextran in the abluminal compartment for C6 only, BAEC only, and Mix groups. No statistical significance was supported between either the time points or the experimental groups. (Note: these values have been normalized by their fold increase over the maximum intensity value for the 4 kDa Dextran determined in the initial permeability experiment). (Note: concentration values for the “No Cells” groups at the 15 and 20 minute time points exceeded the maximum y-axis value).
Section 3.1.2.2  Permeability of 250 kDa FITC Dextran

The next set of experimentation was meant to evaluate the diffusion of the 250 kDa Dextran across the cell culture layers. Three replicates of the experimental setup shown in Figure 35 were used in this experiment. Methods for transwell insert preparation were the same as in the 4 kDa study, replacing only the 4 kDa Dextran with the 250 kDa Dextran.

![Diagram](image)

**Figure 35** Same experimental setup as the 4 kDa study, except the 250 kDa Dextran was used to assess monolayer permeability.

P10/12 BAECs were cultured according to the protocol in Appendix 2.3, and then passed onto the luminal surface of 24 of the 36 inserts (as shown in Figure 35). All 3 sets of inserts were then allowed to culture in the incubator for an additional 48 hours to ensure proper adequate monolayer confluency and sufficient interaction between the ECs and the glial cells in the Mix groups. Following this culture period, the cell culture media in the luminal compartment of the inserts was aspirated and replaced with 500 µl of 250 kDa Dextran. A 250 µl sample was taken from the abluminal compartment of each well at the time points designated in Figure 35.
Each sample taken was immediately pipetted into a well in the ELISA plate, and analyzed for maximum brightness on the Microplate Spectrofluorometer. The results of the experimentation with the 250 kDa Dextran are shown in Figure 36. Each of the values presented have been normalized by their percent increase over the data collected using the quartz cuvette fluorometer. It is also important to note that these samples did not sit in the refrigerator before being analyzed on the Spectrofluorometer. However, after speaking with the supplier of the Dextran, there should be no issues regarding the comparison of these samples with those from the previous 4 kDa experiment, which did sit in the refrigerator.

**Figure 36** Change in concentration of 250 kDa Dextran in the abluminal compartment for C6 only, BAEC only, and Mix groups. No statistical significance was supported between either the time points or the experimental groups. (Note: these values have been normalized by their fold increase over the maximum intensity value for the 250 kDa Dextran determined in the initial permeability experiment).
Section 3.1.2.3  Calculation of Permeability Coefficients for Transwell Insert Studies

These experiments involving the 4 and 250 kDa Dextrans provided data regarding the permeability of each compound across the membrane of the transwell inserts as a function of time. This information, which was collected as arbitrary units of brightness, was normalized by the brightness of each fluorophore per unit volume (au/µl). To determine the apparent permeability coefficient using Eq. (3.0), the normalized transport rate of each dextran in au/µl was converted to µg/min using Eq. (3.2), and then input into the $P_{\text{app}}$ equation (simplified values and units are shown in Eq. (3.3)).

$$\left(\frac{dQ}{dt}\right) \left(\frac{100 \, \mu g}{1 \, ml}\right) \left(\frac{1 \, ml}{100 \, \mu l}\right) = \mu g/\text{min}$$  \hspace{1cm} 3.2

3.3

The mean values for each time point in each experimental group were averaged as a transport rate per minute and input into Eq. (3.2) to determine the changes in permeability. The values shown in Table 3 indicate a decrease in the permeability coefficient due to the presence of cells cultured on the inserts, and also reflect a significant difference between the transport rates of each Dextran.
Table 3 | Diffusion of 4 and 250 kDa Dextran across transwell inserts

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>4 kDa</th>
<th>250 kDa</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Cells</td>
<td>126 ± 25.6 E-6</td>
<td>30.7 ± 8.17 E-6</td>
</tr>
<tr>
<td>C6 only</td>
<td>41.7 ± 16.5 E-6</td>
<td>3.38 ± 1.14 E-6</td>
</tr>
<tr>
<td>BAEC only</td>
<td>37.3 ± 17.1 E-6</td>
<td>2.62 ± 0.87 E-6</td>
</tr>
<tr>
<td>Mix</td>
<td>30.2 ± 9.82 E-6</td>
<td>3.04 ± 1.14 E-6</td>
</tr>
</tbody>
</table>

Section 3.2  Discussion and Conclusions

This chapter analyzed the functional attributes of the BBB cell types in static culture, through determination of the permeability of two distinct molecular weighted Dextrans. This experimentation provided an alternative method for assessment of the extent to which tight junctional proteins are being expressed. More importantly, this data can be used to infer the extent to which these tight junctional proteins are able to interact and inhibit paracellular transport of different sized compounds across the artificial BBB. However, it is also important to realize that the decreases in permeability observed could also be due to the BAECs and C6s physically clogging the pores, thereby inhibiting the amount of Dextran able to pass through the membrane.

Initial experimentation with these transwell inserts was without cells, and aided in determination of the permeability coefficient of the microporous membranes themselves. Diffusion through non-seeded inserts of both the 4 and 250 kDa Dextran over a time period of 25 minutes was then calculated using maximum brightness values obtained using a Spectrofluorometer. This information helped to provide a baseline diffusion coefficient across the membrane prior to experimentation involving cells.
Further experimentation involved the seeding of C6 cells only, BAECs only, and a Mix group onto the inserts. The concentration data for 4 and 250 kDa Dextrans was collected using a Microplate Spectrofluorometer (distinct from the previous fluorometer used), and as such, the data was normalized by the percent increase in maximum brightness over the first round of experimentation. These maximum brightness values for each experimental group at each time point were then used to quantify the permeability coefficients of the transwell inserts seeded with BAECs only, C6s only, or a co-culture of BAECs and C6s.

The data describing the 4 kDa Dextran is readily compared to values established in the primary literature\textsuperscript{24}, which have established a $P_{app}$ of $93\pm5$ E-6 cm/s for an insert not seeded with cells, $2.7\pm0.2$ E-6 cm/s for an insert seeded with endothelial cells, and $1.5\pm0.7$ E-6 cm/s for an insert seeded with both endothelial cells and astrocytes\textsuperscript{24}. As evident in table 3, data collected in the previous experiments is larger than the primary literature values by close to 30 cm/s in each experimental group, but remains in the same order of magnitude. Again, the large standard error surrounding each of the values collected in this research (when compared to the values established in the primary literature)\textsuperscript{24} suggest that a large enough sample size was not evaluated to obtain consistently significant values of the permeability coefficient.

It is also important to discuss that the membranes from the transwell inserts used in the previous experiments were not imaged or analyzed for cell monolayer confluency. This is critical to mention because if there is not a confluent monolayer of ECs and/or astrocytes, it would be much more simple for both the 4 and 250 kDa dextrans to pass from the top to the bottom compartments of the insert. Confirmation of monolayer confluency can be achieved through analysis of white light images of the transwell insert membrane. These images could then be analyzed using imageJ to determine the percent of surface area covered by cells. This
analysis would provide evidence of monolayer confluency, and further support the data
suggesting an inhibition in permeability due to the presence of the ECs and astrocytes.

While this data does not support a significant difference between experimental groups,
it does lay the groundwork for further experimentation involving regulated diffusion across a
layer of cells. Specifically, this work aided in the development of a permeability testing
protocol (provided in Appendix 2.9) that can be applied to future experimentation involving
dynamic BBB models.
Chapter 4 – Discussion and Conclusion

Section 4.0 Overview

The experimentation involving IF protocol development along with the permeability assessments have created a solid foundation for BBB research in the Tissue Engineering Lab at Cal Poly. The final staining protocol that was developed provides the ability to visually assess the development of the BBB in-vitro through fluorescently labeling tight junctional proteins. This allows for assessment of BBB characteristics not only in static culture conditions, but also in a dynamic environment such as a perfusion bioreactor system. Additionally, the permeability studies provided the first functional assessment of the BBB models established in this lab. These experiments provided the initial look at diffusion across a monolayer of endothelial cells, astrocytes, and co-culture of ECs and astrocytes. This data can be used to speculate on barrier formation in a tubular scaffold, and the way with which these cells will behave in a more dynamic environment, assuming that the static characteristics translate well to a dynamic system. Overall, these experiments have provided groundwork for the development of a dynamic model of the BBB in a custom perfusion bioreactor system.

Section 4.1 IF Protocol Development

The first requirements of assessing the establishment of the BBB in-vitro was the development of an Immunofluorescence protocol that allowed for discrete staining of tight junctional proteins. Through labeling of these BBB specific proteins, it is possible to assess not only the formation of the BBB in culture, but also changes in the extent to which this barrier is formed determined by the volume of cells expressing these tight junctional proteins.
It was important to investigate the expression of the ZO-1 and Claudin-5 proteins because this information can be directly correlated with previously established values found in literature\textsuperscript{18,25,65,101,102}. Because establishment of tight junctional proteins and the ability to label these proteins is so vital to the establishment of an \textit{in-vitro} model of the BBB, the development of an adequate IF protocol to stain for these tight junctional proteins was the first priority of this research.

IF Protocol version 1 (Appendix 2.0) had proven to be unsuccessful at staining for tight junctional proteins. Therefore, elements from a separate IF protocol (Appendix 2.1) that had been developed for labeling PECAM-1 proteins on hUVECs were incorporated into a hybrid IF protocol (IF Protocol version 2) for BBB research (Appendix 2.2). The success of the stain for ZO-1 on BAECs and hUVECs indicated that it was appropriate to continue experimentation involving tight junctional protein labeling in static culture conditions.

While IF protocol version 2 was sufficient to show the presence of tight junctional proteins in static culture, it was not clear whether this protocol would be able to show upregulation of protein expression as the endothelial cells formed a more confluent monolayer. The next set of experimentation investigated whether tight junctional protein expression would be upregulated as adjacent endothelial cells came into closer contact in higher confluency culture environments.

Qualitative analysis of the images obtained from IF staining of BAECs showed an upregulation in the expression of ZO-1 in a monoculture of BAECs as the monolayer became more confluent. This supports the idea that tight junctional proteins will be expressed in larger amounts on endothelial cells that are in close contact with each other.
The confluency experiments confirmed the use of the IF protocol to determine upregulation of tight junctional protein expression in response to increases in monolayer confluency. It was then necessary to determine other environmental cues that aid in upregulation of tight junctional protein expression. One of the most critical factors to establishment of the BBB is the interaction between the different cell types. Specifically, the interaction between the endothelium and the glial cells plays a crucial role in barrier formation. These experiments investigated the change in tight junctional protein expression as a function of BAEC and C6 co-culture. Additionally, these experiments were divided into three different groups depending upon the orientation of each cell type. The experimental groups were monocultures of BAECs and C6s, BAECs grown on top of C6s, C6s grown on top of BAECs, and a homogeneous mixture of the two cell types.

The first co-culture experiment investigated the changes in ZO-1 expression when BAECs are cultured on top of a layer of C6 cells. The experimental groups included BAECs only, C6s only, and three groups of BAECs on top of C6s. The fluorescence imaging revealed positive ZO-1 expression in BAEC monoculture, and residual staining in the C6 only group. The observed fluorescence in the C6 only group could mean that the IF protocol was allowing for nonspecific binding of the primary antibody, or it could mean that the ZO-1 protein was being expressed on the C6 cells. Therefore, it was necessary to determine if other tight junctional proteins were being labeled on the astrocytes, to either confirm or rule out the issue of nonspecific binding. Additionally, en-face analysis of the co-culture images revealed a slight increase in the brightness of the fluorophores, suggesting an increase in ZO-1 expression. It is important to note that the images of the co-culture group show a moss-like coating of the ZO-1 protein on the layer of cells, possibly indicating a change in cell
morphometry or a limitation in the resolution of the fluorescence microscope. Due to the lack of similar references in literature, it was assumed that these issues relating to the imaging were a function of the limitations of the microscope.

In the following experiment, an IF stain was performed using IF protocol 2 to get a better understanding of the changes in tight junctional protein expression a function of endothelial cell and glial cell co-culture. The critical piece of information gained from this study was the fluorescence observed on monocultures of C6 cells. In every case, C6 cells showed positive IF staining for ZO-1, PECAM-1, and Claudin-5. As each of these proteins is specific to endothelial cells, it was necessary to investigate the reason behind this fluorescence. After some examination, it was decided that fluorescence in C6 monocultures following IF staining of tight junctional proteins was due to nonspecific binding of the primary antibody. This was due to issues associated with the blocking buffer that was part of the current IF protocol. It is also important to note that the occurrence of nonspecific binding of the primary antibody suggests that the previous results are not necessarily indicative of the formation or upregulation of tight junctional proteins. Specifically, the results suggesting an increase in tight junctional protein expression due to both monolayer confluency and co-culture with astrocytes could in fact be due to nonspecific binding. Because the blocking buffer used in IF protocol version 2 was allowing for this nonspecific binding, investigations into a different blocking buffer were necessary.

To correct for this nonspecific binding of the primary antibody, three different blocking buffers were used in the IF protocol and compared for fluorescence. Bovine serum albumin, normal goat serum, and normal donkey serum were used as blocking buffers during an IF stain for Claudin-5 on C6 cells. This stain was performed on C6 cells to investigate the
ability of the blocking buffer to block for nonexistent proteins on C6 cells. Additionally, Claudin-5 was stained in this study, as ZO-1 expression has been observed on non-epithelial cell types\(^9\), and may not be the strongest indicator of endothelial cells.

Once each of the experimental groups was stained using a different blocking buffer, the groups were imaged on the multiphoton laser scanning microscope. These images showed that BSA was unable to block for nonspecific binding of the primary antibody, while NGS and NDS revealed no fluorescence. Further examination of these two blocking buffers led to the replacement of BSA with NDS in the IF protocol. This choice was mainly due to the compatibility of the donkey serum with the donkey anti-rabbit secondary antibody used.

Following the replacement of BSA with NDS in the staining protocol, it was then necessary to perform a co-culture experiment with ECs and glial cells to determine the efficacy of the finalized IF protocol in staining for tight junctional proteins. This study included BAEC and C6 monocultures along with a Mix group. Confocal image analysis revealed positive Claudin-5 expression in the BAEC only and Mix groups. The C6 only group showed no fluorescence in either the positive or negative control groups, indicating that the NDS was sufficient to block for nonspecific binding on both glial cells and ECs.

This final proof of concept stain supports the use of this IF staining protocol for purposes of labeling tight junctional proteins in static mono- and co-cultures. This protocol can subsequently be used to determine the presence of tight junctions not only in these static culture conditions, but also in dynamic models of the BBB. This IF protocol can be used to identify the extent of tight junction formation in \textit{in-vitro} models of the BBB through histological sectioning and staining of the seeded scaffolds.
Section 4.2 Permeability Studies

Following the development of a protocol to image the formation of tight junction proteins, it was necessary to investigate the functional attributes of these junctions. Specifically, permeability of two different molecular weighted Dextrans across monolayers of BAECs and C6 cells was investigated. This data provided information on the changes in membrane permeability due to the presence of BAECs alone, C6s alone, and a co-culture of both BAECs and C6s. To culture these cells, permeable transwell inserts were used to assess transport of 4 kDa and 250 kDa Dextrans.

To gain an accurate understanding of the transport across a monolayer of cells, it was first necessary to understand the transport of a known compound across the membrane on which the cells are grown. This experimentation involved the addition of the 4 and 250 kDa Dextrans to the luminal port of 12 non-seeded transwell inserts. Diffusion of the Dextran was assessed at 5-minute intervals to determine a transport rate per minute. Graphs of the data show a gradual increase in the concentration of Dextran over 25 minutes. More importantly, the data shows a significant difference in permeability between the two different Dextrans, supporting the hypothesis that a larger sized molecular weighted compound will not as readily cross the membrane.

Once the transport rates of both Dextrans across an unseeded permeable insert were established, it was necessary to introduce cells to the system to influence the formation of the BBB. For the experiment involving the 4 kDa Dextran, the data was analyzed using a one-way ANOVA test, due to the comparison of multiple variables across multiple time points. This analysis revealed no statistically significant differences between the 5, 10, 15, and 20-minute time periods for each of the experimental groups. Additionally, there were no significant
differences between each of the groups (C6 only, BAEC only, and Mix) at each time point. Therefore, no inferences can made as to which experimental group resulted in the least diffusion across the transwell insert. However, at each time point, the data shows a trend suggesting that the C6 only group allowed the most Dextran to pass through the insert, while the Mix group allowed the least amount of Dextran through, possibly due to high number of cells that were able to clog the pores of the insert. This trend is true for all time points. Additionally, there is a trend showing an increase in the amount of Dextran present on the abluminal chamber of the transwell insert as time increases from 5 to 20 minutes. This lack of statistical significance (and large standard error bars) coupled with data that trends in the expected manner suggests that the sample size for this experiment was not large enough to provide an adequate representation of the experimental situation.

The same protocol was applied to the 250 kDa Dextran. Data collected from this experiment was also analyzed using a one-way ANOVA test, and yielded similar results. There was no statistically significant difference between any of the 5, 10, 15, and 20-minute time points. Additionally, there was no significant difference between the C6 only, BAEC only, and Mix experimental groups at each time point. Similar to the 4 kDa experiment, the data does show a positive correlation between the concentration of Dextran in the abluminal chamber of the insert and time. The data also shows a trend suggesting that the C6 only group allows the greatest amount of Dextran to pass through the membrane, while the Mix group allowed the least amount to diffuse through. Again, this lack of statistical significance between the time points and between the experimental groups combined with the fact that the data from the 4 kDa experiment showed similar trends, suggests that the lack of statistical significance is due to a deficit in the necessary sample size.
This data was, however, able to provide useful and relevant information regarding the permeability coefficients (Table 3) of the transwell insert both without cells, and in the presence of the three treatment groups. The coefficients shown in Table 3 from Chapter 3, which take into account the initial concentration of the Dextran in the luminal chamber, the surface area of the permeable membrane, and the final concentration of the Dextran in the abluminal chamber, describe the rate at which a given compound is able to diffuse across a permeable membrane. These values collected in the 4 kDa and 250 kDa experiments are comparable to those found in literature.

Section 4.3 Future Work

While this research presents a step forward in our lab’s understanding of modeling the BBB, there is a significant amount of future work that would enhance the data collected here and solidify further research into an in-vitro model of the BBB. The first step would be the enhancement of the imaging performed to detect the formation of tight junctions. As discussed in Chapter 1, ZO-1 and Claudin-5 are only two of the proteins that form the barrier to paracellular diffusion. Therefore, the staining and imaging performed in Chapter 2 would be greatly strengthened through the addition of other primary antibodies tailored to label other BBB membrane-bound proteins. Additionally, it has been suggested that the ZO-1 protein can be found on astrocytes and other non-epithelial cell types. Therefore, while the use of the ZO-1 antibody does suggest the formation of tight junctions in mono- and co-cultures of endothelial cells, overall tight junction formation would better be assessed through the use of one or more additional primary antibodies, such as ZO-2, ZO-3, Claudin-3, Claudin-12, and the Occludin family.
The next future step would include the use of a TEER system to measure the electrical resistance of each experimental group in the permeability studies from Chapter 3. TEER measurement involves the use of small electrodes to induce a current across a cultured membrane. The resistance experienced by this current as a result of EC-EC junctions is then measured, to provide evidence of the “tightness” of each cell junction and overall monolayer confluency. The majority of in-vitro BBB models found in the primary literature will evaluate the TEER value for tissue-engineered construct. Therefore, TEER in these models is well characterized, resulting in a wealth of peer-reviewed data, which provides a wealth of information with which to compare experimental values. This type of analysis was not included in this research due to cost restraints, but would provide a much more in-depth investigation into the ability of our lab on campus to establish a functional model of the BBB.

The use of a TEER measurement system, such as the EVOM2 Epithelial Voltohmmeter, in a static BBB model (such as those found in transwell insert studies) is well characterized, and involves the placement of two electrodes on a cultured monolayer of endothelial cells. A current is then run across the monolayer, and the resistance is calculated. However, the incorporation of a TEER measurement system into a dynamic model of the BBB is much more difficult. To provide reliable data, it would be necessary to determine both axial and transluminal TEER values through a tubular construct. This would most likely involve the use of permanent electrodes incorporated into the bioreactor itself for continuous, real-time TEER monitoring.

Another piece of future work, which directly applies to the data gathered in this research, would be the re-evaluation of the permeability coefficients of the transwell inserts to
the 4 kDa and 250 kDa Dextrans with a much larger sample size. This experimentation will hopefully provide a general affirmation of the data collected in the studies from Chapter 3, and reduce the variability indicative of experiments using too few replicates.

Additionally, it is necessary to determine the inhibition in permeability is due to formation of tight junctions, or if it is due to the cells physically clogging the pores of the transwell insert membrane. This can be accomplished one of two ways: either through RNA sequencing to determine the relative amount of tight junctional proteins that are being expressed, or through the use a control group using non-tight junction forming cells. The RNA sequencing method would follow the same protocol as established in the 4 and 250 kDa Dextran studies, but would then involve the removal of the cells from the transwell membrane to determine RNA expression of ZO-1, Claudin-5, and PECAM-1. This would help to determine if these proteins are being upregulated in the co-culture conditions established on the transwell insert membrane. A more simplistic approach would be the use of a separate cell type, such as 3T3 Fibroblasts, in the same experimental procedure. These fibroblasts would be seeded onto the transwell membrane in the same method as the ECs and C6s were, but would not be expected to form tight junctions. This would allow the comparison between tight junction forming groups and non-tight junction forming groups to determine if the changes in permeability across the insert were due to BBB formation or just clogging of the pores by the cells themselves.

The final piece of future work would include the addition of a shear element to the co-cultures of ECs and astrocytes. This would most likely come in the form of a tissue-engineered blood vessel. The BAECs and C6 cells used in this research would be pressure sodded onto a microporous cylindrical scaffold housed inside of a perfusion bioreactor
system. Specifically, the type of perfusion bioreactor system developed by Amin Mirzaaghaeian\(^{105}\) in addition to the porous PLGA scaffolds developed by Deven Patel\(^{106}\) would enable the culture of such a dynamic *in-vitro* BBB model. Translation from static culture conditions into a dynamic model such as the one developed in Cal Poly’s Tissue Engineering lab would provide not only a robust model of the BBB, but one that enables sectioning, imaging, and functional analysis of the cultured tissue. This is in direct contrast to the use of multiple capillary tubes for scaffolds, as a seen in the CellMax system, which does not facilitate straightforward analysis due to restraints around the scaffold material and overall bioreactor design. The use of the bioreactor system developed at Cal Poly would allow for the formation of a confluent endothelium inside the tubular construct, while also allowing for the interaction of the endothelial cells with astrocytes and the introduction of a shear stress element to the ECs. Additionally, it would be prudent to incorporate a custom TEER measurement system into the dynamic model, to allow for real time monitoring of monolayer confluency and intercellular adhesion. This next step would be one of the final pieces of experimentation to support the formation of a functional model of the BBB in our lab.

**Section 4.4 Conclusion**

The research presented here describes an assessment of the extent to which tight junctions can be formed in static mono- and co-culture situations with ECs and astrocytes, in addition to an assessment of the permeability of cells in culture. Chapter 2 presented the steps used to establish a practical protocol for immunohistochemical staining of cell surface proteins indicative of tight junction formation. This protocol was used to evaluate the expression of ZO-1 and Claudin-5 in mono- and co-cultures of BAECs and C6 cells to
establish a metric for determination of BBB formation in static culture. The next set of experiments in Chapter 3 introduced a more functional assessment of these proteins. These studies investigated the ability of these cells (both separately and in co-culture) to decrease the permeability of known compounds across the transwell insert membrane. In this case, the compounds used were fluorescently labeled 4 kDa and 250 kDa Dextrans. The diffusion of these compounds through the transwell inserts was used to evaluate the permeability coefficient of the cultured BBB.

This research presents a step forward for the Tissue Engineering lab at Cal Poly in terms of its ability to accurately and efficiently model the phenotypic and functional attributes of the BBB. While the efforts put forth in this research provide pertinent information regarding modeling of this barrier, there is still much experimentation to be performed to fully understand and adequately mimic the functional characteristics of the BBB.
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Appendix 2.0 IF Protocol Version 1

1. Fix cells in 4% Paraformaldehyde (PFA) for 30 mins at 4°C.
2. 5 mins. PBS
3. 15 mins. 0.3% Triton X-100 diluted in PBS
4. Rinse slide with PBS, then do a 2x5 min. wash with PBS
5. 60 min. in blocking buffer at room temp.
6. Dilute primary antibody (usually 1:50) in working buffer and add 200µl to each slide. Cover with foil and leave at 4°C overnight.
7. Rinse with PBS, then do 3x10 min. PBS washes
8. Rinse with working buffer
9. Add secondary antibody diluted (usually 1:400) in working buffer for 60 mins. at room temp.
10. Rinse with PBS, then do 3x10 min. PBS washes
11. 1 min. DAPI
12. 5 mins. PBS
13. 5 mins. dH$_2$O.
14. Mount with mounting medium and coverslip

Blocking buffer recipe:
50mM Glycine, 2% donkey serum, 2% BSA
Appendix 2.1  IF Protocol for PECAM-1 stain on hUVECs

1. Remove cell culture media and fix cells in 10% Formalin for 15 minutes.
2. 3x PBS washes for 5 minutes each.
3. Permeabilize cells in 0.2% Triton X-100
4. 3x PBS washes for 5 minutes each.
5. Block for non-specific binding using 6% Bovine Serum Albumin (BSA)
6. Incubate in Primary Antibody and 6% BSA for 50 minutes.
7. 3x PBS washes for 5 minutes each.
8. ** Steps 8-12 are LIGHT SENSITIVE**
9. Incubate in secondary antibody and 6% BSA for 50 minutes.
10. 3x PBS washes for 5 minutes each.
11. Counter stain using Bisbenzimide for 15 minutes.
12. 1x PBS wash for 5 minutes.
13. Remove coverslips from each well, mount to glass slides using mounting medium, and allow to dry for 24 hours.
Appendix 2.2 IF Protocol Version 2

1. Remove cell culture media and fix cells in 10% Formalin for 15 minutes.
2. 3x PBS washes for 5 minutes each.
3. Permeabilize cells in 0.2% Triton X-100
4. 3x PBS washes for 5 minutes each.
5. Block for non-specific binding using 6% Bovine Serum Albumin (BSA)
6. Incubate in Primary Antibody and 6% BSA for 24 hours.
7. 3x PBS washes for 5 minutes each.
   a. **Steps 8-12 are LIGHT SENSITIVE**
8. Incubate in secondary antibody and 6% BSA for 50 minutes.
9. 3x PBS washes for 5 minutes each.
10. Counter stain using Bisbenzimide for 15 minutes.
11. 1x PBS wash for 5 minutes.
12. Remove coverslips from each well, mount to glass slides using mounting medium, and allow to dry for 24 hours.
Appendix 2.3  Cell Culture and Passage Protocol

Cell Information
Type:          Passage No.

Materials
___ 1. Pipettes  ___ 4. Trypsin
___ 2. Pipette Aid  ___ 5. DCF-PBS
___ 3. Cell Culture Media

Cell Culture
___ 1. Precondition the flask by adding 12 ml of cell culture media per 75 cm² of surface area. (1 T75 flask = 75 cm²)
___ 2. Place flask in incubator for 20 mins.
___ 3. Thaw one vial of cells into a T75 flask.
___ 4. Check cell confluency using microscope (pass cells at 80-100% confluency).
___ 6. Rinse the surface of the flask with the cells and break up clumps.
___ 7. Pass cells into new flask(s) and place in incubator. (Note: 1:2 or 1:3 pass maximum for ECs).

Cell Passage
___ 1. Thaw trypsin in water bath.
___ 2. Warm predetermined amount of media in water bath (3 ml of media per 75 cm² of surface area plus x ml for an easy 1:2 or 1:3 separation).
___ 3. Remove old media and rinse with DCF-PBS.
___ 4. Lyse cells using 3 ml of trypsin per 75 cm² of surface area (smack the sides of the flask to encourage the cells to come off the surface).
___ 5. Add equal amount of fresh cell culture media to flask (for ECs, add 2x the amount of trypsin used).

Notes:

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Date          Cell Culture and Passage Protocol          Initials

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91
Appendix 2.4  DAKO Autostainer IF stain for ZO-1, Claudin-5, and PECAM-1 using IF Protocol Version 2

Section 2.0  Autostainer Experimentation

Due to the volume of IF staining necessary to obtain the right sample size, the use of an automated staining machine (Dako, Cat. No. S380031) was considered. This machine allows for a maximum of 48 slides to be stained according to a predetermined protocol, which is programmed in by the user. The protocol that was programmed into the autostainer software was the IF protocol from (Appendix 2.4). This system should allow the simultaneous staining for PECAM-1, Claudin-5, and ZO-1 across all experimental groups (BAECs alone, C6s alone, BAECs grown on top of C6s, C6s grown on top of BAECs, and a Mix group).

Section 2.3.1  PECAM-1 Test Run

Before running the large-scale experiment in the autostainer, it was necessary to ensure that the protocol had been programmed in correctly, and that the system would accurately stain each slide to ensure successful fluorescence imaging. Therefore, an IF stain for PECAM-1 was performed on HUVECs, due to the robust procedure previously established for this stain.

The positive control group showed ZO-1 expression, while the negative control did not. This indicated that the autostainer successfully executed the programmed IF protocol and produced usable images on the widefield fluorescence microscope.
Figure 37: IF stain images from test run on Autostainer. (A) Primary+Secondary antibody stain positive for ZO-1 expression. (B) Negative control group showing lack of ZO-1 expression. (C) BBI imaging showing location of cells.

Section 2.3.2 Co-Culture Experimentation

Due to the success of the test run on the autostainer, the IF protocol (for what?) (Appendix 2.2) was programmed into the software. For this co-culture experiment, both cell types were arranged into monoculture groups and also into the orientations shown in Figure 38. Cell counts used for each of the treatment groups are listed in Table 2. To construct a more complete picture of the formation of BBB tight junctional proteins, an additional stain was added for Claudin-5. The treatment groups, including the positive and negative controls, for each of the ZO-1, PECAM-1, and Claudin-5 primary antibodies are shown in Figure 39. For the groups with C6 cells on the bottom of the BAECs, the C6s were cultured for 24 hours before adding BAECs. The same was performed for the groups with BAECs on the bottom, allowing the ECs to culture for 24 hours before adding the C6s.
Figure 38 | Orientation of cells used in co-culture experimentation (BAECs grown on top of C6 cells, C6s grown on top of BAECs, and Mix groups). The eye indicates direction through which these cells were imaged using the Multiphoton Laser Scanning Microscope.

<table>
<thead>
<tr>
<th>BAECs on top of C6s</th>
<th>C6s on top of BAECs</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>C6</strong></td>
<td><strong>C6</strong></td>
</tr>
<tr>
<td>Count 1</td>
<td>1.07E+06</td>
</tr>
<tr>
<td>Count 2</td>
<td>1.01E+06</td>
</tr>
<tr>
<td><strong>BAEC</strong></td>
<td><strong>BAEC</strong></td>
</tr>
<tr>
<td>Count 1</td>
<td>5.25E+05</td>
</tr>
<tr>
<td>Count 2</td>
<td>6.67E+05</td>
</tr>
<tr>
<td><strong>Mix</strong></td>
<td><strong>Alone</strong></td>
</tr>
<tr>
<td>Count 1</td>
<td>5.27E+06</td>
</tr>
<tr>
<td>Count 2</td>
<td>5.04E+06</td>
</tr>
</tbody>
</table>

Table 2 | Cell counts indicating the concentration of cells added to each well of each experimental group. (Units are in cells/ml)

After a final co-culture period of 24 hours, the coverslip on which each experimental group was cultured was mounted to a glass slide (face up) using mounting adhesive. To prevent the cells from desiccating, PBS was pipetted onto each of the slides after they were mounted inside of the autostainer. Once the system had finished performing the IF stain, the majority of the coverslips were blown off of the slides. This made imaging very difficult, and only images for Claudin-5 staining of the BAEC on top of C6s and the Mix groups was possible (Figure 40).
Figure 39] Location of each experimental group for IF stain for ZO-1, PECAM-1, and Claudin-5. This experiment investigated the expression of these three cell surface proteins when BAECs and C6s are co-cultured (BAECs grown on top of C6 cells, C6s grown on top of BAECs, and a mixture of the two cell types).

The usable slides were imaged (Figure 40) revealing typical staining for the antibody and cell nuclei (both BAECs and C6s). Although no useful data regarding brightness of protein expression was obtained in this experiment, this test did show positive expression of Claudin-5 in co-culture groups for the first time. However, due to
**Figure 40** IF images from initial co-culture experimentation using the autostainer. (A, E) Primary+Secondary antibody groups showing positive ZO-1 expression. (B, F) BBI stain for cell nuclei. (C, G) CT Red stain for BAECs and (D, H) CT Violet stain for C6s.

The issues associated with the current protocol for using the autostainer, the following co-culture experimentations were conducted manually.
Figure D1: The autostainer with all components. 1 is the DI water and buffer carboys, 2 is the waste carboy, 3 is the PC, 4 is the slide rack, and 5 is the reagent rack.

Programming a Protocol

1. Make sure autostainer is plugged in and turn on the computer. After it starts up open the Autostainer software. Login.
Username: a
Password: a

**Figure D2.** Main Menu (Dako Autostainer Handbook, 7).

2. Click ‘Program’. You should now be on the Programming Grid page.

**Figure D3.** Programming Grid page (Dako Autostainer Handbook, 12).

![Protocol Template Design](image)

**Figure D4:** Protocol Template Design page (Dako Autostainer Handbook, 15).

4. Arrange the general order of your protocol on the right box using the options on the right box.
   - The first step should be ‘Switch’ to ensure that all waste goes to the hazardous waste carboy.
   - PBS washes can be programmed with the ‘rinse Buffer’ action.
   - Each step should be followed with a ‘Rinse Buffer’ step to clear the reagent off your samples.
   - Fixing steps should be performed before samples are brought to the autostainer.

5. Click ‘Reagent Volume’ on the top right of the screen and select your desired dispense volume.

6. Click ‘Save’ and name it something meaningful so you do not lose it.

7. Click ‘Use Template’. You should now be back on the Programming Grid page.

8. Click ‘Slides’ on the top left and set how many slides you want to stain.

9. Set up reagents and incubation times. Click ‘Edit Lists’ and select the appropriate reagent category.
Figure D5: Reagent addition menu for reagents and antibodies (Dako Autostainer User, 69 and 71).

10. Fill out the reagent name, abbreviated name, and incubation time. Hit enter.

11. Repeat steps 9 and 10 until all reagents and antibodies are defined.

12. To set the correct reagent to each step of the protocol, click on the gray box of the desired step on the first slide. A list of available reagents will appear. Select your desired reagent. The software will prompt you if you want all slides to have the same reagent. Click yes/no. Note: incubation time is associated with the incubation time as defined in step 10, it must be changed in the ‘Edit Lists’ menus, not on the Programming Grid.
13. Repeat step 12 for each reagent on each slide.

14. Save your protocol. Click ‘Next’ on the bottom of the Programming Grid page. Note the run time, PBS required, and DI water required.

Figure D6: Setting reagents to each step of protocol (Dako Autostainer Handbook, 19).

Figure D7: Reagent Layout Map (Dako Autostainer Handbook, 31).
15. Take a picture or write down the order and volumes of the reagents as they are displayed on the Reagent Layout Map.

![Load Slides](image)

**Figure D8:** Slide Layout Map (Dako Autostainer Handbook, 33).

16. Click ‘Next’. Take a picture or write down the order of the slides as they are displayed on the Slide Layout Map.

17. On the top left of the Slice Layout Map the reagent drop position of for all of the slides can be selected. The drop position can be changed on individual slides as well by clicking on the bottom, middle, or top of the slide. A yellow bar indicates where the reagent will be dropped onto the slice.

![Slide loading orientation](image)

**Figure D9:** Slide loading orientation (Dako Autostainer User, 83).
18. Programming is now complete. Either save and shutdown the computer or prepare reagents and samples.

**Sample and Reagent Preparation**

19. Bring the information from steps 14, 15, and 16 above along with the reagent racks and carboys to wherever you will be preparing the reagents and samples.

20. Prepare tissue samples how you normally would. Perform fixing procedure. Attach tissue to coverslips. Mount coverslip to slide at location where you set the drop location to in step 17, above. Make sure the bottom of the coverslip and top of the glass slide are completely dry before mounting. Allow at least 20 minutes for the mounting adhesive to dry. Label the slides so you know which position to put the sample in inside the autostainer (from step 16). After 20 minutes a small amount of PBS should be placed onto the samples to prevent drying out. Keep the samples wet until the autostainer protocol is started.

21. Make the required amount of PBS as determined in step 14 and put in the buffer carboy. Fill the DI water carboy with the required amount of DI water as determined in step 14. Prepare the reagents and antibodies and put them in the reagent rack in the correct positions (from step 15). Reduce exposure to air and light using the tube caps and tin foil if necessary. Use the following equation for diluting stock solutions to the required concentrations.

\[ C_1V_1 = C_2V_2 \]

**Starting the Staining Run**

22. Bring carboys, reagents, and tissue samples to the autostainer. Turn on the computer, open up your saved protocol, and hit ‘Next’. Load the reagents as outlined by the Reagent Layout Map and place into the correct block in the autostainer. Click ‘Next’. Load the slides as outlined by the Slide Layout Map in
the orientation outlined in Figure C8. Put the slide racks into the autostainer. Put the caps back onto the buffer and DI water carboys, making sure they are attached to the correct inputs. Click ‘Next’.

23. Make sure all of the waste tubes are firmly placed into waste carboys. Make sure that the total volume used in your protocol will not overflow the carboy!

24. Click the ‘Prime Pump (Buffer)’ button and wait until the autostainer completes the priming procedure. Repeat until the buffer flows out of the autostainer dispenser with no air bubbles. This may take several tries. Make sure the tubing is not pinched and that the intakes are immersed in the buffer.

25. Repeat step 23 but with the ‘Prime Pump (Water)’ button (only if your protocol uses DI water).

26. Remove the caps and tin foil from the reagent tubes (turn off lights if you have to), close the lid of the autostainer, and click ‘Start Run’.

27. After the completion of the stain remove your samples and image them at the appropriate wavelengths. Again, avoid light exposure.

28. Pour some DI water into the autostainer sink, clean the metal mesh sink guard if necessary. Clean all reagent tubes, empty the waste carboys, and shut off the computer. Dispose of your samples appropriately.
Appendix 2.6  Protocol for IF Stain for ZO-1, Claudin-5, and PECAM-1 on C6 only, BAEC only, C6/BAEC, BAEC/C6, and Mix Experimental Groups

1. Trypsinize BAECs and C6s and plate onto separate coverslips and allow to culture for 24 hours.

2. Trypsinize BAECs to plate on top of C6s, C6s to plate on top of BAECs, and a group of BAECs and C6s to mix together, and allow to culture for 24 hours.

3. Remove cell culture media and fix cells in 10% Formalin for 15 minutes.

4. 3x PBS washes for 5 minutes each.

5. Permeabilize cells in 0.2% Triton X-100

6. 3x PBS washes for 5 minutes each.

7. Block for non-specific binding using 6% Bovine Serum Albumin (BSA)

8. Incubate in Primary Antibody and 6% BSA for 24 hours.

9. 3x PBS washes for 5 minutes each.

   a. **Steps 8-12 are LIGHT SENSITIVE**

10. Incubate in secondary antibody and 6% BSA for 50 minutes.

11. 3x PBS washes for 5 minutes each.

12. Counter stain using Bisbenzimide for 15 minutes.

13. 1x PBS wash for 5 minutes.

14. Remove coverslips from each well, mount to glass slides using mounting medium, and allow to dry for 24 hours.
Appendix 2.7 Results from IF Stain for ZO-1, Claudin-5, and PECAM-1 on C6 only, BAEC only, C6/BAEC, BAEC/C6, and Mix Experimental Groups

The BAEC only groups were positive for PECAM-1, Claudin-5, and ZO-1 expression as indicated by the primary+secondary groups (Figure 41). CT Red images showed no significant staining, which is expected as only C6 cells were stained with CT Red. Composite imaging of the antibody stains and BBI indicate localization of protein expression to the cell membrane. The negative control groups shown in Figure 42 did not show protein labeling, indicating a lack of nonspecific binding.

The C6 only groups were also positive for PECAM-1, Claudin-5, and ZO-1 expression as indicated by the primary+secondary groups (Figure 43). Cell Tracker images were positive for C6 cells, which is expected, as this is a monoculture of C6 cells. Composite images reveal localization of protein expression to the cell membrane. The expression of these EC-specific proteins suggests nonspecific binding of either the primary or the secondary antibody. We can assume that the primary antibody is nonspecifically binding, as the negative control groups shown in Figure 44 indicate a lack of nonspecific binding on the part of the secondary antibody.

The BAECs grown on top of C6s group also showed expression of PECAM-1, but showed a trend toward increased fluorescence of the ZO-1 and Claudin-5 antibodies (Figure 45). Because these slides were imaged from underneath (Figure 19), the CT images indicate the presence of C6 cells. Again, composite imaging of the protein expression and nuclear staining indicates localization of protein expression to the cell membrane. The negative control groups shown in Figure 46 indicate a lack of nonspecific binding.
The C6/BAEC group also indicated PECAM-1 expression and an upregulation in the expression of Claudin-5 and ZO-1 (Figure 47). This group showed less CT fluorescence, as would be expected since these slides were imaged from the bottom. The composite images of primary+secondary and BBI groups show protein expression localized to the membrane. Again, the negative control groups shown in Figure 48 indicate a lack of nonspecific binding.

The final group consisting of BAECs and C6s mixed together were also positive for PECAM-1, and showed an upregulation in expression of Claudin-5 and ZO-1 (Figure 49). As the C6s were mixed in with the BAECs, the CT images revealed the presence of C6 cells. Cell surface proteins are localized to the membranes, as confirmed by the composite images. The negative control groups shown in Figure 50 indicate a lack of nonspecific binding.
**Figure 41** IF staining for BAEC only positive control groups. (A-D) indicate PECAM-1 primary antibody group, (E-H) indicate Claudin-5 primary antibody group, and (I-L) indicate ZO-1 primary antibody group. Positive control groups (A, E, I) indicate positive protein expression (B, F, J) show cell location, and composite images are shown in D, H, L. Location of C6 cell nuclei seen in CT images (C, G, K).
Figure 42| IF stain images for BAEC only groups. Negative control groups (A, D, G) indicate a lack of nonspecific binding of the antibody. BBI images (B, E, H) reveal nuclei of both cell types, while CT images (C, F, I) show location of C6 cells.
Figure 43 | IF staining for C6 only experimental groups. (A-D) indicate PECAM-1 primary antibody group, (E-H) indicate Claudin-5 primary antibody group, and (I-L) indicate ZO-1 primary antibody group. Positive control groups (A, E, I) indicate positive protein expression (B, F, J) show cell location, and composite images are shown in D, H, L. Location of C6 cell nuclei seen in CT images (C, G, K).
Figure 44| IF stain images for C6 only groups. Negative control groups (A, D, G) indicate a lack of nonspecific binding of the antibody. BBI images (B, E, H) reveal nuclei of both cell types, while CT images (C, F, I) show location of C6 cells.
Figure 45| IF staining for BAEC on top of C6 experimental groups. (A-D) indicate PECAM-1 primary antibody group, (E-H) indicate Claudin-5 primary antibody group, and (I-L) indicate ZO-1 primary antibody group. Positive control groups (A, E, I) indicate positive protein expression (B, F, J) show cell location, and composite images are shown in D, H, L. Location of C6 cell nuclei seen in CT images (C, G, K).
Figure 46| IF stain images for BAEC on top of C6s groups. Negative control groups (A, D, G) indicate a lack of nonspecific binding of the antibody. BBI images (B, E, H) reveal nuclei of both cell types, while CT images (C, F, I) show location of C6 cells.
Figure 47 | IF staining for C6s on top of BAECs experimental groups. (A-D) indicate PECAM-1 primary antibody group, (E-H) indicate Claudin-5 primary antibody group, and (I-L) indicate ZO-1 primary antibody group. Positive control groups (A, E, I) indicate positive protein expression (B, F, J) show cell location, and composite images are shown in D, H, L. Location of C6 cell nuclei seen in CT images (C, G, K).
Figure 48 | IF stain images for C6s on top of BAECs groups. Negative control groups (A, D, G) indicate a lack of nonspecific binding of the antibody. BBI images (B, E, H) reveal nuclei of both cell types, while CT images (C, F, I) show location of C6 cells.
**Figure 49** IF staining for Mix experimental groups. (A-D) indicate PECAM-1 primary antibody group, (E-H) indicate Claudin-5 primary antibody group, and (I-L) indicate ZO-1 primary antibody group. Positive control groups (A, E, I) indicate positive protein expression (B, F, J) show cell location, and composite images are shown in D, H, L. Location of C6 cell nuclei seen in CT images (C, G, K).
**Figure 50** IF stain images for Mix groups. Negative control groups (A, D, G) indicate a lack of nonspecific binding of the antibody. BBI images (B, E, H) reveal nuclei of both cell types, while CT images (C, F, I) show location of C6 cells.
Appendix 2.8  IF Protocol Version 3

1. Remove cell culture media and fix cells in 10% Formalin for 15 minutes.
2. 3x PBS washes for 5 minutes each.
3. Permeabilize cells in 0.2% Triton X-100
4. 3x PBS washes for 5 minutes each.
5. Block for non-specific binding using 3% Normal Donkey Serum (NDS)
6. Incubate in Primary Antibody and 3% NDS for 24 hours.
7. 3x PBS washes for 5 minutes each.
   ** Steps 8-12 are LIGHT SENSITIVE**
8. Incubate in secondary antibody and 3% NDS for 50 minutes.
9. 3x PBS washes for 5 minutes each.
10. Counter stain using Bisbenzimide for 15 minutes.
11. 1x PBS wash for 5 minutes.
12. Remove coverslips from each well, mount to glass slides using mounting medium, and allow to dry for 24 hours.
Appendix 2.9 Permeability Testing Protocol

1. Open pack of 12 sterile transwell inserts inside the laminar flow hood.
2. Add 1.5 ml of media to the lower compartment and 0.5 ml of media to the upper compartment. Allow to precoat for 24 hours.
3. Transfer and invert each transwell insert to a well in 2 6-well plates. Note: do not discard 12-well plates with media.
4. Trypsinize C6 cells, and spin down to a concentrated solution.
5. Add 2-3 drops of C6 solution to the inverted transwell insert membrane.
6. Leave cover off of 6-well plates and allow cells to adhere for 90 minutes.
7. Place cover on 6-well plates and place in incubator for 2 hours.
8. Invert inserts again so they are in the correct orientation, place back into 12-well plate, and add trypsinized BAECs to upper compartment.
9. Following a 48-hour culture period, aspirate media from the upper compartment, and replace with 500µl of FITC Dextran at a concentration of 100µg/ml.
10. Sample media from lower compartment at 5-minute intervals, placing each sample in one well of an ELISA plate.
11. Once all samples are collected, place ELISA plate into Spectrofluorometer to obtain brightness values for each well.