# Design of a novel *ex vivo* murine brain slice model for analysis of pericyte morphology in diabetes

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On my honor as a University Student, I have neither given nor received unauthorized aid on this assignment as defined by the Honor Guidelines for Thesis-Related Assignments

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# Design of a novel *ex vivo* murine brain slice model for analysis of pericyte morphology in diabetes

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**Abstract**

Individuals with diabetes mellitus experience chronic hyperglycemia that increases inflammation and oxidative stress inside the body, often contributing to macrovascular and microvascular complications that decrease the quality of life of diabetic patients. A previous *in vivo* study has demonstrated specific pathological phenotypes of murine retinal pericytes, outer-lying cells of capillaries, wherein pericytes detach from their native capillaries and migrate or "bridge" to distal capillaries. The observation of bridges and morphological changes of these pericytes are believed to be primary drivers behind the development of retinopathy over time. Pericyte and endothelial cell dysfunction, as well as greater prevalence of neurodegenerative pathology in diabetic populations, inspired the development of an *ex vivo* murine model that allows for observation of brain microvasculature. 200um slices from the frontal, temporal and parietal lobes were cultured for 48 hours and utilized to observe pericyte specific morphological changes in hypoglycemic (5 mM), normoglycemic (33 mM) and hyperglycemic (100 mM) conditions. Using the ImageJ image processing software, average pericyte number, length and area were measured within each brain hemisphere and glucose concentration for three fields of view (n=54). The average pericyte number portrayed that the total number of pericytes decreased across all glucose conditions over time, representing tissue and cell death over time. Normoglycemic conditions maintained tissue viability most optimally, with a 17.7% difference in number from the start to end of the culturing period. Pericyte length remained consistent irrespective of glucose or time changes, as did pericyte area. A two-way ANOVA was applied to all quantification metrics. The differences in pericyte length and area were not significant ($p>0.05$) while pericyte count was ($p<0.05$). These results displayed that the novel protocol allows for pericyte visualization in the brain and maintains tissue viability for up to 48 hours.

Keywords: Diabetes mellitus, hyperglycemia, pericyte morphology, brain slice protocol

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## Introduction

### *Diabetes Background and Segmentation*

Diabetes mellitus is a metabolic condition characterized by a high concentration of glucose in the blood (hyperglycemia) that stems from a suboptimal performance of insulin, one of the main hormones in the body responsible for blood glucose regulation. Diabetes mellitus refers to a group of diseases, the most prevalent of which being type 1 diabetes mellitus (T1DM) and type 2 diabetes mellitus (T2DM). T1DM is an autoimmune disease in which, due to a combination of genetic predisposition and

environmental factors, insulin-producing beta cells of the pancreas are targeted by the immune system of the patient, resulting in insufficient insulin production.[1] T2DM is categorized by compromised insulin sensitivity, rather than compromised insulin production. Patients with T2DM, typically due to excess body weight, experience reduced insulin secretion and abnormal production of other hormones that contribute to insulin resistance.[2] The commonality between T1DM and T2DM is a chronic state of hyperglycemia that increases the risk for blood vessel necrosis and subsequent macrovascular complications, such as heart disease, heart attack, and stroke, and microvascular complications, such as nephropathy, neuropathy, and retinopathy.

### *Alarmingly High Health Expenditure of Diabetes*

Diabetes mellitus (henceforth referred to as diabetes) has become one of the world's largest public health crises with an incidence of 451 million individuals worldwide and has a total annual expenditure of $327 billion on the US healthcare system as of 2017, the highest health expenditure of all chronic diseases.[3,4] The impact and cost of diabetes is only expected to rise, with 693 million individuals expected to be diagnosed by 2045.[5]

### *Microvascular Pathology in Diabetes*

The inability to control glucose levels in diabetes results in chronic hyperglycemia, which increases inflammation and oxidative stress through accelerated production of reactive oxygen species, and increases the likelihood of abnormal cellular signaling and epigenetic regulation.[6] This stressful environment has been known to compromise vascular integrity in many organs of the body such as the retina, kidneys, and nervous system, leading to the classic microvascular complications of diabetic retinopathy, nephropathy, and neuropathy.[7]

One of the most valuable constituents of the microvasculature is the capillaries, small (5-10 um in diameter) blood vessels that facilitate gas and nutrient exchange in blood between arterioles and venules. Capillaries are composed of an inner layer of vascular endothelial cells (EC) that regulate regional blood flow and fluidity, and outer-lying pericytes (PC) that adhere to the basement membrane of capillaries and integrate and coordinate signals from surrounding cells to influence the functionality of ECs through juxtacrine and paracrine signaling with ECs such as stability, permeability, proliferation, permeabilization and contractility.[8,9]

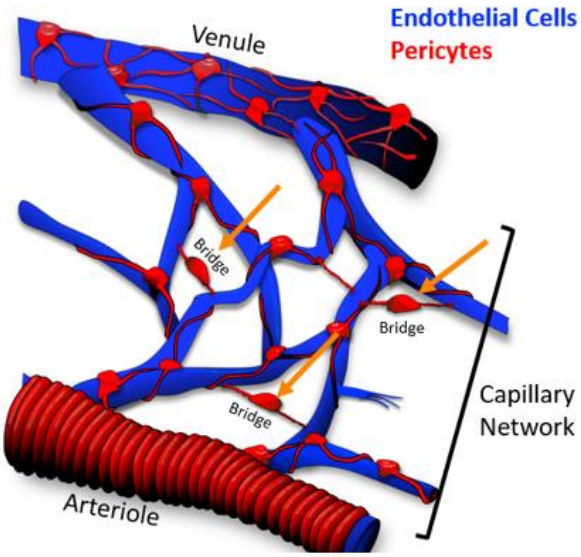


**Figure 1:** A representative image of capillary networks connecting arterioles and venules. The capillaries are composed of endothelial cells (blue) and pericytes (red). The endothelial cells allow for nutrient and gas exchange to tissues and pericytes integrate and coordinate signals to adjust endothelial cell functionality such as proliferation and contractility. The capillary network also highlights the pathological effect of hyperglycemia that leads to the formation of pericyte bridges (orange), a hallmark of microvascular dysfunction.

Previous work in the Peirce-Cottler lab has shown that in the retina, hyperglycemic environments lead to abnormal migration of PCs, resulting in pericyte (PC) detachment from their native capillaries and formation

of PC “bridges" from native capillaries to other capillaries in close proximity (Figure 1).[10,11] The formation of PC bridges in the retina can lead to the limited immune function, decreased angiogenesis, and vessel necrosis that is found in diabetic retinopathy.

### *Research Motivation of the Brain Slice Model*

Being that the retina is an extension of the brain and contains the same 1:1 ratio of ECs to PCs, one motivation of this design project lies in determining whether capillaries of the brain exhibit similar pericyte morphology as in the retina.[12] Diabetic retinopathy is a well-studied complication of diabetes and chronic hyperglycemia, but effects of diabetes on the brain are less-established areas of research. There is, however, a higher prevalence of neurodegenerative disease in the diabetic population than in nondiabetic people.[13] Establishing a connection between a known complication of diabetes affecting the brain and the pericyte phenotypes demonstrated in the diabetic retina would indicate substantial progress in the area of microvascular complications of diabetes. Thus, we introduce a protocol for an *ex vivo* murine brain slice model that preserves brain tissue for up to 48 hours and allows researchers to observe PC morphology, in hopes that this protocol can serve as a foundation for future research on PC dynamics within hyperglycemic environments and subsequent regenerative medicine techniques to restore normoglycemic capillary structure.

## Materials and Methods

### *Animal Preparation*

Animal experiments were approved by the University of Virginia Institutional Animal Care and Use Committee and were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Genetically modified Myh11-DreERT2 ROSA floxed STOP TdTomato mice were aged until 6 weeks before they were placed on a tamoxifen diet for 17 days. These mice were genetically engineered in the Owens Lab to express fluorescent pericytes after consumption of a tamoxifen diet. Tamoxifen deactivates the stop codon that controls suppression of pericyte expression. When knocked out, pericytes display the TdTomato gene, allowing for red fluorescent pericyte visualization. Only male mice were used as female mice with the same genetic modification do not display the red pericyte phenotype following the tamoxifen diet. Adult mice were necessary for this protocol unlike most brain slice protocols in literature that use mouse pups.[14–16] Mouse pups are ideal for the neural plasticity and resiliency of brain tissue, however the rationale behind the decision to focus on adult mice was due to the introduction of tamoxifen. Since it takes tamoxifen 17 days to take effect, it would not be feasible to use mouse pups. Upon diet completion, euthanasia was performed and the *ex vivo* protocol enacted.

### *Murine Brain Extraction*

The brain extraction procedure was adapted from an existing protocol of Dr. Thomas Papouin and Dr. Philip G. Haydon.[17] Large scissors, fine scissors, forceps, and a microspatula were cleaned in ethanol and sterilized using a hot microbead sterilizer (Thermo Fisher Scientific NC9779311) prior to brain extraction. The mouse was quickly decapitated with large scissors and the skull exposed with a large coronal incision down through the midline (Figure 2A). Auditory conducts were cut on each side and the skin was lifted toward the nose to improve visibility of the skull. An incision starting caudal to the cerebellum and ending adjacent to the olfactory bulbs was made with fine scissors. A lateral

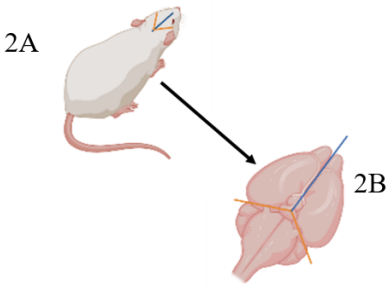


**Figure 2:** A representative image of the murine brain extraction procedure. Two main incisions made with fine scissors enabled brain extraction and isolation without damaging the integrity of the tissue. The first, represented with a blue line (2A), was a large incision down the midline that exposed the skin and allowed for better visibility of the brain. The second, represented with the two orange lines (2A), was a series of lateral incisions that separated the brain from the surrounding optic and cranial nerves. After extraction, the tissue was transferred to dissection media and further separated into left and right hemispheres rostral to the cerebellum (2B).

incision was also made from the base of the skull through the jaw bones for better exposure. The brain was lifted with a microspatula, and the optic nerve on the ventral side and the cranial nerves on the caudal side were both severed. After extraction, the brain was transferred to a dish containing 10mL of ice-cold dissection media. Dissection media (composed of 50 mL HBSS, 0.1415mL HEPES, 0.3g glucose, 0.0175g NaHCO3 and 0.5mL PenStrep) was used to remove the cerebellum (Figure 2B), which was discarded. The left and right hemispheres were then separated along the interhemispheric fissure using forceps.

### *Acquisition of 200um Slices*

A single hemisphere of the brain was placed on a sterile stage of a tissue slicer (Stoelting Co. 51425). The tissue slicer was equipped with a double-edged razor blade cleaned in ethanol and sterilized using a hot microbead sterilizer (Thermo Fisher

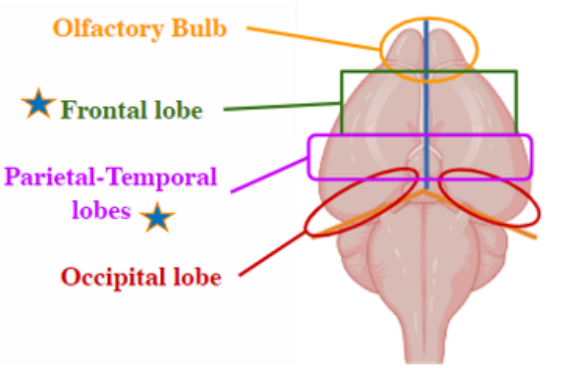


**Figure 3:** 200 um slices were acquired using a tissue slicer and double-edged razor blade and generated from the olfactory bulb to the occipital lobe. The primarily location of slice origin were the parietal, temporal and frontal lobes due to prevalence of neurodegenerative pathological effects in these regions for diabetic individuals. The slices were formed as the force of gravity generated by the falling blade sliced through the tissue which was than rolled onto the sides of a fine hair brush and transferred to culture media.

Scientific NC9779311). Slices were performed from the olfactory bulb toward the occipital lobe via the force of gravity and were primarily collected from the parietal, temporal and frontal lobes (Figure 3). After slicing, each slice was carefully transferred from the blade to the petri dish of culture medium using the sides of a fine hair brush. Slice thickness was optimally determined as 200 um as a result of pilot studies in which 300 and 400 um slices prohibited proper nutrient exchange through the mechanism of diffusion within the brain tissue, which resulted in tissue viability being confined to a single day.

***Novel Culture Technique Design***

After slices were transferred into culture media, each slice was transported into separate wells of a 6-well plate under the culture hood utilizing a modified pipette tip. The left and right columns of the plate consisted of slices from the left and right hemispheres of the brain, respectively. Each row of the 6 well plate held a specific glucose concentration of culture media (Figure 4). The top row held culture media with a concentration of 5mM, representing the hypoglycemic test group. This value is within the physiological range of blood glucose concentration; however, when culturing tissue, a greater quantity of glucose is required to maintain tissue viability. 5mM represents the standard amount of glucose in culture media without any additional added glucose. 33mM served as the normoglycemic test group, as this value was the standard glucose concentration used in literature to maintain brain slice viability and was the second row.[15] Lastly, the hyperglycemic group was represented by the 100mM glucose concentration culture media within the last row. This value was determined by roughly tripling the normoglycemic concentration to mimic hyperglycemic conditions.

Culture media consisted of 24.39mL MEM, 12.2mL supplemented HBSS, 6.1mL HIHS, 6.1mL FBS 5%, 0.65mL HEPES, 0.65mL penicillin-streptomycin, 0.312g glucose.

Each well consisted of a precise orientation of culture media, millipore membrane, and brain slice. In order to facilitate gas exchange and nutrient diffusion within the brain slice, the slices were placed on top of the millipore membrane. A pilot study was conducted in order to determine the ideal orientation for the brain slices relative to the culture media and millipore membrane. Half of the brain slices were cultured with the culture media and slices below the millipore membrane, while the other half were cultured with the culture media below the millipore membrane and the slices on top. The tissue cultured with the slices on top displayed greater viability than the slices that were below, likely due to the lack of gas exchange below (Supplementary Figure 1). Furthermore, placement of slices above the millipore membrane allowed for greater consistency in imaging consistent field of views due to lack of tissue movement.

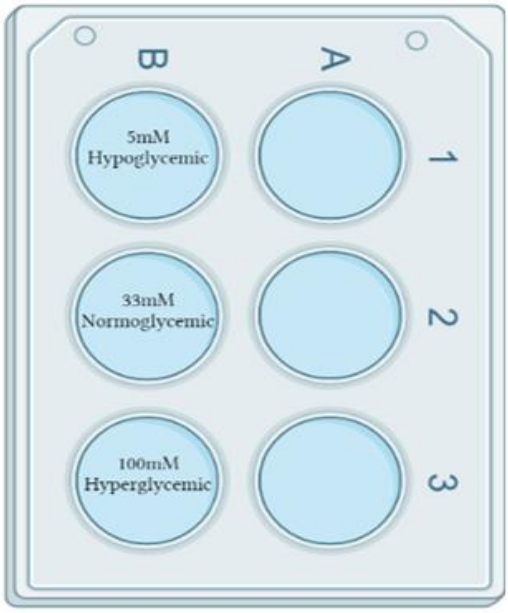


**Figure 4:** After brain slice acquisition from each hemisphere, the slices were cultured in 1 ml of culture solution. The left column slices were from the left hemisphere (**B**) while the right column slices were from the right hemisphere (**A**). Each row was representative of the three variable glucose conditions: hypoglycemic (**1**), normoglycemic (**2**) and hyperglycemic (**3**).

The quantity of culture media was decided by using a pilot study to investigate the effects of 0.75mL, 1mL, and 1.25mL culture media on brain slice viability. The brain slices cultured with 0.75mL culture media did not survive as long as 1mL and 1.25mL. Since there was no difference in viability between the 1mL and 1.25mL conditions, 1mL was chosen as the ideal culture media amount.

The culture media was replaced using a micropipette every 24 hours to promote healthy nutrient exchange within the tissue. The millipore membrane orientation prevented direct contact between the media and each brain slice, allowing for simple culture media replacement. By changing the culture media, brain slice viability was extended to 48 hours post-harvesting.

***Fluorescent Image Acquisition Process***

Serial imaging was performed utilizing a fluorescence microscope with a 20X objective and 10X magnification (DMI8 Leica Microsystems Wetzlar Germany). Image acquisition occurred at 0, 24 and 48 hours of three fields of views (FOVs) within each brain slice from both hemispheres of the brain and in all glucose conditions. 54 images were acquired, with 6 per glucose condition (18 total) at each time point. The focus of imaging was to highlight capillary structures (<15um in diameter) and capture changes in pericyte morphology. Pilot studies were utilized to identify vascular smooth muscle cells within veins and arterioles (>15um in diameter). In the final experiment, arteriole structures were used to localize specific FOVs of highly dense capillary presence while avoiding fluorescence saturation of the larger vessels themselves by increasing magnification from 10x to 20x to target the capillary pericytes. These specific capillary FOVs were imaged and utilized for quantification of the pericyte morphology changes attributed to varying glucose conditions. A representative image of left (top row) and right (bottom row) brain slices serial imaged over 48 hours in low glucose conditions are displayed in Figure 5 with fluorescent red pericytes articulated with white arrows.

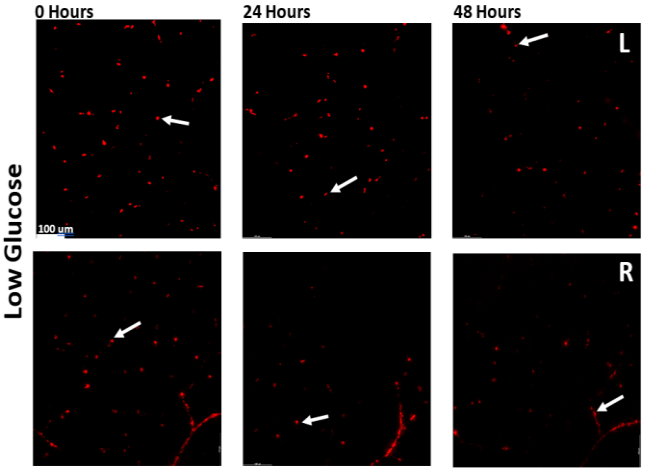


**Figure 5:** A Leica Thunder Fluorescent microscope equipped with a 20x objective was utilized to image 3 fields of view under 10x magnification within both left and right hemisphere slices under all variable glucose conditions over 48 hours. A total cohort of 54 images were acquired. The figure displays left hemisphere slices (top) and right hemisphere slices (bottom) under low glucose conditions over 48 hours with fluorescent pericytes displayed as red dots and accentuated by the white arrows. The protocol design resulted in the ability to observe red pericytes within brain tissue that can be analyzed according to quantification metrics for morphological changes to pericytes due to variable glucose levels.

**Results**

A cohort of 54 fluorescent images was randomized and independently analyzed using the ImageJ image processing tool for three separate metrics of pericyte morphology: pericyte count, pericyte length and pericyte area (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA).[18] Each metric was performed by only one group member so as to maintain the consistency of data collection.

### *Pericyte Count*

Pericytes were counted manually using the ImageJ cell counting tool for each FOV within the randomized image cohort (Supplementary Figure 2). All observable pericytes were counted for each image regardless of color intensity. A manual counting method was used rather than an automatic method such as intensity thresholding because automatic methods were shown to exaggerate the pericyte number on images that included vessel branches. After manual counting was completed, average pericyte number was computed for each glucose concentration over the 48-hour time period. The average pericyte number decreased over time for each glucose concentration (Figure 6). A two-way ANOVA was performed and the difference in average pericyte number for each glucose concentration over time was found to be significant ($p<0.05$). A percentage difference in pericyte number was calculated for each glucose concentration from the starting point to the end of the 48-hour period (22.7% for hypoglycemic conditions, 17.7% for normoglycemic, and 21.6% for hyperglycemic). These results indicate that this novel protocol enables tissue viability in each glucose concentration for up to 48 hours, and the lowest percentage difference in the normoglycemic wells supports conclusions from previous literature indicating that 33mM of glucose is the ideal concentration for tissue viability.

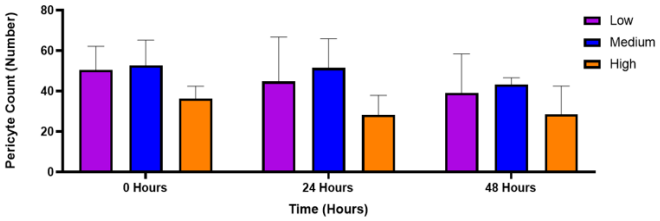


**Figure 6:** Visual representation of the average pericyte number for each glucose concentration over a 48-hour time period. Average pericyte number decreased for each glucose concentration over the 48-hour time period. Percentage difference in pericyte count from the initial time point to the end of the experiment was calculated (22.7% for hypoglycemic conditions, 17.7% for normoglycemic, and 21.6% for hyperglycemic).

### *Pericyte Length*

The secondary metric analyzed to determine the effects of hypoglycemia, normoglycemia and hyperglycemia on pericyte morphology was pericyte length. Within the cohort of 54 randomized images, 12 random pericytes were chosen and measured across the longest portion of length in micrometers (Supplementary Figure 3). It was determined that pericyte length decreased slightly in low and high glucose conditions over 48 hour but overall the pericyte length across all three glucose conditions remained relatively consistent (Figure 7). The percent decrease over 48 hours was 14%, 2% and 9% in hypoglycemic, normoglycemic and hyperglycemic conditions. A two-way ANOVA was performed and was not significant ($p>0.05$). Therefore, no claims can be made about the development of pericyte bridges or deviation from normal pericyte morphology based on the metric of pericyte length after exposure to variable glucose conditions in a 48 hour period.

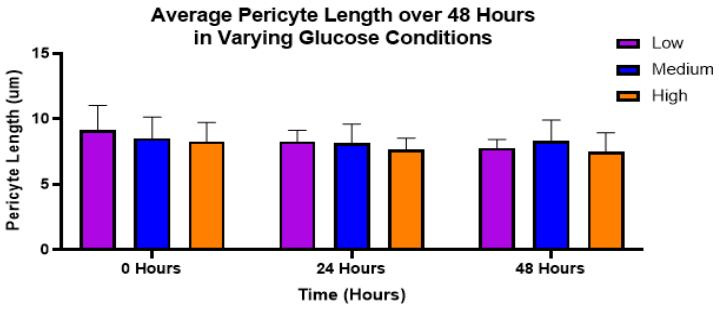


**Figure 7:** Pericyte length in low and high glucose conditions decreased slightly while pericyte length in medium glucose conditions remained relatively the same over 48 hours. The percent decrease over 48 hours was 14%, 2% and 9% in hypoglycemic, normoglycemic and hyperglycemic conditions. A two-way ANOVA was performed and was not significant ($p>0.05$). Therefore, no claims can be made about the development of pericyte bridges or deviation from normal pericyte morphology based on the metric of pericyte length after exposure to variable glucose conditions in a 48-hour period.

### *Pericyte Area*

Pericyte area was measured in order to quantify the pericyte bridging that is expected in hyperglycemic conditions. Each

image was thresholded using ImageJ in order to map out the total area covered by the fluorescent pericytes (Supplementary Figure 4). That total area was then divided by the cell count reporting the average area per pericyte in micrometers squared. Based on the results, the hypoglycemic and normoglycemic conditions remained relatively stable in average pericyte area across 48 hours. Hyperglycemic conditions displayed a 196% increase at 24 hours in average pericyte area and then a subsequent

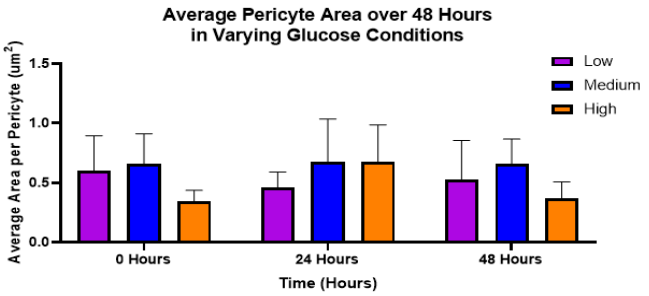


**Figure 8:** Pericyte area in low and medium glucose conditions remained relatively the same over 48 hours. Hyperglycemic conditions displayed an increase at 24 hours in average pericyte area and then a subsequent decrease at 48 hours. A two-way ANOVA was performed and was not significant ($p>0.05$). Therefore, no claims can be made about the development of pericyte bridges or deviation from normal pericyte morphology due to pericyte area changes due to variable glucose exposure.

55% decrease at hour 48 (Figure 8). This was not expected, as pericyte bridging is expected to increase across the first 48 hours. This decrease is something that should be explored in future research. A two-way ANOVA was performed and was not significant ($p>0.05$).

## Discussion

### *A Note on Brain Slice Selection*

In this protocol, slices were exclusively extracted from the frontal, parietal and temporal lobes. These lobes were prioritized due to both ease of slicing mechanics and an interesting potential connection between diabetes and neurodegenerative disease. Slicing mechanics were easier in these portions because the slicer blade experienced less resistance and mechanical disruption upon transfer from blade to culture media damaging the tissue was reduced. Additionally, the prevalence of neurodegenerative diseases such as Alzheimer's, Parkinson's and dementia is higher in the diabetic population than in nondiabetic people.[19,20] Specifically within *in vitro* murine models, diabetic neurodegenerative disease development results in blood brain barrier damage that is localized within the frontal, occipital and parietal lobes, as well as the thalamus and midbrain.[21,22] Therefore, the protocol was designed to study these areas of greater neurodegenerative prevalence under variable glucose conditions where pericyte bridging may occur with the greatest frequency in these highly affected areas. The impact of diabetes on the brain microvasculature is an understudied area and this novel protocol allows for visualization of pericyte morphology from regions affected by diabetes. With future work, this protocol can offer more information on the pathology of microvascular complications due to diabetes.

### *Discussion on Results*

The pericyte metrics analyzed were expected to represent that the pericyte bridging that occurs in diabetic conditions of the retina also occurs within the brain when only glucose conditions are varied. The results did not specifically support these expectations. Hyperglycemic media conditions were expected to demonstrate the highest rate of pericyte dropout of all groups, as well as the highest increase in pericyte length, as pericytes migrate from native capillaries. The results did not support these specific expectations. It is possible that variation in the pericyte count adversely affected the average pericyte area as total pericyte area was divided by pericyte count to normalize the data to the specific slice and glucose condition the data

was drawn from. However, it is difficult to draw specific conclusions about interactions between endothelial cells and pericytes without endothelial cell visualization.

It is important to recognize that all data analysis was performed solely on images with fluorescent red pericytes. Therefore, any metrics that were sought for analysis only tell a partial picture of how the pericyte morphology may change over 48 hours. The development of microvascular and macrovascular complications of diabetes develop over time. Prior *in vivo* studies of induced diabetes often require at least 7 days of exposure to hyperglycemia before negative consequences are observed.[10] Thus, the exposure to glucose conditions for 48 hours may be too short of a time frame to recognize the morphological changes of pericytes seen in prior work. Furthermore, an *ex vivo* study allows for highly specific control of exposure of the brain cells to specific variable changes (glucose only) however diabetes pathological development is often driven by the secondary effects of inflammation and oxidative stress. Therefore, any conclusions drawn from the results of the novel *ex vivo* model may be hindered by the inherent design constraints that may need further exploration to better mimic diabetic pathology accurately.

The novelty of this protocol is that it enables pericyte visualization for up to 48 hours and draws a connection to brain microvessels in simulated diabetic conditions. This work can be used as a foundation for further research into the pathology of microvascular complications of diabetes.

**Future Protocol Expansion**

Examining the challenges faced developing this protocol, there are many future work improvements that can be made to further explore diabetes' impact on the microvasculature. One challenge was that this protocol only examined the effect of varied glucose concentrations on the microvasculature. Diabetes is a complex condition that contains additional secondary consequences which affect the microvasculature. Some examples include an increase in oxidative stress and an increase in inflammation. One way in which we could begin to quantify these secondary metrics is by staining for inflammatory cytokines. A future experiment that would allow us to better visualize the exact effects of diabetes of the mouse brain would be to use a streptomycin model to induce diabetes pre-euthanasia.

Another challenge in the development of this brain slice protocol was achieving long-term tissue viability. There were three main changes that were made that improved the brain slice viability to 48 hours. The first was reducing the brain slice widths from 300um to 200um. By reducing the width, this reduced the distance the culture media would have to diffuse through the tissue. Another change that was made was setting the volume of culture media to 1mL following the first pilot study. The last change was made by adjusting the orientation of the millipore membrane relative to the culture media and brain slices. These three changes improved brain slice viability from 24 hours up to 48 hours. Future work that could be implemented into the protocol is the use of microfluidics to achieve longer slice viability. Liu et al. demonstrated that perfused drop microfluidics can be used to keep brain slices alive for up to 9 days.[23]

One of the biggest challenges in labeling endothelial cells is that the CD31 stain fixation process before staining ends tissue viability. If the tissue is stained on hour 0, there is not an opportunity to see progression of the tissue on hour 24 or hour 48. One way this problem could be

addressed is by an intravascular injection of fluorescent lectin before euthanization. Fluorescent lectin has been shown to demonstrate high uptake in capillary endothelial cells in the lung, ovary, and anterior pituitary.[24] However, this method of endothelial cell labeling showed little to no uptake in most of the brain, which is consistent with our results from attempting this procedure (Supplementary Figure 5). Future work would benefit from an optimized procedure for endothelial cell labeling in the brain.

## Conclusion

We have designed a novel functional protocol for an *ex vivo* murine model to simulate hypoglycemia, normoglycemia and hyperglycemia within the microvasculature of the brain. This model allows for the visualization of pericytes within murine brain slices and maintains tissue viability for a minimum of 48 hours. Future collaborators can follow the protocol and utilize tamoxifen-induced mice to acquire 200um cultured brain slices from both hemispheres in variable glucose conditions. These slices then can be imaged and analyzed over a 48 hour period. Further exploration into staining for inflammatory cytokines, utilizing microfluidics or the use of lectin in highlighting endothelial cells will all offer greater opportunities for optimizing the novel protocol to display a more robust representation of diabetes. Therefore, through future exploration, this novel protocol has the potential to provide further insight into the impact of diabetes on microvascular dysfunction in the brain.

## End Matter

### *Author Contributions and Notes*

The authors contributed equally to this work.

The authors declare no conflicts of interest.

### *Acknowledgements*

We would like to first acknowledge the Biomedical Engineering (BME) Department at the University of Virginia for their organization of the BME Capstone design project. We would also like to acknowledge all members of the Peirce-Cottler Lab for guidance that was instrumental in the creation and completion of this work, including graduate students Kareem El-Ghazawi and Ramon Castellanos-Sanchez, and lab manager Anthony Bruce.

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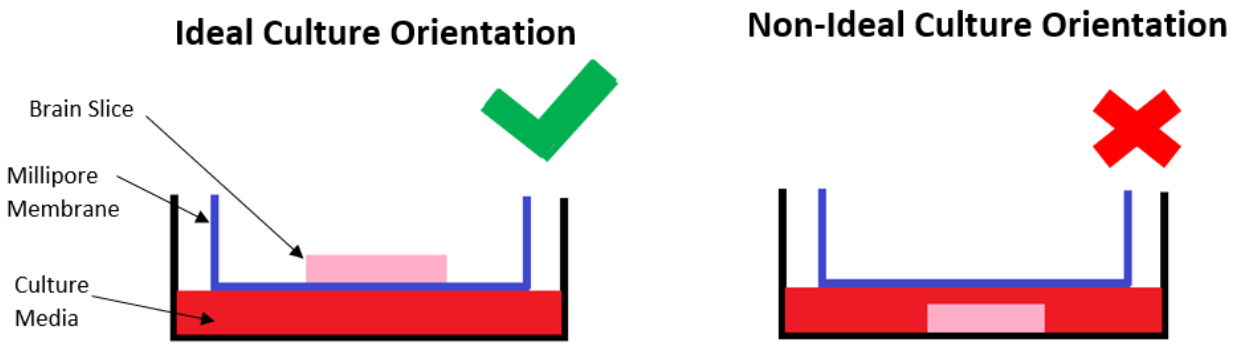
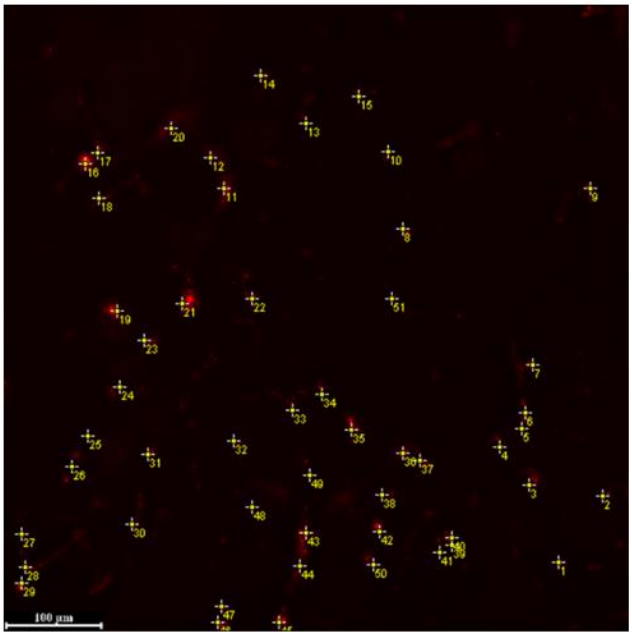
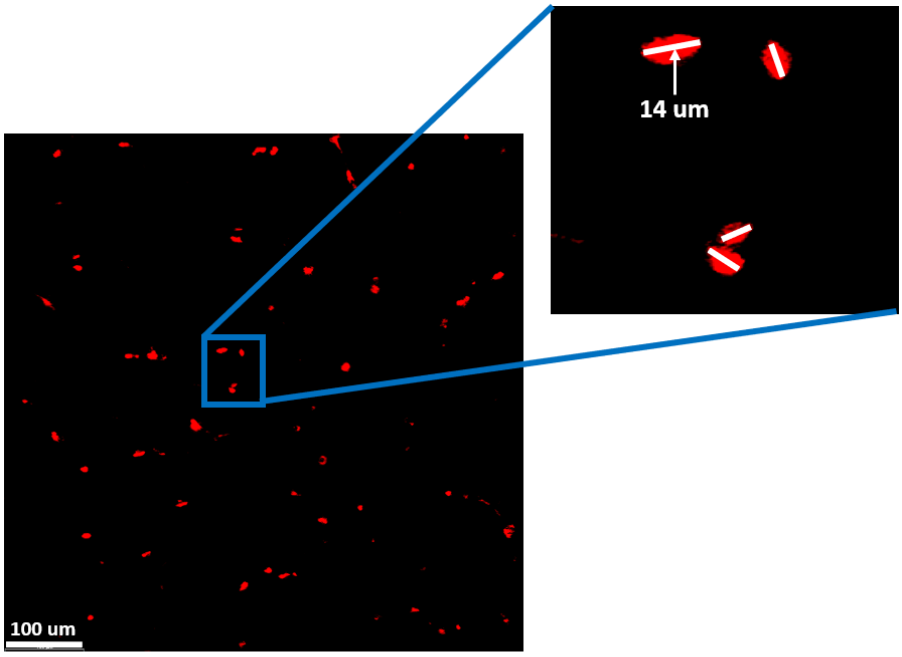
## Supplementary Figures



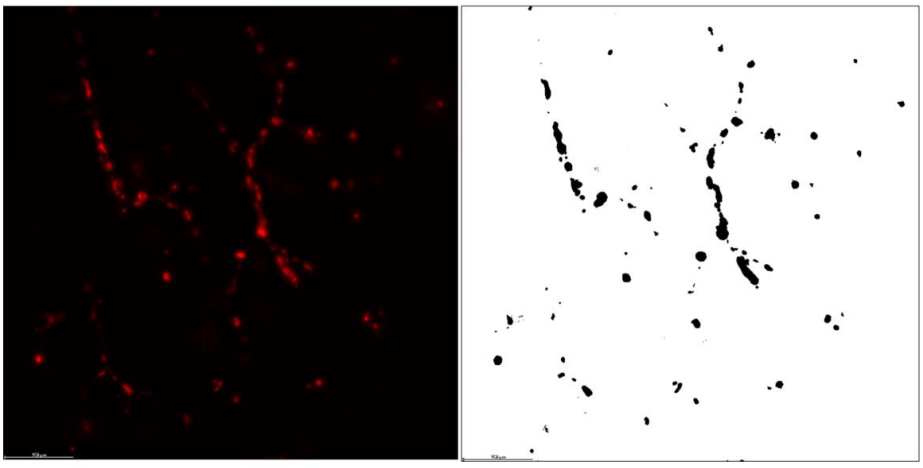

**Supplementary Figure 1:** A pilot study was performed to determine the optimal orientation of culturing elements. The study determined that the brain slice on top of the Millipore membrane above culture media was ideal. The brain slice above the Millipore membrane allowed for proper nutrient and gas exchange and improved tissue viability as well as easy culture media replacement.



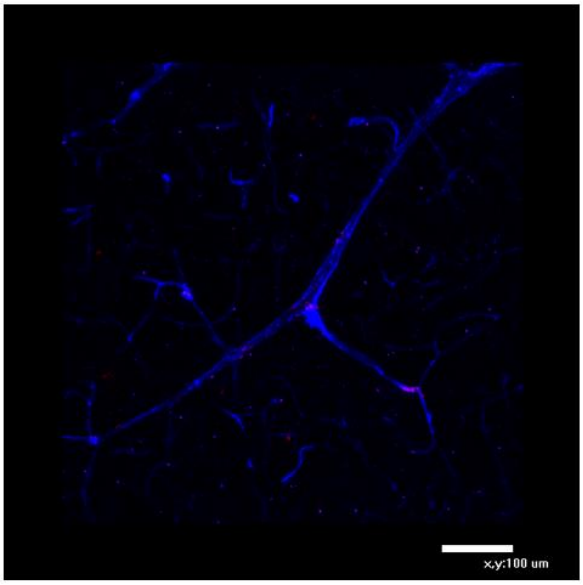
**Supplementary Figure 2:** Representative image of the manual pericyte counting process. All visible TdTomato fluorophores representing pericytes were manually identified with the cell counter tool on ImageJ.




**Supplementary Figure 3:** Representative image of the process of acquiring pericyte length on a left low glucose brain slice. The longest distance of a pericyte was measured in micrometers and represented as pericyte length. 12 random measurements were made across all 54 images to determine the effect of variable glucose conditions on pericyte morphology.



**Supplementary Figure 4:** Representative image of the ImageJ thresholding tool highlighting total pericyte area. The left panel displays the original image while the right panel displays the thresholded image.




**Supplementary Figure 5:** An attempt of a fluorescent lectin endothelial cell Staining procedure. Myh11-DreERT2 ROSA floxed STOP TdTomato mice underwent an intravascular injection of fluorescent lectin via the jugular vein before euthanasia. Endothelial cells are expressed with blue fluorophores and pericytes with red TdTomato fluorophores. Visibility of vascular networks is low and this pilot study resulted in the lowest pericyte visibility of all experiments with Myh11-DreERT2 ROSA floxed STOP TdTomato mice.

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