**Project Description**

**1. Overview**

The purpose of my CAREER proposal is to develop a program that integrates teaching and research to begin designing and understanding two- and three-layered *in vitro* constructs of normal and diseased human subretinas. My long-term vision is to understand the delicate interplay among the layers of the outer retina and, most importantly, to achieve this vision with my students. I am passionate about understanding the relationships of the tissues within the outer retina because of its significant role in vision and the impacts of vision loss on people of various ages, genders, races, and ethnicities. Vision loss can manifest through many means, including acute or chronic malignancies and accidents. Although many people are affected, there remains a gap in our knowledge about the fundamental causes of vision loss, ways to prevent, and treat this loss. The majority of previous studies have been performed on individual tissue layers *in vitro* or on animal models. While these studies have provided a vast amount of information about the outer retina, it still remains difficult to replicate the complexity among the layers of the human outer retina *in vitro*.

**I hypothesize that designing and validating two-layered and three-layered models of the outer retina will improve the information available for understanding the causes of vision loss and for developing new strategies to prevent and treat vision loss compared to one-layered cultures and animal studies**. This CAREER award will position me to build upon my current research progress, to acquire new skills and techniques, to expand my network to develop two- and three-layered constructs of normal and diseased outer retina, and to integrate this research with teaching. My objectives for this project are:

1. Design two-layered constructs of normal and diseased subretinal structures;
2. Model two-layered and three-layered constructs using computational software and develop simulations to inform experimental work; and
3. Design three-layered constructs of normal and diseased subretinal structures.

To work towards these objectives, it will be essential that students participate so that my research and teaching goals are fully integrated. Opportunities to solve straightforward problems, such as how to visualize a diseased retina, as well as unbounded questions, as proposed in the research objectives, will be provided to various students. I envision my CAREER project will strategically enhance my teaching and networking capacity over this five-year period. To do this, I will integrate broader impact elements, including:

1. Materials for building retinal imaging devices for 8th / 9th-grade students ; 
2. New tissue engineering class module to determine ideal substrates for growing healthy, polarized, pigmented retinal cells; 

3. Retinal research experience for undergraduates recruited from the Society of Women Engineers and a mentored research experience with industry;

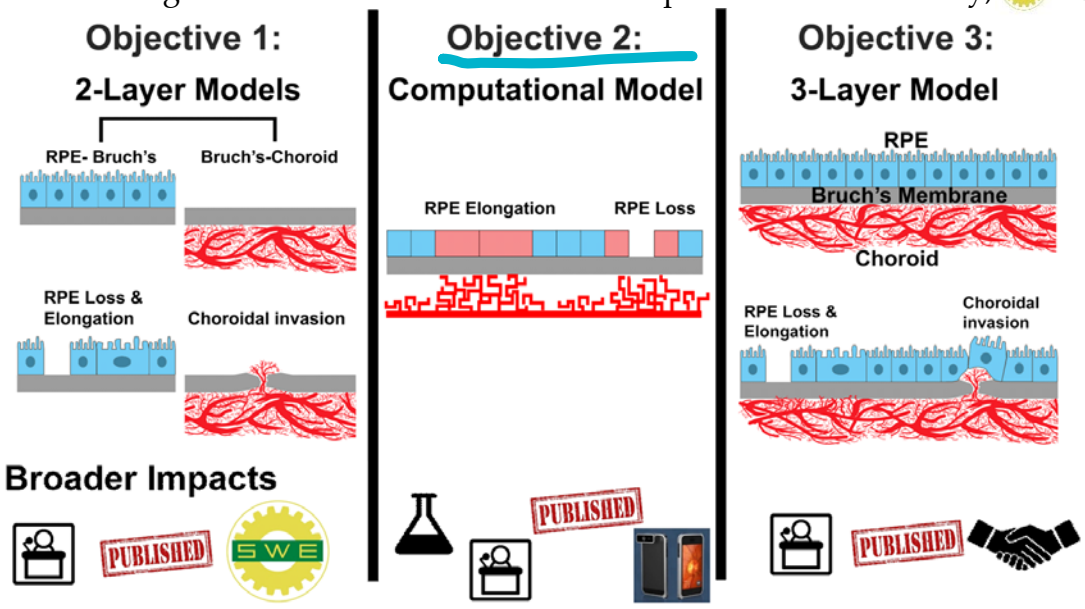


**Figure 1**. Schematic outlining research and broader impacts of CAREER proposal.

## 2. Rationale and Background

The supportive tissues of the outer retina include the retinal pigment epithelium (RPE), the acellular Bruch's membrane (BrM), and the choroid (**Fig. 1**). These layers sit below the photoreceptors and provide indispensable functions such as mediating selective transport between the retina and the blood supply. The polarized and pigmented RPE layer blocks extraneous light and releases pro- and anti-angiogenic factors that protect the integrity of photoreceptors and choroidal cells. The choroid provides the overlying cellular layer with oxygen and nutrients. The BrM functions as a reciprocal filter between the RPE and the choroid.

**Intellectual Merit:** These separate layers are well-defined, but their relationship to each other and their role in blinding diseases, such as age-related macular degeneration (AMD), myopia, and retinopathy of prematurity, is not well-understood. **For this CAREER proposal, accurate, *in vitro* models of the normal and diseased human subretina will be designed to begin to understand vision loss.** My team will elucidate these fundamental gaps in the knowledge by developing two- and three-layered models and by integrating instruction with underrepresented populations.

The field of retinal research has certainly benefited from animal models, *ex vivo* studies, and *in vitro* experiments. These approaches have been effective in understanding specific aspects of some retinal diseases and in testing potential treatments. However, due to the limited availability of human models, molecular mechanisms of initiation and progression of retinal disease remain unclear. The majority of current *in vitro* studies use single-layer models of the RPE or choroid and do not account for the complex interplay

between the RPE, BrM, and the choroid. Based upon the preliminary data gathered by my group, we will focus on developing two- and three-layered constructs of the subretina to better understand the relationship between its layers and bridge the gap between one-layered cultures and animal and human studies.

### *2.1 Preliminary Data*

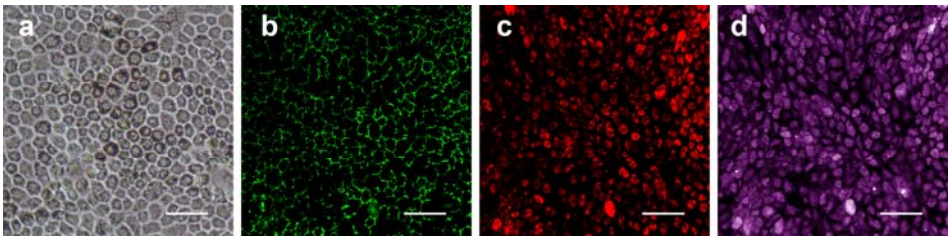


**Figure 2.** Isolated primary RPE cells show characteristics of native RPE. Cobblestone morphology and pigmentation confirmed by brightfield microscopy (a). Immuno-cytochemistry results show ZO-1 (b), VEGF (c) and RPE-65 (d) expression. Scale bars: 100 μm.

We have generated foundational preliminary data that has informed our hypothesis and goals for this proposal. RPE cells have specific indicators of health and behavior that must be maintained within any construct *in vitro*. These indicators include their characteristic cobblestone morphology and pigmentation, tight junction formation (i.e. zona-occludens 1, ZO-1), expression of both pro-angiogenic factors (vascular endothelial growth factor, VEGF) and RPE-specific proteins (RPE-65, **Fig. 2**). These properties are essential for RPEs and will be verified within our constructs.

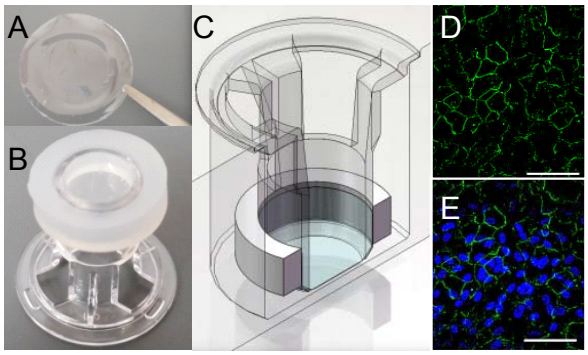


**Figure 3.** Spider silk membranes support RPE cell growth. (A) Spin-coated spider silk membranes are optically clear and thin. (B) Membranes are mounted in empty Transwell inserts. (C) Schematic of assembled device. RPE cells grow on spider silk membranes and express tight junction proteins (ZO-1=green, D; E, nuclei = blue). Scale bars = 100 μm.

To support polarized RPE behavior *in vitro*, RPE cells can be grown on membranes that simulate BrM. Commercially available and commonly used Transwell inserts made of polyethylene terephthalate (PET) neither mimic key properties of the BrM nor support appropriate RPE growth. We explored spider silk protein films as a BrM replacement. These films are made by spin coating a mixture of spider silk proteins to create

membranes with a thickness of ~3-8 μm (similar to normal and aged BrM *in vivo*). RPE cells were grown on spider silk membranes, with initial results demonstrating that spider silk membranes support their attachment and proliferation (**Fig. 3**).

The third component of our preliminary work was to determine if endothelial cell growth due to RPE changes could be replicated within our lab. Several retinal conditions, including wound healing and drusen formation, lead to mechanical stretching of the RPE [1–3]. Endothelial cells responded to the cell growth media from mechanically elongated RPE cells media by forming more capillary-like tube structures *in vitro* (**Fig. 4**), indicating potential endothelial tubal overgrowth and neovascularization in response to the changing protein expression profile of the RPEs.

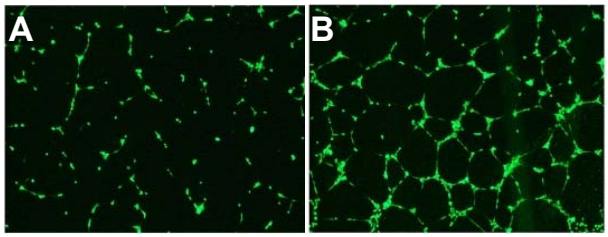


**Figure 4.** *In vitro* angiogenesis assay demonstrates endothelial tube formation resulting from changes in proteins expressed by mechanically elongated RPE cells (B), compared to normal media control (A).

Finally, we have simulated disease states *in vitro*. To understand the role of RPE cell-cell contact on disease initiation and progress, we designed methods to control RPE growth. RPE cells were patterned to grow in 100-300 μm diameter circular colonies so smaller colonies had a higher proportion of cells without a neighboring cell and therefore without intercellular junction formation. VEGF expression per cell increased significantly when more cells did not form intercellular junctions (**Fig. 5** [4,5]).

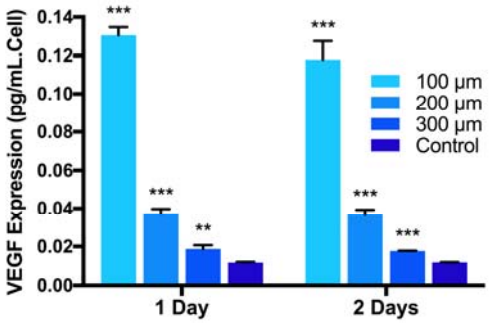


**Figure 5.** Micropatterning demonstrates that VEGF expression per cell increases with smaller patterns. * p<0.05, ** p<0.01, *** p<0.001 [4]

Our preliminary data demonstrate that we have the capabilities to move from one-layered experiments to two- and three-layered constructs to better understand retinal function and disease. The following tasks are associated with each of our objectives.

## 3. Approach

**3.1 Objective 1: Design two-layered constructs of normal and diseased subretinal structures.** RPE cells are polarized and selectively pump angiogenic factors, proteins, and ions through BrM to maintain the retina and choroid [6,7]. A normally functioning BrM is crucial for proper ion and protein transport between the RPE and choroid. Therefore,

to construct a realistic model of subretinal tissue, developing a BrM with physical characteristics similar to *in vivo* tissue is imperative. Previous studies have employed biocompatible membranes made of a variety of materials, such as collagen [8], polymers [9], and silk fibroins [10]. However, whether these synthetic membranes can support the growth of both RPE and choroidal cells is not clear.

To perform this objective, synthetic BrMs will be developed using biomaterials including collagen, silkworm, and spider silk fibers. Then, RPE or choroidal cells will be cultured on either side of the synthetic membrane. Once normal conditions are verified within the two-layered model, this subretinal tissue model will be interrogated to replicate a variety of disease or injury scenarios to begin to identify the mechanisms of disease within two-layered constructs. We will test how RPE health and protein expression changes following RPE loss and RPE elongation and then determine the roles BrM has in controlling blood vessel growth and neovascularization.

### 3.1.1 Develop Two-Layered Normal Subretinal Tissues

**Task 1**. Grow primary RPE cells on simulated BrM. In this task, primary RPE cells from human donor eyes (Lions Eye Bank) will be grown on various materials to mimic BrM – a laminar sheet with five distinct layers made of several structural proteins such as collagen, laminin, elastin, and fibronectin, along with other molecules like glycosaminoglycans [11]. In young, healthy specimens, BrM is thin, ranging from 3 to 5 μm, and biochemically static [11–13]. We will evaluate various recipes of BrM to produce a freestanding membrane that can be housed within a Transwell insert (**Fig. 3C**). Potential components include synthetic, human or animal decellularized BrM, and extracellular matrix and protein formulations (**Table 1**). Optimal RPE support will be evaluated by quantifying growth rates, visualizing cell morphology, verifying polarization and microvilli formation, and imaging RPE65, an RPE-specific protein (**Fig. 2**). Other BrM parameters will be assessed, including permeability and thickness.

**Table 1**. List of potential materials for BrM replicas

| ***Biocompatible Material*** | ***Benefit*** |
|---|---|
| Collagen IV | Facilitate endothelial cell movement, largest component of BrM |
| Elastin | Provides vascular compliance and antiangiogenic barrier functions |
| Fibronectin | Contributes to elastic properties |
| Laminin | Biologically-active component of basal lamina, facilitate endothelial cell movement |
| Silkworm silk proteins | Glue-like, commercially-available |
| Spider silk proteins | Tunable, robust mechanical properties |

**Task 2**. Simulate choroidal blood vessel growth. In this task, choroidal endothelial cells isolated from human eyes will be cultured with a hydrogel containing various proteins, such as collagen and elastin, to support their growth under membranes developed in Task 1. Endothelial cells recruit fibroblasts and pericytes during endothelial tube formation [14,15]. Thus, to produce a more accurate model of the choroid, a mixture of choroidal cells containing pericytes, fibroblasts, and endothelial cells will be isolated from human eyes. The cell mixture will be inspected by immunostaining endothelial, fibroblast, and pericyte markers, followed by confocal microscopy. The capability of the choroidal tissue digest to form endothelial tubes will be examined by growing the cells on hydrogel-coated plates and comparing the tube formation potential with that of choroidal endothelial tubes isolated whole from human donor eyes. The pure population of endothelial cells will be isolated from the choroidal tissue digest using CD-31 (an endothelial-specific protein) antibody-coated magnetic beads that can purify a specific cell type from a cell mixture by placing the beads in a magnetic field.

### 3.1.2 Develop Two-Layered Diseased Subretinal Tissues

RPE cells are largely responsible for elevated levels of a variety of pro-angiogenic factors during retinal disease [16]. Although the mechanisms leading to increased expression of angiogenic factors in the RPE are unclear, a large amount of evidence suggests that mechanical stress and intercellular junction formation are involved in regulating the expression of angiogenic factors [17–19]. The contact between RPE cells is lost under pathological conditions, such as geographic atrophy [20,21], drusen formation, or choroidal neovascularization (CNV) in AMD [22].

**Task 1**. Determine how losing RPE cell-cell contact leads to vision loss. Previous studies in the PI's lab and current literature [4,5,18,19] suggest that the reduced contact between RPE cells can lead to increased expression of angiogenic factors, such as VEGF. However, whether this lack of cell-cell contact can lead to increased angiogenesis and CNV remains unknown. Using our two-layered subretinal tissue model, we will determine how RPE cells affect angiogenic protein expression. Two methods will be used: a) Scratching RPE monolayers to replicate injury and b) Decreasing RPE cell-cell contact to replicate RPE degeneration during ocular disease

Scratching will be performed by scoring the RPE monolayer growing on the optimized BrM (3.1.1) to create cell-free areas. To develop realistic *in vitro* micropatterned models of the diseased retina, fundus images of eyes with RPE degeneration will be obtained from primary literature and from our consultant (see letter, **Fig. 6A**). Images will be processed using ImageJ to calculate the percentage loss of cell-cell contact by measuring the border of degenerated areas. Modified images will be generated by connecting all zones of degeneration to produce an interconnected pattern with the same area and perimeter of degeneration as the original image (**Fig. 6B-C**). These images will be used to design

photomasks with micron-size features using computer-aided (CAD) software (**Fig. 6D**). PDMS stencils will then be made to block the adhesion of RPE cells to certain areas. RPE cells will be seeded onto stenciled tissue culture inserts and grown for 4 weeks to mature and to form intercellular junctions. Using this method, an *in vitro* model of RPE degeneration with the same level of intercellular junction loss as in diseased eyes will be developed. Unpatterned, two-layered constructs models will serve as a control.

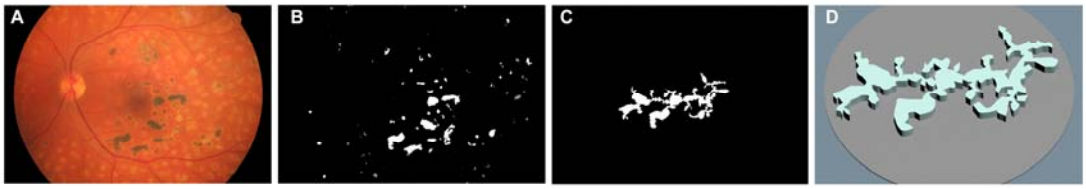


**Figure 6.** Schematic for making PDMS stencils based on fundus images. (A) Fundus image of an atrophic retina; (B) Image of degeneration only; (C) Modified image covering same length of degenerated edges; (D) PDMS stencil produced based on (C).

**Task 2**. Determine how RPE elongation leads to vision loss. The goal of this objective is to apply mechanical stress to the two-layered RPE-BrM construct to determine how mechanical stress and RPE elongation contributes to angiogenesis. Mechanical stress will be applied to the RPE-BrM layer by increasing the pressure in the apical chamber of the construct. A silicon membrane with an access port will be used to seal the apical chamber that will be connected to a syringe pump to control the pressure in the chamber. Different levels of mechanical stress and different rates of increasing mechanical stress will be applied to the RPE monolayer to replicate the following *in vivo* scenarios: a) Chronic increase in mechanical stress at a slow rate due to drusen formation, and b) Acute increase in the intraocular pressure due to new blood vessel formation.

Brightfield and fluorescence microscopy will be used to characterize changes in endothelial tube formation by adding spent RPE media to choroidal cell culture. These changes include increases or decreases in the number and length of endothelial tubes (**Fig. 4**). RNA microarray analysis will also be performed on RPE cells to identify the angiogenic factors involved in mechanical stress-induced neovascularization.

**Task 3**. Investigate the role of BrM in choroidal invasion. With age and during retinal diseases, BrM undergoes structural and compositional changes [1,23]: its thickness increases (**Fig. 7**), its porosity decreases, and many proteins and lipids begin to deposit on the BrM. However, it is unclear whether these changes directly or indirectly induce new growth within the choroid. In this task, the BrM within the BrM-choroid model will be modified to replicate

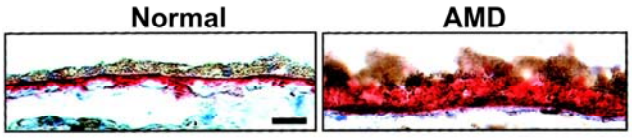


**Figure 7.** Thickening of the BrM occurs during age-related macular degeneration. BrMs in red [1].

the following pathological conditions: a) Increased thickness of BrM as collagenous layers thicken and inner elastic layers become thinner and more brittle [24,25]; b) Reduced permeability of BrM leading to BrM breaks; and c) Deposition of drusen components on BrM. A summary of how each change will be implemented is in **Table 2**. At the end of each of these subtasks, the effect of changing the BrM on potential angiogenesis will be assessed by measuring the quantity and size of choroidal endothelial tube formation using immunostaining and confocal microscopy (**Fig. 4**).

**Table 2**. Approach for replicating diseases-like states within BrM replicas

| *BrM Property* | *Methodology* |
|---|---|
| Increase BrM thickness | - Reduce thickness of the inner cross-linked membrane<br>- Thicken proteinaceous layers by adjusting spin-coating speeds or by increasing the initial volumes or concentrations of protein |
| Reduce BrM permeability | - Reduce pore size and quantity of synthetic BrM by adjusting the methods of synthesis, such as electrospinning parameters<br>- Introduce physical breaks by pressing BrM with a needle |
| Deposit drusen components on BrM | - Treat BrM with drusen components: apoE, clusterin, and TIMP-3<br>- Replicate BrM calcification by adding calcium |

**Expected Results.** We expect the porous BrM made from new materials will support the growth of RPE cells. Choroidal cells will be in a 3D hydrogel, so we anticipate that they will produce a network of endothelial tubes underneath the BrM. Under the disease conditions, we expect that losing cell-cell contact and experiencing higher levels of mechanical stress will drive RPE cells to overexpress pro-angiogenic factors. We also expect that endothelial tube formation will increase due to changes in BrM because of changes in media permeability and gas exchange.

**Potential Problems and Alternative Solutions.** We expect to see polarized, pigmented human RPE cells on BrM, but if this does not occur, cells will be cultured for longer periods of time (8-10 weeks) to induce polarization [26–29]. In normal conditions, primary human RPE cells form a confluent monolayer on PET Transwell membranes within a week. If similar results cannot be achieved by growing RPE cells on BrM models, each BrM model will be treated with a mixture of matrix proteins to enhance cell adhesion and proliferation. Sometimes, a high concentration (>30%) of fibroblasts or pericytes inhibits endothelial tube formation [14,15]. If tube formation is not achieved, pure populations of pericytes and fibroblasts will be used instead of varied concentrations added from the human donor eyes. Finally, drusen deposits extracted from human donor eyes as described previously [30] may be added to the synthetic BrMs if necessary.

**3.2 Objective 2: Model two-layered and three-layered constructs using computational software and develop simulations to inform experimental work.** With the complex

interplay between physical and molecular behaviors within the layers of normal and diseased subretinal tissues, it will be imperative to simplify and identify important factors prior to additional experimental work (Objective 3). To do this, we will work with Dr. Zhen Zhang (key personnel) to design computer modeling and analysis based on results gathered from the two-layered constructs. We will use formal verification since it can deliver provable guarantees by applying automated and rigorous mathematical analysis. *Probabilistic model checking* (PMC) is such a method for analyzing quantitative properties of systems that exhibit probabilistic behaviors, such as the two- and three-layered constructs. The second objective of this proposal is to develop formal models for these constructs, perform design space exploration through PMC, and then use the correct behavioral analysis to guide experiments to improve efficiency.

To model real systems, it is preferable to use a continuous-time Markov Chain (CTMC) model of time, where the delays between transitions can be any arbitrary real values. The transition delays in this type of model are assumed to be exponential distributions. There are two types of analyses, the transient and steady-state behaviors. Transient analysis reports the probability of being in each state of the Markov chain at a particular time instant. Steady-state analysis outputs the corresponding probability in the long-run. PMC extends these analyses by defining a probability space over the set of all paths through the model [31–33]. For systems with both discrete and continuous randomness behaviors, such as normal and diseased tissues, an Markov Automata (MA) [34] is a natural choice.

**Task 1**. Model and validate normal and diseased two-layered constructs. Probabilistic behavioral models for two-layered RPE-BrM and BrM-choroid constructs will be developed and analyzed. For the RPE-BrM model, CTMC models for normal-to-diseased RPE cells will be constructed from experimental data collected from two-layered constructs. Specifically, these models should reflect how reduced contact and mechanical stress for RPE cells contribute to the increased expression of pro-angiogenic factors. For BrM-Choroid, the focus is on modeling new choroidal growth due to changes of permeability, thickness, and drusen components of BrM.

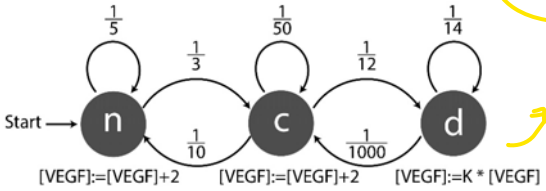


**Figure 8**. RPE cell and VEGF expression modeling with computer simulations.

As an illustrative example, **Figure 8** shows a simple RPE cell model that switches between normal and diseased states. It consists of three states, namely, "normal (n)", "critical (c)", and "diseased (d)". Starting from the normal state, the rate of switching to the critical state is $\frac{1}{3}$, while the rate of staying at the normal state is $\frac{1}{5}$. Once the cell is at the critical state, it has a relatively high likelihood of switching to either the diseased state or back to the normal state. But

once it is in the diseased state, it is extremely unlikely that it can switch back to the critical state, and hence its rate is set to $\frac{1}{1000}$. Note that, at the diseased state, the rate of VEGF production is an integer multiple of that under the normal condition. For simplicity, this rate is assumed to remain unchanged when the RPE cell switches to the critical state. Identifying "critical" states experimentally is a challenge; however, using a computer-based model will provide necessary information and guidelines for further experiments.

One necessary property to verify is the probability that the VEGF concentration c reaches a level (constant $VEGF_{max}$) to cause choroidal invasion within a time bound $T$ of interest. This property is expressed as the following formula:

$$P_{=?}\,[\Diamond\,[0,T]\;c\;>=\mathrm{VEGF}_{\max}]$$

**Table 3** shows the verification results of this transient property using the model of **Fig. 8**. Based on our existing experimental data [4], the VEGF production rate, expressed as multiples of the normal VEGF production rate from the control group, are measured on 93%, 53%, and 33% of cells with a free edge. The time bound T is allowed to vary from 50 to 200 with an increment of 25. A high percentage of cells with a free edge is likely to cause VEGF expression to reach the maximum. Indeed, at $T = 50$, the probability of reaching $VEGF_{max}$ is already more than 50% for samples with 93% of the cells experiencing contact loss, compared to less than 4% for samples with 54% contact loss. Furthermore, in all three cases, the probability that VEGF expression reaches the maximum increases significantly if sufficient time is allowed, as evidenced by the high probability values at $T = 200$. This result is partly due to the lack of VEGF degradation in this basic example. All results presented in **Table 3** were generated by the PRISM probabilistic verification tool [35] in less than 2 minutes on a laptop with a 2.2 GHz dual core processor and 16 gigabytes of RAM. We expect necessary linear interpolation of parameter values in the model, due to the discrete nature of measurement data from experiments. In addition, when transition rate information is not fully available to build a CTMC model, a nondeterministic choice will be used instead, effectively lifting it to a MA model. Analysis of such models provides the boundary probabilities for the property under verification.

**Table 3**: Probabilities that [VEGF] reaches maximal value for given contact loss percentage.

| *RPE Cells with Contact Loss* | *[VEGF] Increase* | *Probability* | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | T=50 | T=75 | T=100 | T=125 | T=150 | T=175 | T=200 |
| 33% (n) | 2x | 4.4366 E-4 | 0.013 | 0.081 | 0.2406 | 0.4580 | 0.6647 | 0.8172 |
| 54% (c) | 3x | 0.0384 | 0.2211 | 0.5043 | 0.7457 | 0.8899 | 0.9581 | 0.9856 |
| 93% (d) | 13x | 0.5431 | 0.8308 | 0.9478 | 0.9858 | 0.9964 | 0.9992 | 0.9998 |

**Task 2**. Model normal and diseased three-layered constructs. This task composes the two-layered formal models of RPE-BrM and BrM-choroid constructs to form a three-layered model. For this model, it is crucial to model the spatial and physical effects of BrM, along which locations with high VEGF concentration are more likely to induce choroidal invasion (**Fig. 9**). We are also interested in modeling behaviors of VEGF leakage, as they can increase the rate and degree of severity of invasion. Spatial modeling will be realized by creating arrays of the two-layered RPE-BrM and BrM-choroid constructs, and modifying the BrM interface to form a three-layered model. The probabilistic verification tools considered for this stage are the MoDest toolset [36] and the Storm tool [37]. The MoDest toolset has rich data type support to provide concise description of array-based three-layered models. It also supports recursive generation of new processes, making it possible to model the growth of blood vessel cells. Storm has efficient analysis support for the analysis of large CTMC and MA models. Since both tools share a common language (JANI [38]), it is possible to use MoDest for modeling and Storm for the analyses.

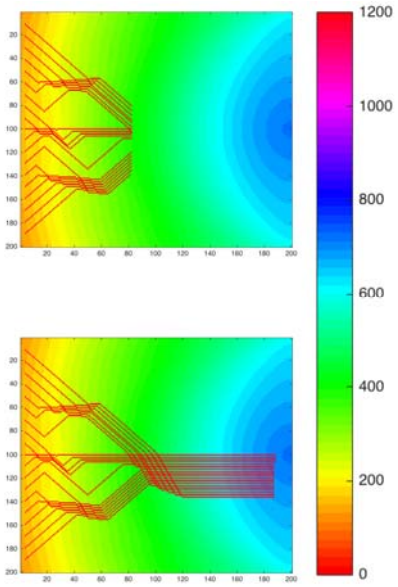


**Figure 9**. Analysis simulating choroidal growth towards higher [VEGF].

**Expected Results.** We expect the PMC will validate the two-layered constructs in Objective 1 and guide the experimental design in Objective 3. Using a CTMC model will allow multiple inputs and time points to be incorporated within the analysis, allowing us to understand the important parts of the design and the constructs' outputs, such as morphology and protein expression.

**Potential Problems and Alternative Solutions.** Complexity of the formal models, specifically the resulting state space, may increase significantly for the three-layered constructs, which may hinder both the capability and efficiency of our analysis. To alleviate this problem, we plan to apply both state space reduction (e.g., abstraction [39], partial-order reduction [40], etc.) and efficient state storage [41], that are readily available in the selected software tools. Another alternative is to apply stochastic simulation-based techniques [42], which are not limited by the size of state space but can still provide accurate approximations of the property checking results.

**3.3 Objective 3: Design three-layered constructs of normal and diseased subretinal structures** In this objective, we will build a three-layered construct, building upon Objective 1 and the parameters tested from the modeling analyses in Objective 2. To

develop a realistic model of the RPE/BrM/choroid complex, multiple cell types will be co-cultured to develop three-layered tissue constructs and provide a clearer model of mechanisms of different stages of ocular diseases.

### 3.3.1. Develop Normal Three-Layered Model of RPE, BrM, and Choroid

Within this construct and *in vivo*, the development of the choroid depends on RPE maturation [43]. Therefore, it is important that the RPE cells are completely differentiated and polarized before co-culturing with choroidal cells. RPE cells will be cultured for at least 4 weeks prior to addition to the construct to ensure their maturity, which will be verified with fluorescence and SEM imaging to validate polarization and the formation of apical microvilli.

**Task 1**. Fabricate microfluidic device to support three-layered normal construct. A microfluidic device will be fabricated to mimic the three-layered structures of subretinal tissue (**Fig. 10A**). A fibrin gel will be added to the top chamber of the device (channel 3) to support 3D vascularization. RPE cells will be grown on the optimized synthetic BrM from Objective 1 on top of the fibrin gel matrix. Choroidal cells will be mixed in fibrin gel and added to the middle channel (channel 2) to initiate angiogenesis. The bottom channel (channel 1) will be used as the culture media reservoir for endothelial cells (**Fig. 10B**). RPE medium will be added to channel 3 from the top separately. Polymer micro-posts will separate the channels of the device to prevent any leakage, while the gaps in between the posts will allow media to reach channel 2 and endothelial tubes to penetrate channel 3. Sprouting of endothelial tubes (**Fig. 10C**) will be monitored by brightfield and confocal microscopy. To characterize the 3D structure of the choroid, models will be stained with fluorescently-labeled endothelial-specific antibodies (anti-CD31 and anti-CD34) and imaged using confocal microscopy.

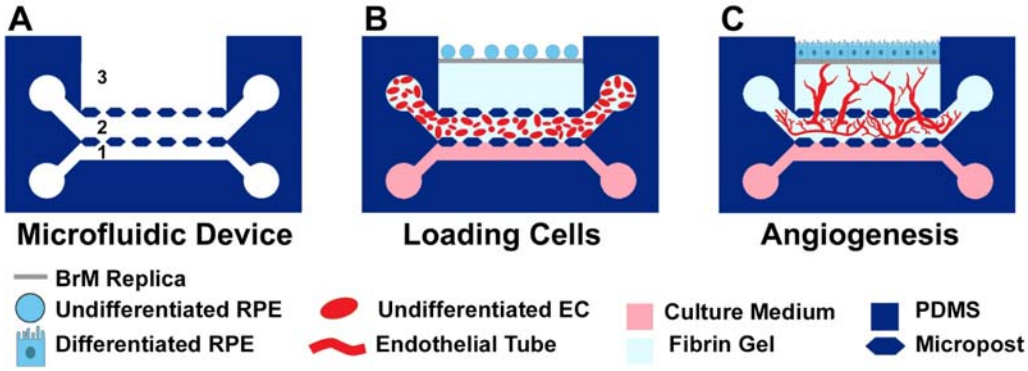


**Figure 10.** Top down view of three-layered construct. (A) Microfluidic device will be made with a depth of ~500μm. (B) Fibrin gel will be added to channel 3 and fibrin gel-embedded choroidal endothelial cells will be injected to channel 2. Culture media will be added to channel 1. (C) A complete model with endothelial cells sprouting towards the RPE.

### 3.3.2. Develop Diseased Three-Layered Model of RPE, BrM, and Choroid

During retinal disease, drusen formation or choroidal vasculature invasion can lead to RPE death and stretching of the RPE. Our preliminary studies show that both RPE loss and RPE stretching elevates VEGF expression (**Fig. 5**), suggesting that these events trigger or accelerate CNV. We will demonstrate the versatility of the normal, three-layered construct developed in Objective 3.3.1 to mimic RPE loss and elongation due to disease.

**Task 1.** Replicate RPE loss. To mimic RPE loss, RPE cells will be patterned on the BrM replica of the subretinal model. Using our stencil micropatterning method (**Fig. 6**), stencils will be placed on the BrM replica and RPE cells will be seeded around the stencil. Once RPE cells adhere to the BrM replica and differentiate, stencils will be removed to leave cell-free areas resembling areas of RPE degeneration. Different sizes of stencils will mimic different levels of RPE degeneration. The rate of endothelial tube formation will be compared with that in normal models to determine whether RPE degeneration leads to increased angiogenesis within the three-layered constructs.

**Task 2**. Replicate RPE elongation. To model RPE elongation, BrM from Objective 1 will be attached to PDMS walls of a three-layered subretinal model from 3.3.1. Two channels will be added near both BrM ends (**Fig. 11A**). By applying vacuum to these channels, membranes with RPE cells will be stretched (**Fig. 11B**). Using this device, we can control the level and rate of RPE stretching to mimic different numbers and sizes of drusen and levels of vascularization.

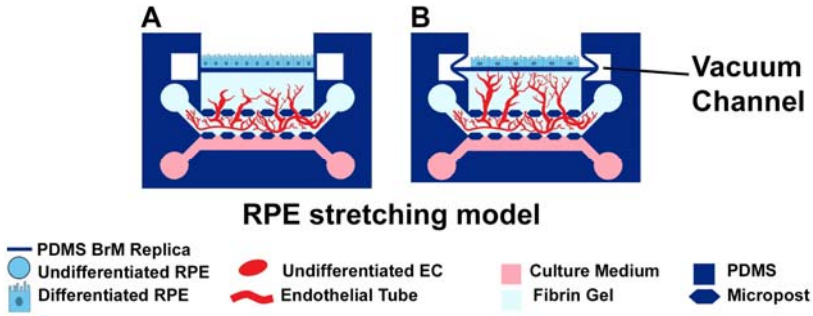


**Figure 11.** RPE stretching model. (A) Two channels will be added to both ends of the PDMS membranes with RPE cells. (B) Vacuum will be added to stretch the PDMS membrane and RPE cells.

**Task 3**. Replicate BrM malfunction. Diseased BrMs with increased thickness, reduced permeability, and drusen components will be used in microfluidic models to determine the effect of changes in the BrM functions on angiogenesis in the choroid.

Within each of these tasks, changes in angiogenic potential due to RPE loss and elongation will be monitored using brightfield and confocal microscopy. To identify the angiogenic factors responsible for increased angiogenesis, qRT-PCR will be performed on RPE cells. Alterations in the behavior of the RPE and choroidal cells will be monitored to find correlations between BrM thickness and new blood vessel formation. RPE detachment from the BrM or cell death and increased endothelial tube formation would indicate a shift towards new blood vessel growth.

**Expected Results.** Upon the completion of this objective, correlations will be found among the rate of increase in mechanical stress, loss of intercellular junctions, and choroidal angiogenesis. The selective transport of RPE-derived growth factors that are essential for the growth of endothelial cells through the BrM will support the growth of choroidal cells and formation of capillary tubes. Moreover, a list of angiogenic factors with increased expression during mechanical stress and RPE cell-cell detachment will be identified. We expect to see increased endothelial tube formation and pro-angiogenic growth factor expression in disease models. Choroidal cells will be in a 3D fibrin gel, so we anticipate that choroidal cells will produce a 3D network of endothelial tubes underneath the RPE. Since the BrM replica will be an accurate model of *in vivo* BrM, it is expected to prevent the invasion of endothelial tubes to the RPE under normal conditions. According to our previous studies, RPE cells with a free edge and elongated RPE cells produce higher levels of VEGF. Therefore, we expect to observe longer endothelial tubes and vessel migration around the edges of RPE-free areas models and elongated RPE cells.

**Potential Problems and Alternative Solutions.** Based on previous work, RPE cells grow on micropatterned surfaces and secrete varying levels of angiogenic proteins. If they do not, other methods of patterning will be used, including growing cells on lines of varying thicknesses where thinner lines mimic increased RPE degeneration. Exogenous factors that promote cell growth (like fibronectin) or inhibit cell growth (blocking agents, i.e. Pluronic F-127) can also be introduced into and around the micropatterns.

## 4. Timeline

| Objective – Task | | Year 1 | | | | Year 2 | | | | Year 3 | | | | Year 4 | | | | Year 5 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 |
| 3.1 2-layered construct | Normal | x | x | x | x | x | x | | | | | | | | | | | | | | |
| | Diseased | | | | | x | x | x | x | x | x | | | | | | | | | | |
| 3.2 Computational model | | | | | | | | x | x | x | x | x | x | x | x | | | | | | |
| 3.3 3-layered construct | Normal | | | | | | | | | | | | | x | x | x | x | x | x | | |
| | Diseased | | | | | | | | | | | | | | | x | x | x | x | x | x |
| Broader Impacts | | | | | | | | | | | | | | | | | | | | | |
| Benefits to Society | | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x |
| Underrepresented | | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x |
| Teaching, Training, Learning | | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x |
| Dissemination | | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x |

### Broader Impacts

**Benefit to Society**—My CAREER will benefit society in several critical ways. Initially, it will evolve the current state for *in vitro* models from one-layered to two-layered and three-layered models for normal and diseased human subretinas. The significance of this

is that this will potentially allow for improved understanding of the relationships of the tissues within the outer retina, its role in vision, and the impacts on vision loss. This fundamental knowledge may be used to improve models for prevention and treatment. Long-term, the CAREER will allow me to build my networks and publications in my field. I will expand my multidisciplinary collaborations to utilize the strength of computer modeling to develop *in vitro* models.

**Underrepresented**—My CAREER will focus on women in engineering through the Society of Women Engineers (SWE) and graduate recruitment. Currently, SWE at Utah State University has 85 members. As a female researcher and engineer, I believe it is critical to share my knowledge with, mentor, and coach women seeking to enter the profession. Since my research and teaching are integrated, I will recruit undergraduate and graduate women to my lab and will provide financial and mentoring support for their development as future female researchers. In addition, as the faculty advisor to SWE, I will work with SWE's student leadership to promote and participate in SWE's Evening with Industry and Community Night (**Fig. 12**). In addition, I have worked with Roche to secure an industry internship for my graduate student. This renowned lab will provide tremendous credibility to the students who are able to participate.

**Figure 12.** SWE Evening with Industry provides an opportunity for industry to meeting potential new female engineers.

**Teaching, Training, Learning**—My graduate students and I will develop workshop materials for building retinal imaging devices to share with 8$^{th}$ and 9$^{th}$-grade students participating in USU STARS! (GEAR-UP), a STEM-focused program that encourages underrepresented and first-generation students to enter into STEM professions. In addition, I will develop a new tissue engineering class module to determine ideal substrates for growing healthy, polarized, and pigmented retinal cells. This module will provide an opportunity for the one-layered model, two-layered model, and three-layered model similarities and differences in normal and diseased models to be studied.

**Dissemination**—The CAREER is an essential mechanism for dissemination in peer-reviewed journals and conferences, networking, and co-publishing with undergraduate and graduate students. We will attend the Association for Research in Vision and Ophthalmology (ARVO) conference and submit to disseminate our findings annually. I will attend the Biomaterials and BMES conferences as well. In addition, we will publish in peer-reviewed journals such as Biomaterials, Annals of Biomedical Engineering, IOVS, and potentially JEE. Attending these conferences and publishing will allow me to build my network and to develop potential future collaborators.