TEMPLATE

Cover page

Please include the following details in your application's cover page:

- Team Lead's name and institution/organization/company
  Rebecca Carrier, Northeastern University
- Team Lead's academic appointment or department, if applicable
  Professor, Chemical Engineering Department
- List of team member's names and affiliated institution/organization/company
  - Michael Young, Schepens Eye Research Institute
  - Joyce Y Wong, Boston University
  - Joydip Kundu, Northeastern University
  - Petr Baranov, Schepens Eye Research Institute
  - Mike Ferguson, Boston University
- Indicate if your submission is a solution most applicable to disease modeling or drug testing
  - solution applicable to disease modeling (Age Related Macular Degeneration)
- Indicate if you are applying to the trainee category
  - No

Comprehensive Description of the Proposed Solution (6 page maximum)

Abstract: Recently, methods to produce 3D retinal organoids, potentially tremendously useful tools for developmental and regenerative research, from iPSCs and ESCs have been developed. However, current approaches are limited in their ability to create retinal organoids that recapitulate the complexity and functionality of the retina. In particular, current retinal organoids cultures lack proper spatial organization of a retinal pigmented epithelium (RPE) relative to neural retina and organization of retinal layers. In addition, current retinal organoid systems lack vasculature, and thereby are limited in availability of nutrients and oxygen supply to the culture system, constraining the size of the organoids that can be grown and the disease states that can be meaningfully explored. To address the shortage of spatial control within developing retinal organoids, we propose to create a biomimetic gradient of chemical and physical cues recapitulating those present during retinal development, rather than an isotropic environment lacking spatial variation in cues. To overcome the lack of vascularization and aid in establishment of these gradients, a bioengineering approach using microfluidic chambers will be employed to enable in situ development of vasculature driving the flow of fluid through a biomimetic hydrogel-based “choroid” on which retinal organoids will be grown. The hydrogel properties, including chemical composition and stiffness (elastic modulus of the retina is 0.431 MPa), will be systematically varied to promote the selective attachment and proliferation of the retinal pigment epithelium cells within growing organoids, and the spatiotemporal control afforded by the microfluidic platform will be used to impart a gradient of signaling cues across the growing organoids to allow proper orientation of layers recapitulating a physiologically relevant laminated retina. The engineering of a system enabling examination of interaction of blood vessels and retinal tissue during organoid development and culture offers high potential for understanding development of the eye and modeling retinal diseases including age related macular degenerations (AMD).

Background: The production and study of stem cell-derived organoids - 3D tissues that develop as the result of a complex genetically encoded self-assembly program and recapitulate the cell populations, micro-anatomy, and functions of a given organ - is an exciting new field in developmental biology providing unprecedented insights into human development, disease, and function (Kretzschmar & Clevers, 2016; Turner, Baillie-Johnson, & Martinez Arias, 2016). Eiraku et al. reported for the first time the dynamic, autonomous formation of the optic cup (retinal primordium) structure from a three-dimensional culture of mESC aggregates (Eiraku et al., 2011). mESC derived retinal epithelium formed hemispherical epithelial vesicles that became patterned along their proximal–distal axis while the flexible distal portion progressively folded inward to form a shape reminiscent of the embryonic optic cup, with stratified neural retinal tissue (Eiraku et al., 2011). However, the cone differentiation in the developed neural retina is rare in mESC culture. Nakano and his coworkers demonstrated the optic cup structure formation by self-organization in human ESC (hESC) culture (Nakano et al., 2012). The neural retina in hESC culture is thick and spontaneously grows into multilayered tissue containing both rods and cones (Nakano et al., 2012). This technology provides significant advantage over existing 2D differentiation methods: the spatiotemporal recapitulation of the development allows for proper formation of all major retinal cell types. The development of optic cup from hESC requires an exhaustive differentiation strategy involving initial dissociation followed by re-aggregation of human ES cells, followed by addition of extrinsic chemical factors and long duration culture under high oxygen conditions. Zhong et al. demonstrated that human induced pluripotent stem cells (hiPSC) can, in a highly autonomous manner, recapitulate spatiotemporally each of the main steps of retinal development observed in vivo and form three-dimensional retinal cups that contain all major retinal cell types arranged in layers (Zhong et al., 2014). Serious limitation of all the protocols discussed so far is the heterogeneity within and between organoids and the practice of manual dissection or mechanical scraping for isolation of optic cup like retinal organoids. Völkner et al. developed a procedure utilizing mESC for the

effective generation of large, 3D-stratified retinal organoids that does not require evagination of optic-vesicle-like structures (Volkner et al., 2016). Lowe et al studied retinal organoid morphogenesis in hESC and hiPSC-derived cultures allowing isolation of large quantities of retinal organoids (Lowe, Harris, Bhansali, Cvekl, & Liu, 2016). Long-term cultures of the retinal organoids autonomously generated stratified retinal tissues, including photoreceptors with ultrastructure of outer segments, and required minimal manual manipulation (Lowe et al., 2016). The majority of protocols described utilize animal-derived components in the medium and animal-derived substrate or feeder layer. Reichmann et al developed a two-step xeno-free/feeder-free (XF/FF) culture system to efficiently differentiate hiPSCs into retinal cells that relies only on adherent hiPSCs cultured in chemically defined media, bypassing embryoid body formation (Reichman et al., 2017). **While there has clearly been a progressive advancement of retinal organoid culture techniques, current retinal organoid cultures lack proper spatial organization of a retinal pigmented epithelium (RPE) relative to neural retina structure and organization of retinal layers. In addition, current retinal organoid systems lack vasculature, and thereby are limited in availability of nutrients and oxygen supply to the culture system, constraining the size of the organoids that can be grown and the disease states that can be explored.**

In the past few years, a class of microfluidic devices that can form perfusable capillary beds by mimicking angiogenesis and vasculogenesis *in vitro* has emerged (Bogorad et al., 2015). In general, the devices (hereafter referred to as *vascularization devices*) contain a *sealed chamber* (microvessel chamber) in which perfusable capillaries self-assemble. A major limitation of the current devices is that the microvessel chamber is only 100um tall - a volume far too small to accommodate even immature organoids. To overcome this limitation and enable culture of retinal organoids on the capillary bed, a vascularization device in which the top wall of the microvessel chamber is removed has been fabricated based on a design by Dr. Steven George and colleagues **Fig. 1 and 2** (appendix section) (Alonzo, Moya, Shirure, & George, 2015; Hsu, Moya, Abiri, et al., 2013; Hsu, Moya, Hughes, George, & Lee, 2013; Moya, Alonzo, & George, 2014; Moya, Hsu, Lee, Hughes, & George, 2013; Wang et al., 2016).

A schematic of how to use the device is shown in **Fig. 1 and 2** (see appendix section). Briefly, hydrostatic reservoirs are used to create a pressure difference that drives culture media through an inlet into a long microfluidic channel. The microfluidic channel is connected to the hydrogel/capillary-containing microvessel chamber by two “pores” at two points along the long microfluidic “flow channel” - one point near the inlet and another near the outlet. Near the inlet, the pressure in the flow channel is similar to the inlet pressure. Near the outlet, the pressure is similar to the outlet pressure. Thus, a pressure gradient is established across the hydrogel in the microvessel chamber, driving flow from the inlet pore to the outlet pore (and through the capillaries that will self-assemble and connect to the pores).

Current organoid culture methods are complicated by the nearly exclusive dependence on matrigel as the 3D matrix (Kleinman & Martin, 2005), with or without presence of extrinsic factors. Matrigel has complex and variable composition, not amenable to modifications, and poses risks of immunogen and pathogen transfer (Hughes, Postovit, & Lajoie, 2010). In the retina, ECM plays a key role in regulating retinal development and cellular differentiation (Place, Evans, & Stevens, 2009). Here we propose to use methacrylate modified ECM components (collagen I/IV, hyaluronic acid, gelatin) to define the microenvironmental parameters (chemistry, stiffness) that govern hiPSCs behavior and retinal organoid formation. To assess whether a 3D gel matrix is sufficient for hiPSC expansion and organoid formation, we will embed aggregates formed from the hiPSCs into photo-crosslinked collagen I methacrylate (Col I-MA), Hyaluronic acid methacrylate (HA-MA), and gelatin methacrylate (Gel-MA) hydrogels of varying stiffness. Addition of laminin and collagen IV, either as intact protein or through functionalization of the gels with appropriate peptides, will be used to further mimic the choroid basement membrane. The macula, the central retinal region where the fovea is located, is particularly prone to

degenerative diseases, including age-related macular degeneration. Researchers have shown that addition of retinoic acid (RA) to the retinal organoid culture induces the formation of photoreceptors (mainly the rod cells in the absence of cone cells) from iPSCs. Thus, the organoids derived from hiPSCs lack the formation of a fovea-like structure. It was recently demonstrated that timed and local inhibition of RA synthesis, and/or local application of Fgf8, induces formation of cone cells (da Silva & Cepko, 2017). This approach will be utilized to promote induction of a fovea in the proposed system. Thus, vascularized retinal organoids with a well-defined macula will be developed to enable meaningful in vitro modeling of macular degeneration disease (like AMD).

Technologies and protocols needed to develop the model systems:

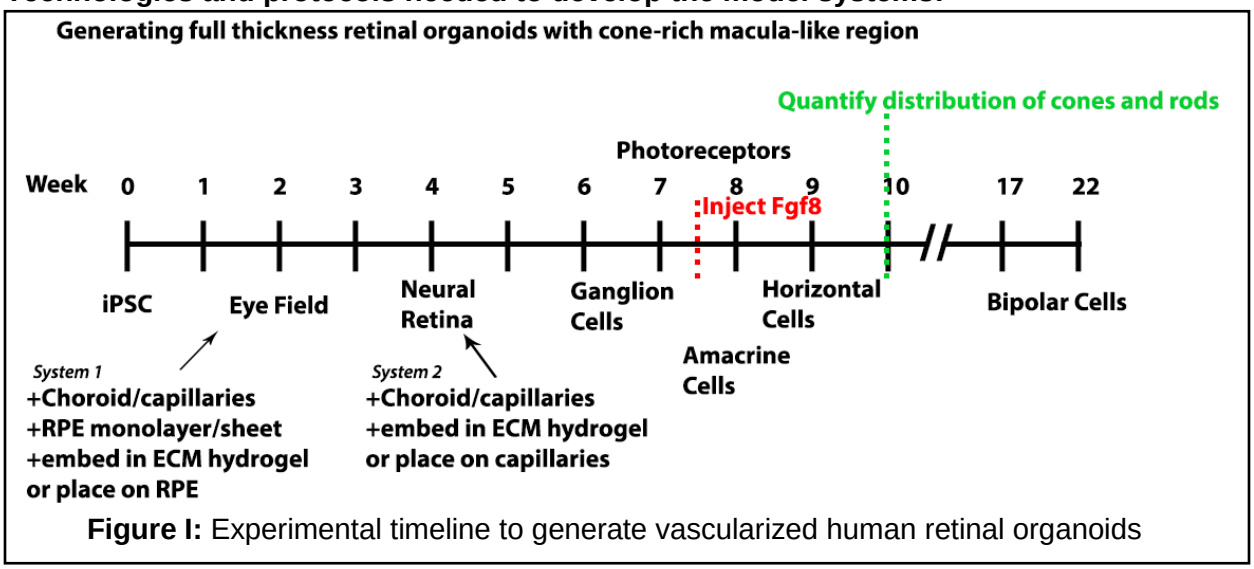

Figure I: Experimental timeline to generate vascularized human retinal organoids

Summary (**Fig I**): We propose to develop an organoid-microvessel co-culture system, where the capillary bed is developed in a biomimetic gel within a microfluidic device either before the introduction of organoid-forming retinal cells, or concurrently with retinal organoid formation. The stiffness and chemical composition of the gel within which the microvessels are formed, as well as the gel within which the organoid-forming cells are introduced, will be systematically varied to mimic gradients present within the developing eye. In addition, the composition of the medium that is perfused through the microvessel system (representing choroid microvasculature) is different from that which rests on top of the organoid culture (representing vitreous composition), such that biomimetic chemical gradients of signaling factors can be established. It is anticipated that biomimetic gradients of chemical and physical cues recapitulating those present during retinal development created by the co-culture of microvessels with retinal organoids will promote robust organization of tissue. The stiffness of the gels will be tailored by altering the polymer content, degree of crosslinking, and crosslinking time. The organoid-microvessel co-culture system within a microfluidic device as described above will be of two types: **System 1:** endothelial cells will undergo vasculature formation in biomimetic gels, a confluent layer of hiPSC derived RPE will be cultured on top of the vasculature gels, and finally EF (eye field) domains developed after 2 weeks of differentiation of hiPSC will be embedded within ECM gels laid on the top of the RPE layer, where retinal organoid formation will occur. **System 2:** endothelial cells will undergo vasculature formation in biomimetic gels, and neural retina (NR) domains developed after 4 week of differentiation of hiPSC (including RPE) will be layered in modified ECM gels on top of the vascularized gels, where retinal organoids will mature. Gel composition and stiffness will be modified in such a manner as to mimic the properties of the choroid basement membrane, to induce attachment and growth of the RPE portion of the organoids at the gel-gel interface. The microfluidic platform will generate an

exposed capillary bed that may integrate with retinal organoids, and could thus be used to perfuse them with various perfusates, which may lead to improved growth, maturation, and function of organoids.

Microfluidic device fabrication: A microfluidic device capable of forming a perfusable an exposed capillary bed has already been developed (**Fig. 1 and 2,** see appendix section). The device can be made from PDMS using a soft lithography-based method or from polystyrene using a hot embossing based method (**Fig. 2 and 3**, see appendix section)). This is significant since PDMS is well known to absorb hydrophobic molecules such as retinoic acid. Further device modification and protocol development will need to occur to successfully apply the technology towards creating vascularized retinal organoids. Perfusion at a higher pressure will be extremely desirable, if not necessary, for increased growth and proper perfusion of *in vitro* vascularized retinal organoids. A closed loop circulation consisting of a pressurized reservoir and peristaltic pumps will be implemented to achieve physiologic perfusion pressures (**Fig. 4**, see appendix section). Finally, it is noted that pressure within the vessel containing the organoids can also be controlled to explore the impact of intraocular pressure on organoid function and disease models.

Vascularization within microfluidic device: Human umbilical vein endothelial cells (HUVEC, Lonza CC-2519) or stem cell derived endothelial cells will be used and cultured in the EGM-2 Media (Lonza). Endothelial cells encapsulated within a biomimetic ECM (photo-crosslinked collagen I methacrylate (Col I-MA), Hyaluronic acid methacrylate (HA-MA), or gelatin methacrylate (Gel-MA)) gel will be loaded into the microvessel chamber. Choroidal fibroblasts and/or pericytes may also be added to the gel (Prasain et al., 2014). EGM-2 media (with VEGF/bFGF) will then be perfused across the microvessel chamber. Capillaries that span the microvessel chamber will start to form on days 2-3. All cultures will be maintained at 37 °C in a humidified atmosphere containing 5% CO_2.

Retinal Differentiation /hiPSC-Derived Retinal Cell Cultures: The 3D retinal organoids will be generated from Human iPSCs following the protocol of Reichman et al (Reichman et al., 2017). In brief, hiPSCs will be expanded in Essential 8 medium to 70–80% confluence in cell culture dishes coated with rhVTN-N (Thermo Fisher Scientific). At this time, defined as day 0 (D0), hiPSCs will be cultured in chemical defined Essential 6 medium (Thermo Fisher Scientific). After 2 days, the medium will be switched to E6N2 medium composed of Essential 6 medium, 1% CTS (Cell Therapy Systems) N2 supplement (Thermo Fisher Scientific), 10 units per ml Penicillin and 10mg/ml Streptomycin (Thermo Fisher Scientific). The medium will be changed every 2–3 days.

hiPSC-derived RPE (hiRPE) cell cultures: The hiRPE cell cultures will be developed as described by Reichman et al (Reichman et al., 2014). In summary, hiPSCs will be expanded to confluence in iPS medium. At this point, defined as day 0 (D0), confluent hiPSCs will be cultured in iPS medium without FGF2. After 2 d, the medium will be switched to a “proneural medium” composed of DMEM:Nutrient Mixture F-12 (DMEM/F12, 1:1, L-Glutamine), 1% MEM nonessential amino acids, and 1% N2 supplement (Life Technologies). The medium will be changed every 2–3 d. On D14, identified neural retina NR-like structures will be isolated using a needle with the surrounding pigmented cells. The identified pigmented patches will be cut at D14 without the nonpigmented budding structures and transferred onto 0.1% gelatin-coated plates, noted as passage 0 (P0). hiRPE cells will be expanded in the proneural medium, and the medium will be changed every 2–3 d.

Organoid-microvessel co-culture system 1: After second week of hiPSC differentiation, self-formed eye field domains will be isolated, using a needle, and encapsulated within methacrylate modified ECM (collagen I/ IV, hyaluronic acid, gelatin) gel with varying stiffness. The gel loaded with 10-20 eye field domains, or, alternatively, the eye domains alone, will then be gently placed on top of the confluent layer of hiPSC derived RPE growing on vascularized gels within the microfluidic chamber. The organoids will be grown in the ProB27 medium supplemented with 10

ng/ml of animal-free recombinant human FGF2 (Peprotech) and the medium will be changed every 2-3 days. The composition and stiffness of the microvasculature gel as well as the organoid culture gel will be varied to explore the impact on organoid formation. The vasculature medium is supplemented with the VEGF and PDGF to drive proper endothelial cell and pericyte differentiation.

Organoid-microvessel co-culture system 2: On the fourth week of differentiation, identified self-formed neural retina domains will be isolated, using a needle, and encapsulated within methacrylate modified ECM (collagen I/ IV, hyaluronic acid, gelatin) gel with varying stiffness. The gel loaded with 10-20 neural retina domains, or, alternatively, the eye domains alone, will then be gently placed on top of the vascularized gels within the microfluidic chamber and grown in the ProB27 medium supplemented with 10 ng/ml of animal-free recombinant human FGF2 (Peprotech) and the medium will be changed every 2-3 days. For long-term suspension culture, the medium will be supplemented with 10% fetal bovine serum (Gibco), 100 mM Taurine (Sigma) and 2mM GlutaMAX (Invitrogen) beginning on D42, unless otherwise noted.

RA treatment and fgf8 treatment: To promote photoreceptor maturation and fovea like structure formation within the organoid-microvessel co-culture system, the culture system will be injected locally with 1 mM all-trans RA (Sigma) and fgf8 at various time windows, subsequently, RA will be inhibited by using inhibitors, promoting the cone formation with the application of fgf8.

Immunohistochemistry: Cells growing on adherent conditions will be fixed in 4% paraformaldehyde (PFA; Sigma) for 15 min. Retinal organoids will be fixed in 4% PFA for 30 min. Tissue cryopreservation, sectioning and immunohistochemistry will be performed as described (Zhong et al., 2014). Antibodies against the following proteins will used: LHX2, RX SOX1, VSX2, OTX2, recoverin, Caspase 3, HU C/D, BRN3, TUJ1, PROX1, CRALBP, rod opsin, L/M-opsin, S-opsin, phosphodiesterase 6- α and -β, human retinal guanylate cyclase 1, GT1α, rod cyclic-nucleotide-gated channel α-subunit and β-subunit, PAX6 , AP2a and SV2 . Secondary antibodies to be used included the corresponding species-specific Alexa Fluor-488-, -546- and -647-conjugated antibodies (Molecular Probes). DAPI (40, 6-diamidino-2-phenylindole) was used for nuclear counterstaining (Molecular Probes). Fluorescence images will be acquired with an LSM 510 confocal microscope (Zeiss).

Longitudinal analysis of hiPSC differentiation: The longitudinal analysis of the hiPSC differentiation will be determined following reported procedure (Zhong et al., 2014). The percentage of NR domains will be evaluated by counting the number of VSX2-positive colonies among DAPI-positive colonies on D12, D16 and D20. Colonies containing 45 VSX2-positive colonies will be considered NR domains. To trace the morphological progression of NR and RPE domains, plated aggregates will be individually outlined using a microscope objective marker (Nikon) and imaged every other day from D17 to D25 under an inverted microscope (Nikon). Growth of RCs in long-term culture will be investigated by imaging every 15 days from D45 until D90 under inverted microscope. The length of the longest axis of RCs will be measured using ImageJ. To determine the time of generation of the major retinal neuronal cell types, a minimum of five RCs will be collected each week from W5 to W13, then every other week until W17, and once a month thereafter. Cell-type-specific markers will be used for immunohistochemical identification as described above.

Model system that will be amenable to disease modeling.

The system described here is the attempt to create a proper model of AMD that incorporates all major components: RPE, neural retina and vasculature (**Fig II**, see feasibility Assessment section). This approach became possible by combining 3D culture system with microfluidics setup. The co-culture of vascularized beds (mimicking the choroid) and retinal organoids in close association with a layer of polarized RPE cells makes the proposed system suitable for disease modeling, as it contains essential anatomical features contributing to disease. In addition, as the microfluidic platform is low-cost and scalable, the system is amenable to high content screening.

Retinal degenerative diseases such as AMD impact millions of people, and current treatments only slow the disease progression. AMD is characterized in its early stages by the presence of extracellular deposits, known as drusen, that accumulate between the basal surface of the RPE and Bruch's membrane, an extracellular matrix complex that separates the neural retina from the capillary network in the choroid (Johnson et al., 2011). Although many animal models are available for AMD research, most do not recapitulate all aspects of the disease, hampering progress. However, the use of cultured RPE cells in AMD research is well established and, indeed, some of the more recently described RPE-based models show promise for investigating the molecular mechanisms of AMD and for screening drug candidates (Forest, Johnson, & Clegg, 2015). Primary cultures of human RPE (hRPE) and iPSCs can be used to generate RPE cells that are particularly effective tools in AMD research because they closely model the function and metabolic activity of native RPE (Ablonczy et al., 2011; Jin et al., 2011). In vivo, the apical RPE surface interfaces with retinal photoreceptors, and its basal surface attaches to Bruch's membrane, a specialized structure composed of collagen, laminin, elastin and fibronectin (Booij, Baas, Beisekeeva, Gorgels, & Bergen, 2010). This porous ECM allows for selective metabolite exchange to occur between the retina and its primary blood supply, the choriocapillaris (Johnson et al., 2011). The vascularized retina organoids growing in microfluidic platform will be cultured in close association with a single layer of polarized RPE cells. Disturbance of this normal polarized configuration of RPE cells is associated in retinal disease conditions. RPE cells grown on porous supports form subcellular deposits that contain drusen-associated molecules and activated complement proteins when exposed to human serum (Johnson et al., 2011) or subjected to peptide based complement-system inhibitors (Gorham et al., 2013). By combining an RPE monolayer with retinal organoids, or with a modeled choroid capillary bed, we will be able to study the interaction between cells contribute to the disease.

Innovation statement.

The proposed approach integrates expertise of and technology developed by our team to enable a system incorporating a vascularized choroid layer in a microfluidic device. This system will allow the introduction and variation of spatial and chemical cues imparted by the difference in perfusion media vs. that on top of the organoids, varied chemical and physical properties of the gel forming the microvasculature as well as the organoid culture gel (which may be eliminated altogether), and varied pressure between perfusion medium (representing vasculature) and medium on top of organoids (representing intraocular pressure).

Using this particular microfluidic organoid vascularization device has many advantages over other *in vitro* vascularization methods (e.g. 3D bioprinting). Rather than being composed of hollow channels lined with endothelial cells that are relatively static, the vessels formed by our device recapitulate development, self-assemble, and are capable of significant remodeling similar to that observed *in vivo* (**Fig 5 and 6**, see appendix section). Moreover, the microfluidic device is low cost, portable, simple to use, and scalable. This is in sharp contrast to bulky, complex, and expensive bioprinters that are incapable of high throughput fabrication.

The vascularization technology developed is modular and applicable to all human organs and tissues rather than being confined to one specific organ or tissue system. Moreover, the vascularization technology is not restricted to simply vascularizing organoids. For example, explanted human tissue may be able to anastamose to the exposed capillary bed and enable perfusion for long-term culture in normal or diseased physiological studies. The proposed system and device can also be used to study human vascular development, function, and disease. For example, a quantitative understanding of the effect of blood pressure in angiogenesis and vascular remodeling is lacking due to inadequate, inaccessible, and impractical *in vitro* or animal model systems.

Biographical Sketches

Carrier, Rebecca L, Professor, Northeastern University

The overall idea of the challenge is to develop well organized and functional retinal organoids that will be used to model retinal degeneration in AMD. Rebecca has extensive experience with multidisciplinary project teams and analysis of cell response to physical and chemical cues presented by extracellular matrix that will enable her to successfully lead the project team to achieve this goal. Her graduate work in cardiac tissue engineering was focused on biochemically and structurally characterizing cell-polymer constructs cultured in different bioreactor environments. Her industrial experience in drug delivery has given her a broad background in materials processing and an appreciation for what it takes to develop a feasible, therapeutically useful product. As a professor, and a PI or co-PI on a number of foundation, industrial, NSF- and NIH-funded projects, she has built a research program based on cell and tissue engineering and drug delivery. Through these projects, she has developed techniques for fabrication and analysis of cell response to biomimetic biomaterials for regenerative medicine, including extracellular matrix-based materials. In particular, her research team have explored interphotoreceptor matrix (IPM), as well as whole decellularized retina as biomaterials. This work has been conducted in collaboration with Drs. Young, Baranov and Kundu, who are members of the Project Team. In summary, she has the experience to successfully coordinate the efforts of and work with the research team to develop solutions for the challenge. Most relevant article*: Kundu, J., Michaelson, A., Baranov, P., Chiumiento, M., Nigl, T., Young, M. J., & **Carrier, R. L.** 2017. Interphotoreceptor matrix based biomaterial: Impact on human retinal progenitor cell attachment and differentiation. J Biomed Mater Res B Appl Biomater. doi: 10.1002/jbm.b.33901.*

Young, Michael J, Associate Professor of Ophthalmology, Harvard Medical School

Michael's laboratory is focused on retinal transplantation of stem cells for the treatment of retinal diseases. His research team isolate stem cells from the neurosensory retina, expand them in culture, and then graft them into the diseased eye, where they differentiate as photoreceptors and restore light-induced activity. His research involved both rodent and porcine donors and recipients. His research team has isolated and characterized human retinal stem cells, and shown that these cells are also capable of generating photoreceptors following grafting to the degenerating retina. During the last 5 years, work in his lab has established that neural stem or progenitor cells overcome the barrier to morphological integration present in the mature mammalian retina. His research group has now embarked upon a series of studies in the pig retina, with the goal of establishing functional connectivity between donor retinal stem cells and the mature, diseased host retina. This approach will allow him to make important steps toward his goal of functional restoration of vision. Most relevant article: *Klassen, H., Warfvinge K., Schwartz P.H., Kiilgaard J.F., Shamie N., Jiang C., Scherfig E., Prather R.S., **Young M.J**. 2008. Isolation of progenitor cells from GFP-transgenic pigs and transplantation to the retina of allorecipients. Cloning and Stem Cells. **10** (3): 391-402. 2.*

Wong, Joyce Y, Professor, Boston University

Dr. Wong's research focuses on developing biomaterials to detect and treat disease. She uses a biomaterials science and engineering approach that seeks to understand interrelationships between composition, structure, processing, and properties - namely cell phenotype and cell behavior. Dr. Wong has developed several in vitro models to study both normal development of tissues and progression of disease (including the use of induced pluripotent stem cells) that enable the systematic investigation of biophysical parameters that affect cell behavior. She has been studying factors controlling vascular smooth muscle and endothelial cell phenotype for vascular tissue engineering applications. Using micropatterning and microfluidics, her laboratory developed several platforms that enable the investigation of interactions between cancer cells and niche cells. Related to the work proposed here, her laboratory has used a model of angiogenesis-on-a-chip (or blood-vessels-on-a-chip) to evaluate the performance of ultrasound-

triggered drug delivery carriers. Because her research approach is fundamental in nature, it can be applied to other disease systems as is proposed here. Most relevant article: *Y. Park, C. Zhang, S. Kim, G. Mohamedi, C. Beigie, J. Nagy, R.G. Holt, R.O. Cleveland, N.L. Jeon, and* ***J.Y. Wong*** *(2016) "Microvessels-on-a-Chip to Assess Targeted Ultrasound-Assisted Drug Delivery," ACS Applied Materials & Interfaces, 8 (46), pp 31541–31549*

Kundu, Joydip, Associate Research Scientist, Northeastern University

Joydip's experience in tissue engineering has given him the expertise to successfully carry out this challenge idea. Since joining Northeastern University, he has been actively working on the development of decellularized matrix-based biomaterials to deliver retinal progenitor cells. In collaboration with Schepens Eye Research Institute, he has been investigating the cellular response to these matrices and their ability to integrate within the host retina to restore vision. His previous experience as a graduate student and postdoc has afforded him a broad set of scientific skills and expertise relevant to tissue engineering and biomaterials, with particular strength in analyzing cell response to materials. Northeastern University and SERI has provided him the opportunity and resources to develop specific expertise in retinal tissue engineering and regenerative medicine, with a particular focus on the analysis of retinal progenitor cell response to materials developed from extracellular matrix in the context of retinal regeneration strategies. Most relevant article: ***Kundu, J****., Michaelson, A., Talbot, K., Baranov, P., Young, M., Carrier, R.L. 2016. Decellularized retinal matrix: natural platforms for human retinal progenitor cell culture. Acta Biomater. 31: 61-70. doi:10.1016/j.actbio.2015.11.028.*

Baranov, Petr, Investigator, Schepens Eye Research Institute

Petr's work is focused on the discovery and translation of novel therapies for degenerative eye diseases. He has extensive experience in several areas of the proposal. During his postdoctoral training at Schepens Eye Research Institute, he has worked with various animal retinal degenerative models using mouse and human induced pluripotent stem cells. He has ongoing collaborations with academia (Northeastern University, University of California San Diego, University of Iowa, Massachusetts Institute of Technology, University of Goias, Brazil) underline his ability to function as a key team player. And the work with industry (Reneuron, GlaxoSmithKline) gave him unique experience on the translation of cell therapy and pre-clinical development process. Currently, he is investigating the cell transplantation and neuroprotection using iPSc-derived retinal organoids and animal models of retinal degeneration. In summary, his unique set of skills, including clinical training and extensive collaborations have prepared him to execute the proposed project. Most relevant article: ***Baranov, P.****, Tucker, B., Young, M.J. 2014 Low-oxygen culture conditions extend the multipotent properties of human retinal progenitor cells. Tissue Eng Part A.; 20(9-10):1465-75*

Ferguson, Mike, Graduate student, Boston University

Mike is a highly interdisciplinary scientist and engineer who strives to apply insights gained from studying stem cells, developmental biology, engineering, and regenerative medicine to work towards generating *in vitro* human models of disease. Mike has 4 years of experience working with human stem cell derived organoids and is currently an MS student in bioengineering and biomedical engineering at Boston University, having spent the past year in Dr. Joyce Wong's lab independently developing a novel microfluidic device that can be used to vascularize and perfuse human stem cell derived organoids entirely *in vitro*. Mike earned his BS in cell and molecular biology at the University of Michigan - Ann Arbor, where he spent nearly 3 years working in the lab of Dr. Jason Spence on numerous projects with the first stem cell derived lung organoids. While in Dr. Spence's lab, he independently developed the first fetal human lung organoids and used this novel culture system to identify critical and unexpected developmental differences between mice and humans. Most relevant article: *Dye B.E., Hill D.R., Ferguson M.A.H., Tsai Y.H., Nagy M.S., Dyal R., Wells J.M., Mayhew C.N., Nattiv R., Klein OD, White E.S., Deutsch G.H., Spence J.R. In vitro generation of three-dimensional lung organoids from human pluripotent stem cells. eLIFE 2015. 4 e05098*

Feasibility Assessment

Evaluation Criterion 1. Cell Type, Structure, Viability, and Function

•Cell Types :The organoid-microvessel co-culture system will promote blood vessel formation (representative of the choroid), incorporate an RPE, and guide retinal organoid development using an established protocol that has demonstrated the presence of photoreceptors, bipolar cells, ganglion cells, horizontal cells, and amacrine cells (Zhong et al., 2014). Moreover, a transient localization of Fgf8 will be used in an attempt to produce a greater number of cones. Immunohistochemistry will be used to assess the presence and maturity of the cells in our system. Our samples will be compared to fetal human retina.

Retinal pigment epithelium (RPE) cells will either be formed using an established protocol (Reichman et al., 2017; Reichman et al., 2014) during the retinal organoid formation process (4-week post hiPSC differentiation) or they will be included in the organoid-microvessel co-culture system as a confluent layer of hiPSC derived RPE growing on a vascular network. In developing the choroid-like layer, choroidal fibroblasts and hiPSC derived endothelial cells (ECs) will be used (Prasain et al., 2014). Pericytes can also be added. It is expected that the presence of choroidal fibroblasts and RPE will help differentiate the ECs to choroid specific endothelium.

By including all of the major retinal cell types, as well as a choroid-like layer and RPE, we expect to maintain the crosstalk between layers and obtain a well-organized, laminated structure. The co-culture of a capillary bed capable of extensive remodeling (including angiogenesis) and retinal organoids in close association with a layer of polarized RPE cells makes the proposed system suitable for disease modeling like age related macular degeneration (AMD), as it contains essential anatomical features contributing to this disease.

•Structure: A bioengineering approach using microfluidic based platform will be used to enable in situ development of vasculature, driving the flow of fluid through a biomimetic hydrogel-based “choroid” on which retinal organoids will be grown. The limitations of spatial control within developing retinal organoids will be overcome through the creation of a biomimetic gradient of chemical and physical cues recapitulating those present during retinal development, rather than an isotropic environment lacking spatial variation in cues. The chemical composition and stiffness of the biomimetic hydrogel, will be systematically varied to promote the selective attachment and proliferation of the RPE cells within growing retinal organoids, and the spatiotemporal control afforded by the microfluidic platform will be used to impart a gradient of signaling cues across the growing organoids to allow proper orientation of layers recapitulating a physiologically relevant laminated retina.

•Viability: The protocol incorporates microfluidic platform based organoid-microvessel co-culture system that promotes interdependence and impact of the vasculature on the retinal organoid formation. The presence of a perfusable vasculature will greatly enhance the delivery of nutrients and oxygen to the developing retina, in addition to allowing communication and biomolecule exchange between the retina, RPE, and capillaries similar to the in vivo situation. Oxygen concentration plays a crucial role in the eye and influences retinogenesis. Retinal organoid formation will take place over a period of 22-24 weeks to develop all the five types of neuronal retinal cells. Growing the organoids with a perfusable vascular system should help prevent the development of a necrotic core with organoids and promote better cell viability as compared to current culture systems.

•Functional characterization of cell types: The composition of the medium that is perfused through the microvessel system (representing choroid microvasculature) is different from that which rests on top of the organoid culture (representing vitreous composition), such that biomimetic chemical gradients of signaling factors can be established. It is anticipated that biomimetic gradients of chemical and physical cues recapitulating those present during retinal development created by the co-culture of microvessels with retinal organoids will promote robust organization of tissue and enable all cell types remain functional. Metabolic labeling and functional characterization of retinal neurons will be used to validate that the retinal organoids

developed in the microvessel co-culture system remain functional. Light responsiveness and synaptic transmission will be measured to recapitulate the functional maturation of photoreceptors.

Evaluation Criterion 2. Robustness and Reproducibility

To help us validate whether or not the protocol is truly transferable and reproducible, the protocol will be executed in 4 independent labs at 3 different institutions and the blueprint of the device design would be available for other labs. Furthermore the microfluidic vascularization device and associated technology (e.g. closed loop perfusion system) is cheap and portable (especially compared to say bioprinting). The ability to mass produce the device using materials that are compatible with organoid media will significantly aid in making the proposed protocol reproducible, transferable, and easy to use. For example, the microfluidic vascularization device will be fabricated out of polystyrene to overcome limitations of using PDMS (absorbs hydrophobic molecules). Lastly, the vascularization technology presented here is modular and applicable to all human organs and tissues rather than being confined to one specific organ or tissue system. This should help advance the organoid field as a whole.

Evaluation Criterion 3. Scientific applications and uses for models Biology/Disease Modeling

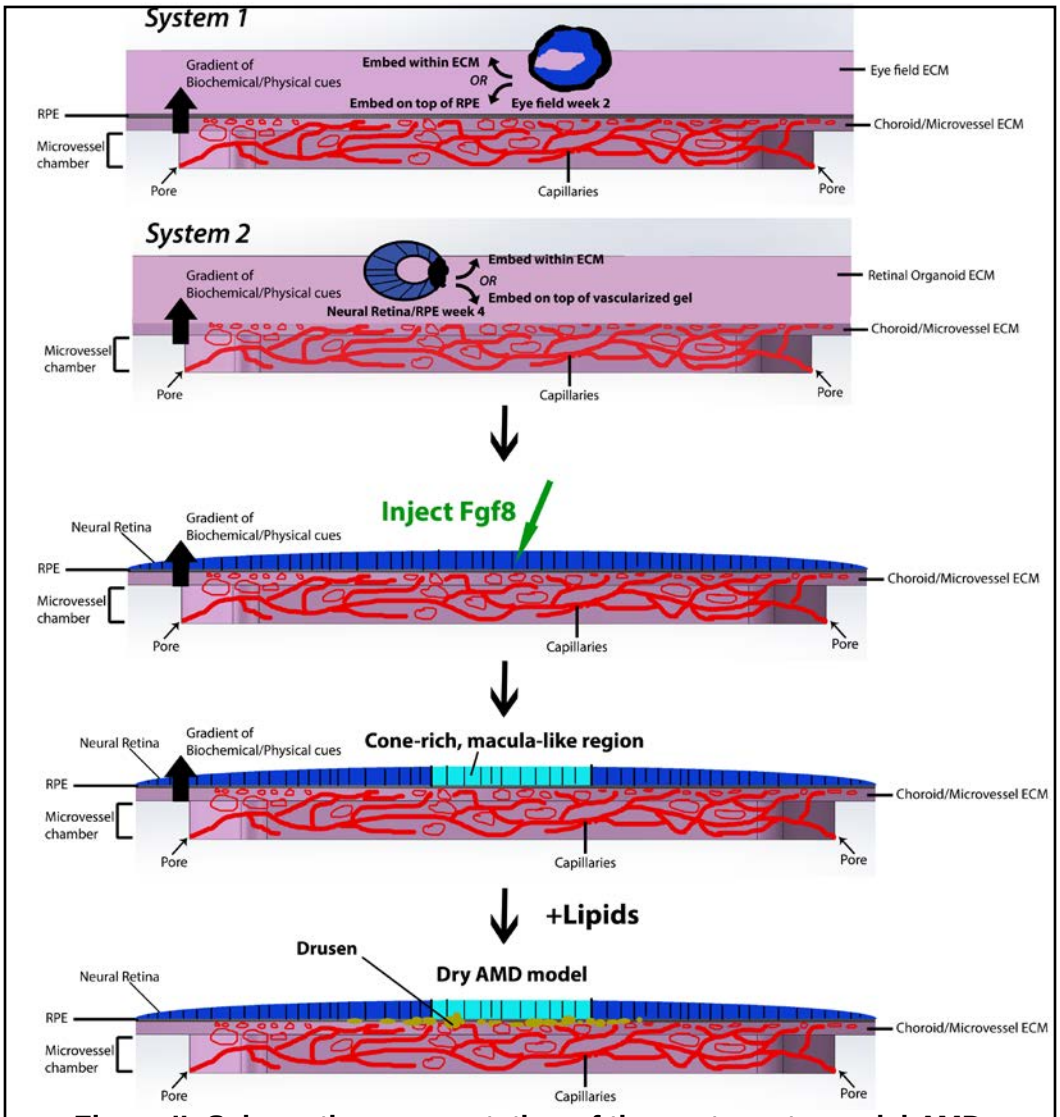

Figure II: Schematic representation of the systems to model AMD

- What aspects of the protocol are in place to improve faithful recapitulation of the biological complexity?

In AMD pathology, the cell types of interest involve the vascular endothelium, the photoreceptors, and the RPE. Till date, no *in vitro* model of AMD contains perfusable blood vessels, photoreceptors, and RPE; and thus cannot recapitulate the disease completely. In our protocol, vascularized retina organoids will be cultured in close association with a single layer of polarized RPE cells. Moreover, we expect that with a transient localization of Fgf8, we can achieve a cone-rich macula-like area. No retinal organoids to date have a cone-rich macula-like region and considering the importance of the macula in AMD, this would overcome a major limitation of current retinal organoid technology. Disturbance of this normal polarized configuration of RPE cells is associated in retinal disease conditions.

- How will this recapitulation be validated?

The vascularized retina organoids in a microfluidic platform can be translated to model AMD disease by exposure to medium containing human serum or lipids or peptide based complement-system inhibitors. The presence of drusen and pigmentary changes within the retinal organoid structure are a characteristic finding of early and intermediate AMD. The formation of drusen and its increased size over time will be used as an indicator for disease progression toward advanced stages of AMD. Furthermore, studies will be conducted to establish drusen formation by analyzing its compositions and different morphologic features in histologic sections.

- How will viability be tested, and how is the disease state expected to affect viability?

It is not expected that the disease state of the model will impact the viability of the organoids. The proposed vascularized retina organoids based in-vitro model of AMD does not utilize any toxic compounds to generate the disease condition. It is noted that pressure within the vessel containing the organoids can also be controlled to explore the impact of intraocular pressure on organoid function and disease models. By combining an RPE monolayer with retinal organoids (layered retinal tissue structure), or with a modeled choroid capillary bed, we will be able to study the interaction between cells and how they contribute to the disease.

Research team: The team has interdisciplinary expertise essential to ensure successful implementation of the idea to develop functional retinal organoids with proper spatial organization and structure. The research team includes chemical, material science and biomedical engineers with expertise in regenerative medicine, neuroscientists with extensive experience in cell based retinal regeneration strategies, and a biologist with expertise in retinal cell biology and regeneration. The idea team includes Dr. Rebecca Carrier, a chemical engineer with expertise in in cell and tissue engineering who has led multidisciplinary teams, frequently bridging academia and industry, focusing on understanding cell response to different natural and synthetic biomaterials as well as other biochemical and physical cues, Dr. Joyce Wong, a biomaterial scientist/ engineer and expert in tailoring cell-material interfaces for tissue engineering and drug delivery through direct, quantitative measurements of biological interactions, Dr. Joydip Kundu, a biomedical researcher with expertise in retinal tissue engineering and biomimetic materials, Mike Ferguson, a bioengineering graduate student with expertise in microfluidic device development for organoid culture, Dr. Michael Young, a neuroscientist at Schepens Eye Research Institute who is a leader in cell-based treatments for retinal degeneration, and Dr. Petr Baranov, an expert in various retinal regeneration strategies and retinal cell biology including development of organoids from iPSCs. Importantly, the project team has demonstrated ability to work together in efforts to develop ECM-based materials for retinal regeneration strategies (Baranov, Michaelson, Kundu, Carrier, & Young, 2014; Kundu et al., 2017; Kundu, Michaelson, Baranov, Young, & Carrier, 2014; Kundu et al., 2016; Park et al., 2016). Carrier will hold monthly research meetings with other team members at NU/ BU/ SERI to review research plan and progress.

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Appendix Section

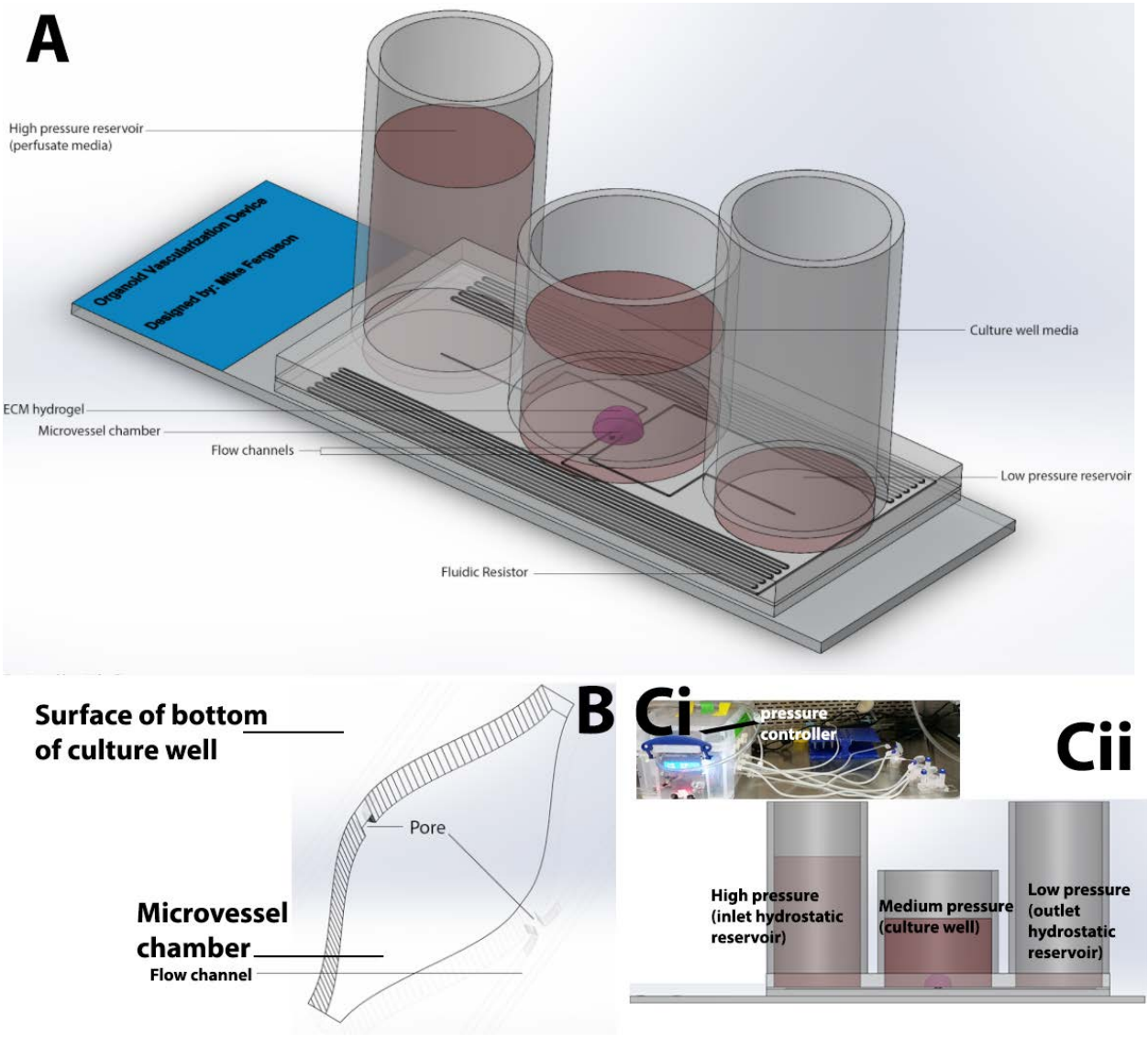

Figure 1 - Cartoon schematic of how to use the vascularization device. A) A droplet of extracellular matrix hydrogel containing ECs and optionally stromal cells is pipetted into the microvessel chamber. **B)** A cartoon of the exposed microvessel chamber, which is situated at the bottom of the culture well (see A). The bottom of the pores and flow channel are sealed by a layer of PDMS (not depicted). **Ci)** A low cost (<$100) custom pressure controller is used to

initially load the flow channel with media. **Cii)** Media is added to the reservoirs and culture well at varying heights. One reservoir (the high pressure reservoir) is filled with media to a height corresponding to a desired flow driving pressure. The culture well is filled with media to half the height of the high pressure reservoir. The pressure of the culture well determines the pressure inside the microvessel chamber. Only a very small amount of media is added to the other reservoir (the low pressure reservoir). In sum, a pressure gradient is established across the device such that media flows across the microvessel chamber and through the hydrogel at a very low velocity. It is critical to ensure that the height of the media in the culture well is halfway between the high pressure and low pressure media reservoir heights so that fluid flows across the microvessel chamber rather than up and out of it.

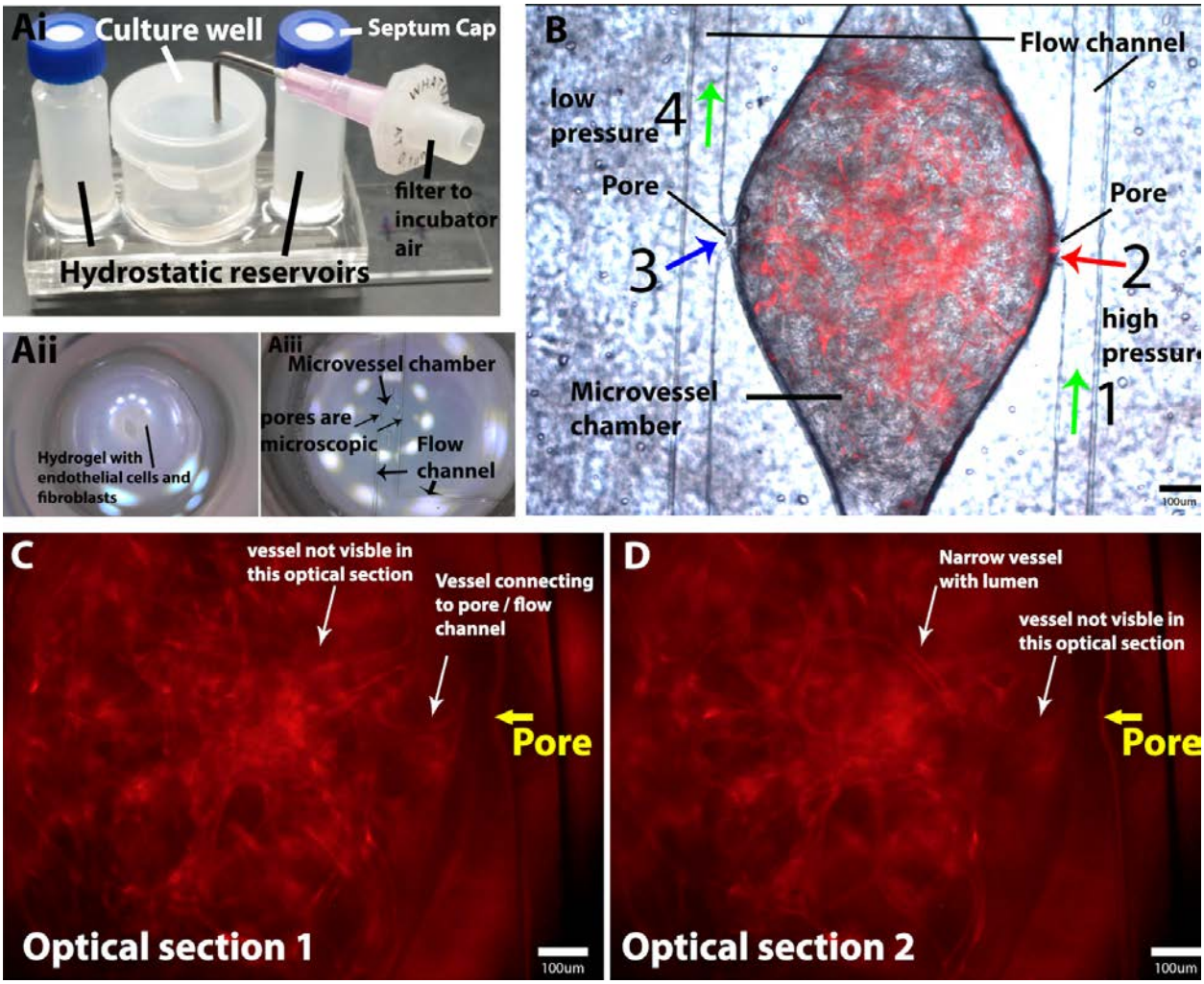

Figure 2 - Real life diagram of vascularization device. Ai) The whole device is made of autoclavable materials. **Aii)** Looking down into the culture well onto a fibrin droplet with ECs and fibroblasts that was pipetted into the microvessel chamber. Organoids can be embedded within or on this gel. Using a larger gel volume will coat the entire bottom of the culture well and reduce the distance from the surface of the “droplet” to the microvessel chamber. **Aiii)** Same view as Aii, but without the hydrogel droplet. **B-D)** Images of microvessels within the microvessel chamber are shown. The images are looking up at the bottom of the microvessel chamber. RFP expressing HUVECs are mixed with NHLFs within a fibrin gel. Image B is 6.5

days old and C,D are 9.5 days old. **B)** Arrows 1 and 4 indicate the direction of flow within the the flow channel. Arrows 2 and 3 indicate the high pressure and low pressure inlet and outlet pores respectively. Flow across the chamber is from right to left. **C,D)** Two different optical sections of the same sample are shown. The white arrows point to two different parts of the same vessel. The 3D nature of the vessel network and the high density of cells make it impossible to capture the entire vessel network structure in a single image using epifluorescent microscopy. The yellow arrow points to the high pressure inlet pore.

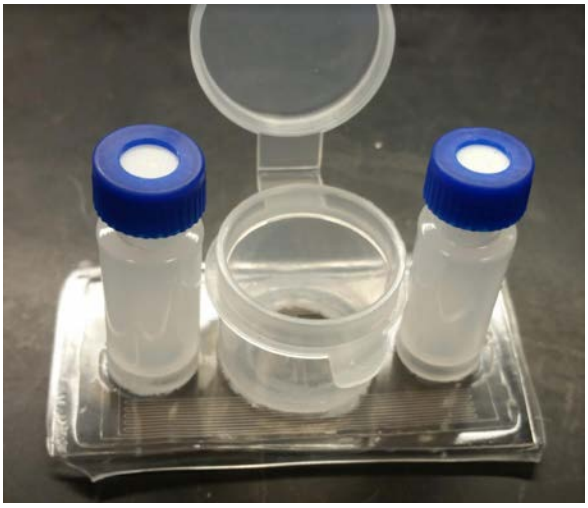

Figure 3 - The vascularization device can be fabricated out of polystyrene using hot embossing.

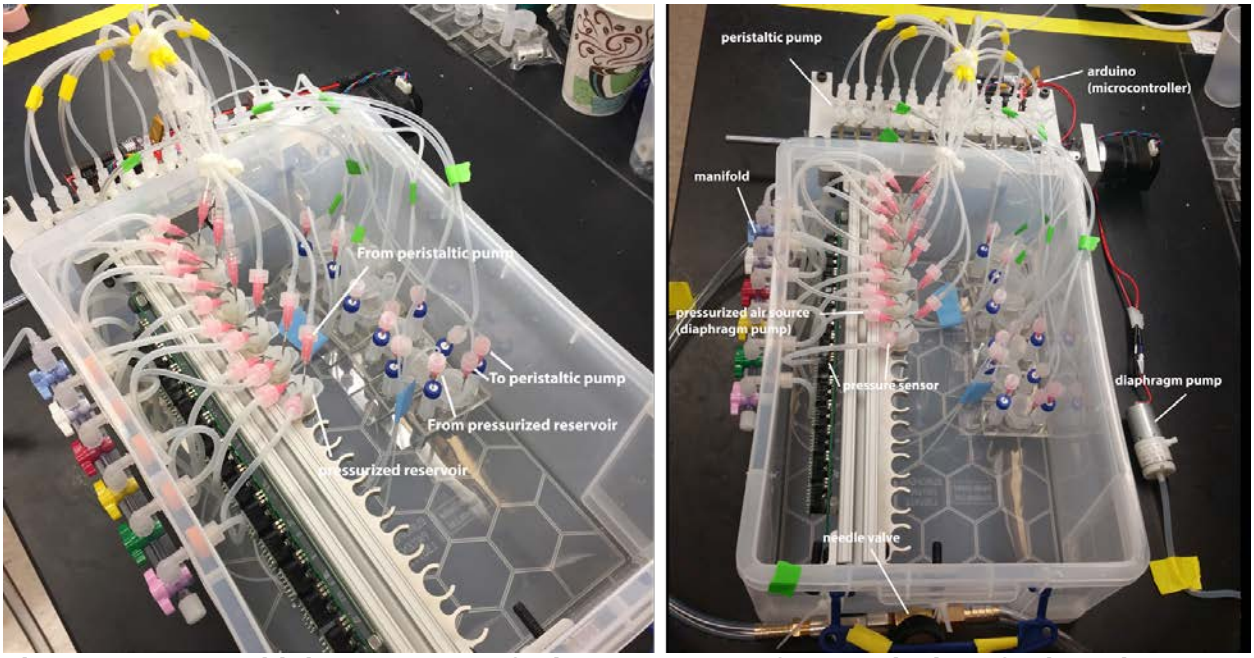

Figure 4 - Custom high pressure perfusion setup to perfuse 12 devices for less than $300. A diaphragm pump continuously pumps air into a sealed reservoir (5mL syringe) partly filled with media. The air above the media becomes pressurized, imparting a pressure onto the

media and driving flow into the inlet of the vascularization device. Two independent tubes are connected from a peristaltic pump to the culture well and the outlet reservoir. The peristaltic pump recirculates any media from the culture well or outlet reservoir to the pressurized reservoir. The slight increase in pressure of the pressurized reservoir when the peristaltic pump is pumping is negligible. A custom array of pressure sensors is used to monitor the pressure of the pressurized reservoir. An arduino microcontroller deals with the logic and sensor readings. Each device has its own independent circulation to prevent cross contamination. Use of a custom multichannel peristaltic pump and pressure sensor board reduces the cost of the system significantly. Comparable systems could easily cost over $10,000. A non-airtight lid (not pictured) is placed over the assembly to aid in preventing contamination.

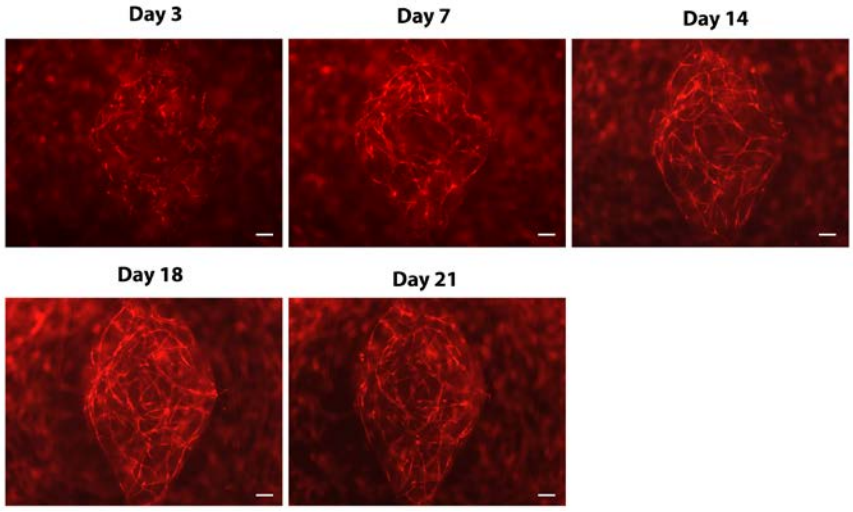

Figure 5 - Capillaries in the vascularization device remodel over time. Images are of the microvessel chamber of the same sample from day 3 to 21. HUVECs are expressing RFP and were mixed with NHLFs. Considerable remodeling is observed. Scale bars are 100um.

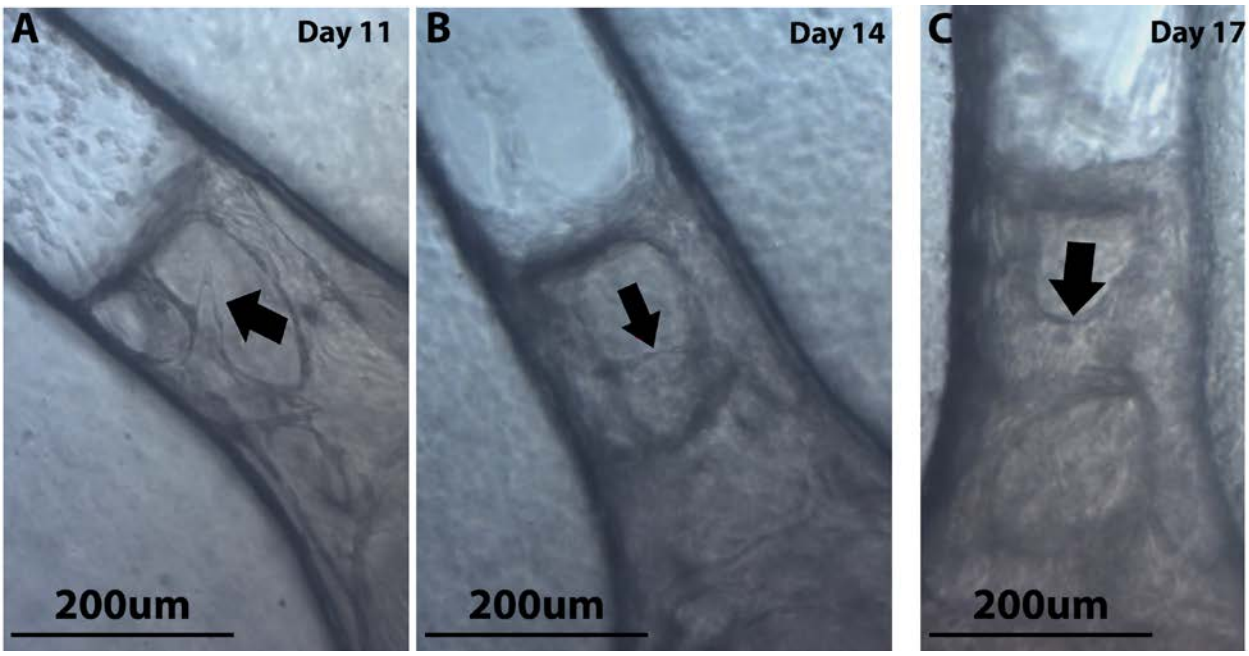

Figure 6 - Microvessels are capable of angiogenesis and anastomosis. Age is in upper right. **A)** An angiogenic-like sprout is observed. **B)** The sprout connects with the opposite vessel. **C)** A new vessel with clear lumen is formed.