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August 1, 2017

Division of Receipt and Referral
Center for Scientific Review
National Institutes of Health
Bethesda, MD 20817

To whom it may concern,

I am pleased to submit the enclosed retinal organoid challenge application.

Title: Engineering vascularized human retinal organoids for disease modeling and functional testing

FOA: NOT-EY-17-006

Team Lead: David Gamm, MD, PhD (Associate Professor, University of Wisconsin, Ophthalmology and Visual Sciences; Director, UW McPherson Eye Research Institute).

Team Members: William Murphy, PhD (Professor, University of Wisconsin, Biomedical Engineering; Director, UW Stem Cell and Regenerative Medicine Center); Nader Sheibani, PhD (Professor, University of Wisconsin, Ophthalmology and Visual Sciences); Chris Sorenson, PhD (Senior Scientist, University of Wisconsin, Pediatrics); Bikash Pattnaik, PhD (Assistant Professor, University of Wisconsin, Pediatrics and Ophthalmology and Visual Sciences); Melissa Skala, PhD (Professor, University of Wisconsin, Biomedical Engineering).

Requested Assignment (IC and/or SRG): National Eye Institute

Team's solution is most applicable to disease modeling (although drug testing is also applicable).

I have no requests for reviewer exclusions.

Thank you for your attention to this application.

Sincerely,

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I. COMPREHENSIVE DESCRIPTION OF THE PROPOSED SOLUTION

Recent studies have demonstrated a remarkable ability to use human pluripotent stem cells (hPSCs) to form "self-assembling" 3D neural retinal (NR) organoids that mimic what is found in the human eye[1-7]. However, numerous limitations exist that reduce their potential to mirror normal and pathologic behavior of their *in vivo* counterparts (Table 1). **We propose to build a prototype culture and monitoring system that builds upon existing protocols and technologies to introduce a perfused micro-vasculature to the inner layers of NR organoids in order to support full thickness tissue structure and function**. If successful, these next-gen organoids would provide greater predictive value for therapeutic discovery and toxicological screening and establish a novel platform to model inner retinal vascular diseases such as diabetic retinopathy (DR).

Table 1. Limitations of hPSC-derived 3D NR organoid culture systems and proposed solutions.

Limitation	Proposed solution
Loss of inner NR cell types and laminae over time that are normally supplied by a separate microvasculature network	Incorporation of a perfusable, hPSC-derived inner NR microvasculature
Poor morphological and compositional reproducibility between individual NR organoids	Use of synthetic biomaterials and microfluidics to create uniform, reproducible culture environments for each organoid with full thickness metabolic support
Low NR organoid production efficiency	Use of hPSC lines and protocols that consistently generate large organoid populations (hundreds per 6 well plate)
Inability to model inner microvascular retinopathies or test effects of therapeutics or toxins on inner NR	Build perfusable inner microvasculature networks using physiologically relevant hPSC-derived cell types (vascular endothelial cells and pericytes)
Limited potential to assay NR organoid function in real time	Design microfluidic system to accommodate microvascular perfusate analysis, electrophysiological recordings, and noninvasive functional imaging
Relative incompatibility to high throughput analysis	Employ scalable and versatile culture platforms

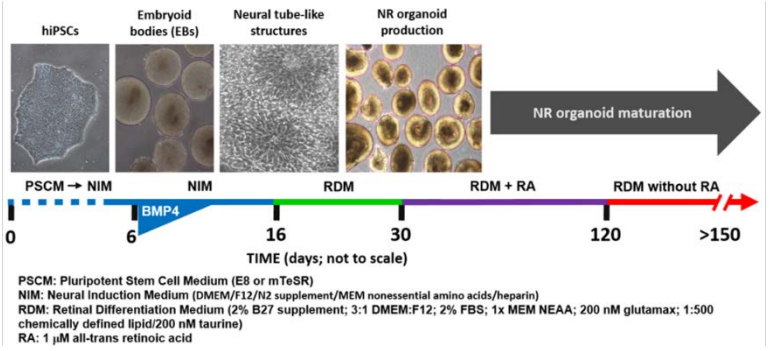


Fig. 1. Updated hPSC 3D NR organoid protocol. Schematic of the scalable hPSC retinal differentiation protocol used to isolate large populations of early 3D NR organoids (which can be performed under Current Good Manufacturing Practice (cGMP)-compliant conditions (UW Waisman Center Biomanufacturing Facility).

A. Describe existing methods or technologies that will be used, combined, or built upon. This proposal seeks to adapt and apply a vascularized tissue assembly strategy to improve upon NR organoid culture methods. Existing methods and technologies that will be brought to bear are listed below in this section, along with the corresponding PI laboratory.

1. <u>hPSC NR organoid production and early differentiation (*Gamm lab*)</u>. Techniques are in place to generate hPSC-derived NR organoids that generate all major classes of NR cells from a common NR progenitor cell (NRPC)[1-4, 7, 8]. Our base protocol was originally described in 2009[4], and has subsequently been refined to enhance NR organoid production efficiency and promote early full thickness laminar

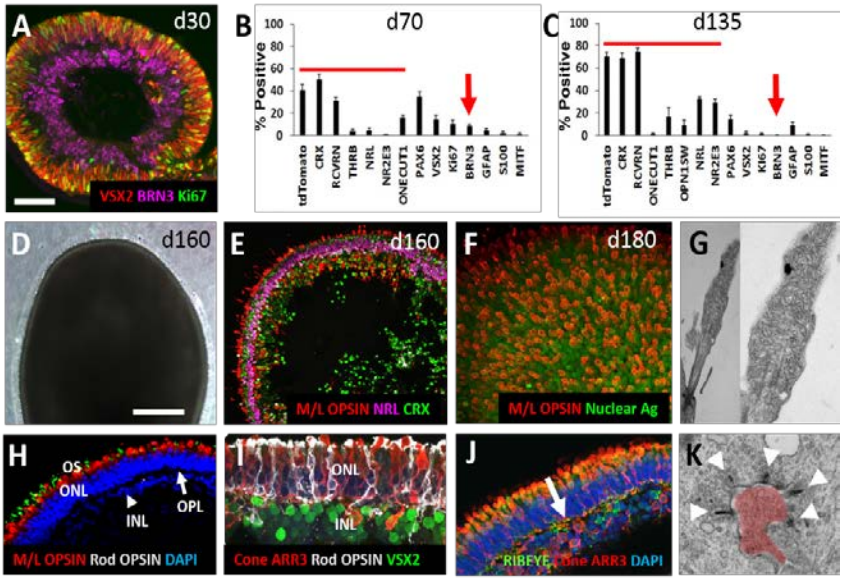


Fig. 2. hPSC-derived NR organoids maintain a highly differentiated outer PR layer but lose inner NR cells and structure with time. A) hiPSC NR organoids at d30 contain an outer layer of VSX2+/Ki67+ NRPCs and a prominent inner layer of RGCs (scale bar=50 μm). **B,C)** Unbiased high content ICC imaging analysis of dissociated organoid cultures (n=3) showing prevalence of cells expressing NR cell type markers at d70 (B) and d135 (C). Note the increase in cells expressing early and late PR markers (*red bars*) and the loss of cells expressing the RGC marker BRN3 (*red arrows*). **D,E)** By d160 (scale bar=500μm), a highly organized outer layer of cones (M/L OPSIN+), rods (NRL+) and PR precursors (CRX+) remain, with some mislocalized PRs found deeper within the organoid. However, the innermost NR layers have largely degenerated by this time point (E). **F)** Confocal image of an NR organoid surface showing distribution of M/L cones. **G)** Transmission electron microscopy (TEM) showing a developing outer segment (OS). **H)** ICC (d200) demonstrating the relationship between the OS and outer nuclear layers (ONL) and the underlying outer plexiform layer space (OPL; *arrow*) and the discontinuous inner nuclear layer (INL; *arrowhead*). **I,J)** ICC (d200) showing expression of PR markers in the ONL (I,J), the bipolar cell marker VSX2 in the INL (I), and the PR synapse marker RIBEYE in the OPL (J; *arrow*). **K)** TEM of the OPL of an organoid (d200) reveals abundant ribbon synapses (*arrowheads*) surrounding a post-synaptic terminal (pseudo-colored pink).

structure (Figs. 1-3). Advantages of these protocols include: 1) the capacity to generate hundreds of organoids per 6-well plate under cGMP conditions in pipeline fashion (Fig. 1), 2) absence of non-retinal tissues, 3) robustness across most hESC and hiPSC lines, and 4) significant organoid growth potential (up to 3 mm in diameter). However, we and others[2, 3, 6, 9] have found that while outer NR laminae thrive long-term in these cultures, internal structure is often disrupted or lost entirely (Figs. 2-4), possibly due to diffusion limits of media components and diminished core metabolic support. Interestingly, this pattern of outer preservation vs. inner disruption mirrors the distributions of the dual retinal blood supplies[10]. Other disadvantages of current systems include: 1) internal mislocalization of PRs with sporadic rosette formation (Fig. 3), 2) limited to no access to inner NR cell types in intact organoids, 3) no natural egress for RGC axons, and 4) lack of non-NRPC-derived components such as an inner microvasculature.

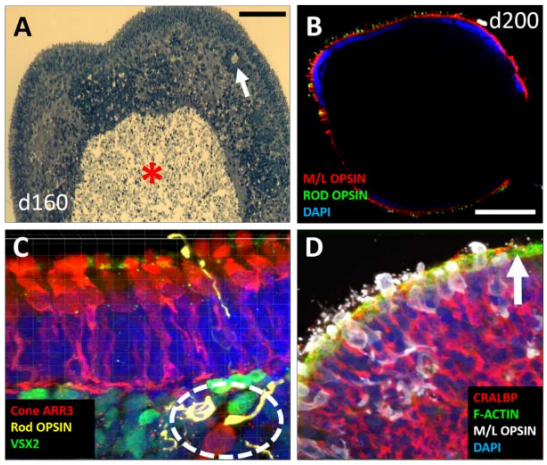


Fig. 3. Loss of inner NR cells and structure over time. A) An d160 NR organoid showing excellent outer organization but rosettes (*arrow*) and cell death (*red asterisk*) internally (scale bar = 100 mM). **B)** ICC showing near-uniform retention of outer NR but few remaining nuclei to suggest maintenance of inner laminae (scale bar=500μm). **C)** Mature PR structure is shown in the outer NR layer with VSX2+ bipolar cells and mislocalized cone and rod PRs (*dotted oval*) in the underlying INL. **D)** Retention of CRALBP+ Müller glia in late stage organoids with prominent F-ACTIN+ outer limiting membrane (*arrow*).

2. <u>hPSC RGC reporter line (*gift from Donald Zack, JHU*)</u>. For the initial prototype system we will employ an H7 hPSC-derived BRN3-mCherry reporter line that labels RGCs throughout differentiation, and thus can be used to non-invasively monitor the status of these cells and their axons, as well as the innermost NR layer in which they reside (Fig. 4).

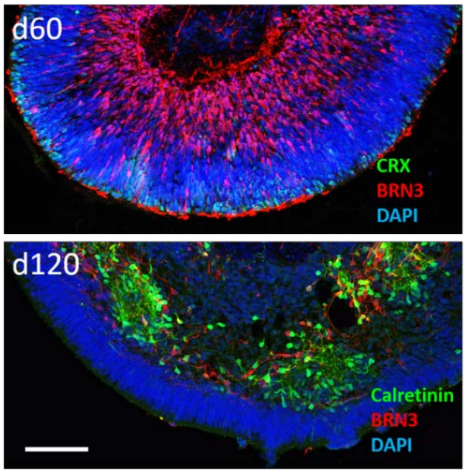
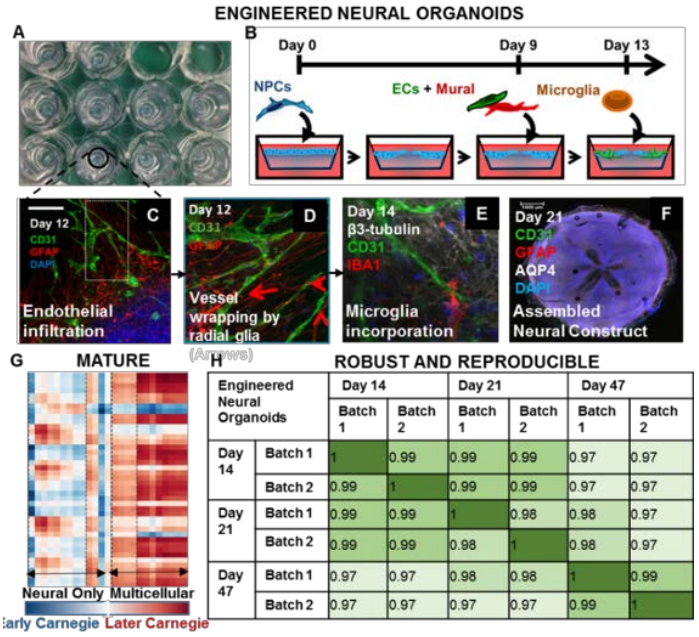


Fig. 4. NR organoids from an hPSC BRN3-mCherry reporter line. ICC of sections from d60 (upper) and d120 (lower) NR organoids showing localization and loss of BRN3+ RGCs over time.

3. <u>Engineered hPSC NR organoids with incorporated microvasculature (*Murphy lab*)</u>. We developed a strategy for assembling "engineered organoids" with multicellularity and 3D organization, while maintaining remarkably high reproducibility and data transferability to biologist and clinician end users (Fig. 5). The approach first derives precursors cells for each lineage of interest, including endothelial cells (ECs) (>90% CD31+, CD34+), pericytes (PDGFRβ+, CD13+ cells), and microglia precursor cells (CD14+, CD68+).[11] The resulting hPSC-derived cells are seeded upon or within customized synthetic hydrogels identified via novel array-



Engineered Neural Organoids		Day 14		Day 21		Day 47	
		Batch 1	Batch 2	Batch 1	Batch 2	Batch 1	Batch 2
Day 14	Batch 1	1	0.99	0.99	0.99	0.97	0.97
	Batch 2	0.99	1	0.99	0.99	0.97	0.97
Day 21	Batch 1	0.99	0.99	1	0.98	0.98	0.97
	Batch 2	0.99	0.99	0.98	1	0.98	0.97
Day 47	Batch 1	0.97	0.97	0.98	0.98	1	0.99
	Batch 2	0.97	0.97	0.97	0.97	0.99	1

Fig. 5. Features and quality control metrics of engineered organoids. Engineered organoids can be made in 96 well plates **A)**, transwells or tissue on a chip devices. **B)** Schematic of the self-assembly process. **C – F)** Addition of ECs and Microglia to the neural constructs **G)** RNAseq data demonstrates increased maturity of engineered neural organoids versus other organoid approaches. **H)** Pearson's correlation coefficient demonstrating reproducibility from batch to batch in engineered organoids.

based technologies[12-17]. Among the thousands of hydrogels evaluated, we have identified many that promote self-assembly of human vascular[18] and neurovascular[11, 19] structures. The uniquely high reproducibility of our approach has led to multiple human screening applications, including predictive neurodevelopmental toxicity screening[11, 19], therapeutic neurotoxicity screening[20], and vascular toxicity screening using a toxcast chemical library[18, 21]. Vascularized organoids can be assembled within microfluidic device geometries, which allow fluid flow and perfusion in a high throughput format. Results to date demonstrate that engineered organoids are robust, reproducible, transferrable to a broad range of end users, and amenable to automated high throughput screening applications.

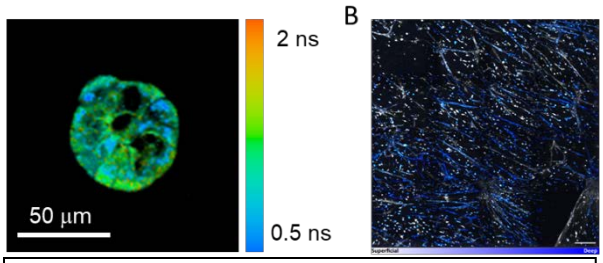


Fig. 6. Optical imaging of organoids. A) Fluorescence lifetime imaging of NAD(P)H in a pancreatic cancer organoid. **B)** NAD(P)H intensity imaging of a vascular organoid, where white indicates superficial vessels and blue indicates deep vessels.

4. Non-invasive metabolic organoid imaging (*Skala lab*). Optical metabolic imaging (OMI) measures autofluorescence from the metabolic co-enzymes NAD(P)H and FAD using multiphoton microscopy. OMI was developed in Dr. Skala's lab, and provides images of living 3D samples without dyes, fixation, or destructive techniques[22-38]. OMI has been used to assess the metabolism of cells in tissue organoids, and to assess the formation of vascular networks (Fig. 6). OMI will be used to monitor vascular formation of NR organoids and metabolism in control and vasculopathic states.

5. Electrophysiological recordings of cultured NR organoids (*Pattnaik lab*). Despite increasing success in generating NR organoids, their functional capabilities remain only cursorily examined. Dr. Pattnaik, an expert in retinal cell and tissue electrophysiology[39-41], has collaborated extensively with the Gamm lab to study structure and function of hPSC-derived retinal cells and organoids[1, 42]. His group will investigate whole organoid electrophysiology by measuring light-induced changes in trans-retinal field potential (*i.e.*, measuring the sum of all light-induced electrical activity of retinal neurons) [1, 43] by recording from organoids using the Ag-$AgCl_2$ needle (*i.e.*, electrode) described in Section IB.

6. hPSC models of angiogenesis and DR (*Sheibani and Sorenson labs*). Angiogenesis is essential for proper retinal tissue development and long-term function, but is ignored in cell and organoid culture systems. The inclusion of vascular components also allows modeling of prevalent diseases such as DR. Our team has studied DR in cell culture and animal models[44-55], focusing on identifying which retinal vascular cells serve as primary targets for the hyperglycemia and vascular rarefaction in DR. 2D culture studies demonstrated that vascular cell types respond differently to high glucose challenge[45, 51]. Although *in vivo* studies have identified pericyte loss as an early marker of DR[56], recent reports also suggest an important role for retinal neuron integrity[57, 58]. Thus, organotypic culture models that recapitulate critical features of *in vivo* cellular organization and communication are highly desirable. Their team utilized hPSC-derived vascular cells to demonstrate formation of appropriate cell-cell and cell-matrix interactions with differentiation into functional blood vessels assessed by permeability, immunofluorescence staining, and high content screening. However, a limitation of this first-generation system is its lack of neuronal components. Recent studies have identified photoreceptors (PRs) as primary sensors of metabolic stress associated with DR[59]. Therefore, the development of NR organoids that recapitulate inner neurovascular organization and maintain communication with PRs would represent a significant advance for DR modeling. Figure 7 shows an example of the system

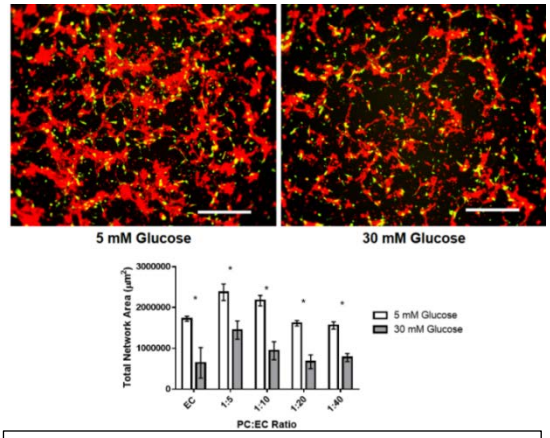


Fig. 7. hiPSC-derived endothelial cells (EC) form capillary-like networks on synthetic hydrogels. The influence of low (5 mM) and high (30 mM) glucose in culture medium on hiPSC-derived EC (Red) and human brain-derived PC (Green) in co-culture. EC network area was quantified under low or high-glucose conditions, and various ratios of PC and EC as indicated. *p < 0.05 between low and high glucose conditions, Dunnett's Test.

developed using a synthetic biomaterial, and the adverse effect of high glucose.

B. Describe the technologies and protocols needed to develop the model systems. We aim to create an enhanced NR organoid model system replete with a perfused inner retinal microvascular network that normally supplies the inner 2/3 of the NR *in vivo*[60]. ***We hypothesize that the presence of an inner retinal microvasculature will facilitate reproducible production and long-term maintenance of full thickness 3D hPSC NR organoids.*** We will combine the protocols and technologies described in Section IA and address a number of challenges (Table 2). Steps involved in the prototype build are discussed below under two milestones that will be addressed in Years 1 and 2, respectively.

Table 2. Challenges to building a perfused inner microvasculature into NR organoids and proposed solutions.

Challenge	Proposed solution
Inability to fix the position of NR organoids within wells or other devices	Impale organoids on a tungsten needle that is centered perpendicularly in each microfluidic chamber, preventing movement and maintaining a constant orientation relative to the underlying vascular cell-seeded hydrogel
Presence of an outer limiting membrane (Fig. X), which acts as a barrier to vascular cell infiltration into NR organoids	Needle penetration through the organoid provides an "injury track" for migration of cells from hydrogel
Inadequate migration and of vascular cells into NR organoids	Needle will be coated with VEGF-A-secreting microbeads encourage vascular ingrowth
Formation of vascular cell-cell interactions that recapitulate the inner blood-retinal barrier	Augment vessel wall integrity by adding hiPSC-derived mesenchymal cells (MC) to the EC + PC capillary-forming mixture (shown by our group to yield capillaries with blood-brain barrier characteristics within vascularized neural constructs [9,11])
Production of patent, anastomosed microcapillaries capable of perfusion	Anastomosis promoted by inclusion of VEGF-A-coated microbeads
Measurement of NR organoid and inner microvascular health and functions	Configure microfluidic device to A) connect tungsten needle to electrophysiological recording device and B) permit metabolic and functional imaging
Scalability and transferability	Although prototype systems may require manual forceps placement of NR organoids onto preaffixed needles, future iterations will overcome this by geometrically registering an array of needle electrodes with an array of organoids (allowed to settle into pyramidal wells in microdevices formed routinely in the Murphy lab)

Milestone 1. ***Incorporating a perfused microvascular network in NR organoids (Year 1):***

a. <u>hPSC NR organoid production.</u> One challenge that we have largely overcome is initial NR organoid scale-up, as we have refined our protocol (Fig. 1) such that it consistently generates hundreds of organoids from a single 6-well plate of hPSCs, including the BRN3-mCherry RGC reporter discussed above.

b. <u>Robust, defined, and scalable assembly of vascularized NR organoids in devices.</u> The Murphy lab provides a range of capabilities to enable design and implementation of devices, including biomaterials synthesis, 3D printing, injection molding, and automated liquid handling. In particular, the Murphy lab has space within the UW "Fab Lab", a rapid prototyping facility that has advanced equipment for 3D printing, micro-CNC, molding, and laser cutting with a full machine shop and an experienced machinist. We will use combinations of hPSC-derived ECs ($CD31^+/34^+/KDR^+$), PCs ($CD22a^+$), customized synthetic hydrogels[61-64], and fluid flow regimes to achieve assembly of perfused vascular networks. Our preliminary studies have shown that 3D microcapillary networks can be formed from hPSC ECs within synthetic hydrogels (Fig. 8)[62, 65]. We will encapsulate 1-10x10^6 hPSC-ECs/mL within 3D PEG hydrogels formed using conditions that support NR organoid growth and survival. PEG hydrogels will be selected that promote capillary network formation as identified in our prior studies[62, 64-66]. To characterize the influence of fluid flow on vascular network assembly we will use microfluidic devices with controllable fluid flow to compare capillary network formation under perfused flow (1-10 µL/sec. flow rate)[67, 68]. Conditions for vascular network formation and anastomosis will also be applied to a microfluidic system in which passive pumping will ultimately enable formation of vascularized NR organoids in a 96-well plate format. Devices we are currently using to generate perfused neural constructs will be reconfigured to house NR organoids as demonstrated preliminarily below (see

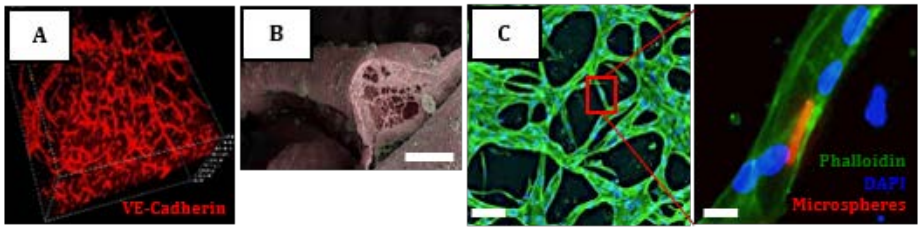


Fig. 8. Perfusable vasculature formation. A) Three-dimensionality of microvasculature (immunostained with VE-cadherin). Image Dimensions: Width 1.27mm, Height 1.27mm, Depth 0.8mm **B)** Lumen formation within endothelial vessel (3-Dimensional), Scale bar: 1µm. **C)** Perfusability of vessels represented by microspheres (Red streak) captured within a portion of vascular network, Scale bar=10µm.

Section III, Fig. 12). Thereafter, fluid flow characteristics and soluble signaling gradients (*e.g.*, VEGF-A[69-71]) will be manipulated to achieve anastomosis between the perfused networks and the embedded endothelium to create a microcapillary network supporting the inner NR.

c. Fixation of NR organoids within microfluidic chambers. A major obstacle to vascularizing NR organoids is the presence of cellular and intercellular barriers to exogenous cell migration throughout differentiation. To facilitate vascular cell entry, we will take advantage of a phenomenon observed in retinal transplantation studies; namely, that donor cells migrate through needle track injuries. Once in the inner NR, cells have the capacity to spread tangentially, and thus should cover the small internal area within organoids. To achieve this in our system, we will engineer a needle into each microfluidic chamber upon which to impale early (d80-100; a time range that precedes inner NR deterioriation) organoids using a forceps (Fig. 9). This modification provides a point of entry for cell migration *and* assures that the injury

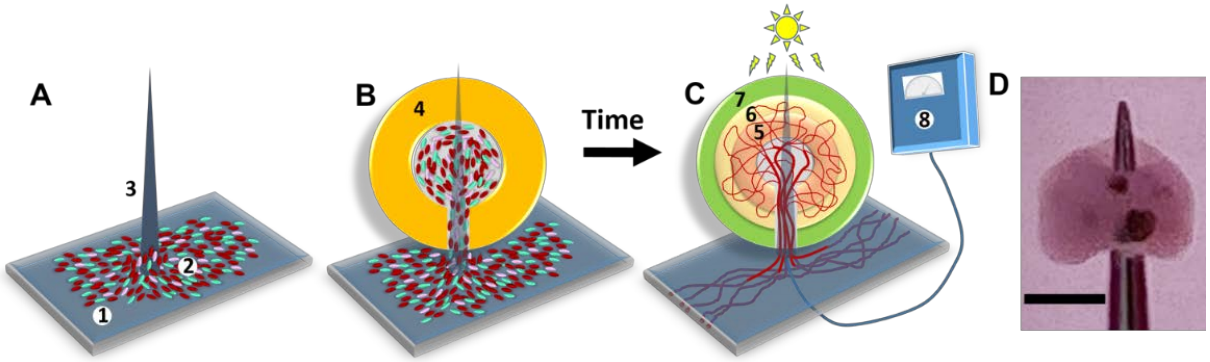


Fig. 9. Schematic depicting concept for generating an inner retinal microcapillary network for NR organoids. A) Microfluidic chamber containing hydrogel seeded with hiPSC vascular cells (2) and an Ag-$AgCl_2$ needle (3) connected to an ephys recording device (8). **B)** Early NR organoids (4) are impaled on the needle, allowing vascular cells to migrate through the injury track into the organoid core. **C)** Over time, patent, perfused microcapillaries form within the organoid allowing growth and maintenance of the RGC (5) and interneuron (6) layers, in addition to the outer PR layer (7) that is fed by diffusion from surrounding media. **D)** NR organoids can be impaled in a controlled fashion (scale bar=500μm). Note that the organoid shown has two small patches of RPE.

track stays stationary and in contact with the vascular cell-containing hydrogel. The needle track may also double as an exit pathway for RGC axons, creating an optic nerve-like structure. Lastly, the needle will serve as a recording electrode and/or can be partially coated to create a localized source for VEGF-A gradients[72-74] to encourage vascular cell migration and capillary anastomosis.

d. Promotion of an inner blood-retinal barrier. We will augment vessel wall integrity by adding hPSC-derived mesenchymal cells (MC) to the EC + PC capillary-forming mixture, which was shown by our group to yield capillaries with blood-brain barrier characteristics within vascularized neural constructs[62, 66].

Milestone 2. ***Functional monitoring of perfused NR organoids and DR models (Year 2):***

a. Perfusate analysis. Cell viability within NR organoids will be assessed via the CellTiter Glo luminesce viability assay and release of lactate dehydrogenase (LDH). The OMI index will similarly be used as an indicator of cell viability, and changes in metabolic index will be monitored throughout the course of the experiment. Organoid size and secretome will be monitored as we have done for neural organoids (Fig. 10). For NR organoids, we will use multiplexed ELISA to monitor secreted proteins known to correlate strongly with DR staging in the vitreous, including IL-6, IL-8, IL-10, IL-13, IP-10, MCP-1, MIP-1beta, PDGF, ICAM-1, and RANTES[75-77].

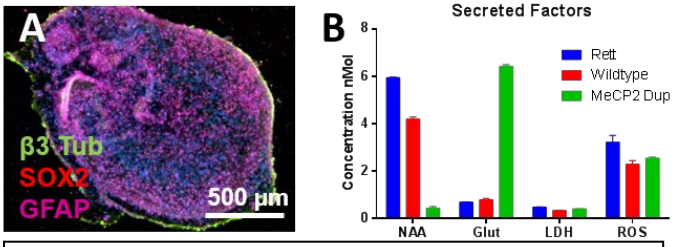


Fig. 10. OMI (A) and perfusate analysis (B) of neural organoids.

b. Electrophysiological recordings. Light stimuli from an LED source will be triggered using the recording program to control intensity, duration, and wavelength of light flashes. Electrical signal output will be amplified using a differential amplifier and stored for off line analysis. If our proposed NR organoid

culture system is successful at supporting and maintaining full thickness synaptic connectivity and tissue polarity, we expect to measure a flash intensity-dependent electrical response (b-wave at minimum). Alternatively, we will record light-evoked currents from exiting BRN3-mCherry+ RGC axons.[78]

c. Optical Metabolic Imaging (OMI). Organoids will be monitored using OMI developed in the Skala lab (Fig. 11). OMI provides a fast, low-cost, non-invasive measurement of cellular health that has already been shown to be an accurate, early predictor of physiological response to drug treatment in 3D organoids[34, 36]. OMI is sensitive to early metabolic changes, achieves high resolution to monitor individual cellular responses to treatment and can be repeatedly performed throughout the course of the study[34, 36, 79]. As a control, drugs that target metabolic enzymes (trasuzumab, paclitaxel) will be introduced that alter OMI indices[80].

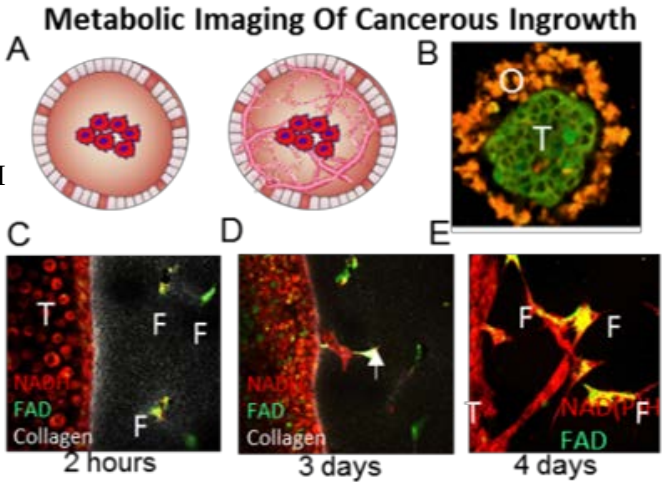


Fig. 11. OMI based tracking of tumor cell invasion into a surrounding organoid.

Validation Procedures: Formation of microvasculature will be characterized via imaging (total vascular coverage area, endothelial network thickness, branching, interconnectivity, and 3-dimensionality) and transport properties (70kDa FITC-dextran flow). Robustness of the models will be determined by measuring vascular permeability under normal and challenged (*e.g,.* histamine, thrombin, VEGF inhibition) conditions, and screening a library of putative vascular disrupting compounds provided by the EPA as part of the ongoing Wisconsin-EPA STAR Center for development of organotypic culture models (Murphy is Center Director)[18, 61, 63]. The presence and location of all major NR cell types will be assessed via ICC at multiple time points up to d200 in culture and control organoids cultured in the absence of vascular cells will be examined in parallel for all experiments.

C. State whether the method will achieve a model system that will be amenable to disease modeling, high content screening, or both. Establishment of an inner vascularized NR organoid system would facilitate *in vitro* modeling of retinal vasculopathies, most notably DR[52, 81]. Furthermore, since our bioengineered capillary networks can be perfused, our format holds unique potential for testing effects of systemic (not just bath) drug administration for the treatment of retinal disorders and for toxicology screening, thus providing the most versatile and clinically relevant *in vitro* drug evaluation platform available for the retina. Inflow perfusate conditions (and bathing medium) will be tightly controlled, and outflow perfusate will be collected and subjected to analysis (*e.g.*, metabolite screening). We will then utilize the microvascularized NR organoid system to create the first human tissue-based model of DR and screen for known agents that modulate the microvascular pathology. Conditions favorable to the development of DR will be imposed upon the system (*e.g.*, high glucose in culture/perfusion medium + low O_2 conditions[82, 83]), followed by monitoring for effects indicative of DR (*e.g.*, 1. dye perfusion analysis for microvascular leakage, blockage, and/or neovascularization, 2. OMI and electrophysiology, and 3. ICC for microvascular cell organization, apoptosis, and proliferation, and NR anatomy). ***We hypothesize that a microvascularized 3D hPSC-NR organoid model can yield a phenotype akin to human DR and predict effects of known agents (deliverable via microcapillary networks) that protect against DR*** (*e.g.*, angiostatin and Vit D [84]).

D. State the disease chosen, if proposing a disease model. DR; however, other inner retinal vasculopathies such as retinopathy of prematurity (ROP) and retinal artery and vein occlusions could also be modeled with our proposed system by adjusting O_2 levels or blocking inward or outward microfluidic flow, respectively.

II. BIOGRAPHICAL SKETCHES

David Gamm, MD, PhD (Associate Professor, Ophthalmology and Visual Sciences; Director, UWMcPherson Eye Research Institute). Inherited and acquired eye diseases that culminate in the degeneration of photoreceptors and retinal RPE (*e.g., RP* and AMD) are a cause of visual morbidity. Human pluripotent stem cells (hPSCs) can be used for modeling retinal development and devising cell-based treatments for these diseases. The aims of the Gamm laboratory are 1) to investigate cellular and molecular events that occur during human retinal differentiation and 2) to generate cells for use in *in vitro* retinal disease modeling and the development of therapies for retinal degenerative disorders. A critical component of their effort is the development of better strategies to support delivery, survival, and function of hPSC-derived retinal cells. They developed the first 3D culture method to generate retinal cells from human hPSCs, which has since yielded key insights into mechanisms of human retinogenesis. In addition, they pioneered the use of patient-specific and gene-modified iPS cells to model retinal disorders and test therapeutic strategies, and have led efforts to adapt this technology for human use.

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William Murphy, PhD (Professor, Biomedical Engineering; Director, UW Stem Cell and Regenerative Medicine Center). Dr. Murphy has experience in the design and applications of engineered human organoids, including brain, liver, and vascular organoids. He has particular experience using synthetic hydrogels to create well-defined environments for organoid assembly, and for generating engineered systems in which organoids can be used for robust screening applications. He has developed synthetic hydrogel arrays for high throughput, 3D cell culture, cell recruitment, and new tissue formation. Applications include human tissues-on-a-chip for toxicity testing, drug discovery, and other tissue engineering applications. He will help lead the development of engineered NR organoids.

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Nader Sheibani, PhD (Professor, Ophthalmology and Visual Sciences). Dr. Sheibani has investigated ocular vascular development and angiogenesis by developing models of DR, ROP, and AMD. He was the first to culture retinal ECs, PCs, and astrocytes from mice, and has also published methods of culturing RPE and choroidal EC. He has determined the impact of various genes and conditions on these cells, in isolation or in co-cultures, and in synthetic hydrogels. He has experience investigating vascular cell communication and biochemical pathways impacted by high glucose conditions. This application builds on his commitment to investigating retinal neurovascular interactions in DR.

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Chris Sorenson (Senior Scientist, Pediatrics). Dr. Sorenson is part of a collaborative group of vision scientists with expertise in ocular vascular biology. The research in her laboratory focuses on the study of ocular vascular changes associated with diseases such as DR and ROP.

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Bikash Pattnaik, PhD (Assistant Professor, Pediatrics and Ophthalmology and Visual Sciences). For the past 15 years, Dr. Pattnaik has focused on understanding electrophysiological circuits in the retina and the pathophysiology of blindness due to ion channelopathies. In collaboration with Dr. Gamm, he is using hiPSCs to study disease mechanisms and therapeutic approaches. His scientific competencies include molecular and biochemical techniques, electroretinography, retina physiology, patch-clamp electrophysiology, and live-cell fluorescence imaging.

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Melissa Skala (Professor, Biomedical Engineering). The non-invasive imaging methods developed in the Skala lab are suited to monitor human tissues on a chip. She has developed and translated optical imaging techniques to quantify cellular metabolism, blood oxygenation and concentration, microvessel network structure and perfusion, and collagen content. Human tumor organoids are one area where these tools have been especially beneficial in designing personalized treatment regimens and in new drug development.

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III. FEASIBILITY STATEMENT

Our multidisciplinary team brings together biologists and biomedical engineers from UW-Madison with uncommon and critical expertise and a highly collaborative track record. Our familiarity with each other and our collective involvement in the UW McPherson Eye Research Institute (Gamm, Director) and/or UW Stem Cell and Regenerative Medicine Center (Murphy, Director) enhance our ability to work effectively as a consortium and accomplish our objectives. This proposal addresses the goals of the Retinal Organoid Challenge by providing an innovative approach for improving existing human NR organoid cultures by:

1. Promoting survival, function, and laminar organization of all major NR cell types.
2. Introducing a heretofore neglected (yet highly relevant) NR tissue component - an inner microvasculature.
3. Improving longevity and reproducibility of NR organoids by providing direct metabolic support to inner laminae.
4. Modeling a common retinal disease (DR) that cannot otherwise be studied using current organoid culture systems.
5. Providing a platform to assess effects of systemic delivery of drugs and toxic agents to the inner retina.
6. Using tools, materials, and assays that are transferrable to other laboratories.
7. Incorporating use of microfluidics or perfusion to enhance culture of 3D NR organoids.

With regard to feasibility and timeline, while we acknowledge the many challenges involved in this proposal, we are confident in the assembled team and their respective expertise, technologies, resources, and dedication. As mentioned in Section IB, we have divided the proposal into two milestones corresponding to Year 1 ("Incorporating a perfused microcapillary network in NR organoids") and Year 2 ("Functional monitoring of perfused NR organoids and DR models"). With regard to functional monitoring within our culture system, we will focus efforts from lowest to highest difficulty as follows: 1) perfusate analysis, 2) electrophysiological recording, and 3) optical metabolic imaging (OMI). Overall, we believe that our approach provides attainable, tiered goals within a reasonable time period for this challenge. Below are listed the judging criteria and the corresponding questions to be addressed by our proposal.

1. Cell Type, Structure, Viability, and Function (50%)

• *What aspects of the protocol ensure that all five neuronal retina cell types (photoreceptors, bipolar cells, ganglion cells, horizontal cells, and amacrine cells) will be produced or included?* Using our hPSC protocol (Section IA), all major NR cell types are generated in a spatiotemporal manner that mirrors normal human retinogenesis. However, the survival and/or laminar organization of NR cell types and layers are not uniform, which is a major driving force for this proposal. Initial use of the BRN3-mCherry hPSC line will allow us to noninvasively monitor the fate of RGCs, a cell type located in the innermost NR layer that degenerates earliest in long-term cultures.

• *Will other cell types be generated or included?* Yes. Vascular cell types (ECs and PCs, as well as MCs to encourage inner blood-retinal barrier formation and microglia) will be incorporated as described in Section IA and B[11, 65, 85, 86] in order to introduce a perfused inner microcapillary network into NR organoids.

• *If the method eliminates a cell type, justify why it is not included.* N/A.

• *What approach (e.g., self-organization or bioengineering with scaffolds, bioprinting, and/or a microfluidic apparatus) is proposed to achieve 3D assembly?* We will utilize both self-organization (initial NR organoid formation and isolation) and synthetic biomaterials and a microfluidic apparatus (following transfer of organoids for vascular cell ingrowth and long-term growth, maintenance, and monitoring). The combination of synthetic biomaterials and a unique microfluidic apparatus results in a highly reproducible vascular self-assembly process, which we have shown to be amenable to high throughput screening[11, 18].

• *What aspects of the protocol ensure that 3D organoids will be properly oriented and have layers recapitulating a laminated retina?* See Section IB (focus of Milestone 1) and "Validation Procedures."

• *Does the protocol incorporate new procedural steps or technologies that aim to increase duration of viability as compared to current protocols?* Yes. As described in Section IA and IB, a main purpose of building a perfused inner retinal microvasculature into NR organoids and developing methods for noninvasive functional assessment is to increase long-term survival of all NR cell types and laminae and to

facilitate repeated testing over time. This approach has already resulted in neural organoids that mimic later stages of brain development when compared to typical cerebral organoids (Section IA[11, 19]), and liver organoids with improved viability and mature hepatocyte function (not shown).

• *Does the solution incorporate novel steps or technologies that may enable all cell types to remain functional through the latest viable time point?* Yes. See answer above.

2. Robustness and Reproducibility (25%)

• *Is the protocol sufficiently clear and detailed to facilitate inter/intra-laboratory utility and reproducibility?* The base NR organoid production and isolation protocol is already developed (Fig. 1). Similarly, the biomaterials and microfluidic apparatus that will be adapted for NR organoid vascularization and monitoring have been described by the Murphy lab (Section I). Novel methods and devices that combine existing technologies and protocols will be tested and refined for widespread use upon completion of the milestones. Importantly, synthetic hydrogels to be used in our studies have been validated in beta testing by several academic and industrial laboratories, including the NCATS Tissue Chip Testing Centers at Texas A&M University (see accepted manuscript describing validation[21]) and MIT. The synthetic hydrogel validation procedures have been successful enough to result in product launches by a start-up company (Stem Pharm Inc.; description in appendix), which licensed the technology from UW-Madison.

• *What other resources will be developed to facilitate transferability?* The characteristic starting materials for the proposed approach will be developed to facilitate transferability. Cell lines will be generated using well-defined protocols from cryopreserved cell stocks. Hydrogels will include simple synthetic components and will be subjected to routine quality control. Microfluidic devices are based on the principle of passive fluid flow, which mitigates the need for pumps and valves that introduce user-dependent complexity into experimental systems. Our previous studies with neurovascular organoids demonstrate that this type of approach is readily transferrable to multiple end-users[11, 18-20], and the ongoing activities of companies that have licensed the core technologies (Opsis Therapeutics and Stem Pharm, see Appendix) indicate that the proposed approaches can be transferred to pharmaceutical drug and toxicological screening environments.

3. Scientific applications and uses for models-biology/disease modeling or HCS (25%)

• *What aspects of the protocol are in place to improve faithful recapitulation of the biological complexity?* By incorporating an inner retinal microcapillary network in NR organoids and subjecting them to diabetic conditions (Section IC), we seek to develop the first hPSC "disease-in-a-dish" model of DR. Exposure of even simple vascular assemblies to high glucose (Section IA6, Fig. 8) results in structural changes, and the presence of support cells such as pericytes significantly influences the vascular response. We anticipate that the presence of a surrounding NR organoid will result in a greatly improved recapitulation of vascular response to diabetic conditions, as well as a clearer view of the NR response to changes in vascular structure and function.

• *How will this recapitulation be validated?* See "Validation Procedures" (Section IB) and the discussion of the DR model (Section IC).

• *How will viability be tested, and how is the disease state expected to affect viability?* Viability and NR cell response in the DR model will be measured as described in Section IC. We will compare readouts to existing animal models and other cell-based culture systems familiar to (or developed by) our team.• *How will the proposed model's amenability to high content screening be enhanced? How will this be tested?* The Gamm NR organoid production protocol routinely generates hundreds of organoids from a single 6-well hPSC plate, and thus is amenable to scale-up. The Murphy lab specializes in assembling human organoids in well-plate based contexts that are directly amenable to high throughput screening (HSC). Our recent studies have assembled multi-cellular organoids in 96-well and 384-well plate models for screening of compound libraries, including the EPA toxcast library. Our proposed studies require a perfusable vasculature with inlets and outlets, and thus are not amenable to assembly in a static well-plate. Thus, in collaboration with Prof. David Beebe at the UW-Madison we have developed a 96-well plate that includes wells that are dynamically fed via "passive pumping". The approach uses surface tension to control fluid flow, and has an open well-plate format that is consistent with HSC applications. Figure 12 shows results of initial feasibility studies, in which we have added NR organoids into microfluidic 96-well plates containing vascular beds. The initial results demonstrate that NR organoids attach to the surface of the

vascular beds (Fig. 12B, D), leading to some ingrowth of vascular tissues into the organoids (Fig. 12E) even in the absence of needle injury. We anticipate that >100 organoids can be simultaneously tested in this format, which will enable assessment of a variety of media conditions associated with DR (*e.g.*, variable glucose concentrations), molecules known to influence vascular tissue formation (*e.g.*, library of tyrosine kinase and metalloprotease inhibitors we previously used to validate vascular models[18, 61]), and compounds that may be toxic to the neural retina (*e.g.*, the EPA's library of putative vascular disrupting compounds[18]).

• *How will the model's ability to recapitulate known retina toxicities be tested?* A three-tiered live functional assessment will be developed in Milestone 2 (Section IB), consisting of 1) NR organoid perfusate analysis, 2) electrophysiological responses, and 3) OMI. See Section IB for a discussion of drugs and conditions that will be introduced to test NR organoid response to light stimuli, known regulators of metabolism and vascular permeability, and conditions predisposing to DR.

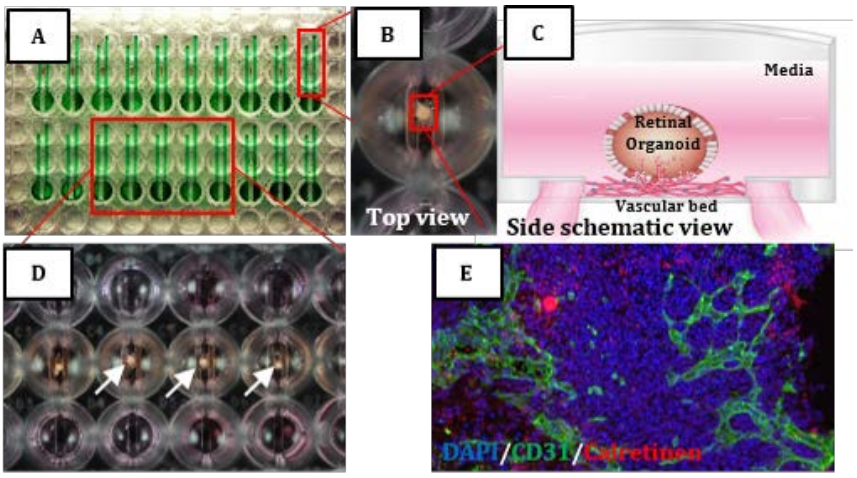

Fig. 12. Preliminary demonstrations of vascularized retinal organoids in 96-well plate devices. A) Assembled microvascular beds within a fabricated microfluidics device. **B)** Top view pictorial representation of the placement of a retinal organoid on top of the vascular bed in the microfluidic device. **C)** Side view schematic of the microvasculature feeding retinal organoids. **D)** Pictorial representation of the placement of several retinal organoids (white arrows) on top of vascular beds in the microfluidic device. **E)** Endothelial cells (CD31) shown in close proximity with inner NR cells (calretinin) within a retinal organoid.

• *What methods will be used to mass-produce the proposed model?* See comments above related to mass production of NR organoids, as well as Section IB. The Murphy lab can readily manufacture both the microfluidic 96-well plate system and the synthetic hydrogels. True mass production of the components of the proposed system will require translation to commercial entities that can achieve economies of scale. Fortunately, our core technologies have been licensed to companies that can robustly generate the cells (Cellular Dynamics International, Opsis Therapeutics), synthetic hydrogels (Stem Pharm), and the microfluidic systems (Salus). While these companies are not integrally involved in the proposed studies, they provide a potential conduit for mass production and broad dissemination of proposed technology. For functional assessment we are emphasizing perfusate analysis followed by electrophysiology and OMI. While relatively new, OMI is very effective at monitoring cell health in organoids, even at depths beyond that of a full thickness laminated retina. Figure 13 shows OMI-based measurement of vascular networks in a microfluidic apparatus.

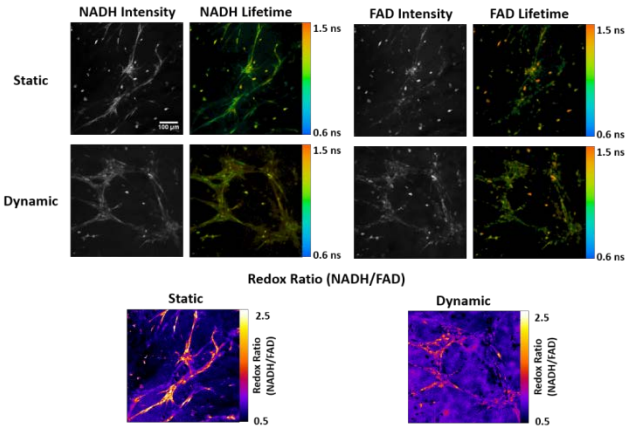

Fig. 13. OMI can non-invasively image vascular beds formed in bioengineered devices. Measurement of NADH lifetime/intensity, FAD lifetime/intensity, and redox ratio results in a clear image of a vascular network in real time, and in the absence of any labels. Model developed by the Murphy lab in conjunction with the Skala lab

4. Other information

• *Supporting precedents:* See Section I.
• *Special resources:* See Fab Lab (Section I).
• *Protections for human subjects:* N/A
• *Compliance with policies related to use of human stem cells:* Yes (SCRO approval for all labs).
• *Biosafety:* Yes (protocol approval for all labs).
• *Use of technologies covered by patents or other intellectual property protection:* Yes (>14 composition and/or methods patents issued or pending).

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V. APPENDIX

Successful completion of the proposed studies will lead to a need for translation of the technology for a broad range of biomedical applications. In particular, there will be a need for robust mass production of components of the technology, and broad dissemination via commercialization and strategic industry partnership. Technology translation will be facilitated by the existence of two start-up companies that are broadly relevant to key components of the proposed studies. Specifically, the proposal team has been involved in co-founding companies that are generating human iPS-derived retinal cells (Opsis Therapeutics) and biomaterials for tissue assembly by human iPS-derived cells (Stem Pharm). While these companies are not directly involved in the proposed studies, their existence is expected to streamline the process of technology translation, which could lead to impactful use of the technology in a relatively near term. A description of the companies and their potential relevance to the ultimate translation of the proposed technology is as follows:

Opsis Therapeutics was founded in 2016 as a partnership between FUJIFILM, Cellular Dynamics International (CDI), a FUJIFILM company, and Dr. David Gamm at the University of Wisconsin, Madison. The company's mission is to develop first-in-class treatments for retinal diseases. The technology to develop these therapeutics is based upon recent advances that enable manufacture of human cell lineages from induced-pluripotent stem cells. The human iPS-derived retinal cells developed by Opsis Therapeutics could be used as a feedstock for generating engineered retinal organoids upon successful completion of the proposed studies.

Stem Pharm was founded in 2015 to offer unique biomaterials for cell and tissue manufacturing. Applications include medical devices for drug discovery, cell therapy, and regenerative medicine. Stem Pharm's synthetic biomaterials support the optimization of cell expansion and differentiation, human tissue formation and cell/tissue delivery applications. The biomaterials developed by Stem Pharm could serve as an ideal framework for assembly of engineered retinal orvanoids upon successful completion of the proposed studies.