

Production of RPE-retina composite with micropatterned vascular networks for Usher syndrome modeling

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Our solution is most applicable for disease modeling

We have chosen to model Usher syndrome (USH), an autosomal recessive genetic disease, in our model retina. Individuals with USH experience loss of both hearing and vision and sometimes have vestibular defects. USH is a rare disease, with a prevalence of approximately 1 in 10,000; however, the loss of both vision and hearing is particularly debilitating. While cochlear implants have ameliorated the impacts of hearing loss, an effective treatment for retinal degeneration is urgently needed. There are three types of USH. USH1 patients have some hearing loss from birth and an onset of Retinitis Pigmentosa (RP) in the first decade. USH2 patients also show early onset hearing loss, but the onset of RP is not observed until the second decade. USH3 patients have a more variable onset and progression of hearing loss and RP. In all USH types, degeneration of rod photoreceptors occurs first, followed by loss of cone photoreceptors, often resulting in total blindness. Considerable effort has gone into the identification of the genetic defects underlying USH, and more than 10 genes have been implicated in the disease to date. Identification of USH genes has led to considerable advances in our understanding of the function of these genes in the inner ear; we know far less, however, about the normal and pathological functions of the USH genes in the retina, primarily because the human USH retinal phenotypes are not present in mouse models of these mutations. Only the Ush1c knock-in mouse has a spontaneous retinal degeneration phenotype.

Producing a model retina *in vitro*, from USH patient-derived iPSCs would be a key step to both (1) advance our understanding of this disease in the retina, and (2) to use as a screening tool to evaluate potential therapies, like viral gene therapy, for effectiveness. If the UshStat gene therapy trial demonstrates clinical efficacy in USH1B participants, it would be useful to dissect the mechanism of photoreceptor stabilization in an *in vitro* model to allow the development of targeted earlier interventional treatments. A human retinal USH disease model could also permit screening of small molecule therapies, particularly at the validation stage. Since most USH disease genes are localized to the photoreceptors, the model must be able to reproduce the essential aspects of photoreceptor development. However, to adequately reproduce photoreceptor cell biology and physiology it will be necessary that the model contains key support cells *i.e.*, Müller glia and retinal pigmented epithelial (RPE) cells. Moreover, some of the USH gene products have been localized to synapses (Table 1), and so the model also needs to contain interconnected interneurons, *i.e.*, bipolar cells and horizontal cells. Therefore, we propose to produce iPSC-derived organoid model retinas with both RPE and most components of the neural retina: the rod and cone photoreceptors, the bipolar, horizontal, and amacrine cells, and the Müller glia.

Table 1. Known gene mutations associated with USH

USH type	Locus	gene name	cellular localization
USH1B	USH1B	<i>MYO7A</i>	Connecting cilium, periciliary membrane, calyceal process and synapse
USH1C	<i>USH1C</i>	harmonin	Outer segment, calyceal process and synapse
USH1D	<i>CDH23</i>	cadherin 23 protocadherin 15	Inner segment, calyceal process and synapse
USH1F	<i>PCDH15</i>		Base of outer segment, calyceal process and synapse
USH1G	<i>USH1G</i>	SANS	Connecting cilium, basal body, calyceal process and synapse
USH1J	<i>CIB2</i>	CIB2	Inner and outer segment
USH2	USH2A	<i>USH2A</i>	Periciliary membrane complex and synapse
USH2C	<i>GPR98</i>	VLGR1	Periciliary membrane complex and synapse
USH2D	<i>DFNB31</i>	whirlin	Periciliary membrane complex
USH3	USH3A	<i>CLRN1</i>	Connecting cilium, inner segment, adherens junction and synapse

The generation of USH model retinas will allow us to study the pathophysiology of the disease from the very earliest stages. We will start with USH1, since these patients present with an early onset of the retinal disease. The model will contain (1) a neural retinal organoid (2) a layer of RPE, and (3) perfused vasculature for both the neural retina and RPE (Figure 1). The neural retina and RPE will be derived from patient-derived iPSCs, while the vasculature will be generated from primary fetal human retinal endothelial cells and choroidal endothelial cells. The vessels will be generated in a microfabricated device that allows us to separately establish the RPE and the neural retina, and then the two layers will be placed adjacent to one another. The ability to perfuse the vasculature allows us to maintain the neural retina and RPE in its natural configuration with their corresponding vascular supplies as an entire unit. We predict that perfusion of the RPE and retina through their own microvessel networks will facilitate their differentiation, and reduce cell death that occurs as the retinal organoids mature by increasing their access to oxygen and nutrients similar to what occurs *in vivo*.

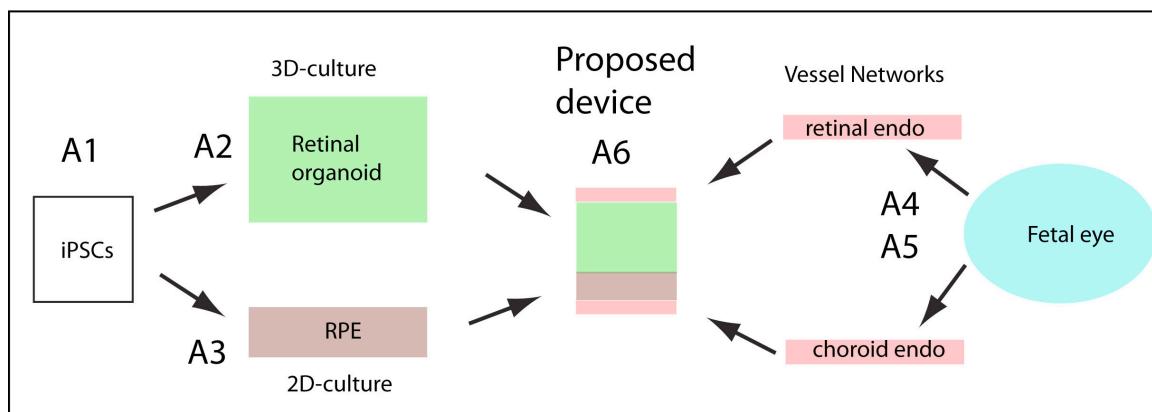


Figure 1. Schematic of proposed of model unit. Retinal organoids and RPE will be derived from iPSCs, and endothelial networks will be cultured from fetal human eyes. Descriptions of each component can be found below in the corresponding section.

A. Creating the model

A1. Producing the iPSCs from USH patients.

We propose to first generate iPSCs from USH patients using a well-established protocol (Okita, Matsumura et al. 2011; Hubbard, Sullivan et al. 2014). The USH patients are genotyped for known USH loci. PBMCs are separated from fresh blood samples using a Ficoll-Hypaque gradient. Erythroblasts are generated by expanding 2 million PBMCs in culture for 9 days in a QBSF-60 serum-free medium supplemented with stem cell factor (50 ng/mL), interleukin 3 (10 ng/mL), erythropoietin (2 U/mL), insulin-like growth factor 1 (40 ng/mL), dexamethasone (1 μ M), ascorbic acid (50 ng/mL), and penicillin-streptomycin. Episomal vectors encoding the reprogramming factors OCT4, SOX2, KLF4, LIN28A, LMYC, and a short hairpin RNA targeting p53 are introduced into the *in vitro* expanded erythroblasts using the Nucleofector II device. After 48–72 hours, transfected erythroblasts are plated onto a layer of irradiated mouse embryonic fibroblasts (iMEFs) in a reprogramming medium. Approximately 12 days after nucleofection, colonies with the morphological characteristics of human embryonic stem cells (hESC) begin to emerge, and the medium is then switched to hESC medium (see Resources). Once colonies with ESC-like morphology grow to a sufficient size, they are manually transferred to separate iMEF-coated culture dishes for expansion in hESC medium.

A2. Producing retinal organoids from iPSCs

The protocol we will use to generate the human iPSC-derived retinal organoids is adapted from existing published protocols (Lamba, McUsic et al. 2010; Nakano, Ando et al. 2012; Ohlemacher, Iglesias et al. 2015; Zhu, Cifuentes et al. 2017). Briefly, undifferentiated iPSCs are maintained on Matrigel-coated plates in mTeSR1 medium. To begin differentiation, undifferentiated iPSCs are treated with Dispase to lift colonies from the Matrigel-coated plates (Day 0) and transitioned into Neural Induction Medium (NIM) containing (see Resources) in suspension in T75 flasks for the formation of embryoid bodies. After 7 days in NIM, embryoid bodies are plated on 6-well TC plates with NIM containing 10% FBS. On Day 8, media is changed to NIM without FBS. Additionally, 2 mM of IWR1, 10 mM of SB431542, 100 nM of LDN193189, and 10 ng/ml of IGF1 are added to the medium for 5–6 days to enrich induction of eye field markers. NIM media is changed every other day until Day 16, when the cells are lifted from the TC plates by manual scraping for the formation of optic vesicle-like structures. Cell aggregates are then grown in suspension in 60 mm dishes in Retinal Differentiation Medium (see Resources). By Day 25, non-retinal neurospheres are manually removed from the dish to allow for the enrichment of retinal neurospheres. At Day 45, retinal organoids are bisected (Appendix Figure 1) and the two halves are transferred to the vascular network.

A3. Producing the RPE

iPSC-derived RPE are differentiated according to established protocols with minor alterations (Appendix Figure 2(Buchholz, Pennington et al. 2013). Briefly, human iPSCs cultured are passaged onto GFR Matrigel- plates in DMEM/F12 basal media with N2, sodium pyruvate, 10% BSA, NEAA, penicillin-streptomycin and 10 mM Y-27632, 50 ng/ml Noggin, 10 ng/ml DKK-1, 10 ng/ml IGF1, and 10 mM nicotinamide. On day 3, the media is changed to N2-supplemented media described previously containing 10 ng/ml Noggin, DKK, and IGF 5 ng/ml Basic Fibroblast Growth Factor, and 10 mM nicotinamide. On day 5, N2-supplemented media containing 10 ng/ml of DKK and IGF and 100 ng/ml of Activin A is added to the cell cultures. From day 7 to 14, cells are treated with N2-supplemented media containing 100 ng/ml Activin, 10 mM SU5402, and 1 mM Vasoactive Intestinal Peptide. From Day 14 onwards, cells are cultured in N2-supplemented media until RPE begins to appear (approximately 4 weeks). RPE cells are either manually picked or trypsinized, depending on the quality of differentiation. The RPE is then plated on Matrigel and cultured in medium containing 5% FBS and 10 mM Y-27632. After reaching confluence, the FBS concentration in the media is decreased to 1%.

A4. Generating retinal and choroidal microvascular endothelial cells:

Human retinal and choroidal microvascular endothelial cells (MVECs) will be isolated from human fetal donors, based on existing published protocol (Browning, Gray et al. 2005; Browning, Halligan et al. 2012). Briefly, matched human retinal and choroidal MVECs will be isolated from anonymized, paired human fetal globes at two to four months gestation. Retina and choroid will be dissected, minced, and dissociated into small fragments in serum-free EBM-2 endothelial growth medium, followed by sieving through a 40 µm cell strainer and digestion in 0.1% collagenase for 30 mins at 37°C. The obtained cell suspension will be plated on gelatin coated T-75 flasks in EBM-2 medium supplemented with antibiotic (Invitrogen), 10% FBS, 100 µg/ml ECGS, 50 µg/ml Heparin, and 40 ng/ml VEGF. Cells will be cultured at 5% O₂ until confluent, followed by sorting on BD FACS-Aria II at the UW SLU Flow Cytometry Facility to obtain two types of endothelial cells—the CD144 positive and CD45 negative populations. After sorting, both endothelial cells will be cultured in EBM-2 supplemented with antibiotic, 10% FBS, 100 µg/ml ECGS, 50 µg/ml Heparin, and 20 ng/mL VEGF, on gelatin-coated plates and used for further experiments.

Retinal and choroidal endothelial cells have been demonstrated to be quite different based on their molecular signatures and response to hypoxia, different VEGF isoforms, and anti-VEGF treatments (Brylla, Tscheudtschilsuren et al. 2003; Stewart, Samaranayake et al. 2011; Mammadzada, Gudmundsson et al. 2016). Recent findings showed, in particular, choroidal ECs provided unique angiocrine factors that promoted the maturation of RPE and the blood-retina

barrier, and generated appropriate RPE basement membrane matrix (Benedicto, Lehmann et al. 2017). Choroidal and retina ECs will be co-cultured with RPE and retina organoids separately.

A5. Engineering retinal and choroidal microvessels to support the integration and growth of RPE and retina in single devices.

Our team has developed soft-lithography based approaches to recreate live microvessel networks *in vitro* with controlled geometry, flow characteristics, and organ-specific microvascular endothelial cells (Zheng, Chen et al. 2012; Zheng, Chen et al. 2015; Ligresti, Nagao et al. 2016; Roberts, Tran et al. 2016). Briefly, a micropatterned silicone rubber stamp is fabricated from a lithographically determined silicone mold and stamped in collagen gel to form an enclosed network in a closed jig device. The networks are seeded with cells for long-term culture and remodeling under perfusion through the device inlet and outlet.

This approach offers the opportunity to create patterned microvessels with diameters ranging between 20 and 500 μm ; is scalable through multi-layered stamping; and maintains capacity for high-resolution imaging and extraction of cells, proteins, RNA, and DNA. When seeded with hPSC-ECs (or vessels derived from human fetal retina), the microvessels form a confluent endothelium along the microchannel and will start to remodel after 24 hours of culture, with significant angiogenesis into the matrix after three days of culture under flow. The vascularity can further be enhanced and stabilized by embedding these endothelial cells in the matrix. This technique also allows perivascular cells to be embedded in the matrix and interact with the endothelium. Importantly, it can be modified to allow multiple layers to be assembled together and the perfusion of each layer to be independently controlled (Appendix Figure 3).

We will further improve and utilize this system to build retinal and choroidal microvessels to support the retina organoids and RPE, respectively, to further mature and assemble the tissues as a single vision device. We will have three major steps: 1) fabricate retinal and choroidal microvessels; 2) culture retinal organoids and RPEs on top of the respective microvessels to allow nutrient support and proper organization; and 3) combine cultured retinal and choroidal layers to form a “sandwiched” model, with the retina in contact with the RPE and both placed between retinal and choroidal microvessels (Figures 1-2). To allow for higher throughput culture, we will modify the microvessel devices from published systems to allow for the culture of multiple organoids per device.

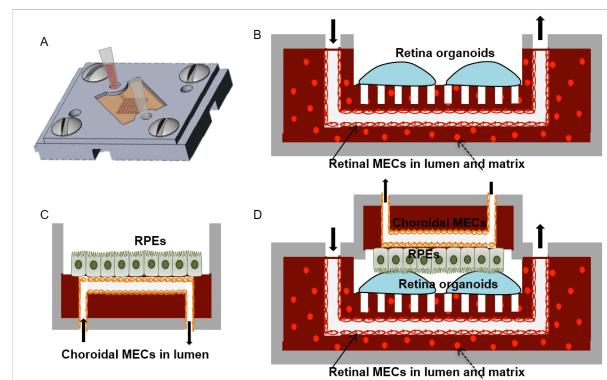


Figure 2. Device Schematics. A. Vessel device that fits in a 6 well plate. B. Retinal micro-vessel devices, with planar + vertical vessels to support organoids in culture. C. Choroidal microvessels co-cultured with RPEs. D. Sandwiched devices to culture retina with RPEs supported by two independent microvessels.

A6. Innovation Statement.

The combination of microvascular networks and both RPE and laminated retinal organoids will for the first time duplicate key features of ocular anatomy that we predict will promote functional maturation to levels not present when these tissues are cultured in isolation. We rely on established protocols that are currently in use by many labs to generate the components of a unique model retinal device.

B. Characterizing the model

B1. Single cell RNAseq to determine the stages of retinal development.

With this technique, we can get a rapid assessment of whether all retinal cell types are generated and what stage of maturation the individual cells reach. For the single cell RNAseq analysis, the organoids from key stages of development will be harvested, dissociated into single cells with 0.25% trypsin at 37C. Every few minutes, the organoids will be gently triturated, the supernatant containing dissociated cells removed, and trypsin inactivated with FBS. The cells are centrifuged for 10 minutes at 300g at 4C and resuspended in PBS containing 0.04% BSA. This solution is then passed through a 20 µm cell strainer and processed immediately for Drop-seq (Macosko, Basu et al. 2015). Cells are loaded onto the Chromium Single Cell A Chip for a targeted cell recovery of 3,000 cells. Droplet generation and barcoding, RT, cleanup, cDNA amplification, and library preparation will be performed according to the 10X Genomics' guidelines. The library is sequenced on the Illumina NextSeq 500. Reads are preprocessed in Cell Ranger, aligned to hg38, and filtered for possible doublets and mitochondrial reads.

Secondary analysis on the remaining cells is performed in R using the package Seurat (Satija, Farrell et al. 2015), and the data visualized using t-Distributed Stochastic Neighbor Embedding (t-SNE). We have already carried out this pipeline for human fetal retina at three stages, with separate data from central and peripheral retina, and these data will provide key benchmarks for the organoid results. The data from one age is shown in Appendix Figure 4 and the cell clusters in the t-SNE plot correspond to known classes of retinal neurons. Many of the genes related to USH were identified in photoreceptor clusters. Even at this relatively early stage of retinal development, most of the USH genes are expressed and so we can study the effects of mutations in these genes from their onset of expression.

B2. Immunohistochemistry (IHC) and *in situ* hybridization (ISH) to analyze the developing retinal organoids.

We have just completed a comprehensive resource study characterizing the development of the fetal human retina using histology and bulk RNA-seq, which will improve our ability to stage the retinal organoids. Human retina develops in a central-periphery gradient, and foveal development is accelerated by several weeks. From these studies on human fetal development we have characterized many antibodies and *in situ* probes to track photoreceptor and synaptic development (Appendix Figure 5). For IHC, organoids will be fixed in 4% paraformaldehyde and embedded in OCT for sectioning. Sections will be incubated in block solution (10% normal horse serum in 10% Triton-X 100/PBS), and then incubated with primary antibodies overnight at 4C, incubated with appropriate secondary antibodies and DAPI (to counterstain nuclei). Slides will be coverslipped using Fluoromount G and images will be taken on the Zeiss 880 confocal microscope.

In addition, ISH will provide information about proteins for which we lack antibodies. For ISH, organoids will be fixed overnight in 60% ethanol, 30% formaldehyde, 10% glacial acetic acid at 4C, sequentially dehydrated and embedded in paraffin. To generate the probes, plasmids will be linearized and anti-sense probe generated using digoxigenin-11-UTP and T7 RNA polymerase. ISH will be carried out as previously published (Ueki, Wilken et al. 2015); this is a robust protocol and we have trained many labs to carry this out. The histology will complement our RNAseq study by providing spatial information.

B3. Serial block face scanning EM (SBFSEM) and 3D reconstruction of organoids.

We will perform SBFSEM on organoids at various stages to obtain high-resolution 3D reconstructions of cell morphology and connectivity within these structures. Briefly, organoids will be fixed in 4% glutaraldehyde/0.1 M cacodylate buffer for several hours, washed in buffer, and processed for SBFSEM, following staining protocols we have established previously (Jorstad, Wilken et al. 2017). Appendix Figure 6 illustrates the excellent ultrastructural

preservation of the organoids. Serial sections at 50 nm z, 5 nm xy pixels will be obtained using the Zeiss/GATAN 3View or FEI VolumeScope (both scopes are available in house), which will provide good resolution of subcellular structures, including pre- (synaptic vesicles, ribbons) and postsynaptic specializations. We have been reconstructing cells and their connectivity in the fetal retina from SBFSEM images (Appendix Figure 7). These data will enable us to compare the developmental progression of the organoids at stages ‘equivalent’ to those of the developing retina *in vivo*. 3D reconstructions of the organoids will enable us to assess: (1) Cellular differentiation of major cell classes of the retina (photoreceptors, horizontal cells, amacrine cells, bipolar cells, retinal ganglion cells and Müller glial cells); (2) lamination of cell bodies and their processes in the correct order; (3) synaptic differentiation (synaptic vesicles, ribbons) and (4) synaptic connectivity (targeting appropriate partners, or miswiring in USH iPSCs).

B4. Evaluate the maturation of physiological properties of the organoids.

We will characterize the functional maturity of individual neurons and of synaptic connections between them, with a focus on comparing these property changes in organoids derived from non-Usher and Usher iPSCs. We will also compare these functional properties with data we have been collecting on fetal human retinas.

We will use suction electrode or patch clamp recordings to determine whether the derived photoreceptors generate light responses (*i.e.*, have operational phototransduction cascades); if light responses are present, we will characterize their sensitivity and kinetics. Electrical properties of bipolar, amacrine, and Müller glial cells will be characterized using patch clamp approaches. We will focus on the magnitudes of sodium, potassium, and calcium currents; voltage-activated sodium and potassium currents increase substantially in neurons during development, while mature glia are characterized by a large non-voltage dependent potassium current. Synaptic transmission will be probed using calcium imaging. Calcium-sensitive dyes will be introduced by electroporation (Oregon Green) or viral approaches (GCaMP6). We will stimulate the retina by patch-clamping individual neurons and manipulating their voltages while monitoring changes in calcium in nearby cells. We will begin with photoreceptors, and will be particularly interested in whether depolarizing a photoreceptor produces opposite polarity responses in different bipolar cells, indicative of development of both ON and OFF bipolar types. We will use a similar approach to test for excitatory synapses from bipolar cells to amacrine cells, and inhibitory synapses from amacrine cells back onto bipolar cells. We have successfully recorded from cells in mouse organoids (Appendix Figure 8), so do not foresee any technical challenges with obtaining recordings. We have also successfully loaded calcium indicators using electroporation into both mouse and primate retina. Our knowledge of retinal signaling, particularly of phototransduction, is sufficiently advanced that any observed differences will suggest hypotheses about which component(s) are altered. This will guide attempts to recover normal responses using gene therapy or small molecule approaches.

C. Evaluating the model for USH phenotypes.

Once we have the model retina well characterized at key developmental milestones, we will begin to characterize the different USH mutations for phenotypes. Many USH proteins are localized to photoreceptors, though some are expressed in other retinal cell types and may affect outer segment morphogenesis and/or synaptogenesis. We can assess changes in gene expression in the USH iPSC-derived model retinas using single cell RNAseq and *in situ* hybridization. We can assess changes in synapse formation with Serial block face SEM reconstructions, comparing USH iPSC derived model retinas with those from control individuals. We can assess changes in electrophysiological properties of photoreceptors and bipolar cells in the USH iPSC-derived model retinas, using Calcium imaging and patch clamp recordings. The combination of these high resolution molecular, morphological, and functional read-outs should reveal phenotypes in the USH model retinas and provide useful models for these diseases for the first time.

Thomas Reh, PhD: Tom Reh has been studying development and regeneration in the retina since establishing his lab in 1985. His group developed some of the first methods for *in vitro* retinal cell culture and used this system to identify key factors in retinal development and regeneration. The Reh lab was among the first to culture human fetal retina and using what they learned from studying retinal stem/progenitors *in vitro* over many years, they developed one of the first protocols for directing hES cells to the retinal progenitor cell fate and expanding these cells. The lab is also well-qualified to carry out molecular analyses of the proposed model, with many publications using microarray, RNAseq, and DNase-I hypersensitivity analyses of the developing retina and hESC-derived retina. With Drs. Wong and Rieke, he received the Allen Distinguished Investigator Award to collaborate on circuit formation in retinal organoids.

Rachel Wong, PhD: Rachel Wong obtained her doctoral training in Visual Neuroscience with A. Hughes and W.R. Levick, studying the development of the mammalian retina. Her postdoctoral training with Carla Shatz at Stanford University, led to the discovery of retinal waves, a pattern of spontaneous activity now known to play a key role in the refinement of retinal projections *in vivo*. Since becoming an independent investigator in 1994, she has been studying the cellular mechanisms that underlie the assembly, disassembly, and reassembly of vertebrate retinal circuits. The Wong lab uses mice and zebrafish as model systems, and they now study the human retina. They use a combination of light microscopy, correlated fluorescence and serial block face electron microscopy, and live cell imaging approaches to address their questions. Her extensive experience using state-of-the art imaging methods to assess the development and regeneration of retinal connectivity, including that of the human retina, will provide the necessary technical and conceptual expertise for determining the precision by which circuits form in retinal organoids.

Ying Zheng, PhD: Ying Zheng joined the bioengineering department of the University of Washington as a faculty member five years ago. Her training and experience are in the field of tissue engineering, microfluidics, and most recently, vascular biology. Her research interest is to develop and understand the integrative functions of living tissue and organ systems in our body. She has been awarded the Scientist Development Grant by the American Heart Association to investigate vascular growth and remodeling under microfluidic control, and the NIH Director's New Innovator Award to develop a marrow microenvironment to further understand the role of different vasculature in the organ-specific functions. Her research utilizes a form of 3D cell culture system with endothelialized, perfused microvessels within a bioremodelable hydrogel (type I collagen), and complements it with engineering technologies (e.g., microfabrication, biomaterial synthesis, and molding), imaging analysis (e.g., fluorescence microscopy, confocal microscopy, and scanning and transmission electron microscopy), and biochemical characterization (e.g., flow cytometry, ELISA, cell culture, genomics and proteomics, etc), to study engineered organ-specific vasculature as a functional unit. The Zheng laboratory has been working with the Reh laboratory at UW to develop vascularized retina by combining engineered microvessel techniques with retinal organoids. She is excited to participate in this 3D retina challenge and believe this collaboration will make a big impact in the field of retinal differentiation and produce retinal organoids that exhibit more mature tissue organization, vascularization, and function than is currently possible.

Fred Rieke, PhD: The broad goal of our work is to determine how the biophysical mechanisms involved in transduction, synaptic transmission, and neural coding enhance and limit the fidelity of signals in the retina. The issues involved are quite general—from the control of G-protein coupled receptor signaling to adaptive mechanisms that match the range of neural responses to the range of input signals. Hence, their results advance our general understanding of neural signaling and computation. The Rieke lab relies on a variety of techniques, including imaging, genetic manipulations, electrophysiology and behavior, and have been collaborating with the

Reh and Wong laboratories for several years now to put these techniques to use to characterize retinal development and progress in *in vitro* generated retinal organoids.

Jennifer Chao, PhD: The primary focus of research in Jen Chao's lab is to develop *in vitro* models of retinal pigment epithelial (RPE)-related diseases. Her lab has successfully generated induced pluripotent stem cells (iPSC) from over twenty subjects with retinal degenerative diseases, as well as from normal controls, and has differentiated the iPSCs into RPE cell lines, characterized, and cultured patient-specific RPEs. Using immunocytochemistry, qPCR, phagocytosis assays (microbeads and bovine rod outer segments), transepithelial resistance measurements, and TEM, they confirmed that their iPSC-derived RPE are similar in structure and function to their *in vivo* counterparts. Her lab has gained substantial experience and acquired the necessary techniques to culture human fetal RPE, efficiently differentiate RPE from iPSCs, and develop tools for dissecting the structure and function of RPE. Her clinical expertise is in medical and surgical retina, with a particular emphasis on patients with retinal degenerative diseases, including RP and Usher Syndrome. She does not anticipate any issues in recruiting Usher type I patients for this project—they have already generated iPSCs from an Ush1B patient in a UW IRB approved protocol. She looks forward to contributing to this project by generating Usher patient-derived iPSC-RPE and culturing the RPE on vascular scaffolds, and by advising on the relationship between the proposed organoids and their clinical relevance and applications.

Akina Hoshino, PhD: Akina Hoshino completed her PhD study at the University of Maryland, Baltimore where she investigated the regulation of prohormone convertases using a variety of molecular and biochemical techniques. During the past 5 years as a postdoctoral fellow in Dr. Tom Reh's laboratory, she has been involved in improving the retinal differentiation protocol in both mouse and human embryonic stem cells using small molecules, growth factors, and miRNAs. She is funded to pursue this work through an F32 research grant (2015–2018) from the NEI. In addition, she has been characterizing the early development (2–5 months gestation) of human fetal retina using histology, long-term explant cultures, and single-cell and bulk RNAseq, to identify similarities and differences between human and mouse retinas. This Challenge builds on her expertise of pluripotent stem cells and human retinal development.

Chi Zhang, PhD: Chi Zhang's graduate research project at the University of Louisville has trained her to combine electrophysiology and engineered viral tools to perform functional characterization of normal and genetically altered circuits of the mouse retina. Her current postdoctoral projects under Dr. Rachel Won's mentorship focus on investigating the maturation of neurons and their circuitry in developing mouse and human retina. In collaboration with the Reh lab, she has been studying the structural organization of retina and retinal organoids using approaches she routinely uses, including immunohistochemistry, serial block face SEM reconstruction and Calcium imaging. Her extensive knowledge and technical expertise using serial EM to reconstruct the developing human fovea, together with her electrophysiology training, enable Chi to readily evaluate the structural and functional arrangements of cells and their connections in the retinal organoids.

Paul Nakamura, PhD: Paul Nakamura obtained his PhD in Neurobiology and Behavior at the University of California, Irvine, where he worked in the lab of Karina Cramer studying the development and reorganization of mammalian auditory brainstem circuits. As a postdoctoral fellow in Dr. Tom Reh's laboratory at the University of Washington, the foci of his research has been small molecule-mediated reprogramming of retinal cells to treat degenerative diseases of the eye, and also directed differentiation of embryonic stem cells. His contribution to this project will include stem cell culture maintenance and differentiation, and histological characterization of stem cell-derived retinas.

Feasibility Assessment (3 page maximum)

A. Describe the ability to execute the proposed solution.

We are well positioned to carry out the production of the proposed model retinas, and the analysis of USH phenotypes in the model.

1. Our group has expertise in generating retinal cells and organoids from both human ESCs and iPSCs with publications extending over ten years. Our lab was among the first to develop protocols for efficient generation of retinal cells from human ESCs and we have relevant cell and organ culture of retina from frog, fish, rat, mouse and human extending more than 30 years. No lab in the world has more experience with retinal cell culture.
2. Our group has expertise in the isolation and cell culture of endothelial cells from mouse and human and in the fabrication of microvascular networks in engineered scaffolds, with publications in PNAS and Nature Communications. The Zheng Lab in our team is a leader in 3D microvascular engineering, and has applied their system to build organ-specific vascular niches and vascularized cardiac tissue, as well as to study endothelial cell biology.
3. Our team has experience in all the proposed methods of analysis of the human organoids.
 - A. Our group has recently completed an extensive characterization of human fetal retinal development with RNAseq, DNase I epigenomic characterization, immunofluorescence and *in situ* hybridization that is currently under review. We have also carried out single cell RNAseq of human fetal retina at three ages, and together these studies will allow us to stage the organoids and ensure that development is proceeding normally, with all relevant cell types generated and differentiating appropriately.
 - B. Our group includes one of the top retinal morphologists in the world, and has relevant experience with serial block face scanning EM and the equipment to characterize the "connectome" of the organoids. We have already made significant progress in reconstructing regions of the developing human fetal retina, and these studies will serve as an important benchmark to compare the circuit development of the retinal organoids, derived from both normal and USH iPSCs.
 - C. Our team includes one of the top retinal electrophysiology labs in the world, with extensive experience in mouse and primate retinal patch clamp electrophysiology. This includes recordings from all major retinal cell types. Our group has already successfully carried out recordings from retinal organoids and human fetal retina.

Taken together, our team has extensive experience with the production of retinal organoids, and RPE from ESCs and iPSCs and the characterization of human retinal development; we are uniquely positioned to realize the proposed solution for this challenge.

B. Innovation and Estimated timeframe.

We currently have all the elements of the proposed solution "up and running" in our labs. The fact that all members of the team are at the University of Washington in Seattle makes the production of the model retinas and the analysis seamless. All of the proposed steps in the generation of the model retinas proposed in this application are established. No new technology needs to be invented, but this is the first time to our knowledge that these elements will be combined such that a perfused vascular network will support the differentiation of both the retina and the RPE. We have already generated iPSCs from an USH patient, so we can run an initial study without delay. All proposed methods, including single cell RNAseq, Serial block-face

scanning electron microscopy (SBFSEM), and electrophysiological analyses are currently in use in the labs of the team members.

C. Protections for human subjects.

Informed consent is obtained from all participants prior to inclusion in the study, and all experiments are conducted according to the principles expressed in the Declaration of Helsinki. Peripheral blood mononuclear cells (PBMC) are obtained from normal control and USH patients and converted into iPSCs under a University of Washington IRB-approved protocol (HSD#: 43143). We have already carried this out for a patient with USH1B and confirmed splicing mutations on each allele of Myo7A.

D. Use of technologies covered by patents or other intellectual property protection.

The Zheng lab has a patent for the blood cell generating bioreactor using this system. (Blood-cell producing bio-microreactor US 20160097033 A1).

E. Evaluation Criteria.

1. Will the proposed new method improve aspects of retinal organoids?

Cell Types: Current protocols of organoid production have shown that most cell types, including ganglion cells, amacrine cells, and even rod and cone photoreceptors are produced in a stage appropriate manner. However, there are two important problems with the current protocols. First, the ganglion cells do not survive well in culture, even as organoids, and second, Müller glia and bipolar cells are not produced in most protocols. Long-term culture of retinal organoids with current protocols allows for very good development of the outer retina, with rods even extending outer segments in some cases. However, the inner retina does not remain organized, and normal circuits in the inner plexiform layer (IPL) fail to undergo normal maturation. We propose that adding a perfused vascular network to the retinal organoids will solve both of these problems. We hypothesize that endothelial cells are necessary to induce proper formation of the Müller glia and subsequently the inner limiting membrane (ILM). With proper Müller glial maturation, this key element of inner retinal support will promote bipolar cell development and correct lamination in the IPL. We have found in preliminary studies that co-culture of embryonic retina with a vascular network improves the ganglion cell survival and supports IPL formation.

Structure: In our proposed solution, retinal organoids and RPE will be generated with protocols similar to those already in use in several labs. The organoids will be cut at 45 days, when ganglion cells are being generated, and then placed on the vascular network in the described device to allow the rest of retinal development to proceed in contact with a vascular network. Although we do not currently have a method to ensure that the organoids will settle with the ganglion cell surface facing the vascular network, we anticipate that approximately half will be correctly oriented. As noted above, the presence of the endothelial cells will promote ILM formation and this should provide better structural maintenance than existing protocols, particularly for the later stages of development. In addition, the perfusion of the microvascular network will ensure that the cells will receive adequate oxygenation and nutrient supply, even when apposed to the RPE.

Viability: For the best USH model, we will need nearly all the retinal cell types and the RPE. The ganglion cells may be dispensable, but rod and cone photoreceptors, Müller glia, RPE, and bipolar cells are all likely important to assess the full range of USH mutations on retinal development and function. Although current protocols are very good at producing many of these cell types, they are limited in the production of Müller glia and differentiated bipolar cells, largely because of disorganization of the inner retina in long-term cultures. In addition, retinal ganglion

cells do not survive well in organoids. We propose that the addition of the microvascular networks of perfused endothelial cells will promote viability of all cells, including the ganglion cells, due to more effective oxygenation and nutrient supply than is possible in diffusion-limited floating culture models. We also propose that signaling molecules from the retinal endothelial cells provide important inducing and survival factors, and these are not normally present in existing protocols.

Functional characterization of cell types: Both the retinal cells and the RPE can be assessed in the proposed device for their state of functional differentiation. The retina can be analyzed by SBFSEM, single cell RNAseq, immunolabeling, and electrophysiological methods to assess the maturation of the cells and their circuits. The combination of the RPE with the neural retina should enable maturation of the photoreceptor outer segments, and physiological characterization can be done by imaging the GCaMP in live organoids in the chambers, or by patch clamp of the retinal cells after acute separation from the RPE layer.

2. Robustness and Reproducibility

We have built our protocol around existing protocols for producing retinal organoids and RPE monolayers that are currently in use in many labs around the world. In addition, the design and implementation of microengineering scaffolds for the endothelial cells is well-established. The unique aspect of our proposal is that we are bringing these elements all together to essentially generate a more complete ocular structure. The Zheng lab would be happy to supply prototype devices like those to be used in our solution, and these can be commercially produced if there is sufficient demand. Human fetal retinal endothelial cells can be banked and distributed to other labs that want to create similar devices. To facilitate transferability, we propose to (1) publish a detailed protocol (2) produce a JoVE video of the methods and (3) create a website where the data from normal human retinal development is made available to the community, analogous to the Allen Brain Institute's site.

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

The combination of RPE and retina, with their normally associated microvasculature provides a closer approximation of *in vivo* retinal development than that which has been achieved to date. We propose that the combination of all these elements, with the ability to modulate the perfusion of nutrients and oxygen, will provide the support needed for full differentiation and viability of all retinal cell types. We will assess the state of differentiation of the cells in the model at 60, 90, 120, and 180 days; we currently have comprehensive data on all but the last time point for human fetal retina, and so it will be straightforward to compare the state of the iPSC-derived retina and RPE at these stages to determine what aspects of normal human fetal development are captured and which are not. We also have carried out patch clamp recordings from the same stages of human fetal retina, and so we can compare the maturation of electrophysiological properties of iPSC-derived retinas with that of normal human at the equivalent stage. USH disease leads to degeneration of photoreceptors, and we should be able to assess this in the proposed model. While this degeneration is in most cases a late event, our model will also allow us to study early events in the disease pathogenesis. Changes in the retinal circuitry, particularly between photoreceptors and bipolar cells, will be assessed with SBFSEM, electrophysiology, and functional imaging; and changes in retinal cell differentiation can be evaluated with single cell RNAseq, immunolabeling, and *in situ* hybridization. We will be able to characterize the disease model with unprecedented resolution.

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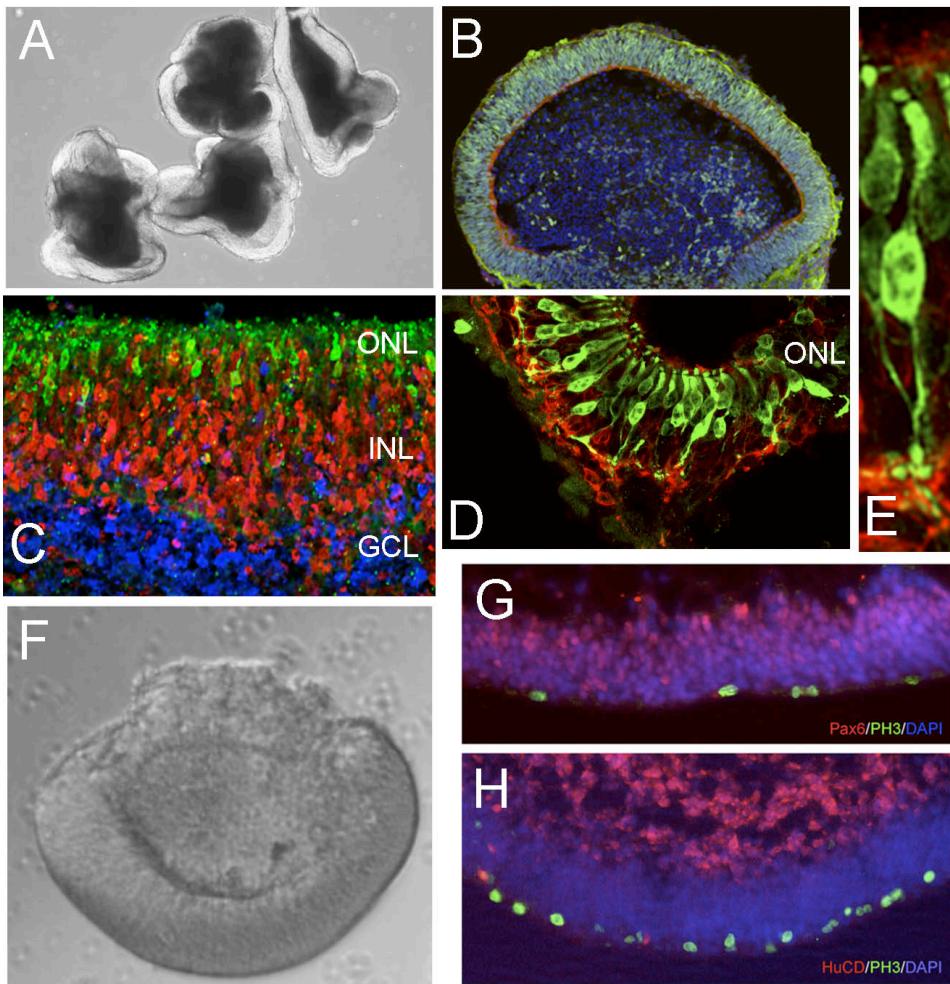
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Appendix for Reh et al Organoid Challenge

8 Figure pages

2 Resource pages

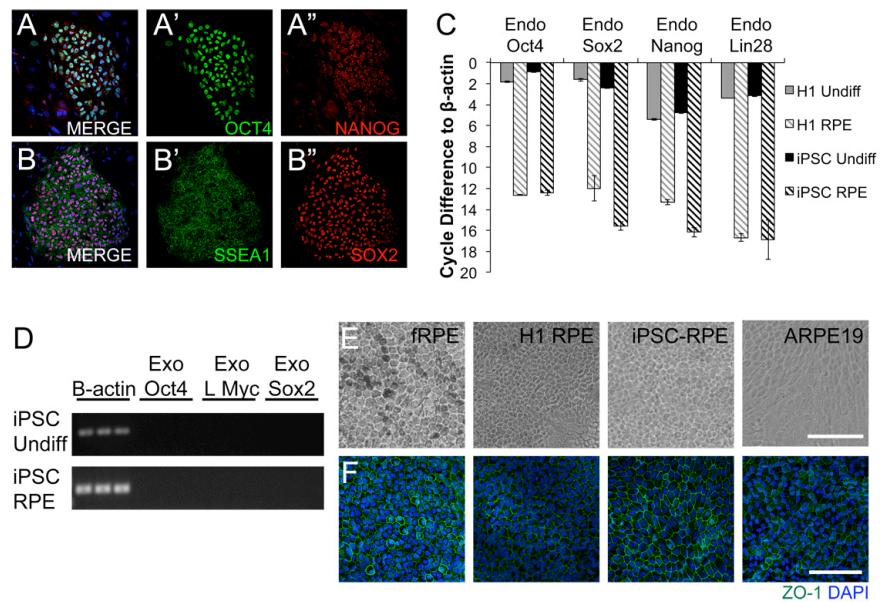
Creating the model; retinal differentiation of embryonic stem cells



Appendix Figure 1. Laminated retinal structures can be generated from mouse and human embryonic stem cells.

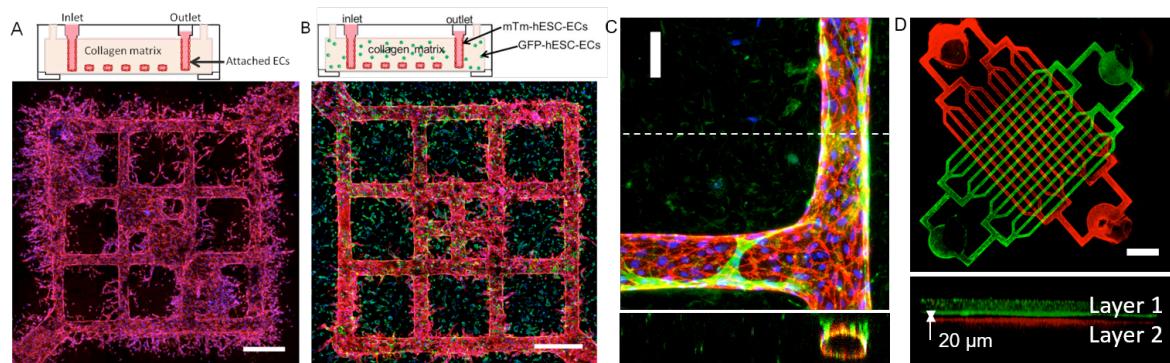
A. ESCs give rise to optic vesicle and retinal neuroepithelial structures. B. The optic vesicles label for retinal markers, like Lhx2 (green). C. After 2-3 weeks, the retinal structures that develop from the optic vesicles form distinct lamination, with recoverin+ (green) photoreceptors, Otx2+ bipolar cells (red) and HuC/D+ amacrine and ganglion cells (blue). D. E. The photoreceptors that develop in the cultures (recoverin+ green) have morphology similar to those in postnatal mouse retina, and form a distinct lamina. F. Human embryonic stem cells induced to form neural retina; the optic vesicles can be dissected from the non-retinal regions of the embryoid bodies and have an appearance similar to optic cups. G.H. Sections from retinal structures derived from human ESCs show good lamination with a HuC/D+ amacrine/ganglion cell layer (red, H) and a neuroblastic layer (Pax6+, red, G). PH3+ mitotic cells are located on the apical surface of the neuroepithelium (green).

Creating the model; generation of iPSCs and RPE differentiation



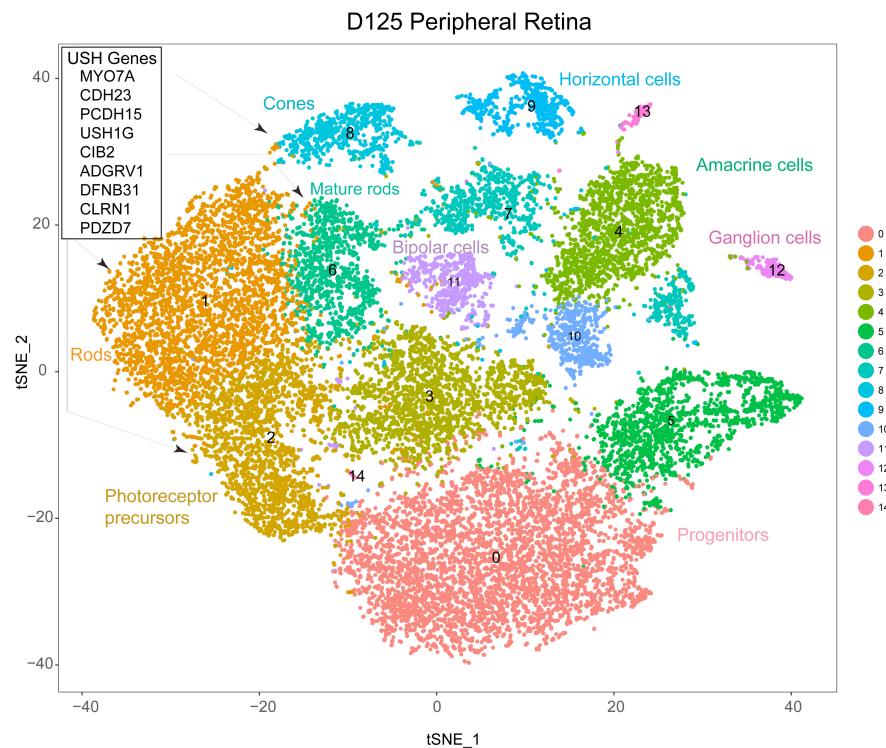
Appendix Figure 2. Patient-derived iPSCs and RPE. iPSCs were generated by transfecting human erythroblasts with episomal vectors (A and B). Upon differentiation of iPSCs and HESC (H1) cells to RPE, endogenously expressed pluripotency genes (C) and exogenous reprogramming genes (D) were downregulated, as detected by qPCR. (E) iPSC- and H1-differentiated RPE cells demonstrated cobblestone-like hexagonal morphology and formation of tight junctions characteristic of native RPE. (E, F, scale bar = 50 μ m).

Creating the model; engineering retinal and choroidal microvessels



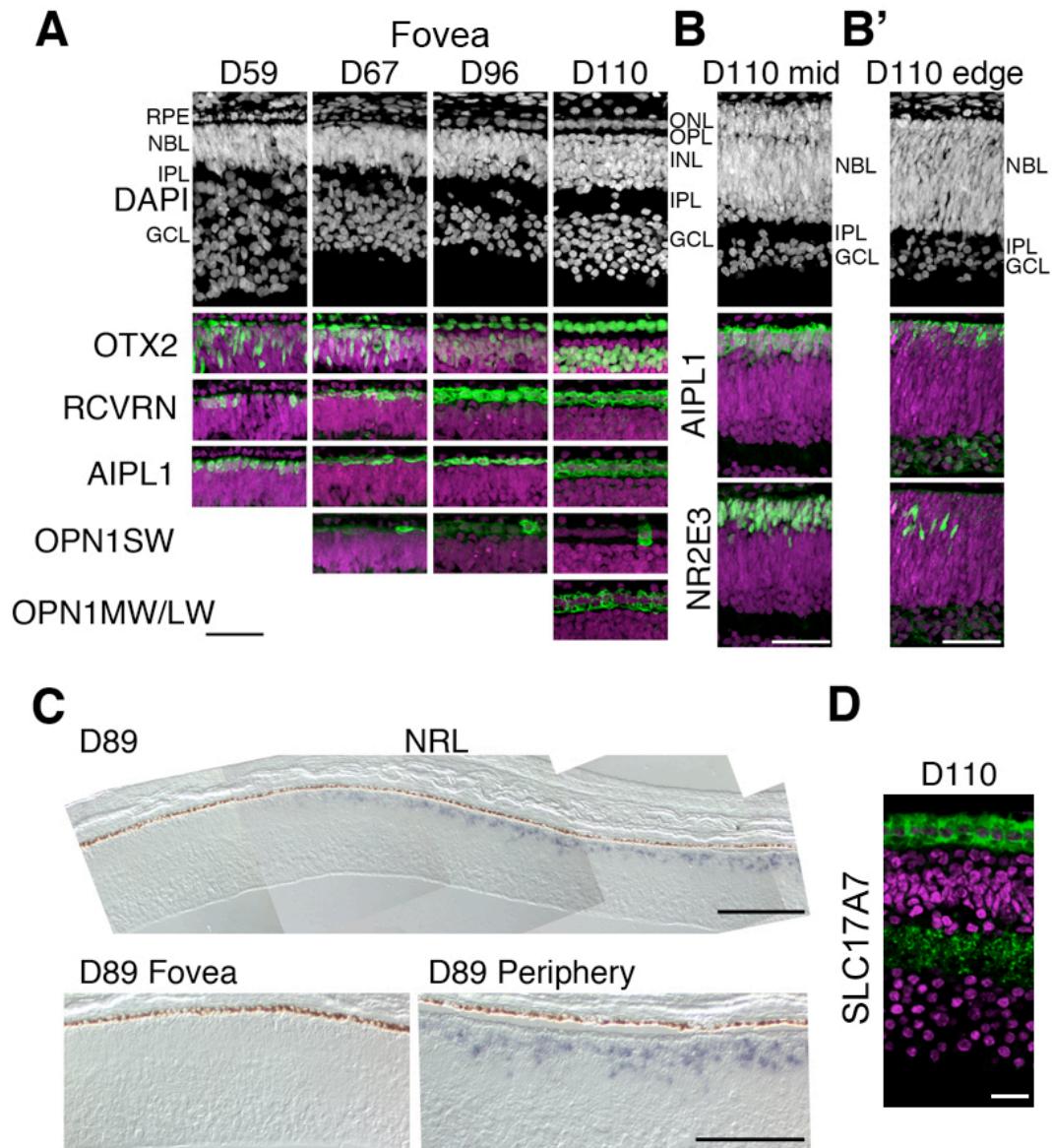
Appendix Figure 3. Engineered microvessels. A. Patterned microvessel network generated via lithography method in collagen gel, cultured with gravity-driven flow for 4 days through the vessel lumen (Palpant, Pabon et al. 2015) with human pluripotent stem cells derive endothelial cells. Scale bar: 200 μ m. B. Lithography defined microvessels readily sprout, connect and anastomose with self-assembled vessels in the matrix. C. Human smooth muscle cells embedded in the matrices migrate and coat the endothelium. Scale bar: 100 μ m D. Engineering thick vascularized tissue by multiple-layer stacking. Two vessel layer were stacked orthogonal to each other with individual flow path with distance of ~20 μ m between them. Red and green are fluorescent beadsperfused in individual channels. Scale bar: 500 μ m

Characterization of differentiation in Retinal Organoids: single cell RNAseq



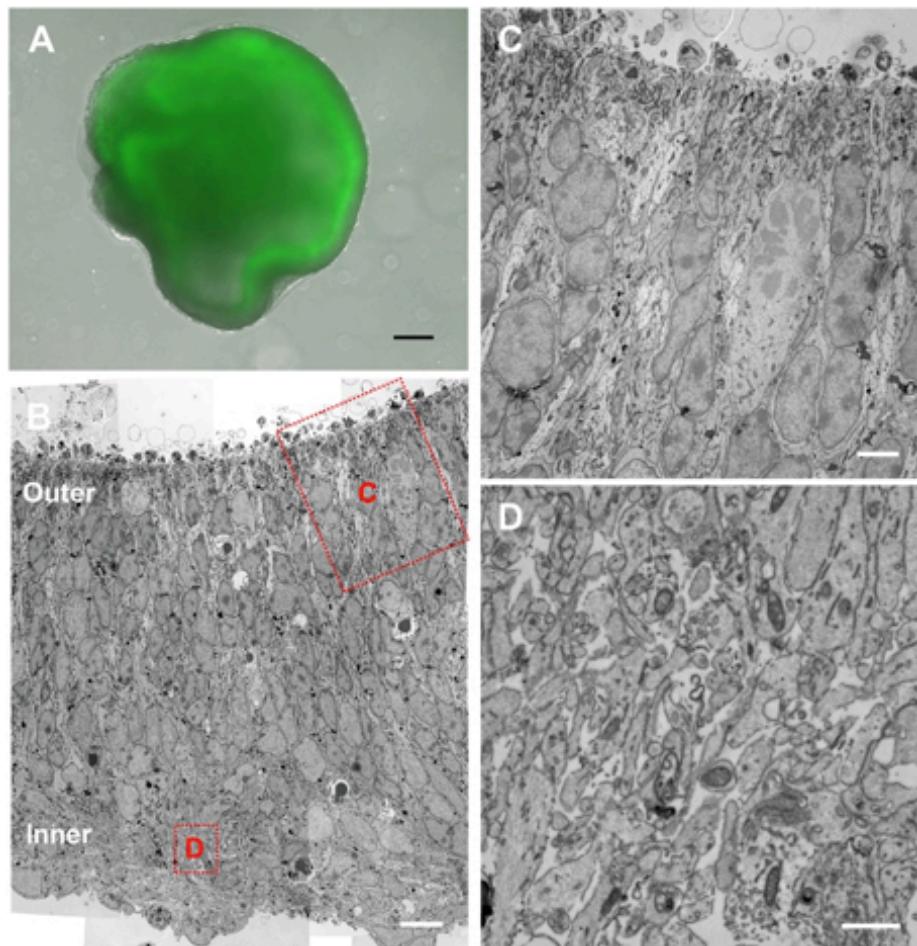
Appendix Figure 4. Single cell RNA-seq analysis of a D125 peripheral retina.
Major retinal cell types were identified by this age, and many of the USH genes were detected in the rod and cone photoreceptor clusters (#1,2,6, and 8)

Characterization of differentiation in Retinal Organoids: Immunolabeling and *in situ* hybridization



Appendix Figure 5. Photoreceptor development in the human fetal retina. (A) D59–D110 fovea were stained with early and late photoreceptor markers. S-Opsin and M/L Opsin expression were first detected at D67 and D110, respectively. (B) The same section from the D110 retina as in A but more temporally. The retinal layers have not yet developed and Nr2E3 expression is barely reaches the temporal edge. (C) *In situ* hybridization using a probe against NRL. NRL mRNA is absent in the fovea but rapidly increases at the peripheral edge and is expressed in the periphery. (D) SLC17A7 staining shows early signs of synapses in the outer and inner plexiform layers of a D110 fovea. Scale bars for A–C: 50 µm; D: 25 µm.

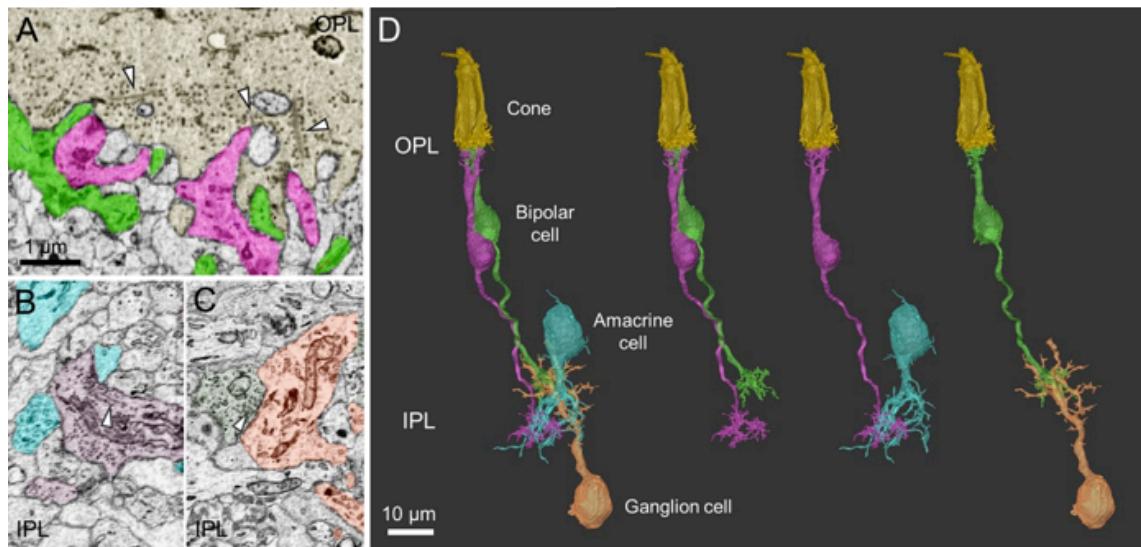
Characterization of differentiation in Retinal Organoids: electron microscopy



Appendix Figure 6. Ultrastructure of 21d mouse organoid.

(A) Fluorescence image of a well-developed 21d mouse organoid with RAX-GFP positive cells. **(B)** Transmission EM section of the same EB in A, which shows the organization and morphology of cell bodies in the putative outer and inner retina. **(C)** Higher magnification image of the area 'C' indicated in B, of the putative outer retina where photoreceptors and progenitor cells are located. **(D)** Higher magnification of the area 'D' indicated in B, of the putative inner retina where ganglion cells and amacrine cells are located. No obvious synapses were detected in either the outer or inner regions. Scale bar for A: 500 μ m; B: 10 μ m; C: 4 μ m; D: 1 μ m.

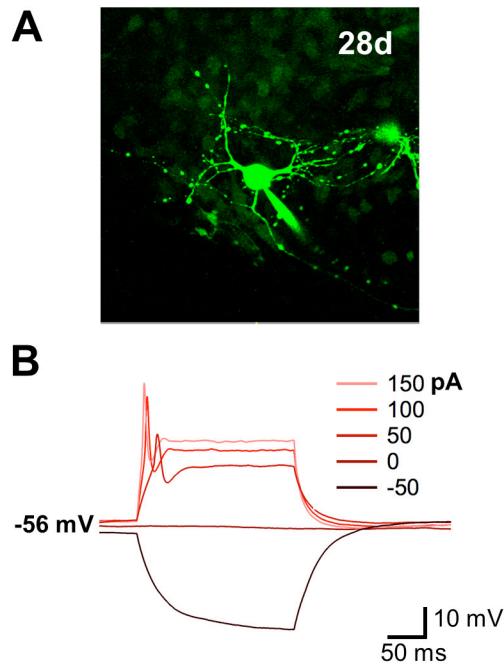
Characterization of differentiation in Retinal Organoids: Serial block face SEM reconstruction of synaptic circuits



Appendix Figure 7. Serial block face SEM reconstruction of a D127 old human fetal retinal fovea.

(A) Example of synaptic contact between a cone photoreceptor (yellow-gold) and the dendritic tips of an ON (magenta) and an OFF (green) bipolar cell in the outer plexiform layer (OPL). Synaptic ribbons are indicated by arrowheads. **(B,C)** Ribbon contacts (arrowheads) between an (B) ON bipolar cell (magenta) and an amacrine cell (cyan), and between an (C) OFF bipolar cell (green) and an OFF ganglion cell (orange). IPL, inner plexiform layer. **(D)** Three-dimensional reconstruction of the cone, ON and OFF bipolar cells, amacrine cell and ganglion cell highlighted in panels (A–C).

Characterization of differentiation in Retinal Organoids: patch clamp electrophysiology



Appendix Figure 8. Intrinsic physiological properties of differentiated cells in 28d mouse retinal organoid. The image of Alexa Fluor 488 dye-filled cell recorded from 28d EB is shown in **A** and the corresponding whole-cell clamp recordings are shown in **B**. **(B)** Voltage changes in 28d EB cells in response to different amounts of current injection

Resources

A1. iPSC production

hESC media: DMEM/F12 containing KSR, MEM NEAA, L-glutamine, sodium pyruvate, sodium bicarbonate, β -mercaptoethanol, 10 ng/ml bFGF, and 1% PenStrep

DMEM/F12 1:1 (Gibco)
Ficoll-Hypaque (GE Healthcare)
QBSF-60 serum-free medium (Fisher Scientific)
pCXLE-hOCT3/4-shp53-F (Addgene #27077)
pCXLE-hSK (to express SOX2 and KLF, Addgene #27078)
pCXLE-hUL (to express L-MYC and LIN28A, Addgene #27080)
pCXLE-EGFP (Addgene #27082)
Nucleofector II device (Lonza, Kit V, Program T-019)
N2 Medium Supplement (Gibco)
Sodium pyruvate (Gibco)
10% BSA solution (Sigma-Aldrich)
MEM NEAA (Gibco)
Knockout Serum Replacement (KSR; Life Technologies)
L-glutamine (Gibco)
Sodium bicarbonate (Gibco)
 β -mercaptoethanol (Sigma)
bFGF (R&D systems)
Essential 8 medium (Life Technologies)

A2. Retinal organoid production

Neural Induction Medium: DMEM/F12 containing 1% N2, 1% MEM NEAA, 0.1 % Heparin, IGF-1

Retinal Differentiation Medium: DMEM/F12 containing 2% B27, 1% MEM NEAA, 1% PenStrep

DMEM/F12 1:1 (Gibco)
N2 Medium Supplement (Gibco)
MEM NEAA (Gibco)
Heparin (Invitrogen)
human recombinant IGF1 (R&D Systems)
B27 (Gibco)
PenStrep (Gibco)
GFR Matrigel Matrix (Corning)
Dispase (Gibco)
IWR1 (Sigma Aldrich)
SB431542 (Stemgent)
LDN193189 (Stemgent)
FBS (Invitrogen)

A3. RPE production

HEPES (Gibco)
Y-27632 dihydrochloride (Tocris Bioscience)
Recombinant Human Noggin (R&D Systems)
Recombinant Human DKK-1 (R&D Systems)
Recombinant Human IGF1 (R&D Systems)
Nicotinamide (Sigma Aldrich)
Basic Fibroblast Growth Factor (Invitrogen)
Recombinant Human/Murine/Rat Activin A (Peprotech)
SU 5402 (Tocris Bioscience)
Vasoactive Intestinal Peptide (Sigma-Aldrich)

A4. Production of Retinal and choroidal microvascular endothelial cells

serum-free EBM-2 endothelial growth medium (Lonza)
ECGS (Millipore)
Heparin (Invitrogen)
VEGF (R&D Systems)
FBS (Invitrogen)
Gelatin (Sigma)

B1. Single cell RNAseq

Trypsin
FBS (Invitrogen)
Single Cell Reagents (10x Genomics, Chromium Single Cell 3' Reagent Kits)
Cell Ranger (10x Genomics)
R (www.r-project.org)