Abstract:

The outer retina, consisting of the light-sensing photoreceptors and the overlying retinal pigment epithelium (RPE), are affected in a number from retinal degenerative conditions. Developing strategies to maintain the function and cellular homeostasis of the outer retina requires a better understanding of the degenerative process. The creation of valid *in vitro* disease model for a number of retinal degenerations would require an organized 3D state such as that existing in the eye in order to mimic its highly-differentiated characteristics. Our solution to this challenge is to combine our expertise in retinal differentiation of iPSCs and combine it with our recent work on using hydrogels as bioscaffolds to promote 3D organization and lamination. This in turn should lead to maturation and generation of organized bilayered retina upon printing over a monolayer of mature RPE. We have previously published work on generating various retinal cells from human pluripotent stem cells (embryonic stem cells and induced pluripotent stem cells) in 2D monolayer culture setting. In this proposed solution, we will develop technologies to generate human retina in a dish by combining human induced pluripotent stem cell technologies with hydrogel based biomaterials. We propose to generate a Gelatin methacrylate (GelMA) based-bioink for three reasons: (i) it is biocompatible with human retinal cells, (ii) it provides support to the retinal cells through ECM adjuvants to promote lamination and (iii) it is extrudable through 3D printers. By optimizing this hydrogel-based bioink, we aim to print human retinal tissue over iPSC-derived retinal pigment epithelial layer to promote tissue interaction which is critical for maturation of photoreceptors.

Long-term implications of this solution include the ability to use the proposed technology to *study both retinal development as well as human disease modeling in a dish*. It also has *high-throughput screening application*s as it is adapted to be used with multi-well 3D bioprinters.

Background:

The outer retina, consisting of the light-sensing photoreceptors and the overlying retinal pigment epithelium (RPE), is critical for our light-sensing functions. Photoreceptor degenerations cause visual impairment in millions of patients in the United States and worldwide. Retinitis Pigmentosa (RP) is a prominent example of such a disorder, a progressive degenerative disease that encompasses a group of inherited retinal degenerations. It has a prevalence of 1 in 4000 and typical symptoms include night blindness followed by decreasing visual fields, leading to tunnel vision and eventually legal blindness or, in many cases, complete blindness. The functional disruption and atrophy of the RPE is a key factor in the progression of age-related macular degeneration, leading to the secondary death of light-sensitive photoreceptors, resulting in significant vision loss (1, 2). The only way to therefore potentially help these patients is to either replace the dead cells with new photoreceptors or to find ways to better understand the degenerative process in order to identify novel drugs to halt or delay the progression of degeneration.

Current methods to study diseases of the eye are limited to either 2D cell culture systems using cell lines or animal models. While each of these strategies is a powerful tool to study retinal development and pathology, each has critical limitations. Cell culture studies suffer from an inability to examine cell to cell interactions that occur in 3D space as well as lack of complete differentiation and maturation of the cells in the dish. Animal studies are costly and must always be understood to be an analogy to human physiology. Development of stem cell and reprogramming technologies to generate specific human cell lineages offer great promise for novel therapies. These allow for gaining a better understanding of the basic biology underlying the degenerative process in order to identify novel therapies which may delay, if not completely prevent the degeneration. However, current stem cell culture systems still suffer from similar issues i.e. lack of complete maturation *in vitro*. While dissociated cultures stem cell-derived retinal cells display rudimentary aspects of morphological differentiation, cells can only acquire highly

differentiated characteristics upon maintenance in their organized 3-dimensional (3D) state along with opposed mature RPE layer. The creation of valid *in vitro* disease models for a number of retinal degenerations indeed require an organized 3D state such as that existing in the living eye in order to mimic its highly differentiated characteristics. Recent efforts have employed the use of human pluripotent stem cells to generate 3D organoids (3-5). These have significant advantages for understanding human tissue. However, again there is a evidence of minimal maturation despite tissue organization due to absence of all the cellular layers required for retinal function and the inability to co-culture photoreceptors in apposition with the RPE layer.

Our previous efforts to create 3D retinas using soft lithography based columns showed promise in re-generating 3D mouse tissue with mature characteristics (6). This technique, however, failed to create the high aspect geometry required to allow for 3D assembly and maturation of human pluripotent stem cell-derived retinal cells. We are now focusing on 3D bioprinting as it is a promising method to arrange cell cultures in a 3D environment in a reproducible way without expensive tools. The key here is the development of a cell encapsulation media that allows the cells to mature and self-organize. For generating 3-D printed human retina, it must be extrudable so that it can be deposited in controlled, well-designed 3D layers over the RPE. It must be polymerizable using a method that is not toxic to cells or significantly alter their expression. In this proposal, we propose to generate such a bioink media for human retinal cells and combine it with stem cell technologies to generate *in vivo*-like retinal tissue. This technology will have a number of future applications including understand of human retinal development as well as modeling diseases in a dish by combining it with iPSC technology and genome engineering. Finally, since it is printable in a stereotypical manner, it is also amenable to medium-to-high throughput screening.

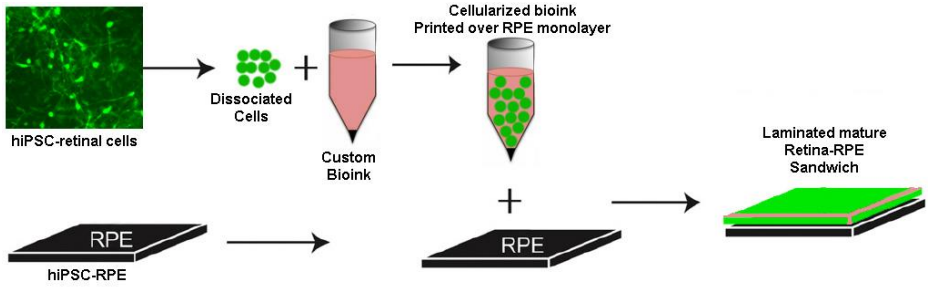

Figure 1: Cartoon showing conceptual steps to be used to generate custom bioink based 3D printed human retina.

Generation of retinal cells from hESCs and hiPSCs.

We and others have published work towards specific differentiation of pluripotent stem cells towards retinal fate(4, 7-10). In order to develop a strategy for the production of retinal cells from human ES cells, we have relied on our current understanding of retinal development. To direct the ESCs to a retinal fate, we treated undifferentiated human ESCs and human IPSCs with a BMP pathway inhibitor, Wnt/beta-catenin signaling pathway inhibitor and IGF-1 (7, 11) Upon culture over the next three weeks, we found that over 80% cells assumed retinal progenitor cell fate (7). Over the next 6-8 weeks, the cells differentiated to various retinal neuron subtypes including photoreceptors, ganglion cells, bipolars, and amacrine cells. For details of gene and protein expression data, please see Lamba et al., 2006, Lamba et al. 2010 and Zhu et al, 2017.

Scaffold-based Bioengineering 3D retina and challenges.

As photoreceptors mature *in vivo*, they extend inner and outer segments which arises as an out-pouching of the cell membrane and is filled up with discs of double folded membrane containing the visual pigment. This however fails to occur in photoreceptors grown in a dish. Lack of complete differentiation and maturation of photoreceptors in vitro, prompted us to look at ways to generate a 3-dimensional culture system. Recently, a number of labs including ours have shown formation of organized optic structures from human and mouse ES and iPS cells (11-14). However, showing outer segment formation has generally been elusive except in a couple of recent reports (5, 15). We tested whether biomaterial scaffolds based on spatial dimensions from

prior retinal progenitor cell tracing studies could help organize dissociated retinal cells by facilitating neural and glial process alignment and polarity. Employing soft lithography techniques, we patterned polymer scaffold with high aspect ratio micropillar features. We measured the effects on mouse retinal cell survival and morphology under various polymer and culture conditions (6). When cultured without scaffolds on RPE, photoreceptors lacked features of maturation, namely the formation of any outer segment disc material. In contrast, when the cells were grown in scaffolds, we found sparse outer segment disc material that had been assembled by photoreceptors near the RPE (Appendix). This suggests that the organization provided by the scaffolds along with a polarized supportive RPE can promote photoreceptor maturation. The application of this technology to generate 3D human retinas turned out to be difficult. The human retinal thickness varies from 100μm at the fovea to 250μm at the rim, averaging around 150μm (16). Developing micropillars to match these dimensions turned out to be a major engineering challenge. The extremely high aspect ratios needed to carry this out often resulted in column tilting, collapse or inadequate micro-well openings/pores leading to poor nutrient exchange in the biomaterial. This lead to poor survival, differentiation, lamination and maturation of human stem-cell derived retina.

Hydrogels for encapsulating retinal cells.

Hydrogel design for tissue engineering must simultaneously control several factors and be tuned to the specific tissue type. The main areas of focus in developing hydrogels of tissue engineering applications include (a) scaffolds must be biocompatible and (b) the gelation scheme must minimize disruption to the encapsulated cells. Further, since many components used in hydrogel design are bioactive, decisions which affect one characteristic, such as viscosity, will likely also affect cellular migration, maturation and potentially function. Recently there has been a lot of interest in using particular **light-activated gelatin methacryloyl (GelMA)**.

GelMA is a popular scaffold molecule because its characteristics are highly tunable by modifying the conditions of synthesis and processing. Altering the molecular structure of GelMA allows great variability on macroscopic factors key to scaffold design such as water swelling, mechanical stiffness, diffusion and degradation(17). Methacrylic anhydride (MAA) readily substitutes the primary amines of gelatin's lysine side groups. The degree of substitution (DS) is defined as the proportion of methacryloyl substituents to the number of terminal primary amines available on the unreacted gelatin molecule. The DS of GelMA affects the physical and chemical characteristics of the gel. The higher the DS, the higher the mechanical strength, but the lower the swelling ratio of the hydrogel. In preliminary work (see appendix), we used GelMA (5-20% DS from Biobots, Inc) to test its ability to support human iPSC-derived retinal cells. The encapsulated cells survived for over 8 weeks establishing the feasibility of GelMA as a backbone molecule for hiPSC-retinal organoid development.

Although there are various methods to deposit cell-laden material, pneumatically extruding filaments, or bioprinting, is one of the most popular. We chose to use filament deposition via pneumatic syringe because it allows us to print viscous gel precursors with high cell densities. We are currently optimizing multi-well printing using the dual-head Inkredible+ bioprinter (Cellink, Inc, Appendix). The primary drawbacks to pneumatic filament printing are low cell viability due to shear stress and we propose to optimize these parameters to allow for high cellular viability of iPSC-derived retinal cells.

Technologies and Protocols to Develop the model:

Stem cell culture. Neuro-retinal and RPE induction

We have extensive expertise in maintenance of pluripotent stem cells, as well as their neuro-retinal and RPE differentiation.

The **human induced pluripotent stem cells** will be maintained in Essential 8 basal medium

(Gibco) supplemented with 1% Essential 8 supplement (Gibco) and 1% Penicillin Streptomycin Amphotericin B (Lonza). Cells will be grown on Matrigel (BD Biosciences)-coated culture plates in a 37°C incubator with 5% CO_2 and 5% O_2.

Validation Steps: Immunocytochemistry: OCT4, NANOG, SOX2, SSEA4

Retinal differentiation was induced in hiPSCs in medium consisting of DME/F-12 1:1 with 10% Knockout Serum Replacement (KSR), 1% Penicillin Streptomycin Amphotericin B, 1% Sodium pyruvate, 1% Sodium bicarbonate, 1% HEPES Buffer, 1% MEM Nonessential Amino Acids and 1% N1 Media Supplement. Additionally, 2nM of IWR1 (Sigma Aldrich), 10µM of SB431542 (Stemgent), 100nM of LDN193189 (Stemgent) and 10ng/µl of human recombinant IGF1 (R&D Systems) is added to the medium for 7 days. Cells will be then dissociated and replated onto 6-well Matrigel-coated plates at a passaging ratio of 1:3 in Neural Stem Cell (NSC) medium comprised of DME/F-12 1:1 (HyClone), 0.5% Fetal Bovine Serum (FBS, Atlanta Biologicals), 1% Penicillin Streptomycin Amphotericin B (Lonza), 1% Sodium pyruvate (Corning), 1% Sodium Bicarbonate (Corning), 1% HEPES Buffer (Corning), 1% MEM Nonessential Amino Acids (Corning) and 1% of N1 Media Supplement (Sigma Aldrich). The neuro-retinal regions were manually separated from any RPE, dissociated and cultured in Matrigel-coated 6-well plates. Cells were maintained in NSC media here-after and serially passaged at 1:3 ratio upon confluency.

For 3D organoid cultures, all the above steps are carried out by using low-attachment culture plates and allowing natural 3D organization starting from cluster 100-150um in size.

For the 3D organogenesis in hydrogels, we will compare whether either of the two protocols are more efficient in generating 3D printed retinas. Finally, we will also assess the effect of differentiation/maturation of cells on 3D organogenesis in hydrogels.

Validation Steps: These will be carried out at monthly intervals at 1, 2, 3 and 4 months of culture.

ICC: Retinal Stem Cell Markers: PAX6, LHX2, CHX10 (VSX2), Ganglion Cell markers: BRN3, HuC/D, Amacrine cell markers: HuC/D, ISL1, PROX1, Bipolar cell markers: VSX2, CABP5, Muller glial markers: CRALBP and GS, Early Photoreceptor markers: OTX2, CRX, PRDM1, THRB, NRL, Mature Photoreceptor markers: Opsins (BCO, GCO, Rod), RCVRN, AIPL1. Q-RT-PCR: All of the above markers. TEM: Transmission electron microscopy to see ultrastructure of mature cells

RPE differentiation will induced in hiPSCs using the same media as above containing the small molecules for the first 7 days. Following this media will be changed to RPE medium that contains MEM/EBSS (HyClone) with 5% FBS, 1% Penicillin Streptomycin Amphotericin B, 1% Glutamax (Gibco), 0.25mg/ml Taurine (Sigma Aldrich), 10µg/ml Hydrocortisone (Sigma Aldrich) and 0.0065µg/ml Triiodo-Thyronine (Sigma Aldrich) and 1% N1 media supplement. The Cell will be serially passaged at 1:3 ratio for up to 7 passages and then allowed to mature in RPE medium containing 1% FBS.

Validation Steps: These will be carried out at monthly intervals at 1, 2, 3 and 4 month post-induction. ICC: OTX2, MITF, ZO1, BEST1, RPE65. Q-RT-PCR: All of the above along with PMEL17, TYR, TTR, SIL1. TEM: Ultra-structural changes to assess maturation including pigment granules, apical microvilli and localization of mitochondria. Functional: Trans-epithelial resistance measurements.

Bio-Ink Design and 3D retinal-Hydrogel organoid development:

Scaffold designs for tissue engineering aim to mimic the natural extracellular matrix (18). ECM varies throughout the body to meet different structural, mechanical and chemical demands, so each ECM-mimic must also match the unique demands for each application. In order to 3D print retinal cell-laden constructs, we must develop a suitable cell encapsulation material (Fig. 2). Candidate materials must be biocompatible, be able to extrude in filaments, and be sufficiently rigid to maintain 3D printed shape until gelation. Characteristics for ideal biomaterial to make 3D

retina are described in Table 1 (Appendix).

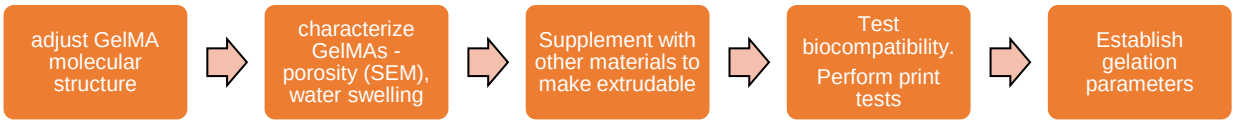

Figure 2: Schematic showing plan for 3D retina-compatible bioink development

GelMA will be used as our base bioink material. In our preliminary work, we have found it to be biocompatible with pluripotent stem cell derived retinal cells (see Appendix). We plan to vary its degree of substitution (DS) to generate a maneuverable bioink. DS which determines the maximum possible hydrogel crosslinking density which, in turn, affects mechanical strength, porosity, and diffusion rates, is affected by length of reaction, feed rate of MAA, and pH of reaction among other factors. We propose to control the DS primarily by limiting the feed rate of MAA. We will produce GelMA with 10%, 20% and 30% DS by reacting Type A gelatin (porcine skin) in 0.1M carbonate-bicarbonate buffer (pH 9) with MAA (25). The ratio of MAA to gelatin will be adjusted to experimentally determine the appropriate feed rates to achieve each DS. We will determine the DS via assays previously described (19) and confirm the results with H^1NMR Spectroscopy (20). Photoinitiators are required to polymerize GelMA hydrogels and several have been used previously including Irgacure, eosin Y, and lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP) (21). In our preliminary test, we have found that LAP (BioKey, Biobots Inc) is biocompatible with retinal cells and we will identify the lowest concentration of LAP (0.1%, 0.2% and 0.5% (w/v) LAP) that promotes polymerization at shortest UV-duration. We will next focus efforts to ensure that we find a range of suitable bioinks that print well. We plan to control viscosity of GelMA-based hydrogels by the addition of support molecules including hyaluronic acid and alginate in various ratios (22). We seek to balance the need for mechanical strength with the need for open architecture allowing good nutrient exchange and room for cells to grow and laminate. These modified inks will be evaluated using the Inkredible+ bioprinter (Cellink, Inc) owned by the lab.

Next, we will optimize the bioink to promote retinal tissue organization using a 3D bioprinter and print over a layer of RPE, the main support layer at the back of the eye critical for photoreceptor function. To do this, we will test biocompatibility, provide ECM support and define printing parameters. Using retinal cells derived from human iPSCs (monolayer and 3D culture protocol), 5-10x10^5 cells will be mixed with 100µl volume of different bioinks with varying ratios of Matrigel (5% - 50%) and plated and polymerized in 24-well plates. Cells will be fixed for analysis one, seven, 14, 30 and 60 days following polymerization. Finally, the cell-bioink mix will be printed over the mature RPE monolayer and polymerized. The 3D engineered retina will be analyzed 3 weeks later by ICC for mature photoreceptor markers and by transmission electron microscopy (TEM) for mature photoreceptor characteristics like disc structures. These will be compared against similar cellularized gels polymerized and cultured in the absence of RPE as well as non-polymerized gels with RPE. Functional analysis to detect light sensitivity will be done using a *ex vivo* ERG unit in the lab from Ocuscience, Inc. The retinal responses to light stimulation will be compared to that of freshly dissected mouse retinas in a dark room.

Validation Steps: These hydrogels will be characterized for porosity (SEM), water swelling, and relative mesh size (inferred from water swelling ratio). SEM studies will be conducted at the Stanford Cell Sciences Imaging Facility (see Appendix). Printability will be tested using extrusion tests, line tests, xy fidelity tests, z-stack tests. We will estimate the degree of polymerization by calculating sol fraction (PBS hydrated weight minus dry weight divided by hydrated weight). For bio-compatibility and cell survival analysis, cells will be stained for TUNEL, activated Caspase 9, γH2A (DNA damage) and total cell number by DAPI. 3D printed retina will be analyzed for retinal maturation and lamination using markers described above on a confocal microscope. TEM for optic disc structures and functional analysis using *ex vivo* ERG unit (HMsERG, Ocuscience, Inc).

High-throughput 3D Printing

The final aspect of this solution is to define high-throughput generation of 3D organoids by multi-well printing. The steps of this would involve multi-well plating of RPE monolayer which we will carry out using multi-channel pipets but could easily be adapted to robotic media dispensers. This will be followed 2-3 weeks later by printing cell-bioink mix on top of the RPE monolayer. We will employ the Inkredible bioprinter in the lab which is a dual head heated printer with HEPA filters and UV LED for polymerization. The LED light can be changed for other polymerizing agents if need be. Our preliminary work has found no significant DNA damage with the 30sec pulse required for hydrogel polymerization. We will define various 3D printer parameters to promote survival and maturation including nozzle diameter, print pressure, viscosity as well as UV duration.

Validation Steps: We will test survival of the cells one- and seven days following extrusion and polymerization as described above. We will determine 3D organization and retinal maturation using ICC and TEM analysis as above.

Innovation Statement:

1. 3D printing of retinal tissue: Many studies exist now which aim to engineer an *in vitro* tissue that closely mimics human *in vivo* tissue. However, the existing body of literature primarily focuses on neural, cartilage, bone, skin, and some muscle tissues (23, 24). There are studies on neural cell encapsulation in 3D scaffolds (25-27), but none focus on organized sensory-neural tissue. Here, we aim to generate organized, laminated tissues against its support layer.

2. Development of a neural retinal-compatible bioink: Previously, our lab and others have sought to construct 3D scaffolding on which to seed cells and other materials. However, there are many challenges inherent in this method. Cells have a long travel distance and generally demonstrate poor adhesion (28). Thus, it is difficult to create uniform cell distributions on the surface of pre-built scaffolding materials(22). Encapsulating the cells directly in the polymerizable bioinks provides a non-intensive and easily applicable solution to these problems. We will focus on generation of an encapsulating ink with ECM adjuvants that retains retina-like characteristics even post-polymerization to allow cells to migrate within the encapsulating media to self-assemble into laminated tissue (Table 1, Appendix, describes key characteristics).

3. Using human pluripotent stem cells to generate in vivo like retinal tissue: Human iPSC-derived cells can closely mimic an *in vivo* human tissue and thus likely provide an effective system to study development and disease. This will allow us to eventually model disease development (Figure 3).

4. Using commercially available dual head bioprinter. We will employ a commercial dual head bioprinter (Cellink Inkredible+) to pattern the neural retina on top of the RPE layer. This will allow for broader applicability and reproducibility as well as make the technology amenable to high throughput applications.

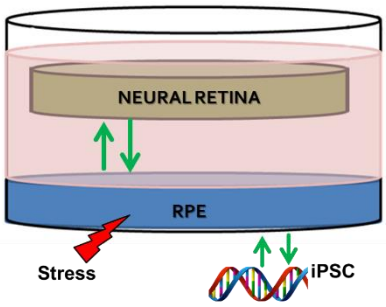

Figure 3: Cartoon showing potential studies that can be carried out using the proposed solution by using different stressors as well as patient iPSCs

Pitfalls:

We do not anticipate many technical issues with the proposed studies. However, it is possible that GelMA may end up not being an ideal base substrate. If so, several other biocompatible molecules including collagen, and hyaluronan also react readily with MAA and we will test them. Also, while we expect UV light required for photoinitiation to be safe for retinal neurons based on our preliminary testing, if it turns out to be an issue, we will test other photo-initiators which are sensitive to blue light. Finally, it is possible that a single ECM adjuvant is not sufficient to promote cellular lamination. In such a case, we will test whether different ECMs-bioink mixes layered one on top of the other allow for better lamination by using our dual head bioprinter