COVER PAGE

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- **Indicate if your submission is a solution most applicable to disease modeling or drug testing:** Our solution is applicable for both, disease modeling and drug testing. In this solution, we provide three independent interconnected and synergistic idea descriptions to address maximal coverage of evaluation points.

- **Indicate if you are applying to the trainee category:** NO

GLOSSARY:
3D-Retinal Organoid Culture (**3D-ROC**); Age-related Macular Degeneration (**AMD**); Artificial Micro-Tissue Approach (**AMTA**); Choroid (**CHR**); Early Stage Embryonic Architecture (**ESEA**); Primary Fibroblasts (**FBs**); Fn-Ln-peptide-alginate (**FLpAlg**); Human Embryonic Stem Cells (**hESCs**); Human Induced Pluripotent Stem Cells (**hiPSCs**); High Throughput Screening (**HTS**); Layer by Layer *M*ulti-*C*ellular Assembly Process (**LbL-MCAP**) for *P*3DTRA *M*odel (***MCPM***); Layer by Layer Multi Cellular Differentiation Process (**LbL-MCDP**); Multicellular spheroid (**MCS**); **Multicellular Spheroid** based Transwell Macro EYE (**MS^{TM}EYE**); Neural Layer (**NL**); Neural Retina (**NR**); **Organoid** based Transwell Macro EYE (**O^{TM}EYE**); Physiological 3D Retinal Spheroid Architecture (**P3DRSA**); physiological 3D-transwell retinal architecture (**P3DTRA**); Photoreceptors (**PRs**); Retinal 3D MCSs (**R3DMCS**); Retinal Cell(s) (**RC**(**s**)); Retinal Ganglion Cells (**RGCs**); Retinal Pigment Epithelium (**RPE**); Basic Primary Human Retinal Cell Types: (i) RPE; (ii) PR; (iii) bipolar; (iv) RGCs; (v) horizontal; (vi) amacrine cells; (vii) Müller glia.

ABSTRACT

In response to the **NEI-3D-ROC** Idea Competition Challenge, we provide description of three major idea **Strategies: I**] taking Organoid and Multicellular Spheroid Models to the next level by incorporation of a new media circulatory system, **II**] provide new alternative method to improve advanced 3D cell culture system addressing the gaps where organoids potentially fail, **III**] describe new retinal architecture layering for problems unsolvable with I and II. These advances are independent, complementary, interconnected, and feasible combinations of already published methods with customized new protocols. The introduced strategies are capable of generating physiological retinal architecture, modeling multiple diseases, and can be utilized as convenient high throughput drug screening platforms. Our innovation and intellectual property (*IP*) is relevant to all "3D culture/organ systems" not just "Eye". It is protected by provisional patent applications (pending). No confidential information provided at idea description level, detailed protocols will be shared/available for research when our solutions are selected for further implementation as a winner.

BACKGROUND

Stem cells, *e.g.*, human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs), will develop into an embryonic optic cup, *i.e.*, 3D-Retinal Organoid (3D-ROC), representing a surrogate model for the human eye with outer wall, retinal pigment epithelium (RPE), inner neuro-sensory layers (neural retina, NR), and showing early stage embryonic architecture (ESEA) (Nakano et al., 2012).

Nakano assumes the existence of NR "intrinsic developmental program" driving invagination to confer a final spherical 3-D ROC topology (Nakano et al., 2012). The absolute requirement of (mouse ESCs (mESCs)) RPE cells for NR invagination to occur was also demonstrated (Eiraku et al., 2011). Interestingly, (i) RPE marker Mitf was expressed at day 18 in human optic cup-excised NR, confirming RPE cells presence (Nakano et al., 2012); (ii) phosphorylated myosin light chain 2, which dominantly present in RPE, was not detected, indicating that hESCs did not recapitulate the morphogenesis as completely as mESCs (Nakano et al., 2012); (iii) another model placing the RPE cells at a center-stage and contradicting the NR-driven invagination was subsequently proposed (Sasai, 2013); while (iv) a computer-simulated model suggested tubular tissue formation (Okuda, Inoue, Eiraku, Adachi, & Sasai, 2015). In these studies, NR-specific culture conditions were used without outer segments (OSs) of photoreceptors (PRs) presence (Nakano et al., 2012). Importance of a well-formed OSs-PRs can explain the inefficient (~0.3%) PRs integration into mouse adult degenerate retina after transplantation. (Decembrini, Koch, Radtke, Moulin, & Arsenijevic, 2014; Gonzalez-Cordero et al., 2013). Sub-retinal grafting of hESC derived NR-structure sheets generated spherical 3D-ROC "rosette"-structures (Shirai et al., 2016), prompting that such grafted sheets are not capable of maintaining the correct topology required for vision. Additionally, clinical applications using hESC/hiPSC or organoid derived cell populations for transplantation has limited efficiency (Ghosh, Wong, Johansson, Bruun, & Petters, 2004). The first Astellas trials indicated that such grafting is safe (Schwartz et al., 2012) with increased visual acuity Phase I/II study (Schwartz et al., 2015). The Phase II PORTRAY (NCT02563782) study was suspended, due to cell line change (Yasukawa, 2016).

These results suggest that **1**) organoid models represent architectures reflecting early/embryonic developmental stages. **2**) Simultaneous differentiation control of Retinal cell type(s) is difficult **3**) The 3D-ROC models' application for clinical trials are premature. **4**) 3D-ROC(s) have limited disease modeling capacity. In response to overcome these critical limitations our team developed three basic Strategy to take the culture systems to the next level, and providing suitable alternative platforms generating physiologically correct retinal architectures for high throughput drug screening (HTS), disease modeling and molecular mechanism studies.

STRATEGY I: IMPROVING EXISTING ORGANOID CULTURES

To elevate the organoid technologies to the next level, we must provide correct micro-environmental spatio-temporal feedback mechanisms, incorporate circulatory systems to facilitate growth, and provide conditions for "tissue architecture" maturation. To achieve all this, the answer is **Strategy I**, which is an eye/retinal biology specific adaptation of NAGB LLC organogenesis project efforts (patent pending).

Macroscopic EYE Model Development (cell resources listed in Strategy II and III**):**
I] Organoid based Transwell Macro EYE (**O^{TM}EYE**). **II] Multicellular Spheroid** based Transwell Macro EYE (**MS^{TM}EYE**) from Strategy II. Both uses similar innovation principles. They are independent and complementary approaches addressing multiple and different aspects of eye biology. The **I]** is a primarily growth, maturation and native 3D-ROC developmental architecture platform, while **II]** is an advanced 3D cell culture approach for standardized drug HTS and disease mechanism analysis (Figure 1). **Both I], II]** use transwell system with ECM mask to ensure media/factor/nutrient gradient and circulation through the lower→upper chambers to advance physiologically correct EYE models and generate mature retinal architectures. The **I]** use developed organoids, the **II]** use R3DMCS, CHR::FLpAlg::RPE/PR/RGC.

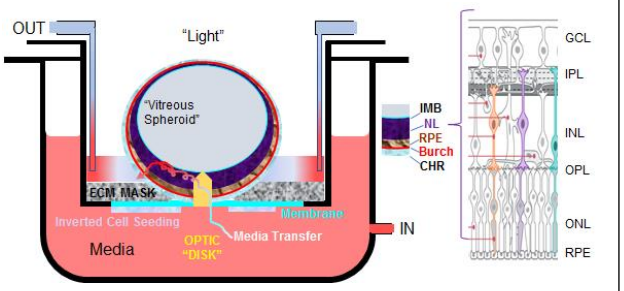

Figure 1. Macro EYE (*our IP*) design principles. Not to scale and for anatomical correctness. CHR/Choroid, IMB/inner mb.

Justification/Rationale: *To advance 3D-ROC at all*, proper ECM Young modulus with circulatory feedback *dynamics must be present*, providing correct spatiotemporal cellular mechano-signaling cues for tissue architecture development. *Any current organoid cultures are missing both.*
Significance/Innovation Multiple: (*our IP*): For example, **1**) the method/engineering of "through the EYE" circulation system, **2**) cell layering, protocols, ECM masks design, **3**) Providing organizational field for "optic disc" equivalent, **4**) optional extra neural field processing layer.

Description: I] Organoids are derived by NEI listed protocols, while **II]** Multicellular Spheroids (MCS) with inverted architecture (RPE outside) described below in Strategy II. In both cases, Choroid is the outer layer. **Purpose:** both approach serve as disease research/drug HTS platform. NOT for clinical purpose.
Major model creation steps as follow (*see relevant feasibility section for more details*):
1) Use modified transwell plates from 18–24 to 96 well depending on the experimental goal.
2) Create "central disc" mask (ECM (Figure 1) to facilitate media transfer. Use HA, Collagen, Gelatin, Cultrex and Matrigel with tunable hydrogels as Qgel, Phytagel to engineer a "central disc" mask.
2) Seed endocyte, pericyte & choroid cells. Position "EYE" on the "disc". Induce "vessel" formation.
3) Pulse media to enhance pressure fluctuation and delivery/transfer through the membrane.
4) Optimize according to Organoids/Spheroids "natural needs/responses" to pressure change dynamics.
5) Confirm that media enters into the structure (live trace staining) and passes through the model.
6) Follow/Confirm circulation effect on retinal architecture growth, differentiation, and maturation.
7) Validate architecture, cell viability, and functionality in disease modeling, and drug HTS.

Expected Results and Justification: 1) The media will penetrate the "EYE" model structures in a pressure dependent manner, allowing to establish nutrition, factor, and regulatory molecular gradients for continuous growth, differentiation, maturation. *In vivo*, cells above ~200-250 μm radius from vessels/nutrition sources are under stressed/hypoxic conditions with different biochemistry, mass-turnover, and signaling, ultimately limiting 3D growth. **2**) We expect "micro vessel equivalent accommodation" of media transport supported by cell structures through the models. **3**) This will promote retinal architecture proper maturation (all major cell types) and multiple disease modeling capabilities. **4**) Organoids have an "intrinsic developmental program" of which proper induction is supported by applied factor sequences. Circulatory system(s) drive dynamic changes and expansion of relevant architecture defining conditions, generate micro-environmental feedback for integrated execution of developmental programs. This solution fill-in a critical research need.

Limitations and Alternative approaches: Strategy I, is a high risk/reward approach in relativistic tissue model engineering. It is a transwell system based approach (not an organ on a chip). The key is a proper circulatory system integration with the model in question. Relevant to all 3D model types. No theoretical limitation known to exist, but might face significant difficulty of optimization issues depending on organ models. Specifically, circulatory system establishment is required to move beyond ESEA with additional cell types incorporation. In the case of failure, feasible alternatives are Strategy II, and III as discussed.

STRATEGY II: PHYSIOLOGICAL 3D-RETINAL SPHEROID ARCHITECTURE SCREENING PLATFORM (P3DRSA)

1. Physiological Retinal Cell Geometry Analysis

The assessment of current state of the art human retinal cell (**RC**) culture systems led to the following observations: **a**) hPSC-derived RPE cells faithfully recapitulated the hexagonal shape and pigmentation – morphological features intrinsic to RPE *in vivo* (Ohlemacher, Iglesias, Sridhar, Gamm, & Meyer, 2015); **b**) the *in vivo* morphology of human cone PRs is typically termed "hexagonal mosaic" (Ahnelt, Kolb, & Pflug, 1987); **c**) The morphology of Retinal Ganglion Cells (RGCs) of macaque monkey, with retina similar to humans, is of hexagonal shape (Rossi et al., 2017); **d**) Assembling the penta-, hexa-, and heptamers in a triangular pattern results in a spherical shell (Caspar & Klug, 1962; Hicks & Henley, 2006), virtually any size either regular or irregular. This finding is striking as it parallels the human cone PR hexagonal mosaic arranged in a triangular pattern (Ahnelt et al., 1987). Thus, the spherical 3D core must be provided to achieve the maximum physiological relevance in human eye modeling and any of the retinal pathologies.

2. Artificial Micro-Tissue Multicellular Spheroid (MCS) – A Core Model

Culturing the RCs in spherical configuration is possible using Artificial Micro-Tissue Approach (**AMTA**). The AMTA is based on monodispersed primary cells self-assembly into spherical 3D cellular aggregates, called multicellular spheroids (MCSs). The biological relevance of this approach is demonstrated by cardiac 3D MCSs https://www.youtube.com/watch?v=yA6ehkDNECk (Kelm et al., 2004). Assembly principles: *First*, a feeder spheroid, made of primary fibroblasts (FBs), is spontaneously formed. *Second*, another type of primary cells is added to this feeder spheroid. *Third*, the added cells form an additional cell layer around the feeder spheroid (Kelm & Fussenegger, 2004). Thus, the 3D FB feeder spheroid can provide the spherical core required by RCs. For example, primary human fibroblasts can be used for the proof of concept stage.

The application of spherical core principle for culturing RCs will yield following benefits: *First*, one of the current challenges in treating glaucoma patients is the inability to generate integration-competent RGCs (Zhao, Wang, & Temple, 2017). RGC and dorsal root ganglion (DRG) neurons share multiple similarities. Mixture of mouse embryonic FBs (MEFs) and DRG cells assembled into 3D MCSs with MEF feeder and peripheral layer of DRG-derived cells. The neurons of peripheral layer formed ganglion-like structure at one pole of the feeder spheroid and coordinated guided axonal outgrowth and innervation of another pole of 3D MEF feeder (Kelm, Ittner, Born, Djonov, & Fussenegger, 2006). These findings strongly suggest that integration-competent human RGCs can be produced utilizing 3D MCS approach. *Second*, human RPE cells form spheroids with organized outer cell monolayer that becomes gradually coated with a thin membrane containing the constituents of native Bruch's membrane. Moreover, collagen IV and lipo-proteins accumulated between RPE monolayer basement membrane and the reticular lamina of elastin (Sato et al., 2013). Thus, the MCS approach can be used for human RPE culturing and monitoring of toxic products accumulation in the space between RPE cells and Bruch's membrane, a molecular hallmark of dry age-related macular degeneration (AMD). *Third*, transplantation of encapsulated FBs promoted the survival of PRs in rat retinas (Uteza et al., 1999). Thus, all major disease-related RCs should be MCS-compatible.

3. Deciphering Viability, Growth, Production, and Integration Requirements of RCs

The capacity of cardiac 3D MCSs to self-organize into a higher order structures forming functional tissue interactions, *i.e.*, beating interconnected "mini-hearts" (Kelm et al., 2004), strongly suggests that this approach can be leveraged to seek similar phenomena using 3D MCSs derived from RC types. We suggest to generate the Retinal 3D MCSs (**R3DMCS**) composed of primary human FBs and following primary human RCs: (i) RPE; (ii) PR; (iii) bipolar; (iv) RGCs; (v) horizontal; (vi) amacrine cells; and (vii) Müller glia cells. The successful application of 3D MCS approach for primary human RPE cells and murine DRG cells strongly suggests that R3DMCSs production is feasible. Furthermore, by co-culturing various R3DMCSs, *e.g.*, RPE-MCS and PR-MCS, under different conditions it will be possible to define the requirements allowing the interaction of these types of MCSs. Such interactions between R3DMCSs will result in functional and architectural integration of RC layers.

To overcome oxygen and nutrients diffusion limits, permeable and aqueous core containing spherical capsules made of natural alginate polymer will be inserted inside the FB feeder. The alginate will be modified with RGD-peptide (Rowley, Madlambayan, & Mooney, 1999). Highly-efficient microfluidic

method allowing large-scale production of size-controlled capsules under mild physiologic conditions is available (Alessandri et al., 2013). The 3D FB feeder spheroid formation, enclosing RGD-modified alginate capsule, will trigger secretion of the ECM preparing it for RCs adhesion. FBs attach efficiently to RGD-modified alginate and promote endothelial cell adhesion (Guerreiro, Oliveira, Barbosa, Soares, & Granja, 2014), implying the feasibility of approach for R3DMCSs production. Tuning the composition of culture medium will lead to formation of viable and functional R3DMCSs. To distinguish R3DMCSs of different type, the RGD-alginate solution will be doped with traces of dextran-conjugated dyes (FITC, TRITC, *etc.*) conferring color to alginate capsules. The proximity of differently colored capsules will be used in a HTS format as read-out for the detection of interactions between various types of R3DMCSs.

4. Capsule-Based MCS Recapitulating the Physiological Laminated Retina

After validation of RCs' requirements for viability and growth, the integration-competent RCs will be used to assemble 3D retinal structures. Following is the simplified assembly sequence: (**i**) generation of RPE-MCS; (**ii**) addition of integration-competent PRs under conditions supporting RPE- and PR-MCS interaction; (**iii**) addition of integration-competent RGCs under conditions supporting PR- and RGC-MCS interaction. The MCS here refers to FB-coated RGD-alginate capsule, *i.e.*, FB::RGD-alginate. Here, three additional RC layers are assembled onto the MCS resulting in RGC/PR/RPE/FB::RGD-alginate R3DMCS.

Successful PR integration with RPE cell layer can be verified by detecting RPE surface sheath formation around the PR OSs using scanning electron microscopy (SEM) (Hollenberg & Lea, 1988). SEM was successfully used for the analysis of MCSs (Blache et al., 2013), and can be used for analysis of transparent RGC layer structure. To measure the PR membrane potential oscillation in response to various stimuli a novel approach is proposed here. The PNA:iGluSnFR fusion protein composed of peanut agglutinin (PNA) and glutamate-sensor protein (iGluSnFR) should be used. PNA covers the cone PRs in their entirety (Blanks & Johnson, 1984), whereas the iGluSnFR works exceptionally well in mouse retina (Marvin et al., 2013). Moreover, PNA does not bind to intact RPE layer allowing to assess its integrity.

The RGC/PR/RPE/FB::RGD-alginate R3DMCS contains the FB layer, which is not physiological. RPE cells attach to fibronectin (Fn) and laminin (Ln) with its basolateral surface. Thus, the alginate can be functionalized with Fn- and Ln-derived peptides (Boateng et al., 2005; Melkoumian et al., 2010) to promote the correctly-oriented RPE layer formation, eliminating the FB layer. This will generate the RGC/PR/RPE ::Fn-Ln-peptide-alginate(**FLpAlg**) R3DMCS consisting of human-only RCs forming physiological laminated retina structure. Such a design will enable deposition of other cells (*e.g.*, choroid) into the core of a capsule. To model wet AMD such RGC/PR/RPE::FLpAlg::choroid(**CHR**) R3DMCS can be transiently incubated in phosphate-buffered saline (PBS), which will dissolve the alginate capsule and remove the barrier between the retina and choroid, triggering pathological state. Antibodies can be deposited into the capsule's aqueous core mimicking human retina drug access from systemic circulation.

The RGC/PR/RPE::FLpAlg R3DMCS contains only relevant human RCs, however, its geometry is inverted. The RCs in laminated R3DMCS structure are oriented outwards, whereas in the human eye, there is an inward orientation of RCs with the RPE being the external layer of the eye structure making the contact with choroid. Thus, the interaction platform should be harnessed to seek the conditions for simultaneous formation of the RPE/PR/RGC structure. FLpAlg will be used to encapsulate dispersed human RPE, PR, and RGC using conditions allowing the formation of correctly laminated retinal layers. Such FLpAlg:: RPE/PR/RGC R3DMCS structure will represent advanced HTS physiological human retinal system. If the condition for simultaneous RPE/PR/RGC cells interaction will not be found, the NR layer generated in 3D-ROC can be used with human primary RPE cells.

5. Innovation Statement

The P3DRSA Strategy is the collection of platforms pursuing three goals: (i) to generate functional mature human RCs in 3D environment; (ii) to elucidate the 3D RC culture conditions allowing the integration, assembly of various (potentially all) mature human RC layers in a HTS format; (iii) to apply the knowledge gained in (i) and (ii) to create the HTS-ready human eye model enabling both disease modeling and drug testing. These goals are achievable due to interdisciplinary approach combining 3D MCS and microfluidic technologies. The R3DMCS, CHR::FLpAlg::RPE/PR/RGC layering support native retinal architecture and Strategy I.

STRATEGY III: PHYSIOLOGICAL 3D-TRANSWELL RETINAL ARCHITECTURE

The "Eye" and physiological 3D-transwell retinal architecture (**P3DTRA**) is a result of positional 3D spatiotemporal stratifying cell/cell and cell↔ECM signaling/communication by SHH/WNT/Notch/TGF-β superfamily driven cues (Kuznetsova, Grirorian, & Aleksandrova, 2011; Kuznetsova, Kurinov, & Aleksandrova, 2014; Mathura et al., 2000; Milyushina, Kuznetsova, Grigoryan, & Aleksandrova, 2011; Milyushina, Verdiev, Kuznetsova, & Aleksandrova, 2012; Wahlin, Campochiaro, Zack, & Adler, 2000). The hESC, hPSC, and hiPSC differentiation generates eyecup organoids (Maruotti et al., 2013; Masuda et al., 2014; Wahlin, Maruotti, & Zack, 2014; Zhong et al., 2014), with Embryonic Stage Equivalent Architectures (**ESEA**). Limitations: **1**) ESEA, incomplete cell type inventory, biased architecture, **2**) Partial signaling-missing/circulatory feedback, **3**) individually variable, low throughput systems, **4)** variability and limited disease modeling capability. To overcome all, **Strategy III** uses Layer by Layer *M*ulti-*C*ellular Assembly Process (**LbL-MCAP**) for *P*3DTRA *M*odel (***MCPM***) *our (IP)*.

Justification/Rationale: transwell membrane (▓) based LbL-MCAP delivers because it provides opportunity to achieve the proper material transport, cell polarity and density/ratio, sequential layering, thickness, functional connectivity, cell-matrix communications, and disease modeling capability.

Significance/Innovation: LbL-MCAP generated P3DTRA addresses all limitations described above simultaneously. Availability of multiple readouts, *e.g.*, cytokine production/signaling spectrum, gene expression profiles, metabolic (RA overdrive/drusen deposits), and cellular processes as autophagy phagocytosis, epithelial to mesenchymal transition (EMT) is possible. There is no other platform, of which would be more robust, flexible, stable, and suitable for high throughput screening and disease modeling.

A]: LbL-MCAP→*MCPM* (pericyte ▓ choroid/RPE→Photoreceptors→extra NL) platform uses 96 well transwell plates (Corning # 3374). A seeding protocol is illustrated on the Figure 2.

Justification/Rationale: The critical point is to get the RPE full polarization because it is fundamental for physiological Bruch's membrane creation (Sato et al., 2013). This will direct/synchronize all the remaining major cell types and layers (PRs, RGCs, amacrine cells and others) in creating the correct P3DTRA (Vecino, Rodriguez, Ruzafa, Pereiro, & Sharma, 2016).

Significance/Innovation: P3DTRA platform can model multiple eye disease mechanisms including AMD, Glaucoma, Diabetic and other Optic Retinopathies. *Our IP* covers architecture layering and matrix embedding methods, applied cellular sequences and number, P3DTRA conditions for functionalization and maturation.

Description and Expected Results: LbL-MCAP use multiple cell types to create P3DTRA (choroid, RPE, progenitor cells, PR, RGCs, bipolar amacrine) the http://www.innoprot.com/en_buscador_detalle.asp?IDsP=80,82,253,368,372&IDsS is a one stop resource. Sponsors cells http://www.xcellscience. com, http://www.neuromics.com/, https://www.reprocell.com/en/ ATCC #ARPE-19 (ATCC CRL-2302™), hTERT RPE-1 (ATCC CRL-4000™) and from ScienCell are also available: http://www.sciencellonline.com/products-services/primary-cells/human-retinal-astrocytes.html, https://www.sciencellonline.com/human-retinal-pigment-epithelial-cells.html. See also (Dutt et al., 1989, 1994; Dutt, Ezeonu, Scott, Semple, & Srinivasan, 1996). Primary photoreceptor cells http://restoresight.org/. **Critical points: Purpose:** LbL-MCAP generated P3DTRA **is not** a clinical cell production platform.

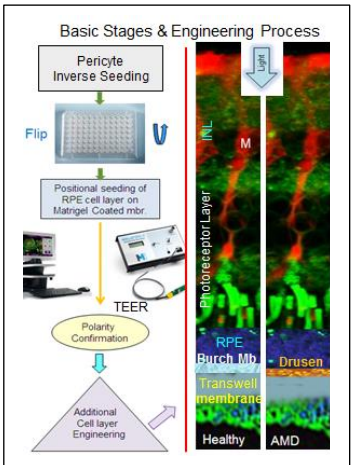

Figure 2. LbL-MCAP process.

- **Protocols:** Detailed protocols available as update when our solution considered for implementation and/or selected as winner of NEI-3DROC Challenge (*see Feasibility section for more details*).
- **Basic Experimental sections:**

1. ECM Coating (For example: Matrigel, biodegradable hydrogels etc.).
2. Cell seeding (Pericyte, choroid, RPE/NL) sequence/functionalization is important.
3. Confirmation of RPE Full Polarization (Conductivity/TEER, multiple timepoints to plateau).

4. Additional NR Layer engineering (*our IP*):
    - ECM embedding choices and 3D layering depends on the cell type in question
    - Only human primary and conditionally immortalized cells/cell lines used (*SBI Cumate*)
5. Additive/mixed layers engineering/ECM embedded vertically laminated architecture (*our IP*).
6. PTRA Confirmation (Confocal cell marker, RNAseq/gene expression, and lineage genotyping).
7. Disease modeling (RA metabolic overdrive, drusen deposits, autophagy EMT and others).

- **Expected Results:** We expect that: **1**) the LbL-MCAP generated PTRA will mimic *in vivo* retina physiological function(s). **2**) RPE full polarization will coincide with Bruch's membrane development, and correctly orients the photoreceptor layer. **3**) The vertical lamination process (*our IP*) will support the additional cell types proper integration and interaction with the photoreceptors. **4**) These aspects support adequate disease mechanism modeling and high throughput drug screening.

Limitations and Alternative approaches: The Strategy III A] section provide solutions addressing the limitations of organoid cultures. Its key limitation is that developmental and cell differentiation cues are more difficult to reproduce here than in the "self-guided autonomous" pure organoids. Therefore, this is a highly complementary approach with a key advantage of the better multiple disease modeling capability. Alternatives: Strategy I & II. Strategy III A] implementation is robust/cheap and suitable for drug screening.

B]: LbL-MCDP is an on-site Layer by Layer Multi Cellular Differentiation Process. As such it is a combination method of Transwell 3D culture approach using Organoid derived cell progenitors with cell lines from Strategy III A] incorporating "on site" cell differentiation and maturation. (Vecino et al., 2016). The LbL-MCDP approach use 96 well transwell platforms of basic cell layers (Choroid/RPE) with neural layer (NL) progenitor cells (organoids) to locally differentiate all retinal cell types "on the spot".

Justification/Rationale: 1) organoid development is incomplete because a) multiple factors and protocols can lead to same result and b) uniform factor concentration can bias local gradients leading to ESEA conservation. **2**) Transwell systems are robust, uniform, reliable high throughput platforms using any desired functional readouts with full parameter control and less variability than organoids. **3**) Transwell assay systems usually *do not incorporate cellular differentiation process.* Our solution is LbL-MCDP.

Significance/Innovation: The key improvement in Strategy III B] vs. I, II, and III A] is that use EMC embedded retina NL progenitor layers from organoids and seed it on the top of fully polarized CHR/RPE membrane, driving complete developmental sequence (Zhao et al., 2017). The innovative primary cells layering supports *in vivo* like physiological, retinal architecture development relevant to drug screening.

Description/Expected Results: The experimental strategy is similar as under III A]. Cell resources are transferable and commercially available as for example NIH approved hESC/H9 (WiCell).

Critical points: Purpose: LbL-MCDP **is** a developmental platform (intention# scaling up organoids).

- **Additional Protocols:** Detailed custom protocols available similarly and as under section III A]. The major difference of LbL-MCDP *vs*. Organoids is the cell population size, accessibility, and layering.
- **Basic Experimental steps:** (similar experimental approach, equipment and requirements as at III A])

1. ECM Coating (see Strategy I, II, III A] and relevant feasibility sections.)
2. Cell type/precursor ECM embedding and layering to generate basic architecture cell layers.
3. Neuronal layer (NL) engineering/proliferation from the bottom (additional differentiation factors).
4. Characterize migration/cellular interactions, integration, and phenotypes with confocal microscopy.
5. The decisions for additional factors used in LbL-MCDP must be determined experimentally.

- **Expected results:** We expect that the NL progenitor cell population "on the spot" differentiation will result in a more physiological architecture with all mature retinal cell types. We expect that the basic cell layers of Choroid and RPE will direct and organize the extra physiological retinal architecture following full polarization. We expect significant cell viability increase, stabilization, disease and drug HTS capacity.
- **Limitations and Alternative approaches:** The main difference between Strategies III A] & B] is that III A] rely mature cells/lines and a more complex layer engineering process, while the III B] rely on simplier layering with induction of on-site differentiation. This way, they are complementary, utilization depending on the disease model in question. Specific cell types easily omitted from the first, while complex interactions are better modeled with the second. Alternatives: Strategy I, II.

FEASIBILITY ASESSMENT

The NAGBL Team Solution potential implementation phase calls for expansion and extended collaborations, with NIH/NEI 3D-ROC challenge competitors, sponsors, and any designated research enterprise/laboratory NEI wishes to incorporate. *As such, our Team is available at any level NEI desires from consultancy to full implementation when this solution is selected for further development/as a winner. Our first collaboration choice is with Professor Kunal Mitra's group (Florida Institute of Technology).*

NAGBL Team has over 30 years of required assay design/tissue engineering expertise (including past academic background). In house basic cell, molecular biology wet lab capability (DNA, RNA, Protein analysis (PCR, Electrophoretic, Western etc.), Thermo Fisher cell culture incubator and hood, tissue-culture microscope centrifuges, fridges, freezers, computers, imaging and analysis software, access to cryostorage, Zeiss Axio II). Additionally, nearby large academic centers (University of Miami, Bascom Palmer Eye Institute, FIU, FAU) and their core facilities are available with advanced instrumentation, imaging, and additional expertise complementation above what an "expanded" Team could provide. Consequently, any level collaborative implementation efforts can be fully secured/executed, and guaranteed.

To take any current organoid/advanced spheroid 3D culture methods to the next level, we must create a circulatory system (among others) with the proper ECM embedding provide the correct spatio-temporal micro-environmental feedback dynamics for physiological retinal architecture development. **To achieve this goal** we propose three independent, complementary, and synergistic/integrative Strategies.

STRATEGY I

Eye anatomy suggests that engineering an "optic disk" equivalent region for media nutrition/regulatory factor intake is a feasible and functional way to ensure that all model regions (organoid/MCS based) could have the right conditions and micro-environmental feedbacks for complete execution of maturation, lineage differentiation program and architecture. Naturally, at this level, neural communication-optic nerve is likely far-fetched to be included (needs full NL correct retinal differentiation and communicating nerve fiber generation first). Our breakthrough is to create a proper substitution for *in vivo* micro-capillary system.

Major steps brief description of central "disk" engineering: Function: transitional material transport.

1) Create perforations at the center of membrane (~8 μm pore size and ~20 μm holes (>20 ea) at ~80 μm apart). Goal: create holes for capillary cross-membrane growth through this central "disk" area.
2) Create ECM mask from biodegradable hydrogel, with exception of the central disk area, which is coated with (collagen/fibronectin/Gelatin/Matrigel and extra VEGF) to support transmembrane penetration.
3) Seed membrane in inverted position with endothelial/pericyte cells (5:1 ratio), while seed "central disk" area in normal position with endothelial/pericyte/choroid (1:4:1 ratio). Confirm confluency on both side.
4) Enhance vessel formation/penetration with ScienCell #8708 3D tubule formation kit. Confirm initiation with markers: tight junction ZO-1, endothelial CD31, VWF, Pericyte NG2 staining + DAPI.
5) Add organoid/MCS, confirm central "disk" full attachment, induce endothelial cell proliferation (VEGF)
6) Introduce media pressure fluctuations to drive more effective capillary penetration.
7) Reduce/eliminate upper chamber nutrition to support media cross-membrane transport
8) Monitor capillary formation/"Eye" penetration (Figure 3 Spheroid vessel induction)
9) Analyze retina architecture/cell types (*confocal microscopy and single cell NGS*) growth, confirm media cross-transport. Compare Strategy I with standard organoids.
10) Confirm *in vivo* equivalent functionality in disease models as Glaucoma, AMD, and Degenerative Retinopathies. **Note**: circulation supports Glaucoma mechanism studies (increased pressure effect on retinal architecture/function and selective cell death).
11) Confirm physiological functionality in drug screening and high throughput assays.

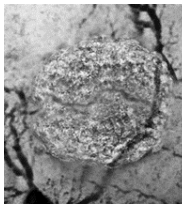
Figure 3

The major/feasible goal of these steps is to engineer a region of transwell membrane which support "vessel" formation facilitating physiological media, nutrition, and factor gradient dynamics through the model. There is no known experimental/technological limitation exist, which would prevent engineering of a transmembrane crossing "vessel" equivalent cellular structures. This will increase cellular viability and supports new advances in disease modeling and high throughput screening platform development.

STRATEGY II

The high-level design of overall validation approach for P3DRSA Strategy can be imagined in this way. First, peptide-functionalized (*e.g.*, Novatach M RGD, NovaMatrix) alginate (Pronova- SLM20, UP LVM, UP MVM; Manucol DH, NovaMatrix) capsules are produced *via* off-chip gelation microfluidic simple and straightforward procedure (Alessandri et al., 2013). Approximately 1,000 capsules are produced per second. Taking into account approximate prices for alginate and FITC/TRITC-dextran required for capsule shell visibility, as well as 45% yield of spherical capsules (roundness>0.8) the price of 1,000 microspheres will be ~ 1$ (10 microspheres/cent). Subsequently, capsules should be distributed to either 96-well or 384-well microplates and dispersed FB cells should be added in plating medium, *e.g.*, $DMEM_{67\%}/M199_{17\%}$ with essential components (Kelm et al., 2004). When the feeder FB spheroids are formed, dispersed human primary RPE cells are added to generate RPE-MCSs. Upon RPE/FB::RGD-alginate assembly formation, additional layers of retinal cells are formed utilizing the conditions elucidated in high-throughput retinal cells integration assay. If three layers of retinal cells are required, then utilizing this approach the RGC/PS/RPE::RGD-alginate assembly is formed. Subsequently, the medium should be changed to maintenance medium, *e.g.*, $DMEM_{78\%}/M199_{20\%}$ plus essential components, and human retinal 3D MCSs should be transferred to glass plates with funnels (made of 1% phytagel) for observation, and functional validation assays should be performed. Upon completion of the validation stage and analysis of the novel data, it will be possible to make the decision on whether to proceed with P3DRSA strategy.

The high-level methodology described above is versatile and flexible, *i.e.*, it can be used with: (i) different types of functionalized alginate, *e.g.*, alginate functionalized with Fn- and Ln-peptides (FLpAlg); (ii) different types of dyes; (iii) various combinations of retinal cells, *i.e.*, enabling modeling of various retinal diseases; (iv) FB layer or without it; (v) various encapsulation strategies, *e.g.*, retinal cell layers' outwards direction (capsule inside) *vs.* inwards direction (retinal cells inside capsule forming RPE-supported NR); (vi) capsule's aqueous core can be impregnated with high-molecular weight drug candidates or choroid cells or any other cell type; (vii) the alginate capsule can be disassembled at the experimenter's will by simply incubating the 3D retinal MCS structure in PBS.

At least two high-throughput biological approaches can be used to identify novel potential targets for retinal disease treatment: (i) siRNA or shRNA gene silencing; (ii) therapeutic or phenotype-modulating transgenes expression. To successfully use both approaches NEI needs the ultra-efficient gene transfer technology platform, which would enable ~100% efficiency of transgene delivery into retinal cells. Due to the fact that spheroids and MCSs are hard to transfect micro-tissues the only option for efficient delivery are viral vectors. A genetically engineered adeno-associated virus of type 2 (AAV2) that, starting from the vitreous humor, traversed several hundred micrometers of dense tissue filled with retinal ganglion cell layer, inner nuclear layer, photoreceptors layer and achieved the RPE cells layer was developed. On its path this AAV2 variant, named 7m8, transduced efficiently all four cell layers achieving pan-retinal powerful transgene expression. In particular, strong gene expression was achieved in the deepest RPE cell layer (Dalkara et al., 2013). If the transduction of only the outermost 3D Retinal MCS is required, then as an alternative a powerful lentiviral system can be used. It has been demonstrated that up to ~90% of MCS outer layer cells can be transduced with VSV-G-pseudotyped lentiviral vectors (Kelm et al., 2004).

To discover novel potential drug targets, however, it is required to clone short hairpin RNAs (shRNAs) targeting human genome into this pseudotyped lentivirus transduction system to obtain a lentiviral library, which can be used in high-throughput screening system. Alternatively, existing lentiviral libraries can be used. For example, a lentiviral shRNA library consisting of ~100,000 vectors targeting ~22,000 human and mouse genes has been published and successfully used in high-content screens (Moffat et al., 2006).

The high-level design of biologic target identification can be imagined in this way. First, either the pan-retinal or the outer layers of human mature retinal 3D MCSs are transduced with either 7m8- or lentivirus-based shRNA library. Once the drug target transcript resulting in required phenotypic response has been identified, the libraries of low molecular weight leads can be utilized to obtain the proof of concept for a potential drug or potential drugs panel. Utilizing this strategy will enable to not only model any retinal disease, but also to molecularly characterize the disease phenotype *via* direct testing of potential life-saving drugs.

STRATEGY III

Transwell system applications and assays are fundamental pharma industry workhorses, with basically any desired/available outcome parameter measurements (such as interaction, activation, enzyme process, gene expression, cellular processes (*e.g.*,migration/invasion, autophagy, immune reactions, live-dead analysis, and wide array of signaling processes (including GPCR)). Consequentially, transwell system applications are guarantee for the desired feasibility/outcomes. **Brief Example protocols**:

- **Coating:**
- 96-well HTS Transwell Permeable support with 8.0 μm pore sizes (Corning # 3374);
- 5x BME Extract/coating solution (Trevigen # 3455-096-02);
- 10x Coating Buffer (Trevigen # 3455-096-03);
- Media: for example, Dulbecco's modified Eagle's medium–nutrient mixture F-12, (Ham) 1:1 (Gibco, Bethesda, MD) supplemented with 10% fetal bovine serum (Hyclone Laboratories, Logan, UT), 10% serum plus supplement (JRH Biosciences, Lenexa, KS), 2mM L-glutamine, 0.075% sodium bicarbonate, penicillin 100 units=mL, and streptomycin 100 mg=mL (Gibco).
- **Seeding:**
- Harvest pericyte with dissociation solution (as for example HyQtase, Hyclone # JQH2447);
- Count, set desired concentration (~ 0.2-0.5x10^5 in 50-75 μl media/well) and seed the Coated Transwell plate (bottom side up, incubate 24 hours. Critical to prevent it from drying out);
- Flip, use Choroid Endothelial cells to seed the inside surface of the membrane in the wells Note: for the best results, optimize cell seeding concentrations of each type individually before the co-culture experiment for fast and complete full polarization;
- Incubate Choroid Endothelial cells with growth media for ~36 hours then coat ~50 μl media/well containing 1x BME/Matrigel/Cultrex at room temperature for 6-12 hours or at 8^{o}C (to enhance induction and formation of Bruch's membrane equivalent layer between Choroid/RPE cells.
- **RPE Layer Engineering Basics:**
- Harvest RPE similarly as pericyte/choroid endothelial cells, count, set seeding concentration similar that was used for choroid endothelial cells. Aspirate media carefully.
- Suspend RPE cell population in ~50 - 75 μl media/0.5x BME/media (growth hormone depleted Matrigel/Cultrex ~50 μl) and layer it over the choroid endothelial cells. Note: The goal is to get a solid confluent fully polarized "cobblestone" layer. Confirm with microscopy/TEER data.
- Refine as needed for optimal results. Engineer additional NL Layers.

EXPERIMENTAL VALIDATION

Strategies/milestones (below) can be treated as separate projects and developed/implemented as such. They are interconnected, mergeable, and synergistic in a way that cells, methods and associated protocols are interchangeable with modifications and fine tuning. Feasibility depends on successful realization of strategy specific milestones following selective experimental logistics. In all three strategies, the physiological architecture confirmation through confocal/cell type specific staining (see refs), TEER measurements, PRs bioactivity measurements and Bruch's membrane, NGS profiling is fundamental. Key points:

-**Strategy I**, committing step is "vessel" induction and transmembrane material transport;
-**Strategy II** committing step is layer assembly and mature Retinal cell types confirmation;
-**Strategy III** committing step is layer assembly and mature Retinal cell types confirmation.

EXECUTION TIMEFRAME and MILEASTONES (MST)

	3 months	3months	3 months	3months	3months	3months	3months
Strategy I	←	—	—	—	→		
Strategy II			←	—	—	—	→
Strategy III				←	—	—	→
MST/St I	1) ECM mask + organoid/MSC setup 2) "Vascularization" 3) Architecture confirmation 4) Disease modeling						
MST/St II	1) Confirmation of MCS compatibility with mature Retinal cells. 2) Retinal 3D MCS integration (assembly) 3) Capsule::RPE/PR/RGC architecture confirmation 4) Disease modeling and HTS drug testing						
MST/St III	1) Basic layering setup 2) RPE/NL architecture confirmation 3) Disease modeling 4) HTS drug testing						

BIOGRAPHYCAL SKETCHES NIH/NEI-3D-ROC NAGBL TEAM

NAME: *Nandor Garamszegi Ph.D.* eRA Commons ID: inactive

POSITION TITLE: Chief Scientific Officer, Research Director *NAGBL Team Captain*

EDUCATION/TRAINING/POSITIONS

INSTITUTION AND LOCATION	Degree	Date	FIELD OF STUDY
Kossuth Institute Miskolc Hungary	BS	1975	Chemistry, Biology
University of Szeged, Hungary	MS	1982	Molecular biology
University of Szeged Hungary	Ph.D.	1986	Genetics, Biochemistry
University of Szeged Hungary (Lecturer/Instructor)		1986-1991	Department of Genetics
University of Minnesota (Postdoctoral)		1991-1993	Lipid Biochemistry
Mayo Clinic Rochester MN (Senior Res. Assoc.)		1993-2004	Cancer Cell Biology
University of Miami (Instructor and Assistant Prof)		2004-2010	Cancer & ECM Signaling
Natural to Artificial Genomes and Biosystems, Founder, Chief Scientific Officer		2010-present	Disruptive Technology Development

A. Personal Statement

"To solve any problem, I am just warming up when/where others quit". What is most exhilarating for me about the scientific research is the disruptive potential and consequential progress by leaps/bounds. This inspire me to bring in out of field/box thinking, new focus, create and implement patentable solutions for clients and different challenges. The fast-evolving dynamic work assignments and environment of a small business combined with high risk/reward projects is an optimal foundation for Disruptive technology.

B. Relevant Experiences and Expertise

Won Age related Macular Degeneration cell culture model development challenge (InnoCentive 9933641). Expertise: State of art Cell, Molecular-, and Cancer Biology, Signal Transduction, Cell-/Cell-Extracellular Matrix communication--focus; crosstalk signaling, and Tissue Engineering. Proficient in advanced DNA, RNA, Protein technologies related to purification, analysis, and engineering. Experience with molecular modeling, NGS technologies and Bioinformatics network analysis/Cytoscape. Disruptive solution development and implementation.

C. Contributions to Science

A] Academic (past)**:** Discovery and analysis of TGFβ Type I receptor C-terminal Signaling regulator and Endocytosis/Farnesyl transferase α interaction SQ box. (Garamszegi et al., 2001) Discovery and analysis of TGFβ Type I & II dimer crosstalk signaling induced by type II collagen (Garamszegi et al., 2010).
B] Business (2011-)**:** Developed and implemented disruptive solutions for companies as **MERCK** KGaA (Oligodendrocyte cell line vector), **Syngenta** (Chromosome transformation method), **DemeRx** Inc. (Noribogain/κ Opioid Receptor interaction characterization), **University of Miami** (Striatal MSN differentiation from hESC), and others.

D. Academic Awards/Completed Disruptive Tech. Development/Innovation Projects.

Publications: https://www.ncbi.nlm.nih.gov/pubmed/?term=garamszegi%20n

2017 –	NAGB LLC is a NASA iTECH Cycle 2 semifinalist https://nasaitech.com/journal
2008 – 2010	**WCA** #66461Y Tissue Metastasis in Osteosarcoma. / *Principal Investigator*
2004 – 2009	**NIH-NCI** R01 #CA-66088Y Chondrosarcoma Metastasis / *Co-Investigator*
2004 – 2005	**ACR** # IRG-98-277-04 Extracellular Matrix Reorganization / *Principal Investigator*
2011&2015	InnoCentive: Global Top Solver https://www.innocentive.com/ar/challenge/topSolvers Disruptive technology development project awards (11) from for example InnoCentive 371122, 845617, 9557945, 9864299, 9932934, 9933009, 9933387, 9933525, 9933641, 9933890, 9933892.
2008	WCA: Madelon Ravlin Memorial Award

NAME: *Andrei Nikonov Ph.D.*

POSITION TITLE: Innovation Consultant *NAGBL Team Member*

EDUCATION/TRAINING/POSITIONS

INSTITUTION AND LOCATION	Degree	Date	FIELD OF STUDY
University of Tartu, Estonia	BS,*s.c.l*	2002	Gene Technology
University of Tartu, Estonia	MS	2004	Gene Technology
University of Tartu, Estonia	Ph.D.	2014	Molecular Virology
University of Tartu, Estonia (Research Associate)		2014-2016	Translational Medicine
Freelance Innovation Consultant		2016-present	Innovation-Driven Research & Development

A. Personal Statement

I successfully solved research and development challenges faced by organizations like **Boehringer Ingelheim**, **Elanco**, and **AstraZeneca** (*e.g.*, novel human disease models suitable for high throughput compound screening, novel gene therapy applications, novel vaccine development, antibiotic alternative treatment options, *etc.*). I am interested in and actively pursue the innovation-driven research and development and translational medicine.

B. Relevant Experiences and Expertise

I am the only winner of the "**Novel Models of Airway Smooth Muscle (ASM) Remodeling in Severe Asthma**" InnoCentive, Inc. challenge 9933777 (Boehringer Ingelheim). Expertise: molecular virology; molecular biology; cell biology; biotechnology; drug delivery; antibody engineering; phage display; application of microfluidic technology for high throughput human disease modeling using FDA- and EMA-approved materials; functional human tissue engineering using multicellular spheroid approach; translational medicine; open innovation; problem solving.

C. Contributions to Science

Discovery of an ancient mechanism for the recognition of viral RNA-dependent RNA polymerase activity by vertebrate host cells: Nikonov, A.*, Mölder, T., Sikut, R., Kiiver, K., Männik, A., Toots, U., Lulla, A., Lulla, V., Utt, A., Merits, A., and Ustav, M. RIG-I and MDA-5 detection of viral RNA-dependent RNA polymerase activity restricts positive-strand RNA virus replication. PLoS Pathogens 2013; 9(9):e1003610 (Nikonov et al., 2013). *Primary corresponding author.

D. Innovation-Driven Research and Development Awards

2017: Measuring the Lipid Composition of a Viral Envelope (InnoCentive, Inc.) https://innocentive.com/ar/challenge/9933914 (to be announced shortly);
2017: Coccidiosis Prevention and Control in Poultry (Elanco & InnoCentive, Inc.) https://innocentive.com/ar/challenge/9933890;
2017: Prevention and Control of Enteropathy/Ileitis in Swine (Elanco & InnoCentive, Inc.) https://innocentive.com/ar/challenge/9933892;
2017: Delivery of Biomolecules to the Gastrointestinal Tract (InnoCentive, Inc.) https://www.innocentive.com/ar/challenge/9933830;
2016: **Top Solver 2016 (InnoCentive, Inc.)** https://www.innocentive.com/ar/challenge/topSolvers;
2016: New Solutions for Targeted Gene Therapy (Boehringer Ingelheim & InnoCentive, Inc.) https://www.innocentive.com/ar/challenge/9933852;
2016: **Novel Models of Airway Smooth Muscle (ASM) Remodeling in Severe Asthma** (Boehringer Ingelheim & InnoCentive, Inc.) https://www.innocentive.com/ar/challenge/9933777;
2014: Selecting for Internalizing Antibodies or Other Specific Cell Surface Target Binders (InnoCentive, Inc.) https://www.innocentive.com/ar/challenge/9932830;

REFERENCES

Ahnelt, P. K., Kolb, H., & Pflug, R. (1987). Identification of a subtype of cone photoreceptor, likely to be blue sensitive, in the human retina. *The Journal of Comparative Neurology*, *255*(1), 18–34. https://doi.org/10.1002/cne.902550103

Alessandri, K., Sarangi, B. R., Gurchenkov, V. V., Sinha, B., Kiessling, T. R., Fetler, L., … Nassoy, P. (2013). Cellular capsules as a tool for multicellular spheroid production and for investigating the mechanics of tumor progression in vitro. *Proceedings of the National Academy of Sciences of the United States of America*, *110*(37), 14843–14848. https://doi.org/10.1073/pnas.1309482110

Blache, U., Silvan, U., Plodinec, M., Suetterlin, R., Jakob, R., Klebba, I., … Schoenenberger, C.-A. (2013). A tumorigenic actin mutant alters fibroblast morphology and multicellular assembly properties. *Cytoskeleton (Hoboken, N.J.)*, *70*(10), 635–650. https://doi.org/10.1002/cm.21120

Blanks, J. C., & Johnson, L. V. (1984). Specific binding of peanut lectin to a class of retinal photoreceptor cells. A species comparison. *Investigative Ophthalmology & Visual Science*, *25*(5), 546–557.

Boateng, S. Y., Lateef, S. S., Mosley, W., Hartman, T. J., Hanley, L., & Russell, B. (2005). RGD and YIGSR synthetic peptides facilitate cellular adhesion identical to that of laminin and fibronectin but alter the physiology of neonatal cardiac myocytes. *American Journal of Physiology. Cell Physiology*, *288*(1), C30-38. https://doi.org/10.1152/ajpcell.00199.2004

Caspar, D. L., & Klug, A. (1962). Physical principles in the construction of regular viruses. *Cold Spring Harbor Symposia on Quantitative Biology*, *27*, 1–24.

Dalkara, D., Byrne, L. C., Klimczak, R. R., Visel, M., Yin, L., Merigan, W. H., … Schaffer, D. V. (2013). In vivo-directed evolution of a new adeno-associated virus for therapeutic outer retinal gene delivery from the vitreous. *Science Translational Medicine*, *5*(189), 189ra76. https://doi.org/10.1126/scitranslmed.3005708

Decembrini, S., Koch, U., Radtke, F., Moulin, A., & Arsenijevic, Y. (2014). Derivation of traceable and transplantable photoreceptors from mouse embryonic stem cells. *Stem Cell Reports*, *2*(6), 853–865. https://doi.org/10.1016/j.stemcr.2014.04.010

Dutt, K., Ezeonu, I., Scott, M., Semple, E., & Srinivasan, A. (1996). Proto-oncogene expression in cAMP and TPA-mediated neuronal differentiation in a human retinal cell line KGLDMSM. *Current Eye Research*, *15*(5), 477–485.

Dutt, K., Scott, M., Wang, M., Semple, E., Sharma, G. P., & Srinivasan, A. (1994). Establishment of a human retinal cell line by transfection of SV40 T antigen gene with potential to undergo neuronal differentiation. *DNA and Cell Biology*, *13*(9), 909–921. https://doi.org/10.1089/dna.1994.13.909

Dutt, K., Waldrep, J. C., Kaplan, H. J., Del Monte, M., Semple, E., & Verly, G. (1989). In vitro phenotypic and functional characterization of human pigment epithelial cell lines. *Current Eye Research*, *8*(5), 435–440.

Eiraku, M., Takata, N., Ishibashi, H., Kawada, M., Sakakura, E., Okuda, S., … Sasai, Y. (2011). Self-organizing optic-cup morphogenesis in three-dimensional culture. *Nature*, *472*(7341), 51–56. https://doi.org/10.1038/nature09941

Garamszegi, N., Dore, J. J. J., Penheiter, S. G., Edens, M., Yao, D., & Leof, E. B. (2001). Transforming growth factor beta receptor signaling and endocytosis are linked through a COOH terminal activation motif in the type I receptor. *Molecular Biology of the Cell*, *12*(9), 2881–2893.

Garamszegi, N., Garamszegi, S. P., Samavarchi-Tehrani, P., Walford, E., Schneiderbauer, M. M., Wrana, J. L., & Scully, S. P. (2010). Extracellular matrix-induced transforming growth factor-beta receptor signaling dynamics. *Oncogene*, *29*(16), 2368–2380. https://doi.org/10.1038/onc.2009.514

Ghosh, F., Wong, F., Johansson, K., Bruun, A., & Petters, R. M. (2004). Transplantation of full-thickness retina in the rhodopsin transgenic pig. *Retina (Philadelphia, Pa.)*, *24*(1), 98–109.

Gonzalez-Cordero, A., West, E. L., Pearson, R. A., Duran, Y., Carvalho, L. S., Chu, C. J., … Ali, R. R. (2013). Photoreceptor precursors derived from three-dimensional embryonic stem cell cultures

integrate and mature within adult degenerate retina. *Nature Biotechnology*, *31*(8), 741–747. https://doi.org/10.1038/nbt.2643

Guerreiro, S. G., Oliveira, M. J., Barbosa, M. A., Soares, R., & Granja, P. L. (2014). Neonatal human dermal fibroblasts immobilized in RGD-alginate induce angiogenesis. *Cell Transplantation*, *23*(8), 945–957. https://doi.org/10.3727/096368913X670183

Hicks, S. D., & Henley, C. L. (2006). Irreversible growth model for virus capsid assembly. *Physical Review. E, Statistical, Nonlinear, and Soft Matter Physics*, *74*(3 Pt 1), 031912. https://doi.org/10.1103/PhysRevE.74.031912

Hollenberg, M. J., & Lea, P. J. (1988). High resolution scanning electron microscopy of the retinal pigment epithelium and Bruch's layer. *Investigative Ophthalmology & Visual Science*, *29*(9), 1380–1389.

Kelm, J. M., Ehler, E., Nielsen, L. K., Schlatter, S., Perriard, J.-C., & Fussenegger, M. (2004). Design of artificial myocardial microtissues. *Tissue Engineering*, *10*(1–2), 201–214. https://doi.org/10.1089/107632704322791853

Kelm, J. M., & Fussenegger, M. (2004). Microscale tissue engineering using gravity-enforced cell assembly. *Trends in Biotechnology*, *22*(4), 195–202. https://doi.org/10.1016/j.tibtech.2004.02.002

Kelm, J. M., Ittner, L. M., Born, W., Djonov, V., & Fussenegger, M. (2006). Self-assembly of sensory neurons into ganglia-like microtissues. *Journal of Biotechnology*, *121*(1), 86–101. https://doi.org/10.1016/j.jbiotec.2005.07.009

Kuznetsova, A. V., Grirorian, E. N., & Aleksandrova, M. A. (2011). [Adult human retinal pigment epithelial cells - a potential source of cells for regeneration retina]. *Tsitologiia*, *53*(6), 505–512.

Kuznetsova, A. V., Kurinov, A. M., & Aleksandrova, M. A. (2014). Cell models to study regulation of cell transformation in pathologies of retinal pigment epithelium. *Journal of Ophthalmology*, *2014*, 801787. https://doi.org/10.1155/2014/801787

Maruotti, J., Wahlin, K., Gorrell, D., Bhutto, I., Lutty, G., & Zack, D. J. (2013). A simple and scalable process for the differentiation of retinal pigment epithelium from human pluripotent stem cells. *Stem Cells Translational Medicine*, *2*(5), 341–354. https://doi.org/10.5966/sctm.2012-0106

Marvin, J. S., Borghuis, B. G., Tian, L., Cichon, J., Harnett, M. T., Akerboom, J., … Looger, L. L. (2013). An optimized fluorescent probe for visualizing glutamate neurotransmission. *Nature Methods*, *10*(2), 162–170. https://doi.org/10.1038/nmeth.2333

Masuda, T., Wahlin, K., Wan, J., Hu, J., Maruotti, J., Yang, X., … Esumi, N. (2014). Transcription factor SOX9 plays a key role in the regulation of visual cycle gene expression in the retinal pigment epithelium. *The Journal of Biological Chemistry*, *289*(18), 12908–12921. https://doi.org/10.1074/jbc.M114.556738

Mathura, J. R. J., Jafari, N., Chang, J. T., Hackett, S. F., Wahlin, K. J., Della, N. G., … Campochiaro, P. A. (2000). Bone morphogenetic proteins-2 and -4: negative growth regulators in adult retinal pigmented epithelium. *Investigative Ophthalmology & Visual Science*, *41*(2), 592–600.

Melkoumian, Z., Weber, J. L., Weber, D. M., Fadeev, A. G., Zhou, Y., Dolley-Sonneville, P., … Brandenberger, R. (2010). Synthetic peptide-acrylate surfaces for long-term self-renewal and cardiomyocyte differentiation of human embryonic stem cells. *Nature Biotechnology*, *28*(6), 606–610. https://doi.org/10.1038/nbt.1629

Milyushina, L. A., Kuznetsova, A. V., Grigoryan, E. N., & Aleksandrova, M. A. (2011). Phenotypic plasticity of retinal pigment epithelial cells from adult human eye in vitro. *Bulletin of Experimental Biology and Medicine*, *151*(4), 506–511.

Milyushina, L. A., Verdiev, B. I., Kuznetsova, A. V., & Aleksandrova, M. A. (2012). Expression of multipotent and retinal markers in pigment epithelium of adult human in vitro. *Bulletin of Experimental Biology and Medicine*, *153*(1), 157–162.

Moffat, J., Grueneberg, D. A., Yang, X., Kim, S. Y., Kloepfer, A. M., Hinkle, G., … Root, D. E. (2006). A lentiviral RNAi library for human and mouse genes applied to an arrayed viral high-content screen. *Cell*, *124*(6), 1283–1298. https://doi.org/10.1016/j.cell.2006.01.040

Nakano, T., Ando, S., Takata, N., Kawada, M., Muguruma, K., Sekiguchi, K., … Sasai, Y. (2012). Self-formation of optic cups and storable stratified neural retina from human ESCs. *Cell Stem Cell*, *10*(6), 771–785. https://doi.org/10.1016/j.stem.2012.05.009

Nikonov, A., Molder, T., Sikut, R., Kiiver, K., Mannik, A., Toots, U., … Ustav, M. (2013). RIG-I and MDA-5 detection of viral RNA-dependent RNA polymerase activity restricts positive-strand RNA virus replication. *PLoS Pathogens*, *9*(9), e1003610. https://doi.org/10.1371/journal.ppat.1003610

Ohlemacher, S. K., Iglesias, C. L., Sridhar, A., Gamm, D. M., & Meyer, J. S. (2015). Generation of highly enriched populations of optic vesicle-like retinal cells from human pluripotent stem cells. *Current Protocols in Stem Cell Biology*, *32*, 1H.8.1-20. https://doi.org/10.1002/9780470151808.sc01h08s32

Okuda, S., Inoue, Y., Eiraku, M., Adachi, T., & Sasai, Y. (2015). Vertex dynamics simulations of viscosity-dependent deformation during tissue morphogenesis. *Biomechanics and Modeling in Mechanobiology*, *14*(2), 413–425. https://doi.org/10.1007/s10237-014-0613-5

Rossi, E. A., Granger, C. E., Sharma, R., Yang, Q., Saito, K., Schwarz, C., … Williams, D. R. (2017). Imaging individual neurons in the retinal ganglion cell layer of the living eye. *Proceedings of the National Academy of Sciences of the United States of America*, *114*(3), 586–591. https://doi.org/10.1073/pnas.1613445114

Rowley, J. A., Madlambayan, G., & Mooney, D. J. (1999). Alginate hydrogels as synthetic extracellular matrix materials. *Biomaterials*, *20*(1), 45–53.

Sasai, Y. (2013). Cytosystems dynamics in self-organization of tissue architecture. *Nature*, *493*(7432), 318–326. https://doi.org/10.1038/nature11859

Sato, R., Yasukawa, T., Kacza, J., Eichler, W., Nishiwaki, A., Iandiev, I., … Wiedemann, P. (2013). Three-dimensional spheroidal culture visualization of membranogenesis of Bruch's membrane and basolateral functions of the retinal pigment epithelium. *Investigative Ophthalmology & Visual Science*, *54*(3), 1740–1749. https://doi.org/10.1167/iovs.12-10068

Schwartz, S. D., Hubschman, J.-P., Heilwell, G., Franco-Cardenas, V., Pan, C. K., Ostrick, R. M., … Lanza, R. (2012). Embryonic stem cell trials for macular degeneration: a preliminary report. *Lancet (London, England)*, *379*(9817), 713–720. https://doi.org/10.1016/S0140-6736(12)60028-2

Schwartz, S. D., Regillo, C. D., Lam, B. L., Eliott, D., Rosenfeld, P. J., Gregori, N. Z., … Lanza, R. (2015). Human embryonic stem cell-derived retinal pigment epithelium in patients with age-related macular degeneration and Stargardt's macular dystrophy: follow-up of two open-label phase 1/2 studies. *Lancet (London, England)*, *385*(9967), 509–516. https://doi.org/10.1016/S0140-6736(14)61376-3

Shirai, H., Mandai, M., Matsushita, K., Kuwahara, A., Yonemura, S., Nakano, T., … Takahashi, M. (2016). Transplantation of human embryonic stem cell-derived retinal tissue in two primate models of retinal degeneration. *Proceedings of the National Academy of Sciences of the United States of America*, *113*(1), E81-90. https://doi.org/10.1073/pnas.1512590113

Uteza, Y., Rouillot, J. S., Kobetz, A., Marchant, D., Pecqueur, S., Arnaud, E., … Neuner-Jehle, M. (1999). Intravitreous transplantation of encapsulated fibroblasts secreting the human fibroblast growth factor 2 delays photoreceptor cell degeneration in Royal College of Surgeons rats. *Proceedings of the National Academy of Sciences of the United States of America*, *96*(6), 3126–3131.

Vecino, E., Rodriguez, F. D., Ruzafa, N., Pereiro, X., & Sharma, S. C. (2016). Glia-neuron interactions in the mammalian retina. *Progress in Retinal and Eye Research*, *51*, 1–40. https://doi.org/10.1016/j.preteyeres.2015.06.003

Wahlin, K. J., Campochiaro, P. A., Zack, D. J., & Adler, R. (2000). Neurotrophic factors cause activation of intracellular signaling pathways in Muller cells and other cells of the inner retina, but not photoreceptors. *Investigative Ophthalmology & Visual Science*, *41*(3), 927–936.

Wahlin, K. J., Maruotti, J., & Zack, D. J. (2014). Modeling retinal dystrophies using patient-derived induced pluripotent stem cells. *Advances in Experimental Medicine and Biology*, *801*, 157–164. https://doi.org/10.1007/978-1-4614-3209-8_20

Yasukawa, K. (2016). *Strategy for creating innovation, R&D meeting 2016*. Astellas. Retrieved from https://www.astellas.com/system/files/RD_2_en_20161213.pdf

Zhao, C., Wang, Q., & Temple, S. (2017). Stem cell therapies for retinal diseases: recapitulating development to replace degenerated cells. *Development (Cambridge, England)*, *144*(8), 1368–1381. https://doi.org/10.1242/dev.133108

Zhong, X., Gutierrez, C., Xue, T., Hampton, C., Vergara, M. N., Cao, L.-H., … Canto-Soler, M. V. (2014). Generation of three-dimensional retinal tissue with functional photoreceptors from human iPSCs. *Nature Communications*, *5*, 4047. https://doi.org/10.1038/ncomms5047