Published in final edited form as:

*Drug Discov Today*. 2021 January ; 26(1): 181–188. doi:10.1016/j.drudis.2020.09.034.

# 3D engineering for optic neuropathy treatment

**Wenjing Xuan**[1], **Aji Alex Moothedathu**[1], **Tuo Meng**[1], **David C. Gibson**[2], **Jinhua Zheng**[1,3], **Qingguo Xu**[1,2,4,*]

[1]Department of Pharmaceutics, Virginia Commonwealth University, Richmond, VA 23298, USA

[2]Department of Ophthalmology, Virginia Commonwealth University, Richmond, VA 23298, USA

[3]Department of Ophthalmology, Guizhou Medical University, Guiyang, Guizhou, China

[4]Massey Cancer Center, Center for Pharmaceutical Engineering, and Institute for Structural Biology, Drug Discovery & Development (ISB3D), Virginia Commonwealth University, Richmond, VA 23298, USA

## Abstract

Ocular disorders, such as age-related macular degeneration (AMD), diabetic retinopathy (DR), retinitis pigmentosa (RP), and glaucoma, can cause irreversible visual loss, and affect the quality of life of millions of patients. However, only very few 3D systems can mimic human ocular pathophysiology, especially the retinal degenerative diseases, which involve the loss of retinal ganglion cells (RGCs), photoreceptors, or retinal pigment epithelial cells (RPEs). In this review, we discuss current progress in the 3D modeling of ocular tissues, and review the use of the aforementioned technologies for optic neuropathy treatment according to the categories of associated disease models and their applications in drug screening, mechanism studies, and cell and gene therapies.

## Teaser:

3D models can reduce animal testing, evaluate targeted drug delivery, and accelerate drug development process; they also have promising applications in cell and gene therapies, especially for retinal degenerative diseases and/or optic neuropathies that are difficult to mimic *in vivo*.

### Keywords

glaucoma; macular degeneration; neuroprotection; ocular drug delivery; ganglion cells

## Introduction

2D *in vitro* systems are important for studying and discovering targets and biomarkers, but are not appropriate for tissue function studies [1]. To recapitulate the 3D environment of *in*

*Corresponding author: Xu, Q. qxu@vcu.edu.

*vivo* tissues, especially for ocular pathophysiology and development, tissue engineering and microfluidic model systems have been developed and are now essential for drug discovery and development during the preclinical stages [2]. 3D miniaturized assays can reduce animal testing, evaluate targeted drug delivery, and accelerate the drug development process. Conventional 3D engineering is an efficient tool to fabricate architecturally and physiologically relevant 3D tissues with defined properties [3], which benefits from: (i) 3D bioprinters with defined 3D geometries; (ii) biocompatible materials and hydrogels as 3D cell support; (iii) bioreactors for providing mechanical and chemical cues; (iv) autologous cells from different stem cell resources; and (v) available noninvasive technologies for high-resolution imaging analysis.

Microfluidic models (100–500 μm width) can provide a mechanical and dynamic milieu to cells [4]. The flow and pressure are crucial aspects for the structure and function of cultured cells. Compared with conventional 2D culture models, this microfluidic technology can more closely recapitulate the human organs, including cell–cell interactions and biomicroenvironment stimuli. In some cases, the combination of conventional 3D engineered models and microfluidic technologies, and the addition of hydrogel (conventional 3D model support) to microfluidic systems can create an advanced channel network where multiple cell types can be introduced [5,6]. This is attributed to close contact among different cell types to capture the dynamic cell–cell interplays in a restrained flow. In addition, the spatiotemporal gradients of chemicals and optical transparency of devices in microfluidic chips allow for real-time monitoring of cell migration and interaction, which can precisely mimic the dynamic process of tissue development, repair, and regeneration.

Ocular disorders, such as AMD, DR, RP, and glaucoma, can cause irreversible visual loss, and affect the quality of life of millions of patients [7]. However, only very few 3D systems can mimic human ocular pathophysiology, especially of retinal degenerative diseases. Retinal dystrophies primarily affect the outer layers of the retina, such as photoreceptors and RPE, but there is increasing evidence that they also affect inner layers, such as RGCs [8–10]. Optic neuropathies can be caused by a variety of mechanisms, including trauma, inflammation, ischemia, and infection [11]. Some optic neuropathies, such as glaucoma, are common and contribute significantly to blindness, whereas inherited optic neuropathies, such as Leber hereditary optic neuropathy (LHON) and dominant optic neuropathy (DOA), are less common but can lead to devastating visual impairment [12]. In this review, we discuss progress in the 3D modeling of ocular tissues, and review the use of the aforementioned technologies for retinal degenerative disease and optic neuropathy treatments according to the categories of the associated disease models.

## RGC disorders toward optic neuropathies

RGC disorders affect the neurons of the eye that feed in to the optic nerve. Thus, the RGC layer is primarily affected by optic neuropathies. In fact, optic neuropathies are always associated with RGC disorders [13–15]. In glaucoma, the common features are optic nerve degeneration and loss of RGCs, accompanied by the thinning of the neuroretinal rim of the optic disc and nerve fiber layer [16]. The relationship between high intraocular pressure (IOP) and RGC death is not fully understood but is likely caused by mechanical stress at the

lamina cribrosa resulting in altered capillary perfusion and, in turn, disrupted axoplasmic flow at the optic nerve head [17]. Although high IOP is an important risk factor, glaucomatous RGC degeneration can occur with normal IOP, especially in primary open-angle glaucoma, which is also associated with other risk factors, such as age, ethnicity, and family history. This suggests that other mechanisms of glaucoma and RGC death exist. Generally, during glaucoma, a variety of signals, oxidative stress, hypoxia, neuronal excitotoxicity, or trophic factor deprivation have been reported to trigger RGC apoptosis [18]. Intrinsically, neurotrophic factors are widely used for the promotion of RGC survival after injury. In addition to brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), glial cell-derived neurotrophic factor, insulin like growth factor-1(IGF-1) and leukemia inhibitory factor might all have effects to delay or prevent RGC death [19,20].

Inherited optic neuropathies represent a group of genetic disorders with optic nerve degeneration, as well as the death of RGCs. LHON and DOA are the two types of mitochondrial disordered inherited optic neuropathies resulting from mutations of genes that encode inner mitochondrial membrane proteins [21]. For both diseases, gene mutations are located in the genome (LHON, mitochondrial genome; DOA, nuclear genome) and encode a dynamin-related GTPase targeted to the mitochondrial inner membrane. This enzyme is involved in many functions, including mitochondrial membrane transport, oxidative phosphorylation, and apoptosis [22]. In the unmyelinated segment of RGC axons, mitochondria are abundant; therefore, mitochondrial abnormalities are strongly associated with optic neuronal dysfunction here. Factors related to RGC degeneration are detailed in Figure 1. Similar situations are also observed in other RGC degeneration diseases, such as RP and AMD [23].

## Cellular structure of the retina

Understanding the retinal structure and function is important for clinicians and scientists who are diagnosing and treating patients who have optic nerve damage in the retina. The retina is a thin, multilayer structure located at the inner surface of eye and mainly comprises neuronal cells derived from ectoderm [24]. Structurally, the retina is composed of various types of cells, such as photoreceptors, bipolar cells, horizontal cells, amacrine cells, and RGCs [25]. RGCs collect input from bipolar and amacrine cells and modulate output from the retina [26]. RGC death, as the final common pathway of degenerative diseases such as glaucoma and other optic neuropathies, can lead to significant vision loss [27]. Furthermore, several glaucoma studies have shown photoreceptor damage and destruction in addition to the primary involvement of the overlying RGC layer [28].

Photoreceptors can be further categorized into rods and cones, and their nuclei are contained in the outer nuclear layer (ONL). Photoreceptors form synapses with interneurons, such as bipolar cells and horizontal cells in the outer plexiform layer (OPL). A variety of retinal neuropathic lesions are related to the degeneration of cone photoreceptors after a relatively short disease duration [29]. Compared with ONL, the inner nuclear layer (INL) is a thicker portion in the central retina comprising the cell bodies of cone bipolar cells, horizontal cells, and amacrine cells. Cone bipolar cells are responsible for the transmission of signals from photoreceptors to the inner retina. Horizontal cells and amacrine cells are interneurons that

help regulate bipolar cell transmission [30]. In addition to these cells, inter plexiform-layer cells (IPL) also share various properties with amacrine cells and project towards the outer retina (Figure 2).

The components of the optic nerve are unique because they are central nervous system (CNS) structures easily accessible from the exterior, and their functions can be readily quantified through imaging and electrophysiological testing. These features assist the study and treatment of neurodegenerative diseases.

## 3D engineering for retinal models

Researchers have already created retinal organoids bearing a striking similarity to the human retina, in terms of cell composition and organization. However, modeling retina is challenging because of its functional complexities. Therefore, only partial functions are capable of being recapitulated in a single model. Retinal organoids and microfluidic systems are two major development methodologies. Figure 3 shows the organoid generation and their different applications in the areas of drug screening, disease modeling, cell therapy, and gene therapy [31,32].

One robust protocol is to pattern anterior neural and retinal fate in *vivo* by the addition of retinal cell generation factors [33]. In this protocol, lefty-A and dickkopf-1 are added to assist mouse embryonic stem cells (ESCs) to grow into 3D serum-free floating embryoid bodies (SFEB). The aggregation of SFEB in the presence of knockout™ serum and materials can promote the formation of optic cup-like structures containing neural retina, which can be self-organized into a rigid continuous stratified epithelium. By contrast, 3D/2D stepwise differentiation protocols have been developed from human/mouse pluripotent stem cell (PSC) lines [34,35]. One example is the isolation of structures displaying the characteristics of optic vesicle (OV)-like structures from human PSC lines [36]. Self-aggregates from human induced PCS (iPCS) colonies were plated to form neural clusters and then manually picked up. This protocol not only promoted the improvement for the generation of photoreceptors in 3D retinal optic cup (OC)-like structures, but also provided a classic representation of an *in vitro* retinal model. Thus, it further allowed the identification of different RGCs based on morphological, phenotypic, and functional characteristics.

In addition, an innovative strategy can generate self-forming neuroretinal structures bypassing 3D embryoid body (EB) formation, with the addition of a porous membrane and other exogenous factors for human iPSC lines [37–39]. In this protocol, a subsequent adherent cell culture step was applied to promote the maturation of RGCs, enzymatic dissociation of retinal organoids, followed by seeding cells onto poly-D-lysine/laminin-coated plates. By contrast, PSCs can be directly cultured into gelatin-coated plates without any preliminary EB formation. The overexpressed biomarkers of RGC, such as *atoh7*, can promote the differentiation of mouse iPSCs into retinal ganglion-like cells, displaying other expression patterns, including Isl1, Brn3b, and Thy1.2 [38]. Using a similar methodology, co-culture of different cells/tissues towards the retina as well as the generation of RGCs are also widely reported [40,41].

Compared with the above-mentioned static culture conditions, organoid development can be hindered by the limited diffusion of oxygen and nutrients. A recent 3D culture system was developed to culture retinal organoids into drug-screening models, deriving from mouse PSCs in rotating-wall vessel (RWV) bioreactors. RWV organoids at day 25 (D25) revealed similar maturation as those at D32 in static culture, closely recapitulating the development of postnatal D6 mouse retina *in vivo.* This process promoted the spatiotemporal development and maturation of retinal organoids at an accelerated speed (7 days ahead) for modeling retinal disease and evaluation of therapies [42].

Even with the wide application of retinal organoids, some limitations still exist to studies of RGC behaviors. The ratio of RGCs decreases over time during *in vitro* culture. As reported in the floating culture of retinal organoids, hindered axon growth outside the structure and the lack of projection targets can both induce RGC death [43]. Thus, a microfluidic technique was developed to assist 3D organoid development. Microfluidics is a system using channels measuring from tens to hundreds of micrometers to process small amounts of fluidics, and it can retain the vasculature structure when co-cultured with RPE cells (Figure 4) [44]. By mixing retinal organoids, precursor cells, and their microenvironment, retinal models can recapitulate part of the retinal function. In a synaptic regeneration study [45], researchers isolated retinal precursor cells and cultured them in a microfluidic chip with multiple arrays of micro-channels to restore the retinal neuronal synapse functions. Mishra *et al.* reported a convenient, diffusion-based microfluidics system that replicated the geometry and scale of mouse and human retinas, called the microRetina (μRetina) [46]. In this case, chemical concentration gradients were monitored with real-time imaging on cell migration, with well-controlled computer simulation and experimental validation.

RPEs in the outermost layer of the retina normally act as a major homeostatic units, regulating both choroid and retina tissues [47]. For example, tight junctions between RPE cells help form the blood–retinal barrier (BRB) and prevent large molecules from entering the retina. By disrupting these intercellular junctional proteins, RPE cell death negatively affects the viability of the photoreceptors and the mechanical integrity of the BRB. To simulate retinal vasculature/RPE interaction and BRB function, an early model comprising a simple co-culture chip with the human RPE cell line ARPE-19 and human umbilical vein endothelial cells (HUVEC) was established [48]. By increasing the complexity of the choroid tissue, Chung *et al.* developed a 3D vascularized ‘Choroid’ tissue with an artificial Bruch’s membrane [49]. This was the first model mimicking choroid neovascularization (CNV) of wet AMD, in which an angiogenesis model that can increase VEGF concentration was established with perfusable blood vessel networks, enabling the observation of pathological retinal angiogenesis. New approaches now seek the combination of organoids and microfluidic technologies for generating multilayered retinal vasculature and RPE models. One novel microfluidic model was developed by integrating more than seven human iPSC-derived retinal cell types [50]. The applicability of the system was demonstrated towards drug screening of chloroquine and gentamicin by reproducing retinopathic side effects. This allowed for the formation of outer segment-like structures and the establishment of *in vivo*-like microenvironment, such as outer segment phagocytosis and calcium dynamics.

## The translational application of pluripotent stem cell technology to optic neuropathies

To form retinal organoids containing all the retinal layers, 3D organoids provide access to their development, which is difficult to obtain via natural modeling [51–53] (Figure 5) and, thus, such organoids can meet the need for spatial focus/resolution, penetration of dyes/lasers for imaging, mimicking of drug diffusion kinetics, and overcoming the difficulty of analyzing measurements from the various cell types present. The production of different PCSs should facilitate the study of 3D models for optic neuropathies and advance the use of PSCs in personalised medicine. The generation of cell types on a large scale, such as RGCs and PSCs for chemical, small interfering (siRNA) and, most recently, guide (g)RNA screening, can be realized by high-throughput methods using the Labcyte® Echo® Liquid Handler [54].

Early studies derived from retinal organoids allowed the examination of disease mechanisms with more native spatial cell arrangement. For example, to study the clinical phenotype of microphthalmia, defects in early retinogenesis were modeled in cells carrying an R200Q mutation in the transcription factor visual system homeobox 2 (VSX2) [55]. This mimics the damaged optic vesicles that express VSX2 at later stages of the disease. During retinal development, early retinal organoids developed from a mutated gene mutation (e.g., USH2A) exhibit lower laminin expression compared with healthy controls. This study provides essential assistance from the molecular level for RP screening and diagnosis [56]. In addition, structural integrity of the retina via gene mutation has also been studied in organoid cultures to recapitulate the retinal phenotype and neural retina [57].

Another important application of 3D retinal organoids is their use in drug screening for ocular disease treatment to both increase the efficiency of differentiation protocols and improve the correction of disease phenotypes. However, there is no currently commercially available *in vitro* ocular models that can mimic *in vivo* conditions of retinal cells because they are not cultured on curved scaffolds. Research in this area is still in its infancy. Scientists used adult rat retinal explant models for screening potential RGC neuroprotective therapies [58]. The models were used to screen potential neuroprotective drugs, including maintenance of neurons *in situ*, control of environmental conditions, and dissociation from other factors, such as IOP. In this study, the direct retinal neuroprotective effects of several clinical ocular hypotensive drugs (betaxolol, losartan, tafluprost, and simvastatin) on RGC survival in adult rat retinal explants were screened efficiently and replicated in adult retinal explant cultures.

All the above-mentioned studies clearly demonstrate the use of patient stem cell-derived retinal organoids for modeling endophenotypes of human diseases in the discovery of the complement system of *in vivo* animal systems. In addition, a cell viability assay after stress-induced cell death was also developed for high-throughput screening (HTS). In one report, T cells were separated from patients with dry AMD and reprogrammed into iPSCs via integration-free episomal vectors. They were further differentiated into RPE cells, which provided an expandable platform for investigating the pathogenesis of dry AMD and *in vitro* drug screening [59]. As a proof-of-concept study, a series of retinal protection agents and

antioxidant supplements were screened, and curcumin was selected as a potential drug candidate for reactive oxygen species (ROS) reduction, which is essential for neuroprotection. In all the cases, drugs were used as both a treatment to induce photoreceptor regeneration *in vitro* and as reagents to enhance the production of PSCs for cell-based therapies.

The literature dedicated to cell therapies designed for RGC disorders is less abundant, especially for retinopathies resulting from photoreceptor and/or RPE cell death. Most studies so far have focused on animal models [60]. Several human clinical trials have been approved for RPE cell replacement [61,62]. Stem cell implantation in patients with advanced dry AMD was reported and lasted for 1 year with no severe adverse events [61]. In March 2018, the London Project to Cure Blindness announced the results of a trial in which two patients with wet AMD received a bioengineered patch containing retinal cells from ESCs. The patch was made by coaxing stem cells to differentiate into RPE, which are damaged in patients with wet AMD [62]. Clinicians were then able to replace a section of damaged RPE with healthy cells by delivering the patch to the base of the retina. Both recipients reported an improvement in their vision. Although these trials are too small to draw a firm conclusion, these promising findings have energized the field of stem cell therapies for the cure of AMD and could be a source of gene-edited cells for transplantation.

Recently, organoids have become a useful research system and have been widely reported for evaluating cell-based therapies. As for human cell transplantation into animal models, cell-cell interactions might be affected because of the donor-host species mismatch [63]. Thus, organoids can be used as *in vitro* recipients for dissociated human photoreceptors. In these cases, tissue-engineered scaffolds can provide physical support vehicles for cell delivery, survival, and integration. In advanced retinal degeneration, the transplantation of laminated retinal sheets represents another approach to treat vision loss [64,65]. In these cases, 3D retinal tissue was separated from organoids and transplanted into the targeted retina to overlay the degenerated retina. However, it is difficult to maintain its morphology and polarity while placing the graft into the recipient eye, which could hinder the connection with host retina [63]. By contrast, a natural and clear outer segment structure can be formed in rats and nonhuman primates via retinal sheet transplantation [66]. This might be because these scaffolds are capable of tailoring to the natural microenvironment surrounding neural tissues of rats and nonhuman primates, thus helping to restore lost axonal connections. However, there is no direct evidence for graft-driven retinal function, especially for the graft itself in the presence of active host retina, to prove the efficacy of photoreceptor therapy.

In terms of gene therapy, scientists developed 3D retinal organoids as models for human optical neuropathies, and a new gene therapy approach to treat a group of eye diseases known as retinitis pigmentosa (RP) has been developed [67]. In this research, investigators developed both gene-edited isogenic *RP2* knockout (*RP2* KO) iPSCs and *RP2* patient-derived iPSCs. Thereafter they used adeno-associated viruses (AAVs) to deliver *RP2* into defectively engineered retina models. Throughout the study, researchers monitored the temporal maturation of CRISPR gene-edited RP2-KO retinal organoids relative to their isogenic controls. This study showed that 3D retinal organoids can be used to model gene-

based photoreceptor degeneration and test potential therapies to prevent photoreceptor cell death [67].

There are also limitations for PSCs for modeling human retinal diseases. Many optic neuropathies result from distinct cell and tissue types, rather than a single primary cell, and their interaction with each other is difficult to model *in vitro*. There are also technical issues, such as cell types, differentiation timing, and culture conditions.

## Current drawbacks of 3D organoids and cellular models

Compared with the construction of the mammalian retina *in vivo*, most *in vitro* organoid systems lack several features, such as proper vascularization, presence of muscle cells and immune cells, among others [68,69]. To mitigate these limitations, endothelial cells and the mesodermal-derived microglia have been incorporated in retinal organoids via external addition, with promising results [70].

However, it is time-consuming for human retinal organoids to follow the embryonic development process, which normally takes around 8–9 months. Shortening the timeline to generate photoreceptor-containing 3D retinal organoids is challenging (e.g., 175 days *in vitro*) [71]. Current protocols still suffer human iPSC cell line variability and difficulties in achieving fully maturated retinal cells [71]. To some extent, the introduction of micromaterials, such as poly(lactide-co-glycolide) copolymer (PLGA) fibers, to support the self-patterned organoids can improve the overall reproducibility of the internal cytoarchitecture of the model [72]. However, *in vitro*-generated organoids might still not mimic the adult *in vivo* retina, which exhibits a more complicated intercellular connectivity and arrangement of rods and cones [73].

Determining how to reproduce the effect of aging to achieve precise developmental timing *in vitro* is crucial to investigate late-stage optical neuropathy diseases, the symptoms of which mainly appeared in the older population. However, there are no approaches that can be applied in the case of 3D retinal tissues. Another major issue is caused by the spheroid organoid structure of retina tissues *in vitro*, whereas the *in vivo* retina is a plane sheet [74]. The lack of orientation surrounding tissues and extracellular matrix can prohibit OC development and peripheral-central specialization [75]. Meanwhile, the passive diffusion pathway of nutrients into the innermost layers also limits organoid growth and viability [76].

*In vivo,* RPE cells are essential for trafficking outer retinal segments and providing nutrition to RPCs during photoreceptor development in retinal organoids. Thus, the complementation of RPE cells and matured retinal organoids can provide a more physiological and mature retina *in vitro* [77]. However, this process is still limited to the generation of sufficient and functionally integrated donor cells.

## Perspectives and future directions

Alternative types of hydrogel material and surface-engineering techniques are applied for the generation of precisely organized tissue structures. The combination of organoids and microfluidic technology to produce ‘human organoids-on-a-chip’ is developing quickly as a

way to build 3D models with high reliability. In general, the integration of hydrogel with human organoids-on-a-chip opens the possibility to advanced 3D models with more precise spatiotemporal control.

Organoid engineering and translation for optical neuropathy treatment are limited by the low reproducibility of such systems. For example, challenges for RGC transplantation include radial extension of RGC axons to the optic nerve head, and the CNS targeting the optic nerve tract [78]. Thus, attachment of RGCs to the host retina cell surface is vital. In most cases, biomaterials or synthetic polymers are essential as a physical support for axon survival and correct regeneration. However, compared with the direct injection of cells into the vitreous cavity, introducing RGC scaffolds into the eye is technically more challenging. In recent research, 3D bioprinting was applied without sacrificing RGC phenotypic features, such as neurite outgrowth and electrophysiological response [79]. Combining the use of specific scaffolds and 3D bioprinting has the potential to control RGC positioning. For neurons that are difficult to manipulate for bioprinting, RGC replacement using multiresponsive self-healing materials can facilitate the development of novel cell therapies [80]. Issues that need to be resolved for this technology include the purification of donor cells, targeting the cell surface, biomarkers for RGC isolation, and immune rejection for transplantation [81]. Other bioengineering approaches, such as imaging, genome editing, and single cell genomics, can also be applied to study organogenesis, diseases, and personalized medicine [82]. Moving from conventional drug development, CRISPR/Cas9 technology and single cell-sequencing on-chip could be developed gradually in academic settings and clinical trials [83,84]. Although many challenges remain, the present methodologies offer big opportunities, and basic understanding for patients with currently untreatable degenerative retinal diseases and optic neuropathies.

## Acknowledgments

This work was partially supported by the National Institutes of Health (R01EY027827), the US Food and Drug Administration (HHSF223201810114C), and the George and Lavinia Blick Research Fund.

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## Highlights

- 3D engineering for recapitulating ocular pathophysiology and retina development
- The development of retina and its 3D model engineering for optic neuropathies.
- The application of pluripotent stem cells in disease modelling, drug screening, and cell and gene therapies
- The perspectives of 3D models in new therapy development for optic neuropathies.

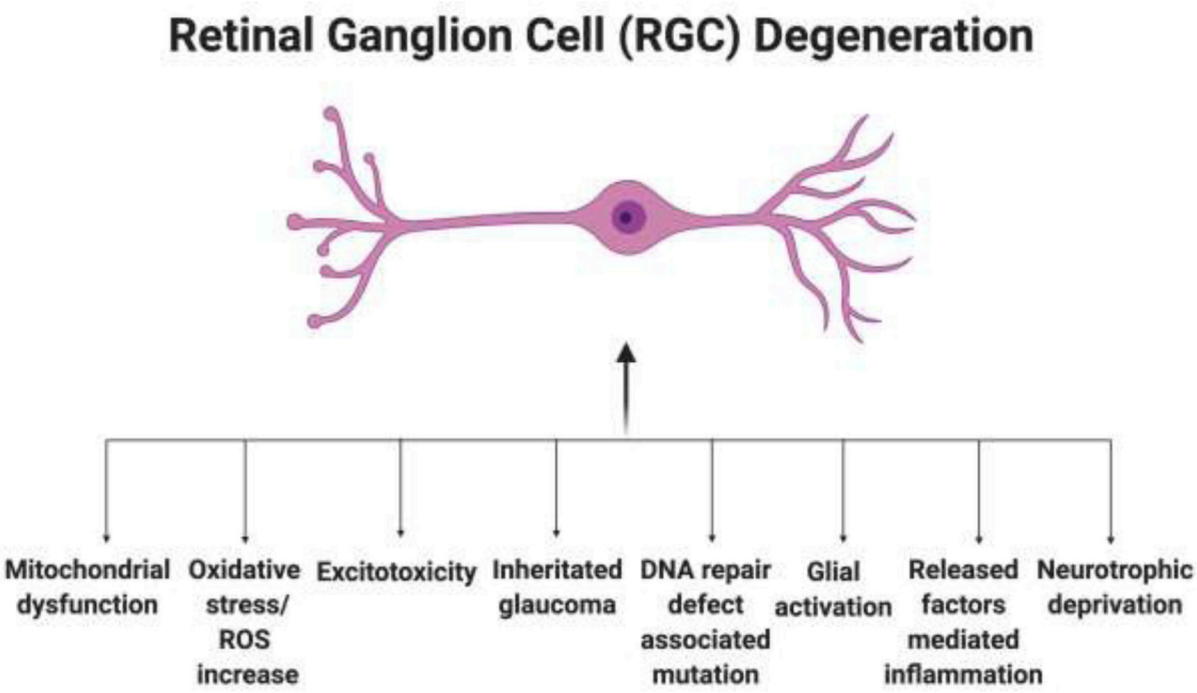


**Figure 1.**
Summary of different cues for retinal ganglion cell (RGC) degeneration. Abbreviation: ROS, reactive oxygen species.

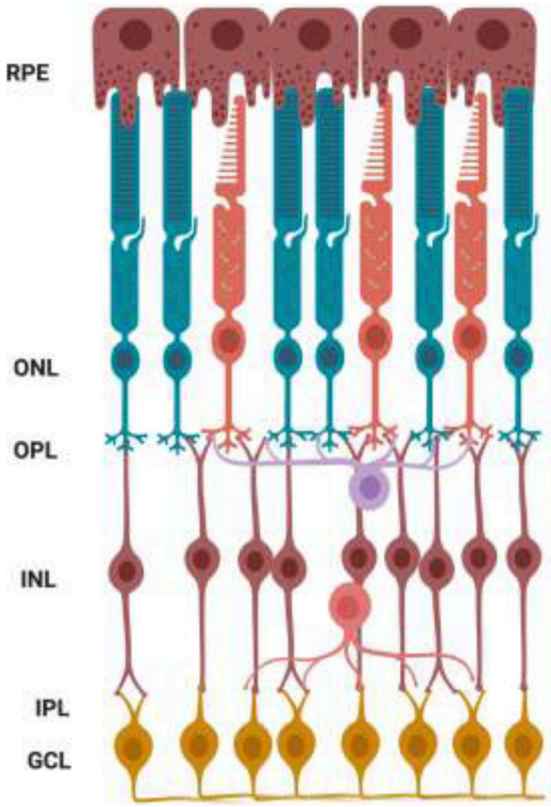


**Figure. 2.**

Schematic of the retina structure. Abbreviations: GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; ONL, retinal ganglion cell; OPL, outer plexiform layer; RPE, retinal pigment epithelium.

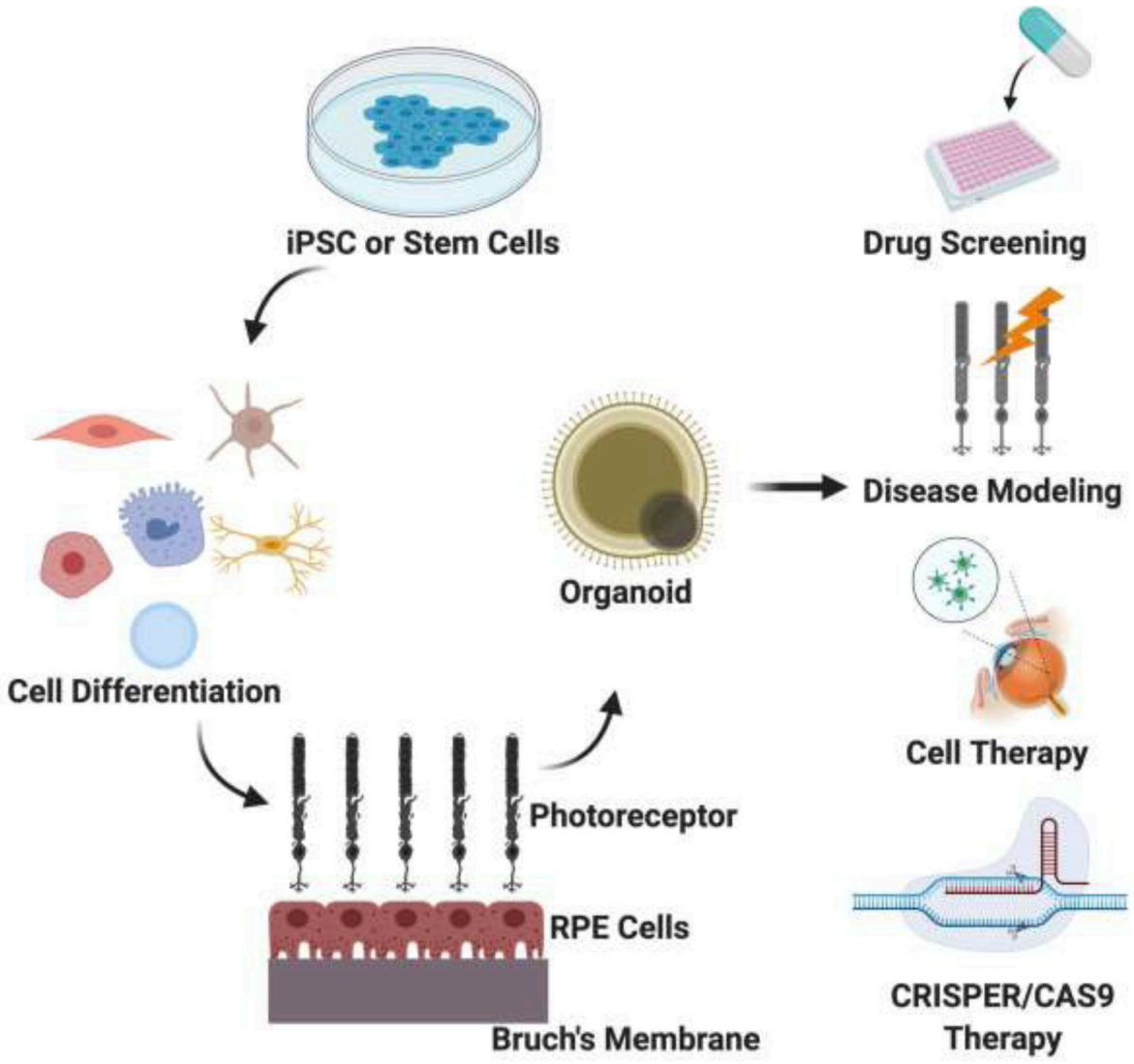


**Figure 3.**
Pluripotent stem cells (PSCs) for organoid generation and their applications, from high-throughput drug screening, disease modeling *in vitro*, and cell therapy to gene therapy (CRISPR/CAS9).

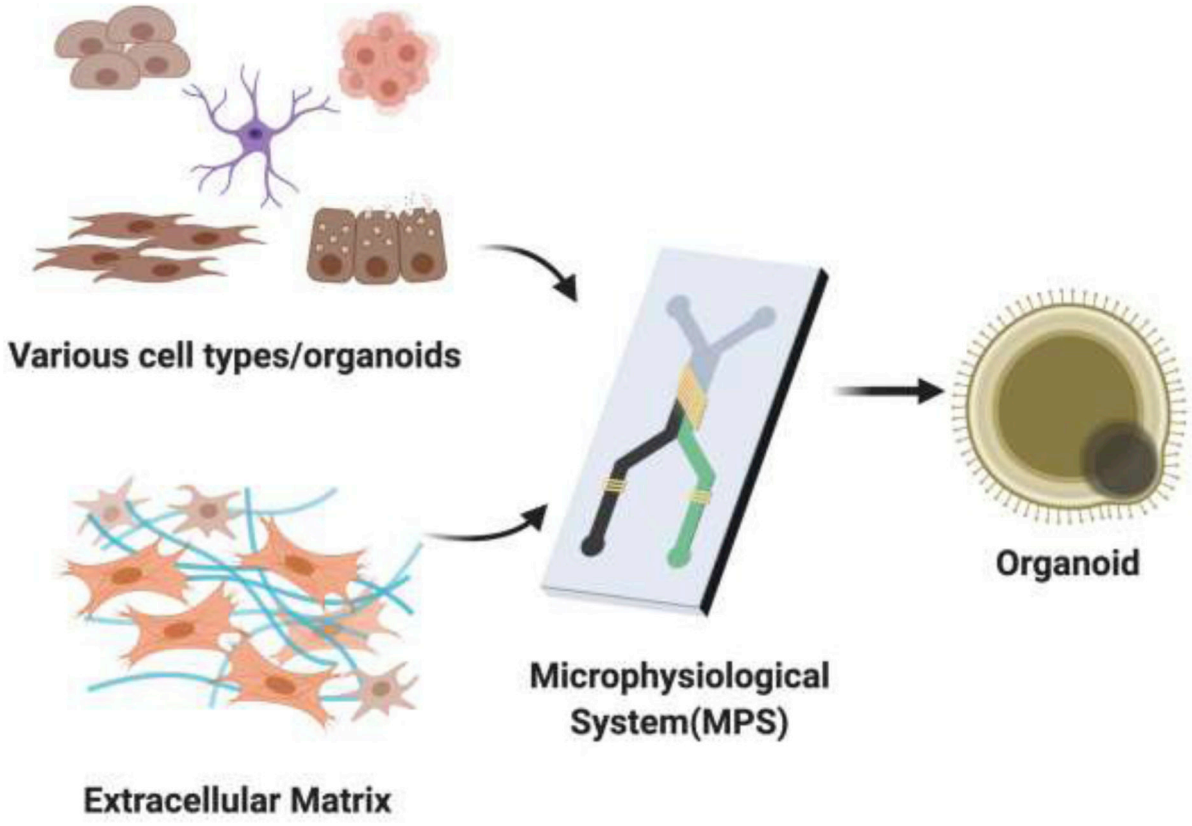


**Figure 4.**
Microfluidic techniques for ocular organoid modeling. Abbreviations; iPSC, induced PSC; RPE, retinal pigment epithelium.

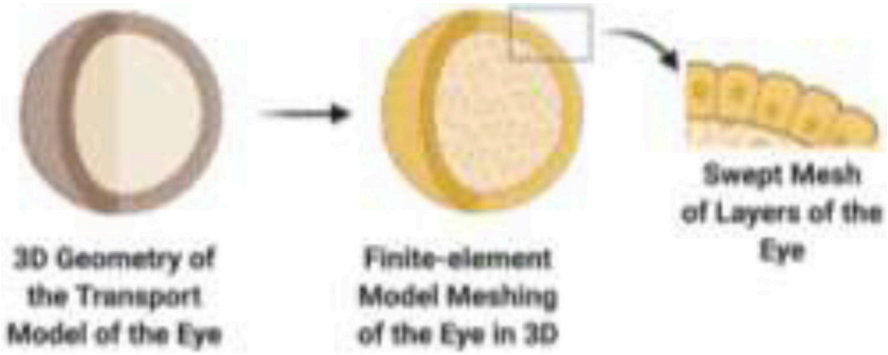


**Figure 5.**

A 3D model generated using COMISOL software. In this 3D model, the posterior segment was categorized as sclera, subconjunctival space, choroid, retina, and vitreous, whereas retinal pigment epithelial cells were modeled as a thin layer between choroid and retina. Reproduced, with permission, from [53].