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Single-cell sequencing in diabetic retinopathy: progress and prospects

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Abstract
Diabetic retinopathy is a major ocular complication of diabetes, characterized by progressive retinal microvascular damage and significant visual impairment in working-age adults. Traditional bulk RNA sequencing offers overall gene expression profiles but does not account for cellular heterogeneity. Single-cell RNA sequencing overcomes this limitation by providing transcriptomic data at the individual cell level and distinguishing novel cell subtypes, developmental trajectories, and intercellular communications. Researchers can use single-cell sequencing to draw retinal cell atlases and identify the transcriptomic features of retinal cells, enhancing our understanding of the pathogenesis and pathological changes in diabetic retinopathy. Additionally, single-cell sequencing is widely employed to analyze retinal organoids and single extracellular vesicles. Single-cell multi-omics sequencing integrates omics information, whereas stereo-sequencing analyzes gene expression and spatiotemporal data simultaneously. This review discusses the protocols of single-cell sequencing for obtaining single cells from retina and accurate sequencing data. It highlights the applications and advancements of single-cell sequencing in the study of normal retinas and the pathological changes associated with diabetic retinopathy. This underscores the potential of these technologies to deepen our understanding of the pathogenesis of diabetic retinopathy that may lead to the introduction of new therapeutic strategies.

Introduction

Diabetic retinopathy (DR) is a prevalent ocular complication in patients with diabetes that presents as a chronic progressive vision-impairing oculopathy characterized by retinal microvascular damage [1]. Statistically, it shows a high prevalence, rendering it a leading cause of blindness in working-age adults. Globally, the International Diabetes Federation estimates that the number of people with DR will rise to 160.5 million by 2045 [2]. In the United States in 2021, epidemiological data indicated that 9.6 million individuals had DR, of which 1.84 million l suffered from vision-threatening diabetic retinopathy [3].

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Based on epidemiological studies, researchers and ophthalmologists have identified DR as a public health issue that causes a heavy economic burden. Current treatments for DR include intraocular antiangiogenic drug injections, retinal laser photocoagulation, and vitrectomy. However, each of these treatments has limitations, and none are capable of fully reversing the retinal damage induced by DR, partly because of the incomplete elucidation of the underlying pathophysiological mechanisms of the disease [4, 5]. Given that the pathophysiological mechanisms and therapeutic targets of DR have yet to be thoroughly clarified, further systematic investigations are required to elucidate the alterations occurring in specific retinal cells during the progression of DR. Furthermore, owing to the involvement of various retinal cell types in DR pathogenesis, a research methodology with

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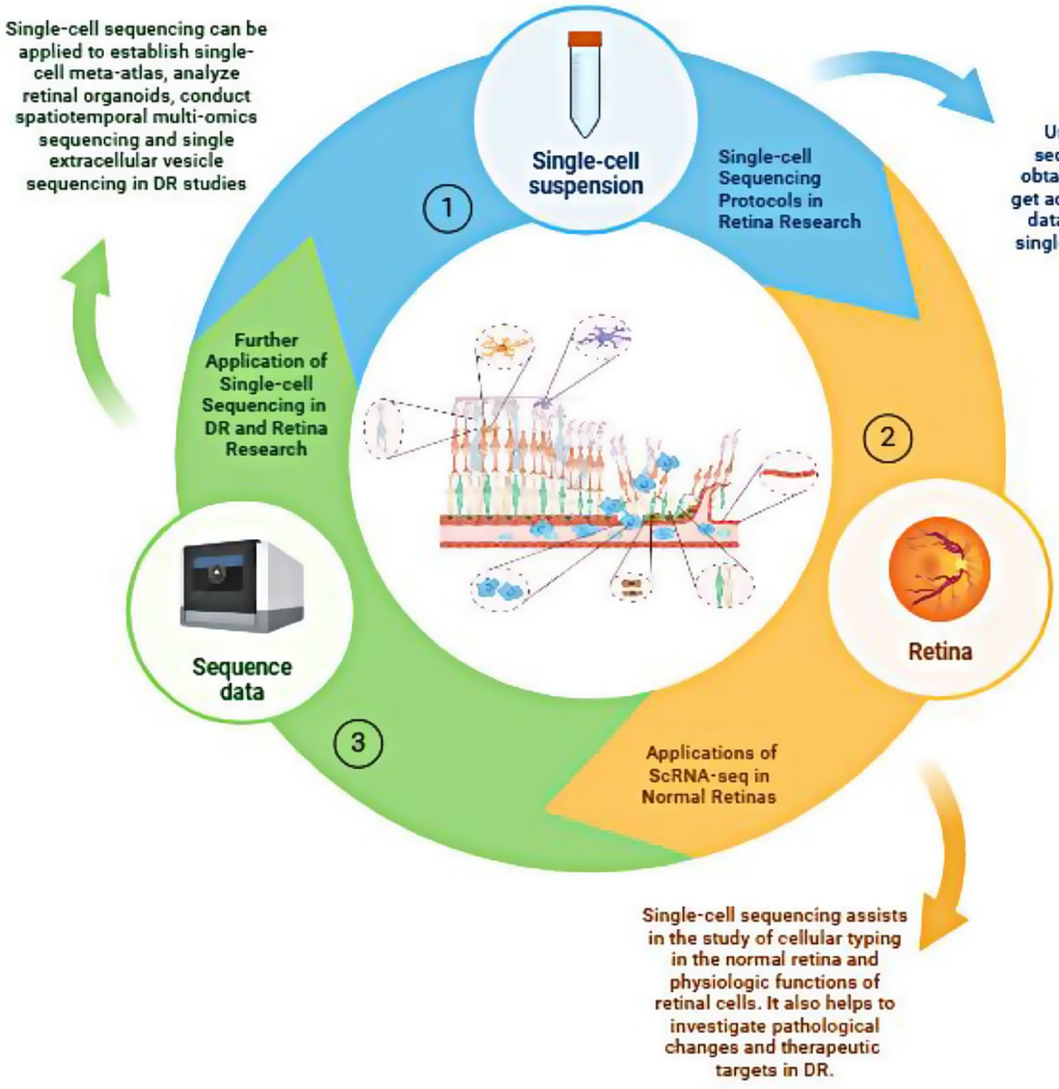
Graphical abstract



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cellular-level resolution is required to dissect the pathogenesis and therapeutic targets of DR.

Advancements in high-throughput sequencing technologies have enabled researchers to conduct large-scale analyses of genes and their products, including DNA sequences, chromatin structures, RNA sequences, and proteins, generating extensive genomic datasets, including genomes, transcriptomes, and proteomes [6, 7]. Traditional high-throughput sequencing obtains molecular samples from numerous cells, thereby providing insight into the overall gene expression of these cells. However, even though multi-omics data analysis has gradually covered nearly all aspects of the central dogma, cell heterogeneity causes the sequencing data of sample resolution alone to not fully satisfy the precise analysis of omics data. In contrast to bulk RNA sequencing (bulk RNA-seq) which provides a quantitative reflection of the overall gene expression level in a sample, single-cell RNA sequencing (scRNA-seq) enables high-throughput omics data acquisition of transcripts from individual cells with a tissue [8]. Consequently, compared to traditional sequencing methods, researchers can extensively use single-cell sequencing to investigate cellular differences and unveil novel cell subtypes and their specifically expressed

genes. Moreover, this method facilitates studies on cellular development trajectories and intercellular communication, thereby deepening our understanding of disease development mechanisms. Building on scRNA-seq, single-cell multi-omics analysis offers a more comprehensive understanding of cellular events and extends various aspects of genetic information transmission and regulation. Additionally, single-cell spatiotemporal genomics technology enables the simultaneous acquisition of gene expression characteristics and spatial distribution data at the tissue level [9, 10]. Studies have shown that the development of DR is related to individual retina cells [11]. Therefore, the unique cellular resolution of single-cell sequencing is well suited for resolving the heterogeneous cellular alterations associated with the pathological changes in DR. In fact, single-cell sequencing is widely adopted in ophthalmology. Studies using this technology introduce novel perspectives for elucidating disease etiology and targeted therapies, which significantly enhance the understanding of the pathogenesis of DR.

Recently, single-cell sequencing has been used to characterize diverse transcriptional profiles and spatiotemporal information associated with cellular function and development across various cell types. This technology underpins our understanding of the cellular heterogeneity implicated in DR-related alterations. With the continuous evolution of single-cell sequencing, novel ways to study DR have emerged, leading to a deeper understanding of its pathogenesis and the potential to build new therapeutic strategies. This paper reviews the application and advancements of single-cell sequencing in research on the normal retina and pathological changes in DR, thereby indicating the research prospects and development directions for single-cell sequencing in DR and retinopathy.

Single-cell sequencing protocols in retinal research

General protocols of single-cell sequencing

The basic protocols for single-cell sequencing involve cell sorting and extraction of target molecules from cells for sequencing analysis. This process begins with sampling, cell dissociation, labeling, forming sequencing libraries for subsequent data processing, and finally ends with analysis to obtain results [12]. Data processing primarily involves reading mapping, normalization, batch correction, and missing data imputation, whereas data analysis involves dimensionality reduction, differential expression analysis, and cell clustering [13]. Studies assessing the performance of different single-cell RNA sequencing methods across various parameters have provided guidance for selecting the most appropriate method for future experiments [14–16]. According to these studies, SMART-seq2 offers lower-throughput sequencing with full-length transcriptome quantification but lacks mRNA specificity. In contrast, 10x Chromium systems provide higher throughput, and are suitable for studying highly heterogeneous tissues and a large number of cells [17].

The first challenge in single-cell sequencing for retinal research is obtaining single cells. The major methods for sorting single cells in samples with high cell counts include flow-activated cell sorting (FACS) and microfluidics, with the 10x Chromium microdrop system being an example of the latter [18]. The 10x Chromium system uses gel beads to capture mRNA from cells, and each gel bead carries hundreds of thousands of oligonucleotide sequences on its surface. The oligonucleotide sequence has four components: a read1 (sequence 1) for sequencing runs, a 10x barcode sequence for identifying the cell of origin, a unique molecular identifier (UMI) sequence for tagging DNA molecules, and a polyT tail of approximately 30 bases for capturing mRNA molecules with polyA tails [19–22]. Gel beads in oils and single-cell suspension are simultaneously added to the channels of the Chromium Chip G in the 10x Chromium "T" cross system to form a gel beads-in-emulsion (GEMs). Theoretically, each GEM contains a single cell, and the RNA from this single cell binds only to one gel bead, thus isolating the mRNA from each cell [22–24].

The second challenge in single-cell sequencing in retinal research is avoiding PCR amplification errors and interference from other molecules to accurately form RNA sequencing libraries. Currently, two main sequencing library preparation methods are used: SMART and TargetAmp. The TargetAmp method, used by the Illumina sequencer, can amplify a small amount of RNA (as little as one cell, approximately 10 pg) to the nanogram level, sufficient for high-throughput sequencing [25, 26]. The 10x Chromium system disrupts cell membranes in GEMs, allowing reverse transcriptase in the aqueous phase to interact with nucleic acid primers bound to gel beads and dNTP substrates, facilitating reverse transcription, and forming cDNAs with barcode tags. The cDNAs are then prepared for Illumina sequencing [22–25]. After sequencing runs, raw data can extract the barcode and UMI of the cells, compare the reference genome to correct the barcode, filter the corrected UMIs, count them, and then differentiate between the barcode of the containing cells and the background barcode according to an algorithm that extracts real single-cell data [27]. This process yields a gene expression matrix for each cell type. Furthermore, cell clustering and data normalization can be performed using various algorithms. Common dimensionality reduction analysis algorithms, such as PCA, t-SNE, and UMAP, are widely used to identify cell groups and marker genes [28, 29].

Specialized protocols of single-cell sequencing in retinal research

The scRNA-seq protocol for the retina largely mirrors that used for other tissues. Rachayata et al. described a detailed methodology for retinal scRNA-seq, including retinal sampling, single-cell isolation, and sequencing data analysis [30]. However, the retina is fragile and sensitive to mechanical forces and enzymatic degradation, particularly in pathological conditions. To address this issue, Fadl et al. designed an optimized scRNA-seq protocol to protect the delicate and complex tissues of the retina during sequencing runs [31]. Retinal dissection and single-cell preparation on ice should be performed to minimize damage. The preparation of single-cell suspensions from methanol-fixed samples is suggested for tissues that cannot be processed immediately. Additionally, antioxidants were incorporated into the single-cell suspension to mitigate the oxidative stress induced by cell preparation. Compared to standard protocols, modifications by Fadl et al. increased cell viability and reduced cell loss, thereby enhancing the accuracy of the single-cell data (Fig. 1) [31].

Applications of scRNA-seq in normal retinal tissue

Retinal cell atlas

Researchers have continually described and updated the retinal atlas by introducing scRNA-seq into retinal studies (Fig. 2). In 2015, Macosko et al. first used Drop-seq to analyze 44,808 mouse retinal cells and identified 39 distinct cell clusters [32]. In 2019, Lukowski et al. used five retinal samples from three human donors and clustered retinal cells into 18 clusters representing photoreceptor, Müller, bipolar, longissimus-free, retinal ganglion, astrocytic, microglial and others cell types [33]. Their study updated human retinal transcriptome profiles, laying the foundation for cell typing in subsequent single-cell sequencing studies of the retina. Furthermore, Chen et al. noted that earlier studies had analyzed only a single dataset with an insufficient number of cells, which did not fully characterize the retinal cell atlas. They performed a joint analysis of eight human and mouse scRNA-seq datasets (containing 276,402 cells) and identified multiple cross-species retinal cell marker genes [34]. These data provide clear insights into the transcriptome profiles of human retinal cell types.

The use of scRNA-seq can also elucidate subregional variations in the retina. Viogt et al. used scRNA-seq to explore the fovea centralis and peripheral retina in three human samples, differentiating the expression of 148 genes between the macular and peripheral retinal cells [35]. Notably, they identified enrichment of the gene encoding beta-carotene oxygenase 2 in peripheral optic cone cells, suggesting that deficiency of this enzyme in the macula may contribute to carotenoid accumulation, resulting in yellow pigment deposition [35]. Peng et al. applied scRNA-seq to the fovea centralis and peripheral retina of *Macaca rhesus* and found similarities in cell types but differences in cell ratios and gene expression in both regions [36]. Similarly, Yan et al. analyzed the fovea centralis and peripheral retina in normal adults using scRNA-seq, reporting nearly identical cellular compositions between these areas but notable disparities in

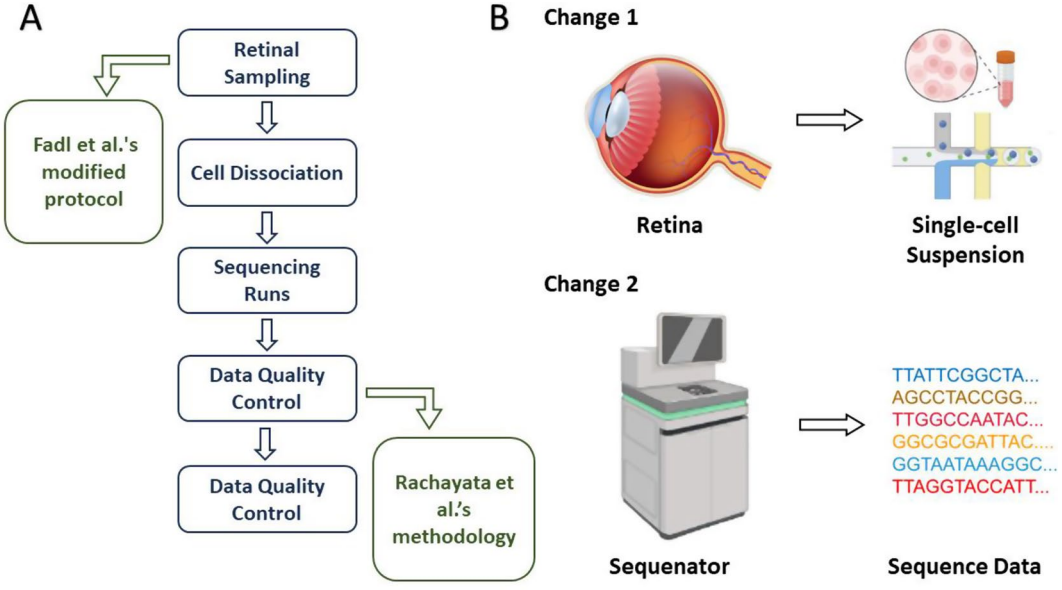

Fig. 1 Single-cell sequencing protocols in retinal research. (**A**) General protocols and specialized applications of single-cell sequencing in retina research. (**B**) Two main challenges in single-cell sequencing in retinal research: how to obtain single cells and how to avoid PCR amplification errors and interference from other molecules to accurately form RNA sequencing libraries

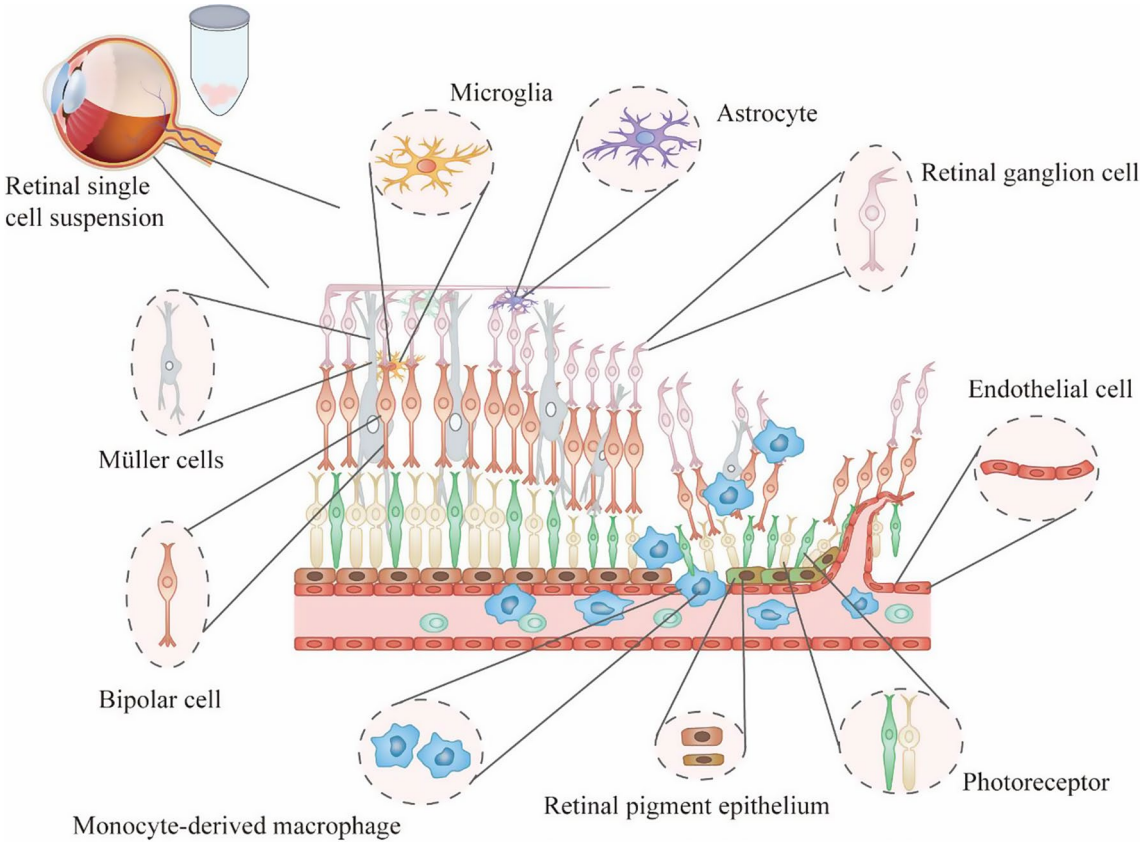

Fig. 2 Retinal anatomy and cell atlas. Using scRNA-seq, researchers clustered retina cells into many clusters, including photoreceptor, Müller, bipolar, retinal ganglion, microglial, astroglial, endothelial, retinal pigment epithelial, and macrophage cells

gene expression and proportions [37]. Collectively, these studies elucidate the mechanistic basis of the specialized functions of the fovea centralis.

scRNA-seq can be used to analyze both the transcriptomes of various retinal cells and their developmental trajectories, making it a powerful tool for investigating the function and development of retinal cells. Hu et al. used scRNA-seq to identify key regulators and cellular markers in the development of human fetal retinal cells, elucidating critical timelines and events in retinal cell formation [38]. Clark et al. used scRNA-seq to analyze the development of the mouse retina and concluded that NFI factors specifically expressed by retinal progenitor cells regulate retinogenesis in the human fetal retina [39]. Lu et al. used scRNA-seq to analyze the retina in 16 time windows across four developmental stages and identified species-specific gene expression patterns during human and mouse retinal development [40]. They also found that ATOH7 expression plays an important role in the regulation of photoreceptors during late retinogenesis [40]. Huang et al. applied scRNA-seq to more than 600,000 cells from nine developmental stages, ranging from 23 weeks of gestation to 81 years of age, across six spatial subdivisions of the human retina to explore regional and temporal gene expression differences from development to senescence [41]. To understand the changes in cellular composition and intrinsic gene regulatory networks during retinal aging, Yi et al. used scRNA-seq to establish molecular maps of retinal aging in both human and rhesus macaques [42]. These studies indicated that retinal development is finely regulated, with different cells exhibiting distinct developmental characteristics and key regulators.

Specific retinal cells

The retina comprises multiple cell types that detect and transmit light signals, thereby maintaining retinal homeostasis through complex interactions [43]. The use of scRNA-seq has identified more than 100 retinal cell subtypes, each with distinct physiological functions and transcriptional characteristics [30]. Therefore, it is essential to study the cell types and functions of each type of retinal cell individually to understand how the overall physiological functions of the retina are distributed among these cells, which, in turn, will elucidate the alterations observed in pathological states.

Human photoreceptors are classified as optic cone or rod cells. These photoreceptors receive light signals and

convert them into electrical signals that are transmitted to bipolar cells. Yi et al. identified two novel classes of rod cells (MYO9A + and MYO9A–) and demonstrated a decline in photoreceptors with aging, notably a significant reduction in MYO9A-rod cells [42]. This suggests that MYO9A plays a crucial role in the anti-aging process in rod cells [42].

Synaptic transmission from photoreceptors to bipolar cells, generates an action potential that is transmitted to retinal ganglion cells (RGCs) [43]. RGC axons traverse the optic nerve, providing the sole conduit for visual information from the retina to the brain. Anaplastic cells are responsible for the complex processing of retinal visual images, particularly the regulation of brightness and darkness. Rheaume et al. used scRNA-seq to analyze mouse RGCs screened by immune antibody coating and identified more than 40 RGC subtypes and their corresponding marker genes [44]. They also discovered that KLP9 gene silencing, particularly in RGC subtypes, enhanced axon regeneration [44]. Similarly, Tan et al. used scRNA-seq to identify 46 RGC species in the mouse retina [45]. Daniszewski et al. identified the transcriptome of human embryonic stem cell-derived RGCs by scRNA-seq [46]. Shekhar et al. identified 15 bipolar cell subtypes by scRNA-seq [47]. Yan et al. analyzed 32,000 anaplastic cells by scRNA-seq and ultimately identified 63 AC isoforms [48].

Many glial cells exist in the retina, among which microglia are a class of active macrophages. Microglia primarily participate in immune processes, including inflammatory responses and antigen presentation, and play a crucial role in maintaining retinal microenvironmental homeostasis [49]. O'Koren et al. identified two microglial subtypes with distinct distribution patterns in the mouse retina using scRNA-seq [50]. They also observed that, under pathological conditions, microglia migrate substantially toward the subretinal space and adhere to the retinal pigment epithelium (RPE) to preserve the structural integrity of the outer retina and RPE [50].

Müller cells are the most prevalent neuroglia in the mammalian retina and support the function of photoreceptors and other retinal neurons by maintaining retinal homeostasis. Extensive research has demonstrated the critical role of Müller cells in DR [51]. Liu et al. used scRNA-seq to examine Müller cells in normal and degenerative macular retinas, revealing a Müller cell subtype with low mitochondrial DNA (mtDNA) expression and reduced protein synthesis in degenerative macular retinas [52]. This subtype, which is characterized by impaired homeostatic regulation, diminished proliferative capacity, and enhanced proangiogenic function, shows alterations in abundance rather than the generation of new subtypes under degenerative conditions [52]. This finding further proves that pathological changes in DR, such as neovascularization, may result from shifts in the abundance of Müller cell subtypes.

The retinal pigment epithelium (RPE), a layer of epithelial cells in the outermost part of the retina, is crucial for maintaining retinal homeostasis [53]. Xu et al. analyzed three samples of human RPE using scRNA-seq and identified two RPE subtypes, macular (specifically expressing ID3) and peripheral (highly expressing CRYAB), each with distinct functions [54]. Voigt et al. conducted scRNA-seq using retinal cells from humans without or with age-related macular degeneration, revealing that choroidal capillaries specifically express regulators of cell cycle genes that respond to complement activation and induce apoptosis in endothelial cells [55].

These studies not only highlight the diversity of retinal cell types but also identify key molecules that regulate behavior (Table 1). Understanding the subtypes and key transcriptional regulators of these cells using scRNA-seq will enhance our understanding of retinal cellular heterogeneity and function.

Applications of scRNA-seq in DR

Pathogenesis of DR

Normal intercellular communication and mutual regulation are essential for maintaining retinal homeostasis. In the pathological state of DR, disruptions in retinal homeostasis can lead to abnormal cell polarization or cell subtype imbalances along with alterations in intracellular transcriptomes and intercellular interactions [56]. Single-cell sequencing enables researchers to identify cell subtypes, analyze transcriptomics, and assess intercellular communication and developmental trajectories. Therefore, the application of single-cell sequencing in various DR models provides a microscopic perspective for understanding the pathological mechanisms of DR (Table 2).

Mouse models are commonly used to study DR. For example, Akimba mouse model, generated by crossing Akita (Ins2) mice carrying a mutation in the Ins2 gene with Kimba (trVEGF029) mice, serves as a valuable tool for studying DR [57]. Van Hove et al. used Akimba mice to investigate DR and identified macroglial subtypes with distinct fibrotic, inflammatory, and gliotic profiles [58]. They also found that macroglia enhance the chemotaxis and activation of immune cells by influencing antigen processing, presentation, and interferon signaling, which, in turn, can augment inflammatory responses and potentially impair the supportive functions of Müller cells [58]. Niu et al. used leptin receptor-deficient db/db mice for scRNA-seq and cell communication studies [59]. They observed that the upregulated RLBP1 expression in Müller cells served as a therapeutic target for mitigating neurovascular degeneration in DR [59]. Sun et al. performed scRNA-seq on streptozotocin induced diabetic mice and

Table 1 Retinal cells found by scRNA-seq

Retinal cell	Cell subtype	Function	Studies and Years
Photoreceptor	MYO9A + and MYO9A– rod cells	MYO9A- rod cells declined while aging, suggesting that MYO9A may play a crucial role in the anti-aging process in rod cells	Yi et al. (2021) [42]
Retinal ganglion cells (RGCs)	40 RGC subtypes in the mouse retina	KLP9 gene silencing, particularly in RGC subtypes, enhanced axon regeneration	Rheaume et al. (2021) [44]
	46 RGC subtypes in the mouse retina	46 RGC subtypes and their corresponding marker genes were investigated	Tan et al. (2019) [45]
Bipolar cell (BC)	15 bipolar cell subtypes	15 BC subtypes and their corresponding marker genes were investigated	Shekhar et al. (2016) [47]
Anaplastic cell (AC)	63 Anaplastic cell subtypes	63 AC subtypes and their corresponding marker genes were investigated	Yan et al. (2020) [48]
Microglia	2 microglial subtypes in the mouse retina	Under pathological conditions, microglia migrated and adhered to the retinal pigment epithelium (RPE) to preserve the structural integrity	O'Koren et al. (2019) [50]
Müller cell	Müller cell subtype with low mitochondrial DNA (mtDNA) expression	This Müller cell subtype diminished proliferative capacity and enhanced proangiogenic function	Liu et al. (2022) [52]
Retinal pigment epithelium (RPE)	2 RPE subtypes, macular (specifically expressing ID3) and peripheral (highly expressing CRYAB)	2 RPE subtypes showed distinct functions in human retina	Xu et al. (2021) [54]

Table 2 Studies on the use of scRNA-seq to analyze the pathogenesis of diabetic retinopathy

Model	Number of cells	Cell type	Findings	Studies and Years
Akimba mice	9,474 cells	Macroglia	Macroglia may enhance chemotaxis and activation of immune cells and augment inflammatory responses	Van Hove et al. (2020) [57]
Leptin receptor-deficient *db/db* mice	51,558 cells	Müller cell	Highly expressed RLBP1 in Müller cells may serve as a therapeutic target to mitigate neurovascular degeneration	Niu et al. (2021) [58]
Streptozotocin-induced diabetic mice	> 14,000 cells	Endothelial cells	Activation of IL-17 signaling pathways in endothelial cells	Sun et al. (2021) [59]
Streptozotocin-induced diabetic rats	35,910 cells	Müller cell	Ctxn3 + Müller cells mediate endothelial damage and angiogenesis through intercellular communication pathways such as Dll4–Notch1 and Vegf/Vegfr2	Wang et al. (2022) [60]
Diabetic cynomolgus macaques (*Macaca fascicularis*)	8,708 cells	Microglia	TNF-α signaling activates microglia in an autocrine manner	Xiao et al. (2021) [61]
Human PDR-FVM	6,894 cells	Microglia	Microglial cells exhibit profibrotic and antifibrotic properties and characteristics	Hu et al. (2022) [62]
Human PDR-FVM	4,044 cells	Pericyte	Pericytes regulate the pericyte–myofibroblast transformation via AEBP1 signaling	Corano Scheri et al. (2023) [63]

PDR-FVM, proliferative diabetic retinopathy-fibrovascular vitreous membranes; RLBP1, retinaldehyde binding protein 1

found that Cirbp, Rmb3, Mt1, and Mt2 were commonly induced across various retinal cell subtypes [60]. Their study also analyzed the transcriptomic changes in different retinal cells under diabetic conditions and experimentally confirmed the activation of IL-17 signaling in endothelial cells in DR [60].

Rat models have also been used to simulate DR and perform single-cell sequencing. Wang et al. analyzed 35,910 retinal cells from healthy and streptozotocin-induced diabetic rats using scRNA-seq and defined two subtypes of Müller cells: Ctxn3– and Ctxn3+ [61]. Their study suggested that Ctxn3 + Müller cells mediate endothelial damage and angiogenesis through intercellular communication pathways, such as those involving Dll4-Notch1 and Vegf/Vegfr2 signaling [61]. In addition, Xiao et al. used scRNA-seq to analyze the retina of diabetic cynomolgus macaques (*Macaca fascicularis*) and found that TNF-α signaling mediated microglial activation in an autocrine manner via intercellular communication [62]. This finding aligns with the study of Niu et al., which demonstrated diabetes-induced microglial activation through intercellular communication [59]. These studies collectively emphasize the importance of Müller cells and microglial alterations in DR.

Some studies have used scRNA-seq to draw a cell atlas of the human pathological environment in DR. Fibrovascular vitreous membranes (FVMs) are significant pathological hallmarks of proliferative diabetic retinopathy (PDR) [63]. Hu et al. performed scRNA-seq on PDR-FVMs obtained by ophthalmic surgery and found that microglial cells originating from the retina exhibited profibrotic and antifibrotic properties [64]. Similarly, Scheri

et al. used scRNA-seq to analyze PDR-FVMs and found that pericytes regulate pericyte–myofibroblast transformation via AEBP1 signaling, suggesting that targeting AEBP1 may prevent scar tissue formation in advanced DR [65].

Pathological changes of DR

Several studies have used scRNA-seq to investigate the pathological changes associated with DR from various perspectives. Vascular pathology plays a crucial role in DR pathogenesis, and pericytes actively maintain vascular homeostasis. Pericyte dysfunction may also contribute to the progression of DR [66]. Xia et al. identified a unique pericyte type associated with capillary dysfunction using scRNA-seq and Col1a1 was identified as a marker gene for this pericyte subtype [67]. Similarly, Xu et al. used scRNA-seq to explore the link between diabetic nephropathy (DN) and DR and discovered that renal mesangial cells and retinal pericytes share common molecular features and undergo similar transformations in response to diabetes [68]. The shared chemokine regulation between these cell types is critical for the coexistence of DN and DR [68]. Taken together, these studies highlight the dysregulation of vascular homeostasis and pericyte alterations in diabetes-related lesions. In addition, the immune system significantly influences the progression of vascular injury in diabetes [69]. Liao et al. used scRNA-seq to profile the circulating immune cells in DR and demonstrated that JunD is a crucial regulatory factor in endothelial dysfunction [70]. Optic nerve injury and ganglion cell apoptosis may also occur in DR. For instance, Becker et al. integrated mRNA and miRNA transcriptome analyses with public single-cell retinal atlases and identified neuronal loss and accompanying transcriptomic changes associated with DR [71].

Some studies have integrated data from single-cell and other sequencing databases. Zhang et al. analyzed two scRNA-seq datasets from the Gene Expression Omnibus (GEO) database and observed that the abundance of Müller, endothelial, microglial, and bipolar cells changed during DR progression [72]. They also noted that the changes in cellular pathways were mainly related to oxidative stress and inflammatory pathways [72]. Similarly, Gao et al. integrated two public bulk RNA-seq datasets and one scRNA-seq dataset from patients with proliferative DR and identified periostin as a potential key factor in DR progression [73]. Using a combined analysis of transcriptome alterations in mRNA and miRNA among both bulk RNA-seq data and public retinal single-cell data, Becker et al. showed that RGC-mediated optic nerve injury is associated with changes in Müller cell-mediated histidine and β-alanine signaling in DR [71]. In addition, by integrating single-cell data from humans and mice, Chen et al. found that bipolar cell subtypes were the most sensitive to pathological changes in DR across all datasets analyzed [34]. These studies show that integrating single-cell data from multiple databases facilitates the elucidation of DR pathogenesis.

Further application of single-cell sequencing in DR and retina research

Human cell atlas and meta-atlas of the retina

Advancements have been made in single-cell transfer sequencing research from the genomic era to the cell atlas era [74]. The Human Cell Atlas aims to establish a comprehensive reference atlas for all human cells [75]. Sequencing data from 33 tissues, 289 donors, and 450,0000 single cells, including the retinal single-cell atlas, are continuously updated in this program [75–77]. Besides the retinal cell atlas updated by Lukowski et al., Swamy et al. developed the single-cell eye (scEiaD) database [33, 78]. The scEiaD database is a user-friendly online retinal atlas that offers data exploration, visualization, and computational tools (R package) for scRNA-seq data analysis [78]. While facing difficulties in dealing with the heterogeneity of data from different sources (e.g., different batches, experiments, and technique-specific variations), single-cell databases integrate data from multiple studies. The evolution and the conserved physiological functions of the retina were investigated by Gautam et al. and Yamagata et al., respectively, who did comparative studies of the human retina with other species, such as pigs and chickens [79, 80]. Additionally, new single-cell sequencing techniques like a single-cell assay for transposase-accessible chromatin (scATAC-seq) have contributed to the construction of a multi-omics atlas of the human retina [81, 82]. In conclusion, the integration of extensive single-cell data continuously evolves the single-cell retina atlas into a multi-species, multi-omics, and multi-dataset meta-atlas accessible online. This information will significantly enhance the ability of doctors and researchers to analyze single-cell retinal data, thereby improving our understanding of retinopathy.

Retinal organoids

Retinal organoids (RtOgs) represent a novel approach for studying retinopathies and offer a promising source for artificial retinal transplantation, both of which benefit research and treatment of DR [83, 84]. Single-cell sequencing is a powerful tool for analyzing RtOgs because of its excellent cellular-level analytical power, and RtOgs have proven to be a good source of samples for single-cell sequencing [85, 86]. Thus, single-cell sequencing and RtOgs, cutting-edge methodologies used in ophthalmology, have significant potential. Currently, established retinal organoids are mainly derived from human embryonic stem cells (hESCs), human pluripotent stem cells (hPSCs), and induced pluripotent stem

cells (iPSCs) [87]. Additionally, Brancati et al. developed EyeSee4is, an online tool for analyzing RtOg spatiotemporal data [85].

To use RtOgs for the research and treatment of retinopathies, including DR, we need to identify similarities and differences between RtOgs generated in vitro and normal retinas developed in vivo [88]. Using scRNA-seq, Sridhar et al. compared the transcriptomes of human fetal retinas and hPSC-derived RtOgs and found similar proportions of various cell types at the same developmental stage [89]. Collin et al. analyzed hESC-derived RtOgs using scRNA-seq and identified nine clusters of RtOgs: five corresponding to the main retinal cell types and four representing mitotic cells and extracellular matrix components [90]. Wahle et al. established iPSC-derived RtOgs and resolved their spatiotemporal profiles using single-cell multi-omic sequencing [91]. They demonstrated the suitability of iPSC-derived RtOgs for scRNA-seq studies because of their similarity with the human retina, as confirmed by immunohistochemical assays and scRNA-seq [91]. The concordance of scRNA-seq data between RtOgs and normal retinas indicates that RtOgs closely resemble the retina and can be used to model retinal development in vivo.

Several studies have investigated the factors that regulate the development of RtOgs. Using scRNA-seq, Mao et al. analyzed human hESC-derived 3D RtOgs in six time windows before and after retinal neurogenesis, suggesting that CCND1 promotes early retinal neurogenesis [92]. Cowan et al. conducted scRNA-seq and comparative analyses of iPSC-derived RtOgs at seven developmental stages across different regions and concluded that the cell types in these organoids mature in vitro through a stable developmental process akin to human retinal development in vivo [93].

Single-cell spatiotemporal multi-omics sequencing

Recently, single-cell sequencing has evolved from single-cell transcriptomic sequencing to single-cell spatiotemporal multi-omics sequencing, which involves stereo sequencing (stereo-seq) and single-cell multi-omics sequencing. Traditional scRNA-seq has two major challenges. First, traditional single-cell sequencing collects single-cell information but loses spatial information, which is crucial for understanding many disease pathologies. The earliest spatial omics technology, such as single-molecule fluorescence in situ hybridization (smFISH), could only perform in situ localization of a few genes [94]. Advances in multicolor fluorescence labeling have enabled the simultaneous detection of hundreds or thousands of genes using techniques such as multiplexed error-robust fluorescence in situ hybridization (MERFISH) and sequential fluorescence in situ hybridization (seqFISH) [95–97]. However, these techniques cannot simultaneously capture genome-wide gene expression. Stereo-seq overcomes these limitations by capturing single cells and their RNAs in tissues using a Stereo Chip and restoring their spatial locations through coordinate ID (CID) [98]. This enables the detection of single-cell histological information in situ. Spatiotemporal omics technology allows the exploration of histological changes in tissue cells and the mapping of disease spatiotemporal atlases, thus enhancing the resolution of pathological alterations in tissues [99]. Although single-cell spatiotemporal genomic technology can only collect spatial transcriptional information at a single time point, computational approaches for pseudotime analyses of scRNA-seq data can reconstruct cellular developmental trajectories, thereby integrating the spatiotemporal gene expression of single cells [100, 101].

Second, while scRNA-seq only collects transcriptomic information, single-cell multi-omics sequencing enables simultaneous analyses of genomes, epigenomes, transcriptomes, proteomes, and other emerging histological modalities at the single-cell level, which enhances our understanding of biological mechanisms and genotype–phenotype relationships [102]. Several single-cell multi-omics sequencing protocols that jointly analyze the genome and transcriptome have been developed, including single-cell triomics sequencing (scTrio-seq), genome and transcriptome sequencing (G&T-seq), and gDNA-mRNA sequencing (DR-seq) [103–105]. These protocols reflect the transcriptional status of the genome and provide cellular genome-transcriptome correlations. Single-cell proteome sequencing protocols using mass spectrometry (MS), such as single cell proteomics by Mass Spectrometry (SCoPE-MS) and single cell proteomics 2 (SCoPE2, a second-generation method improving upon SCoPE-MS), are only suitable for single-cell proteome sequencing and currently fail to synthesize multi-omics information [106]. Cellular indexing of transcriptomes and epitopes by sequencing (CITE-seq) and RNA expression and protein sequencing assays (REAP-seq) utilize similar protocols that associate gene expression with cell-surface protein information at the single-cell level [107]. Single-cell epigenomic sequencing can elucidate DNA methylation, chromatin accessibility, and histone modifications in each cell [108–110]. For example, the single-cell assay for targeting accessible chromatin with high-throughput sequencing (scATAC-seq) provides insights into chromatin accessibility and the interactions of DNA-binding proteins, such as transcription factors, with genomic DNA, thereby elucidating the regulatory networks governing genomic expression in single cells [111].

Therefore, researchers can combine stereo sequencing and single-cell multi-omics sequencing to analyze the spatiotemporal multi-omics information of single

cells comprehensively. In the future, integrating extensive multi-omics single-cell data into public databases will facilitate the construction of multi-omics cellular atlases [112]. This will enable researchers to gain a comprehensive understanding of cellular states and various levels of gene expression and regulation at different spatial locations within a single cell.

Single extracellular vesicle sequencing

Exosomes are extracellular vesicles (EVs) with diameters ranging from 30 to 150 nm that are present in various body fluids, including tears, aqueous humor, and vitreous humor [113, 114]. Exosomes are biomarkers of ocular diseases because they can exist stably in ocular fluids and carry rich genetic information, including omics information on proteins, lipids, mRNAs, non-coding RNAs, and other molecules [115–118]. A liquid biopsy provides information on intraocular lesions reflected by exosomes from the ocular fluids. However, most current methods detect EVs in bulk, neglecting exosome heterogeneity [119–123]. Smith et al. employed Raman spectroscopy to analyze the chemical composition of individual exosomes and identified at least four groups of exosomes with varied functions in one cell line, suggesting that exosomes are more heterogeneous than previously thought [124]. Recent advancements in exosome platforms using single-cell technologies have enabled sequencing of single exosomes (SEVs) [125]. These technologies may lead to a more precise detection of ocular exosomes and improve the accuracy of ocular disease diagnosis [126]. Given the significant role of exosomes in DR-related pathological changes and the absence of highly specific early markers of DR, single-exosome sequencing may be a powerful tool for addressing exosome heterogeneity and enhancing diagnostic accuracy.

Conclusion

Single-cell sequencing is crucial in ophthalmic research because of its detailed cellular resolution, which enables the analysis of individual cell transcriptomes, developmental trajectories, and differentiation origins. DR involves complex mechanisms that affect nearly all retinal cells, making single-cell sequencing particularly useful. Advancements in multi-omics and spatiotemporal genomics have evolved from single-cell sequencing into a spatiotemporal multi-omics sequencing era, enhancing our understanding of DR-induced changes. Integrating single-cell spatiotemporal data using single-cell sequencing of RtOgs and employing single-exosome sequencing links this technology with other advanced research areas, advancing DR diagnosis and treatment. This review summarized recent advancements in single-cell sequencing of normal and DR-affected retinas. We propose that single-cell sequencing will significantly deepen research on DR and other retinopathies and reveal potential diagnostic and treatment directions in the future.

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Declarations

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