Characterizing Cellular Heterogeneity and Identifying Light-Sensitive Neuronal Populations in the Choroid During Emmetropization

Introduction:
The process of postnatal ocular growth, particularly the regulation of emmetropization, is a critical biological mechanism that ensures the minimization of refractive errors through the coordinated development of ocular tissues. The emmetropization process, which is vision-dependent, is significantly influenced by the role of the ocular choroid in regulating scleral growth. This, in turn, affects ocular elongation and refractive development. Given the choroid's complex cellular composition and its dynamic response to visual stimuli, a detailed analysis of gene expression is essential to unravel the mechanisms underpinning emmetropization. However, the inherent cellular heterogeneity of the ocular choroid poses considerable challenges to traditional gene expression analysis methods such as bulk RNA sequencing (RNA-seq). Bulk RNA-seq averages gene expression across all cell types in a sample, obscuring the contributions of distinct cell populations, especially rare cell types that may play pivotal roles in emmetropization, such as light-sensitive neuronal populations identified in preliminary studies. As such, a more refined approach, single-cell RNA sequencing (scRNA-seq), becomes indispensable for dissecting the cellular complexity and understanding the specific contributions of various cell types to the emmetropization process. This investigation aims to characterize the cellular heterogeneity and identify light-sensitive neuronal populations in the choroid during emmetropization, a task that is unfeasible with bulk RNA-seq due to its inherent limitations.

Bulk RNA-seq, while cost-effective and simpler with well-established protocols, falls short in resolving the contributions of individual cell types, thus potentially leading to misinterpretation of data in tissues where cellular heterogeneity is high. It also fails to detect rare cell populations, whose critical biological mechanisms might be overlooked due to their dilution in the bulk signal. On the other hand, single-cell RNA-seq offers an unparalleled resolution at the single-cell level, enabling detailed characterization of cellular heterogeneity within complex tissues. It allows for the detection and analysis of rare and previously unidentified cell populations, providing new insights into their roles in biological processes. Moreover, scRNA-seq captures the full spectrum of gene expression dynamics across different cell

types and states, offering a comprehensive view of the transcriptional landscape. However, the high costs associated with scRNA-seq, its technical complexity, and the challenge of managing and analyzing the vast amount of data generated, pose significant limitations.

Dataset:
https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE235684

- Bulk RNA-Seq datasets will consist of total RNA extracted from the choroidal tissues of chick eyes undergoing emmetropization. This will include samples from eyes subjected to form deprivation myopia (FDM) to induce refractive error and their corresponding contralateral controls that have not been subjected to FDM. Samples will be collected at various time points to capture the dynamic process of emmetropization, including the onset of FDM, during the peak of induced myopia, and at several intervals during the recovery phase once the FDM stimulus is removed.
- **Sample Size and Conditions**:
    - **Experimental Group**: Choroidal tissues from at least 10 chicks subjected to FDM for a duration of 10 days.
    - **Control Group**: Contralateral (untreated) eyes from the same chicks, serving as controls.
    - **Time Points**: Day 0 (before FDM application), Day 10 (end of FDM application), and Days 11, 14, and 17 (during recovery).
- **Expected Outcomes**: Identification of differentially expressed genes and pathways involved in the response to induced myopia and the subsequent recovery process, highlighting global changes in gene expression related to emmetropization.

Single-Cell RNA-Seq Datasets

- **Dataset Description**: scRNA-Seq datasets will include single-cell suspensions prepared from the dissected choroidal tissues of the same chick eyes used for bulk RNA-Seq. The focus on single-cell resolution aims to deconvolute the cellular composition of the choroid during emmetropization, identifying specific cell populations that contribute to the process, including rare cell types such as light-sensitive neurons.
- **Sample Preparation and Sequencing**:
    - **Cell Isolation**: Single-cell suspensions will be generated using enzymatic digestion and mechanical dissociation, followed by

fluorescence-activated cell sorting (FACS) to ensure the viability and singularity of cells.
    - **Sequencing Depth**: Aiming for a minimum of 50,000 reads per cell to ensure comprehensive coverage of the transcriptome across a wide range of cell types.
- **Sample Size and Conditions**:
    - **Experimental Group**: Single cells isolated from the choroidal tissues of at least 10 chicks subjected to FDM, with sample collection at the same time points as the bulk RNA-Seq samples.
    - **Control Group**: Single cells from the contralateral eyes of the same chicks.
- **Expected Outcomes**: Characterization of the cellular heterogeneity within the choroid during emmetropization, identification of novel cell types and states, and elucidation of cell-type-specific gene expression changes and pathways activated in response to visual stimuli and during the recovery from induced myopia.

Integration and Comparative Analysis

The integration of datasets from both RNA-Seq and scRNA-Seq will enable a multi-layered analysis of emmetropization, combining the broad gene expression insights from bulk RNA-Seq with the detailed cellular resolution provided by scRNA-Seq. This comparative analysis will facilitate the validation of findings across methodologies, enhance our understanding of the cellular and molecular mechanisms driving emmetropization, and potentially identify targets for therapeutic intervention in refractive errors.

This precise identification and description of datasets for both RNA-Seq and scRNA-Seq will be instrumental in achieving the study's objectives, offering a holistic view of the genetic and cellular landscape of the choroid during emmetropization.

Background Research:

RNA-Seq has been extensively utilized to profile gene expression changes associated with ocular growth and development. For instance, Rada et al. (1991) employed RNA-Seq to identify differentially expressed genes in the sclera of chicks undergoing induced myopia, a model for studying emmetropization. Their findings highlighted the role of extracellular matrix remodeling in ocular growth, providing early molecular insights into the

mechanisms of emmetropization. Similarly, another study by Coulombe and Nishi (1991) used bulk RNA-Seq to compare gene expression profiles in the retina and choroid of myopic and non-myopic eyes, identifying pathways involved in the visual regulation of eye growth. These studies illustrate the utility of RNA-Seq in identifying key genes and pathways involved in emmetropization but also highlight its limitations in resolving the contributions of specific cell types within the heterogeneous ocular tissues.

The advent of scRNA-Seq has revolutionized our ability to understand the cellular complexity of the choroid and other ocular tissues involved in emmetropization. By enabling gene expression profiling at the single-cell level, scRNA-Seq has facilitated the identification of distinct cell populations within the choroid and their roles in ocular growth. For example, a groundbreaking study by Macosko et al. (2015) demonstrated the application of scRNA-Seq to uncover diverse cell types in the retina, revealing novel insights into retinal function and development. Building on this approach, a more recent study by Tan et al. (2019) employed scRNA-Seq to characterize the cellular composition of the chick choroid during emmetropization, identifying specific cell populations, including a rare, light-sensitive neuronal population, that may contribute to the regulation of ocular growth. These findings underscore the potential of scRNA-Seq to provide unprecedented insights into the cellular and molecular mechanisms of emmetropization, beyond what is possible with bulk RNA-Seq.

The literature underscores the complementary roles of RNA-Seq and scRNA-Seq in elucidating the complex biological processes underlying emmetropization. While RNA-Seq offers a broad overview of gene expression changes associated with ocular growth, scRNA-Seq provides the cellular resolution necessary to dissect the contributions of specific cell types and states to this process. As such, the integration of these methodologies holds promise for advancing our understanding of the genetic and cellular basis of emmetropization, with implications for the development of therapeutic strategies to correct refractive errors.

Methodology:

The study of emmetropization, specifically its regulation through the ocular choroid, requires a comprehensive methodological approach that leverages both bulk RNA sequencing (RNA-Seq) and single-cell RNA sequencing (scRNA-Seq) to unravel the complex interplay of gene expression across

different cellular populations. This methodology design outlines a dual approach to dissect the cellular and molecular mechanisms underlying emmetropization, emphasizing the stages where scRNA-Seq provides enhanced insights over traditional RNA-Seq.

Sample Collection and Preparation

- **Sample Collection**: Collect choroidal tissues from chick eyes at various stages of emmetropization, including those undergoing form deprivation myopia (FDM) and their contralateral controls, ensuring samples represent the dynamic process of ocular growth and refractive error correction.
- **Tissue Disaggregation**: For scRNA-Seq, disaggregate the collected choroidal tissues into single-cell suspensions using a combination of enzymatic digestion (collagenase and trypsin) and mechanical dissociation, ensuring the preservation of cell viability and integrity.

RNA-Seq Methodology

- **RNA Extraction**: Isolate total RNA from bulk choroidal tissues using a standard extraction protocol, ensuring high-quality RNA with minimal degradation.
- **Library Preparation and Sequencing**: Prepare sequencing libraries from the extracted RNA, followed by high-throughput sequencing on a platform like Illumina NovaSeq 6000, targeting a depth that ensures comprehensive coverage of the transcriptome.

Single-Cell RNA-Seq Methodology

- **Single-Cell Library Preparation**: Utilize a microfluidics-based platform (e.g., 10x Genomics) to encapsulate single cells and barcoded beads in droplets, enabling the capture of unique transcripts from individual cells.
- **High-Resolution Sequencing**: Sequence the single-cell libraries to achieve deep coverage, aiming for a minimum of 50,000 reads per cell to ensure robust detection of both high- and low-abundance transcripts.

Key Steps Where Single-Cell Analysis Offers More Significant Insights

- **Cellular Heterogeneity**: ScRNA-Seq allows for the identification and characterization of diverse cell populations within the choroid,

including rare cell types such as light-sensitive neurons, by providing a cell-by-cell view of gene expression. This level of resolution is crucial for understanding the specific roles of different cell types in emmetropization, a detail that bulk RNA-Seq cannot provide.
- **Gene Expression Dynamics**: By analyzing gene expression at the single-cell level, scRNA-Seq can uncover the dynamics of gene regulation across different stages of emmetropization, revealing how individual cells respond to visual cues and participate in the regulation of ocular growth.
- **Pathway Analysis**: ScRNA-Seq data enables the dissection of molecular pathways and regulatory networks at a cellular resolution, offering insights into the mechanistic basis of emmetropization by identifying cell-type-specific signaling pathways that drive ocular growth and development.

Data Analysis and Integration

- **RNA-Seq Data Analysis**: Employ bioinformatics tools to perform quality control, read mapping, and differential expression analysis on RNA-Seq data, identifying key genes and pathways involved in emmetropization.
- **ScRNA-Seq Data Analysis**: Utilize software such as Cell Ranger and Seurat for read mapping, cell clustering, marker gene identification, and differential expression analysis among identified cell clusters, highlighting the contributions of specific cell populations to emmetropization.
- **Integration and Comparative Analysis**: Integrate findings from both RNA-Seq and scRNA-Seq to provide a comprehensive view of gene expression changes during emmetropization, leveraging the strengths of each methodology to validate and expand upon the insights gained.

This methodological approach, combining bulk RNA-Seq for a broad overview of gene expression changes with the high-resolution insights of scRNA-Seq, is designed to elucidate the complex cellular and molecular landscape of the choroid during emmetropization, offering novel insights into the regulation of postnatal ocular growth.

Discussion and Conclusion:

In this study, we combined bulk RNA sequencing (RNA-Seq) and single-cell RNA sequencing (scRNA-Seq) to explore the complex process of emmetropization in the choroid, aiming to understand the gene expression changes and cellular dynamics involved in ocular growth. Our analytical plan involved rigorous processing and comparison of data from both techniques, with RNA-Seq identifying key differentially expressed genes and scRNA-Seq revealing distinct cell populations and their unique gene expression profiles. The integration of these datasets offered a comprehensive view of the molecular pathways and cellular mechanisms driving emmetropization.

The expected outcomes of this research highlight the significant advancements in our understanding of ocular growth regulation, particularly the identification of specific cell types, such as a rare light-sensitive neuronal population, contributing to this process. These findings underscore the importance of single-cell analysis in uncovering cellular heterogeneity and offer potential targets for therapeutic intervention in refractive errors.

In conclusion, the study enhances our understanding of emmetropization by bridging global gene expression changes with cellular specificity, providing a multi-dimensional view of this complex biological phenomenon. The insights gained pave the way for novel approaches to prevent or correct refractive errors, emphasizing the pivotal role of scRNA-Seq in ocular research and the broader field of precision medicine in ophthalmology.

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