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Full Title

Fully Automated Detection, Segmentation, and Analysis of In Vivo RPE Single Cells

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Conflict of Interest

The authors TL, MK, FBC, and CM are involved in a company (EarlySight SA) aiming at commercializing the TOPI technology.

Running title

Automated Analysis of In Vivo RPE Single Cells

Keywords

Retinal Pigment Epithelium; Image Interpretation; Retina; Diagnostic Techniques, Ophthalmological; Computer-assisted image analysis

Abstract

Objective: To develop a fully automated method of retinal pigmented epithelium (RPE) cells detection, segmentation, and analysis based on *in-vivo* cellular resolution images obtained with the transscleral optical phase imaging method (TOPI).

Methods: 14 TOPI-RPE images from 11 healthy individuals were analysed. The developed image processing method encompassed image filtering and normalization, detection and removal of blood vessels, cell detection, and cell membrane segmentation. The produced measures were cellular density of RPE layer, cell area, number of neighbouring cells, eccentricity, circularity, and solidity. Additionally, we proposed coefficient of variation (CV) of RPE cellular membrane (CMD_{CV}) and the solidity of the RPE cell membrane-shape as new metrics for the assessment of RPE single cells.

Results: The observed median cellular density of the RPE layer was 3,743 cells/μm^2 (interquartile rate (IQR) 1,687), with a median observed RPE cell area of 193 μm^2 (IQR 141). The mean number of neighbouring cells was 5.22 (standard deviation (SD) 0.05) per RPE cell. The mean RPE cell eccentricity was 0.67 (SD 0.02), median circularity 0.83 (IQR 0.01), and median solidity 0.92 (IQR 0.00). The median CMD_{CV} was 0.19 (IQR 0.02). The method is characterized by a median image processing and analysis time of 48 seconds (IQR 12) per image.

Conclusions: The present study provides the first fully automated quantitative assessment of human RPE single cells in-vivo. The method provides a baseline for future research in the field of clinical ophthalmology, enabling characterization and diagnostics of retinal diseases at the single cell level.

Introduction

The retina is the vitreal-most ten-layered light-sensitive nervous tissue membrane of the eye. Its role is to convert the received light stimuli into nerve impulses and send them with the optic nerve to the visual centres of the brain. The retinal pigmented epithelium (RPE) is the scleral-most monolayer of pigmented retinal cells.

Although they are located outside of the neurosensory retina, RPE cells play some crucial roles, such as light absorption, epithelial transport, and maintenance of the visual cycle[1–5]. Some RPE cell morphology characteristics, namely cell density, number of neighbours, eccentricity, and form factor, are postulated to differ depending on cell maturation and condition[2,6–9]. Some other studies report RPE cell loss caused by diseases of the eye and aging[10–13].

Although several diagnostic imaging modalities allow for in vivo assessment of the human eye (e.g., optical coherence tomography (OCT)[15–17], scanning laser ophthalmoscopy (SLO)[18,19], and fundus autofluorescence[20,21]) these methods do not allow for the diagnosis of retinal diseases at their early stage because the minuscule changes in RPE cell morphology cannot be detected. Furthermore, RPE layer in vivo imaging at the single cell level is challenging due to several factors, namely, the low contrast between neighbouring cells, motion artefacts, retinal layer non-linearity, and difficulties with the image's focal point identification.

Transscleral optical phase imaging (TOPI), proposed in 2017, is a novel non-invasive, in vivo, high-resolution retinal imaging modality. The use of both adaptive optics and oblique illumination of the retina enhances the contrast of RPE cells[2,9]. The resultant superior imaging resolution enables discerning single RPE cells' cellular membranes.

In this paper, we present a novel fully automated method of RPE cell detection, segmentation, and analysis at both layer-level and single cell level for the TOPI-obtained retinal images.

Materials and methods

Study population

The study population included 11 healthy individuals (4 women and 7 men), from which 14 TOPI-RPE images of the left eye were obtained. The mean age of the examined individuals was 29 years (standard deviation (SD) 8). The conducted study adheres to the tenets of the Declaration of Helsinki. The study was approved by the Ethical Committee of the Swiss Department of Health on research involving human subjects (CER-VD N°2017-00976). Informed consent was obtained from all the participants.

TOPI image acquisition

TOPI relies on high-angle oblique illumination of the retina, combined with a flood illumination adaptive optics fundus camera, to enhance cell contrast and correct for ocular aberrations. Transscleral illumination of the retina was performed using two near-infrared light-emitting diodes (wavelength, λ = 810 nm) located on the nasal and temporal side of the eye. The acquired images encompass a field-of-view of 4.4° x 4.4°. More detailed information on the used TOPI setup has been published previously by our group[2].

A single TOPI-obtained RPE layer image is characterized by a low signal-to-noise ratio (SNR). Therefore, prior to image analysis, the SNR is first increased by acquiring several raw images (around 100 per acquisition), then registered, and averaged into a single TOPI image. Image acquisition and registration were performed following the protocol described in detail by Laforest et al.[2]. The acquired image stacks were exported as .tif files. These images are characterized by a black border, resultant from the registration padding process. To remove such padding, first, the image gradient is calculated. Second, vertical and horizontal borders that presented an average local intensity gradient value below 10% of the image's gradient SD were removed, cropping the image to the final size. The eccentricities

from the fovea of the imaged retinal areas ranged between 2.5° and 13.44° with a consistent field-of-view of 5°. The final TOPI-RPE images were exported as 1975 by 1906 pixels and with a digital sampling between 0.73μm and 1.0μm per pixel. Supplementary Table 1 presents detailed information on imaging parameters, registered images, and imaged volunteers.

Image processing

The fully automated TOPI-obtained RPE image processing and analysis is divided into four stages. First, the images are normalized in terms of contrast/attenuation, unevenness of the RPE layer and noise, and any out-of-focus (*OoF*) areas are discarded. Second, the shadow of retinal vasculature present in the innermost (vitreal-most) retinal layers is detected and removed from the final image. Third, cells are individually detected and segmented. Finally, the fourth and last step consists of characterizing the RPE layer in general and single RPE cells (Figure 1).

Image filtering and normalization

In order to adjust for the unevenness of the RPE layer background, flat-field correction with a two-dimensional Gaussian smoothing kernel (σ 10 pixels) was applied. Subsequently, to clean the image from noise, Butterworth highpass filtering (B_{hpf}) and Gaussian filtering (*Gauss*) were performed. The first-order Butterworth filter used a cut-off radius of 50 pixels. The Gaussian filter used a smoothing kernel with a σ of 20 pixels (Figure 1A). To prevent the filtering out of essential RPE morphology, both in the spatial and frequency domain, the filter sizes, thresholds, and values implemented throughout the image processing and analysis methodology, were obtained experimentally and based on previously published literature in the assessment of ex-vivo and in-vivo morphology of RPE cells. The usual RPE cell size varies between 10 and 14 μm (14 – 19 pixels with a digital sampling of 0.73 μm)[7,26,27].

A lower RPE cell edge contrast characterizes the *OoF* areas compared to the in-focus part of the image. To remove the defocused areas, we implemented the channel-prior method[28] with adaptive gamma correction, which increases the remaining image (*deH*) contrast. The method allows for single-image enhancement without a priori knowledge of its' quality or high contrast standard images. Moreover, the channel-prior method produces a distance map (*Dist*) (an estimation of the haze thickness at each pixel), necessary for the vascular outline approximation.

Detection and removal of blood vessels

The detection of blood vessels is performed by using the four previously obtained images (B_{hpf}, *Gauss*, *deH*, and *Dist*) (Figure 1B). Each image is subjected to Subroutine A (*SubA*). *SubA* begins with square-shaping the image, and its quadtree decomposition (*QuaD*) returns a sparse matrix subsequently reconstructed as a block-map. The *QuaD* threshold is applied at 3*SD of the image. *QuaD* is a common methodology in several fields, including image processing, being used from multiresolution decomposition and analysis[29–31], to compression[32,33] and machine learning[34,35]. Application of *QuaD* for RPE cells segmentation is a novel approach developed specifically for this project. The *QuaD* square blocks of ≥ 8 pixels and ≤10% of the original image size are included in the subsequent image processing. After inverting (image complement), the obtained square blocks maps, small and interconnected structures at their external borders are discarded using morphological filtering (erosion with a discoid element of 4-pixel radius) followed by dilation with the same discoid element. Finally, the last step of *SubA* is reshaping of the resultant mask to the original's image size. Supplementary Figure 1, Block B, presents a more in-depth depiction of the process, where *Gauss* image is an example input.

The *OoF* mask obtained during image filtering and normalization stage is summed with the binary mean of $SubA(B_{hpf})$, $SubA(_{\text{Gauss}})$, $SubA(deH)$, and $SubA(Dist)$, forming the vessel-*OoF* mask (*VOoF*). *VOoF* mask is used to eliminate the infravascular RPE cells from further image processing.

157 ***Cell detection***

158 Cell centre detection is based on the method proposed by Khamidakh et al.[36], henceforth named
159 Subroutine B (*SubB*). In case the distance between adjacent cellular centres is ≤10 pixels, the individual
160 cells are detected as the same cell. We applied *SubB* to B_{hpf}, to the contrast-limited adaptive histogram
161 equalized B_{hpf}, and to the highpass filtered ($\frac{1}{8}$ of the original image sized kernel) B_{hpf}. One more time,
162 cellular centres within ≤ 10 pixels are fused. Finally, cellular centres in the distance of ≤ 10 pixels from
163 the image border are removed to prevent the inclusion of non-fully-imaged cells in the image analysis
164 (Figure 1C). Supplementary Figure 1, Block C, presents a more in-depth graphical demonstration of
165 the cell detection method, with B_{hpf} as its input.

166 ***Cell membrane segmentation***

167 Detection of the cellular membrane at the single cell level begins with convolving B_{hpf} with a
168 discoid structuring element (radius of 4 pixels). The resultant blurring of the image removes any
169 possible local salt-and-pepper noise that might occur during the transformation from the Fourier to
170 the spatial domain. Then, the image is convolved with a star-shaped mask (size 7 pixels). The
171 convolution enhances local vertical, horizontal, and diagonal edges in the image. The final filter is a
172 7x7-pixel Mexican hat. With these three filtering stages followed by zero-crossing in the spatial
173 domain, a binary mask representing the cellular membrane is developed. Finally, the mask is
174 skeletonized and cleaned from sporadic branches, while single pixels are discarded. The inverted mask
175 is convolved with a discoid structuring element (radius of 4 pixels) and re-inverted (Figure 1D). Such
176 a procedure improves the separation of the cells and prevents their possible overlapping. An example
177 of the process of the cell membrane segmentation method is presented in Supplementary Figure 1,
178 Block D, with the B_{hpf} as input. Cellular masks not corresponding with respective cellular centres
179 identified during the "Cell detection" step are subsequently classified as artefacts and discarded from
180 further analysis.

Supplementary Figure 2 presents an example of the outputs and intermediate results obtained throughout the segmentation and analysis process.

Data analysis

Cells with area or centre overlapping with the *VOoF* mask were discarded from the analysis of cellular characteristics.

Using the previously created cellular masks and the original TOPI-obtained image, morphological, and neighbourhood characteristics of individual RPE cells were assessed (Figure 1E). MATLAB *regionprops* function was used to obtain basic morphological characteristics of RPE cells (area, centroid and weighted centroid, eccentricity, solidity, intensity, and circularity). Additionally, assessed characteristics included the coefficient of variation (*CV*) of RPE cellular membrane (CMD_{CV})[37,38], number of neighbouring cells, and the cellular density of the RPE layer. To decrease the possible risk of assessment bias, RPE cells immediately adjacent to the *VOoF* mask were discarded from the number of neighbours' evaluation. A descriptive analysis was conducted for each image. Supplementary Table 2 presents the full list of the assessed metrics, along with their definitions and formulas.

The normality of variables was assessed with Shapiro-Wilk's test ($p > 0.10$) and histogram skewness (skewness -0.5 – 0.5).

The image processing pipeline and the underlying algorithms were developed and tested, as well as data management, on a DELL workstation (DELL XPS 13 9380, Windows 10, 64 bits, 2 1.80 GHz, 16.0 GB RAM) equipped with the MATLAB (version R2019, with Bioinformatics ToolboxTM, Financial ToolboxTM, and Statistics and Machine Learning ToolboxTM). Image registration was performed with ImageJ 1.52 with a modified macro from Laforest et al.[2], with the plugins TurboReg[39]

and Template Matching[40]. For boxplots generation and statistical analysis, we used R studio 1.2.1335 with gmodels, e1071, readxl, and xlsx packages.

Results

Figure 2 presents the resultant analysis maps for an example sample (number 6), where the RPE coverage was 88%.

Characteristics of the RPE layer

In all obtained image samples, the algorithm analysed a significant image area (median 90%, interquartile rate (IQR) 9%). The discarded parts of the image areas were either blood vessels or blurred/hazed. After discarding cells belonging to the *VOoF* areas (example in Supplementary Figure 2 - *VOoF* subpanel), the mean number of cells analysed per sample was 6,864 (SD 869). The median observed cellular density of the RPE layer was 3,743 cells/μm^2 (IQR 1,687) (Table 1, Supplementary Table 3 and 4, and Supplementary Figure 3).

Characteristics of RPE single cells

The median observed RPE cell area was 193 μm^2 (IQR 141). The mean number of neighbouring cells was 5.22 (SD 0.05) per RPE cell. The mean RPE cell eccentricity was 0.67 (SD 0.02). The RPE cell circularity was at a median 0.83 (IQR 0.01). The median CMD_{CV}, denoting the distance of each cellular membrane-depicting pixel to the cellular centroid, was 0.19 (IQR 0.02). The median solidity of the RPE cells was 0.92 (IQR 0.00). The median RPE cell normalized image intensity value was 0.44 (IQR 0.04). The mean pixel distance between morphology-based and intensity-based RPE cellular centroids was 0.29 pixels (SD 0.06) (Table 1, Supplementary Table 3 and 4, and Supplementary Figure 3).

Performance of the image processing algorithm

The median image processing time was 48 seconds (IQR 12) per image. It included digital image transformations, image segmentation and analysis, rendering and saving metrics, figures, and graphs. The most time-consuming stage was image processing and analysis pertaining to single cell detection and cellular membrane identification, accounting for 65% of the total processing time. The second most tedious stage was metrics calculation (23% of the total processing time) (Table 1, Supplementary Table 3 and 4, and Supplementary Figure 3).

Discussion

Comparison of the results with the literature

The aim of the presented research was the development of an algorithm enabling automated segmentation and analysis of in vivo TOPI-imaged RPE cells at the single cell level.

To the knowledge of the authors, fully automated and user-independent in vivo RPE single cell imaging, segmentation, and analysis has not been achieved before, primarily because high-resolution RPE images were not available.

The most akin method presented in the literature was a protocol for the automated segmentation of RPE cells images obtained with adaptive optics SLO, developed by Rangel-Fonseca et al. [43,44]. Some other authors presented semi-automatic protocols for the detection and segmentation of RPE cells based on the localization of cone cell centers[25,45], non-specific for the actual underlying RPE cells layer. Furthermore, unlike ours, none of the three previous methods proposes a fully automated system of vascular outline elimination from the image processing and analysis. Finally, our method discards the *OoF* region automatically.

The observed characteristics of the RPE layer and RPE single cells are comparable with previously published literature (Figure 3 and Supplementary Table 3); however, the published research data on RPE statistics are all based on ex vivo or in vitro observations. It is thus interesting to compare the results between human in-vivo and ex-vivo/in vitro.

The observed median cellular density (3,743 cells/μm^2) was within the range reported previously in the literature (3,000-5,500 cells/μm^2)[44,46,47]. Similarly, the median observed RPE cell area at 193 μm^2 is within the reported studies[27](140-840 μm^2). The mean number of neighbouring cells was 5.22. Since mature, confluent RPE cell colonies or layers, are commonly described in the literature as hexagonal cobblestone conformation, denoting six neighbours per RPE cell[6–8,25,48–50] (Supplementary Table 3),

this result is somehow unanticipated. Further studies in healthy subjects and different retinal eccentricities need to be undertaken.

The observed mean eccentricity at 0.67 was 27% higher than in the literature[2]. The median circularity at 0.83 was in line with the previously published circularity of 0.74 and matching well with the theoretically ideal circularity of 0.84 shown by Bhatia et al. [7](Supplementary Table 3). To our knowledge, both CMD_{CV} and cell solidity were used for the first to characterize RPE cells. Hence, a comparison of our findings with previously published literature was not possible.

Strengths and limitations of the study

The presented image processing method is dependent on the high quality of the TOPI-obtained retinal images. Albeit all images are normalized and filtered, imaging is still dependent on various parameters, both intrinsic and extrinsic to the TOPI setup. Conditional external factors to the TOPI setup include morphological heterogeneity of eye dependent on visual impairment, age, and illumination conditions.

The primary strength of the study is the fully automated analysis of the TOPI images, which allows for reproducibility and replicability of the results. Another strength of the presented image processing algorithm is a short analysis time, acceptable both for research and for clinical purposes (48 seconds per image). All 14 samples (from 11 individuals) were able to be analysed and provide metrics both at the cell and colony level.

The small sample size can somewhat decrease the generalizability of the findings regarding RPE cells characteristics. Moreover, as the whole study population was composed of healthy volunteers, the performance of the developed image-processing pipeline on patients with degenerative diseases cannot yet be assessed.

Significance of the findings and suggestions for future research

To our knowledge, this work is the first fully automated image processing pipeline developed for high resolution TOPI-obtained RPE images both at layer-level and single cell levels.

The presented results are a step towards the possible implementation of automated RPE cell analysis in clinical practice for diagnostics of several retinal diseases. The use of non-invasive, real-time, and fully automated evaluation of the retinal cells can facilitate the work in both ophthalmological research and practice. Furthermore, the RPE cells' assessment method proposed in this paper is time-efficient (median of 48 seconds per image). Finally, the analysis of the retinal cells' morphology both on layer-level and single cell level opens new paths for eye health assessment and follow-up.

In the future, associations between different morphological features of the retinal cells and the type of vision impairment should be assessed. Cross-sectional studies with larger study populations should be conducted to validate and assess the performance of the developed method in adapting to the variability of the in vivo RPE layer. Furthermore, to assess the system and analysis pipeline utility in the follow-up and surveillance measure, prospective cohort studies need to be undertaken.

Conclusions

In this study, a new fully automated image processing method for segmentation and analysis of high-resolution TOPI-imaged RPE cells was presented. It is the first report on the RPE single cells in vivo characteristics. The present study lays the groundwork for future research in the field of clinical ophthalmology, enabling characterization and diagnostics of retinal diseases on the single cell level.

Supplementary Information

Supplementary information is available at Eye's website. Colour figures are available on the online version of the manuscript, as also supplementary figures and tables.

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Titles and legends to figures

Figure 1. TOPI-RPE image processing diagram. From acquisition to single cell masks for an example sample. Cyan boxes represent 9x zoomed areas of the underlying structures. (Other example zoomed regions from each step are presented in Supplementary Figure 3) (For the colour version, please refer to the online version of the article)

SubA: subroutine A.

SubB: subroutine B.

VOoF: Vessel and out-of-focus mask

Figure 2. Sample 6: Analysis results, with final metrics maps. Area of the assessed RPE layer: 88%. CMDCV: coefficient of variation of RPE cellular membrane mask (For the colour version, please refer to the online version of the article)

Figure 3. Spider graph comparison between literature and study obtained parameter ranges. (For the colour version, please refer to the online version of the article)

Data Availability

The image processing codes and datasets generated and or analysed during the current study are available from the corresponding author on reasonable request and subject to the ethical approvals in place and material transfer agreements.

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Author contributions

- FLCS: Study concept and design, analysis and interpretation of data, drafting the manuscript, critical revision of the manuscript for important intellectual content, statistical analysis, and technical and material support;
- TL: Acquisition of data and critical revision of the manuscript for important intellectual content;
- MK: Acquisition of data and critical revision of the manuscript for important intellectual content;
- LK: Critical revision of the manuscript for important intellectual content;
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- CM: Critical revision of the manuscript for important intellectual content, obtained funding, and study supervision.

Abbreviations and Acronyms:

B_{hpf}	Butterworth highpass filtered image
CMD_{CV}	cellular membrane mask coefficient of variation
CV	coefficient of variation
deH	dehazed image
Dist	distance map
FLIO	fluorescence lifetime imaging ophthalmoscopy
FOV	field of view
Gauss	Gaussian filtered image
IQR	interquartile rate
OCT	optical coherence tomography
OoF	out-of-focus mask
QuaD	quadtree decomposition
RPE	retinal pigmented epithelium
SD	standard deviation
SLO	scanning laser ophthalmoscopy
SNR	signal-to-noise ratio
SubA	subroutine A
SubB	subroutine B
TOPI	transscleral optical phase imaging
VOoF	vessel & out-of-focus mask