

Repeatability and Reproducibility of an Automated Image-Processing Software for Quantitative Conjunctival Hyperemia Analysis: A Pilot Study

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ABSTRACT

OBJECTIVE Conjunctival hyperemia reflects the severity of various ocular conditions and is a common side effect of glaucoma medications, posing a significant concern for affected patients. Most existing clinical grading systems are subjective and rely heavily on individual physician interpretation, making interobserver comparison difficult. We developed an automated grading software to objectively and quantitatively assess conjunctival hyperemia. This study aimed to evaluate the repeatability and reproducibility of a newly developed automated software for quantitative assessment of conjunctival hyperemia in glaucoma patients in a pilot validation study.

METHODS In this pilot study, glaucoma patients using at least one topical anti-glaucoma medication and exhibiting conjunctival hyperemia were enrolled. Each patient's conjunctival hyperemia was assessed using the automated software, which segmented images into four regions: blood vessel area, strong red area, weak red area, and non-red area. An automatic region of interest (ROI) registration function was incorporated to enhance measurement consistency. Repeatability was assessed using two measurements from the same image, and reproducibility was tested using separate images of the same eye.

RESULTS Eighty images from twenty glaucoma patients were analyzed (collected between December 1, 2016 to December 31, 2018). Following administration of 10% phenylephrine ophthalmic solution, the percentage of conjunctival hyperemia decreased significantly in all regions ($p < 0.001$). Spearman correlation coefficients for repeated measurements on the same image were 0.884 (blood vessel), 0.982 (strong red), and 0.932 (weak red). Corresponding values for repeated images of the same eye were 0.988, 0.992, and 0.995, respectively. The intraclass correlation coefficient (ICC) for clinical grading by ten ophthalmologists was 0.969 (95% CI: 0.952–0.981).

CONCLUSION This pilot study demonstrates that the proposed automated software provides an objective and quantitative assessment of conjunctival hyperemia with excellent repeatability and reproducibility. The system shows high correlation with clinical grading and may serve as a reliable tool for monitoring conjunctival hyperemia changes in clinical practice.

KEYWORDS repeatability, reproducibility, conjunctival hyperemia, automated, software

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INTRODUCTION

Conjunctival hyperemia occurs in various ocular diseases, including inflammatory conditions (e.g., allergic conjunctivitis, scleritis, uveitis), infections (e.g., conjunctivitis, keratitis), dry eye syndrome, and drug-induced ocular surface disorders (1). The degree of hyperemia correlates with disease severity, making it a key indicator in clinical monitoring. In glaucoma patients, conjunctival hyperemia is a common side effect of topical medications, affecting treatment adherence and quality of life.

Several subjective grading scales, such as the Efron scale, the original Cornea and Contact Lens Research Unit (CCLRU) scale, and the Brien Holden Vision Institute scale, are commonly used in clinical practice (2–5). However, these rely on physician interpretation and suffer from interobserver variability. Currently, many objective conjunctival grading systems have been developed to improve accuracy in clinical assessment, but they are still not widely available in routine clinical practice (6–23).

We developed an automated software tool to objectively evaluate conjunctival hyperemia in glaucoma patients. This software quantitatively assesses hyperemia using pixel coverage in pre-defined regions of interest (ROIs), categorized into blood vessel area, strong red area, weak red area, and non-red area. This pilot study aimed to evaluate the software's repeatability and reproducibility, as well as its correlation with clinical grading.

METHODS

Design

This cross-sectional pilot study was conducted in the Department of Ophthalmology, Faculty of Medicine, Chiang Mai University, from December 1, 2016 to December 31, 2018. The software was created and developed in collaboration with the Department of Computer Engineering.

Participants

We recruited 20 open angle glaucoma patients who were using at least one anti-glaucoma medication and complained of conjunctival hyperemia. The severity of conjunctival hyperemia was evaluated in all patients using both the automated conjunctival hyperemia grading software and assessments by ten ophthalmologists.

The study protocol was approved by the Ethics Committee of the Faculty of Medicine, Chiang Mai University (approval number: 452/2015; approved October 26, 2015). The study followed the tenets of the Declaration of Helsinki. Informed consent was obtained from all participants.

Image collecting and processing

The temporal and nasal bulbar conjunctival images were taken using a Topcon SL-D7 digital-ready slit lamp. We used diffuse illumination, a 30-degree angle between the lamp and microscope arm, brightest light level and 175 Watt/second flashlight. The objective magnification used was 10. All images were saved in red (R), green (G), and blue (B) JPEG format with a resolution of 3264 × 2448 pixels. All images were taken twice with a 10-second interval. After finishing taking the first image, the patients were moved away from the slit lamp and their head position was readjusted before taking the second image. Two images were acquired before and after repositioning to evaluate intra-observer repeatability. The next step was to apply the topical 10% phenylephrine ophthalmic solution which is a strong vasoconstrictor and waited for 30 minutes. We then took another two images using the same protocol. These images were processed using our newly developed conjunctival grading software to evaluate repeatability and reproducibility.

Software algorithm

According to the direction of the lighting sources, there was sometimes reflected light or glare on eye surface inside ROI. The system first identified the glare area by considering R, G, and B values of each pixel in the image. For 24-bit color images which have the maximum R, G, and B values of 255, if R, G, and B were greater than 250, the system marked that pixel as glare pixel and removed it from the image. (Figure 1) Note that, in this step, median filter was applied to refine the shape of glare regions.

The software transformed the input image from RGB Color Space to YCbCr Color Space where Y is a luminance of the pixel, Cb is a blue-difference chroma of the pixel and Cr is a red-difference chroma of the pixel.

Y and Cr of pixels are used to identify blood vessel pixels. Multi-scale morphological top-hat operators were applied to Y Channel to remove

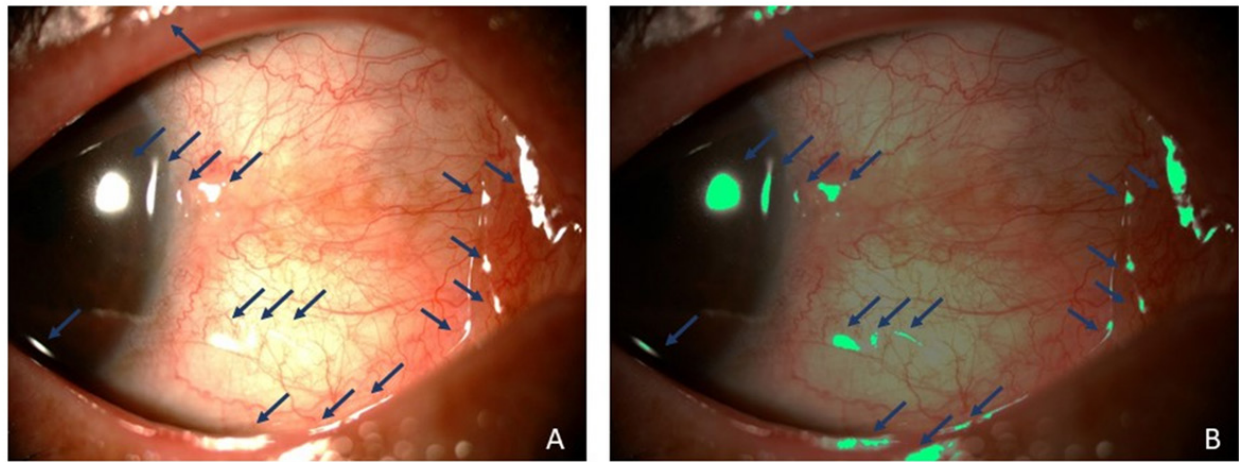


Figure 1. Anterior segment images show (A) light reflection or glare area is identified by pixel detection. (blue arrow) (B) all light reflection or glare area will be removed from the image before starting conjunctival hyperemia grading process.

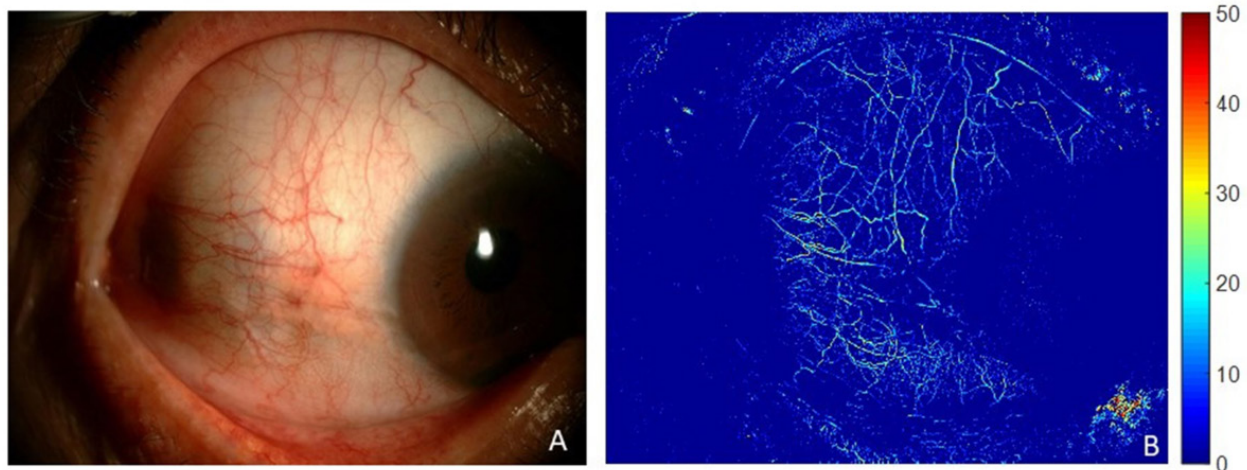


Figure 2. Anterior segment images show before (A) and after (B) the system transforms the input image from RGB Color Space to YCbCr Color Space and compute a blood vessel enhanced image V.

a nonuniform lighting condition (24). Then, the software computed a blood vessel enhanced image V which is defined by this following equation (see also Figure 2)

$$V = \begin{cases} 255 - Y & ; Cr > 0, \\ 0 & ; Cr \leq 0. \end{cases}$$

To obtain blood vessel pixels, an adaptive thresholding was applied to V. Based on assumption that real blood vessel pixels should be connected to other pixels, tiny connected components were considered as random noise and removed. Finally, a morphological thinning operator is applied to the result to improve the shape of blood vessel. (Figure 3)

After the software identified blood vessel, the other pixels were classified according to three redness classes (i.e, strong red pixels, weak red

pixels, and normal pixels). The input image was transformed from RGB Color Space to HSV (hue, saturation, value) Color Space. H channel (hue of pixel in range of [0,1]) was used to classify redness of pixels as

$$Class = \begin{cases} Strong\ Red & ; |0.5 - H| \geq Th_1, \\ Weak\ Red & ; Th_2 \leq |0.5 - H| < Th_1, \\ Normal & ; otherwise. \end{cases}$$

In this work, we set the threshold values as $Th_1 = 0.45$ and $Th_2 = 0.42$. Based on the color scale shown in Figure 4, the hue value of the pure red color is equal to 0 or 1. If we consider the colors with the hue value between [0, 0.05] and [0.95, 1.00], those colors are very close to pure red based on human perception. Colors with hue values near 0.00 or 1.00 correspond to red on the HSV scale; values between 0–0.05 and 0.95–1.00 are

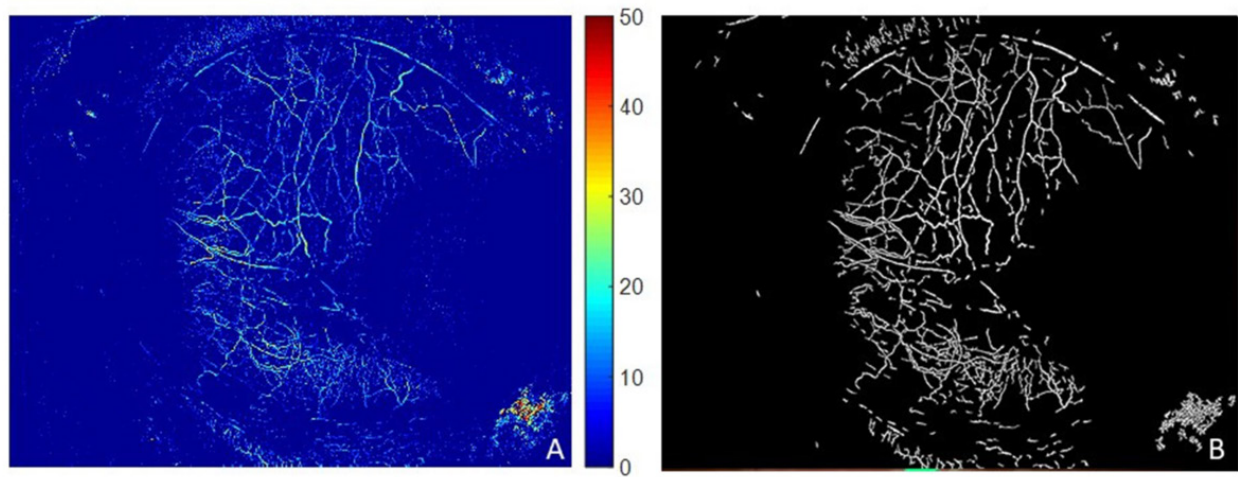


Figure 3. Images demonstrate before (A) and after (B) use of the system, an adaptive thresholding and a morphological thinning operator to improve the blood vessel shape.

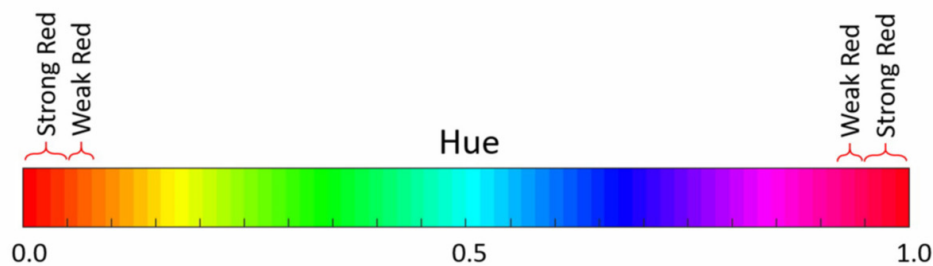


Figure 4. Color scale that illustrates H values and their corresponding colors.

perceptually indistinguishable from pure red. An average just identifiable hue value of pure red is around 0.04, with a standard deviation of around 0.01 (25). As a result, we set the value of Th_1 as 0.45. The visible blood vessels on the white part of the eye (sclera) are bright red or reddish-pink because they are near the surface, allowing us to see the blood's color directly. Depending on the lighting conditions and the camera's white balance, their hue can sometimes appear red-orange. The red-orange and reddish-pink colors have hues of around 0.08 and 0.92, respectively. That's why we set the value of Th_2 as 0.42.

The number of pixels in each area is counted and compared to the number of pixels in ROI, resulting in the area ratio in percentage.

The ROIs on the bulbar conjunctiva were manually chosen in the nasal and temporal areas, which are easily observable by others. We evaluated the percentage of blood vessels and degree of conjunctival hyperemia in the four classes (blood vessel area, strong red area, weak red area and non-red area).

A local feature matching is used for the automatic ROI registration between two images. (26, 27) The algorithm can be described as follows: (Figure 5)

A. Key-points were extracted from both images. For a reference image for which the user has manually selected ROI, the key-points outside ROI will be removed. The key-points are typically defined as corner pixels in the image; therefore, most of the key-points in the reference image are located along blood vessels. However, the key-points in the target image were typically distributed over the whole image (including blood vessels).

B. The system determined the key-points in the target image that most likely matched the key-points in the reference image by comparing Histogram of Oriented Gradients (HoG) (28) of the pixels surrounding the key-points.

C. Based on matched key-point pairs, the system identified a geometric transformation matrix between coordinates in the two images. To find an optimal parameter of the transformation matrix, Random Sample Consensus (RANSAC) was applied (29). RANSAC can determine the optimal

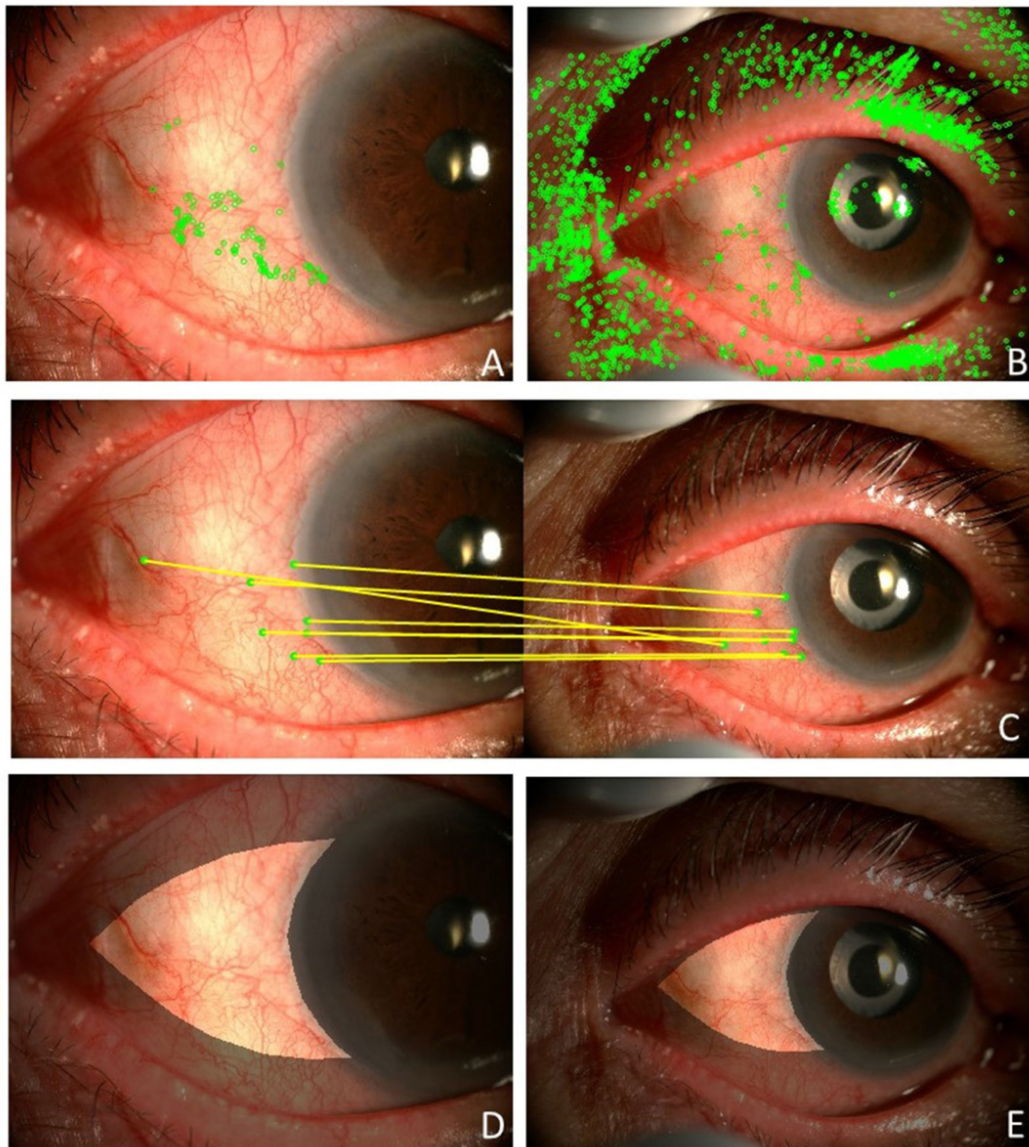


Figure 5. Key-points (green dots) along blood vessel are extracted from the reference image. (A) Key-points (green dots) over the whole image are extracted from the target image. (B) The system determines the key-points in the target image match to the key-points in the reference image by comparing the pixels surrounding the key-points. (C) The coordinates of the pixels in region of interest in the reference image transform to the coordinates in the target image by using the transformation matrix (D, E).

parameters with robustness to any outliers (i.e., incorrectly matched pairs).

D, E. Finally, the coordinates of the pixels in ROI in the reference image are then transformed to the coordinates in the target image by using the transformation matrix.

Clinical grading

Ten ophthalmologists independently viewed and graded each image on a 0 to 4-point scale based on the Efron grading scale (2, 3) without any time limitation. The reference grading scale was shown together with the image being graded on the same page to eliminate the inconsistencies

between computer screen's display.

Statistical analysis

All statistical analyses were performed using SPSS version 23.0 (SPSS Inc., Chicago, Illinois, USA). Normality of data was tested using Shapiro-Wilk test, and it is noted that all data sets showed non-normal distributions. As such, the percentage of conjunctival hyperemia before and after the application of 10% phenylephrine ophthalmic solutions were compared using the Wilcoxon matched pairs test. P value < 0.05 was considered significant.

Repeatability of the software was assessed by comparing two hyperemia percentage measurements from the same image. Statistical tests included the Wilcoxon signed-rank test, the Spearman correlation coefficient (r_s), and the Bland-Altman method.

Reproducibility was evaluated by comparing hyperemia percentages from two separate images taken 10 seconds apart, using the same set of statistical tools: the Wilcoxon signed-rank test, Spearman's r_s , and Bland-Altman analysis.

Reliability of the clinical grading from ten ophthalmologists was shown in intraclass correlation coefficient: The correlation of clinical grading and automated grading was tested by the Spearman correlation coefficient (r_s).

The strength of correlation for Spearman's (r_s) was interpreted as follows: 0.00–0.19: very weak, 0.20–0.39: weak, 0.40–0.59: moderate, 0.60–0.79: strong and 0.80–1.0: very strong.

RESULTS

A total of 80 images were analyzed using our newly developed automated grading software. The average of the median, lower quartile, and upper quartile of the percentage of conjunctival hyperemia before applying 10% phenylephrine ophthalmic solution for each image were 10.21 (6.70, 14.64), 3.20 (0.71, 11.87) and 60.38 (42.47, 72.02) for blood vessel area, strong red area and weak red area, respectively. The average of the median, lower quartile, and upper quartile of the percentage of conjunctival hyperemia before applying 10% phenylephrine ophthalmic solution for each image were shown in Table 1. The percentage of conjunctival hyperemia after the application of 10% phenylephrine ophthalmic solutions was significantly reduced in all areas ($p < 0.001$). (Figure 6)

Software repeatability

The repeatability of software was tested by calculating the percentage of conjunctival hyperemia twice of each image. The median, lower quartile, and upper quartile of the percentage of conjunctival hyperemia for the first measurement were 12.31 (9.50, 15.73), 8.03 (3.21, 28.09) and 51.20 (39.14, 63.93) for blood vessel area, strong red area and weak red area, respectively. The median lower, quartile, and upper quartile of the percentage of conjunctival hyperemia for the second measurement of the same image was 12.38 (9.47, 15.60), 7.75 (3.52, 27.01) and 48.67 (38.88, 62.23) for blood vessel area, strong red area and weak red area, respectively. The Spearman correlation coefficients were 0.884, 0.982 and 0.932 for blood vessel area, strong red area and weak red area, respectively. (Table 2)

Bland-Altman plots of the mean difference conjunctival hyperemia percentage in each area of two measurements on each eye were shown in Figure 7. The mean percentage of conjunctival blood vessel area differences were -0.439% ; 95% confidence interval (CI): -1.359% – -0.420% ; 95% limits of agreement (LoA): -3.329% – -2.451% . The mean percentage of conjunctival strong red area differences were 0.196% ; 95% CI: -0.808% – 1.201% ; 95% LoA: -2.956% – 3.313% . The mean percentage of conjunctival weak red area differences were -0.918% ; 95% CI: -3.269% – 1.434% ; 95% LoA: -8.268% – 6.432% .

Software reproducibility

The reproducibility of software was tested by calculating the percentage of conjunctival hyperemia of two images taken 10 seconds apart. The ROI was manually drawn in the first image by user and was automatically registered into the second image by software. The median and IQR of the percentage of conjunctival hyperemia of

Table 1. The median and interquartile range of the percentage of conjunctival hyperemia before and after applying 10% phenylephrine ophthalmic solutions.

The percentage of conjunctival hyperemia in each area			
	Before ^a median (Q1, Q3)	After ^b median (Q1, Q3)	p-value
Blood vessel area (%)	10.21 (6.70, 14.64)	4.21 (2.86, 10.58)	< 0.001
Strong red area (%)	3.20 (0.71, 11.87)	0.27 (0.02, 1.60)	< 0.001
Weak red area (%)	60.38 (42.47, 72.02)	28.93 (15.65, 44.89)	< 0.001

Before^a, before applying 10% phenylephrine ophthalmic solutions; after^b, after applying 10% phenylephrine ophthalmic solutions; Q1, lower quartile; Q3, upper quartile; %, percentage

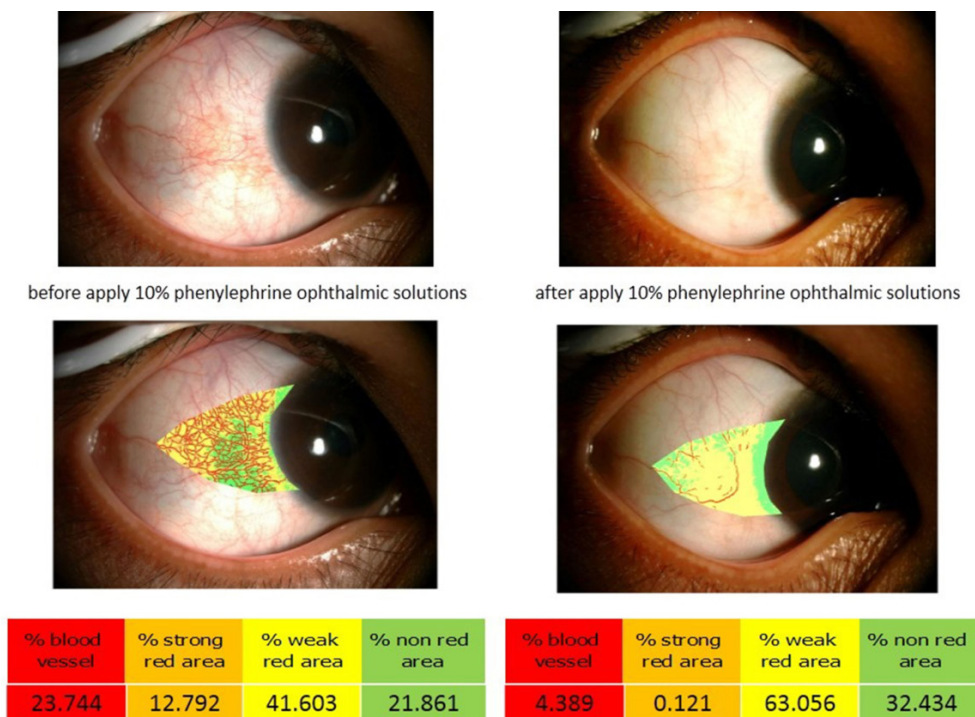


Figure 6. Anterior segment images reveal region of interest of temporal bulbar conjunctiva of right eye. The system evaluates the percentage of blood vessels and degree of conjunctival hyperemia in the four classes (blood vessel area, strong red area, weak red area and non-red area) before (A) and after (B) applying 10% phenylephrine ophthalmic solutions.

Table 2. The median and interquartile range of the percentage of conjunctival hyperemia in each area of twice measurement of the same image.

The percentage of conjunctival hyperemia in each area of twice measurement of the same image			
	1 st measurement median (Q1, Q3)	2 nd measurement median (Q1, Q3)	rs
Blood vessel area (%)	12.31 (9.50, 15.73)	12.38 (9.47, 15.60)	0.884
Strong red area (%)	8.03 (3.21, 28.09)	7.75 (3.52, 27.01)	0.982
Weak red area (%)	51.20 (39.14, 63.93)	48.67 (38.88, 62.23)	0.932

Q1, lower quartile; Q3, upper quartile; rs, Spearman correlation coefficient

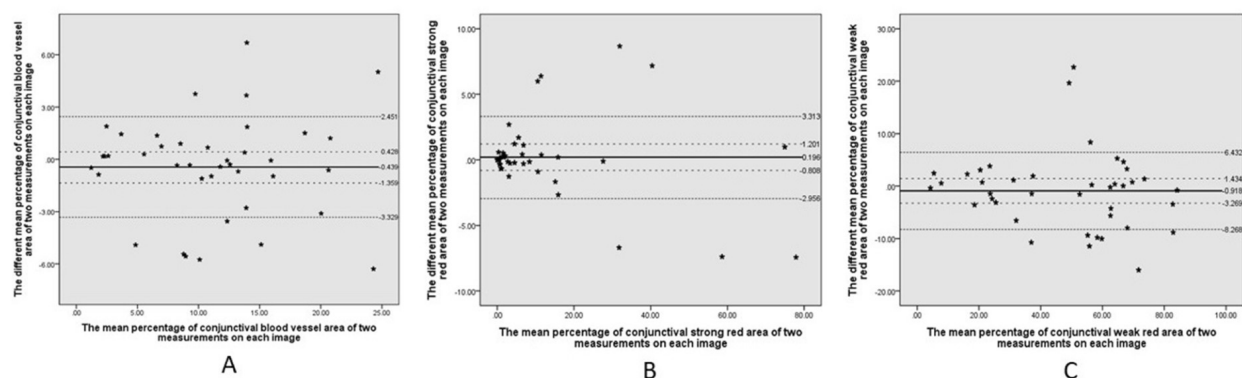


Figure 7. Bland-Altman plots of the mean difference conjunctival hyperemia percentage in each area of two measurements on each image.

- The mean difference conjunctival hyperemia percentage in each area of two measurements
- — — — The 95% confidence limits of mean difference in conjunctival hyperemia measurement
- - - - - The 95% limit of agreement of conjunctival hyperemia measurement

Table 3. The median and interquartile range of the percentage of conjunctival hyperemia in each area of two images of the same eye.

The percentage of conjunctival hyperemia in each area of two images of the same eye			
	1 st image median (Q1, Q3)	2 nd image median (Q1, Q3)	rs
Blood vessel area (%)	10.83 (6.09, 15.44)	11.63 (6.78, 15.00)	0.988
Strong red area (%)	4.76 (0.72, 14.50)	4.256 (1.24, 14.65)	0.992
Weak red area (%)	54.09 (24.29, 64.14)	54.88 (25.88, 65.25)	0.995

Q1, lower quartile; Q3, upper quartile; rs, Spearman correlation coefficient

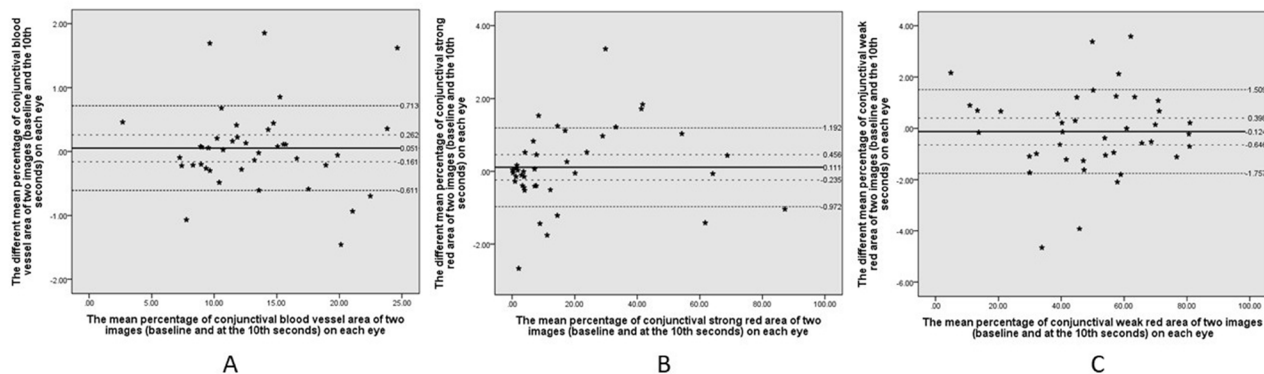


Figure 8. Bland-Altman plots of the mean difference conjunctival hyperemia percentage in each area of two images on each eye.

- The mean difference conjunctival hyperemia percentage in each area of two images
- — — — The 95% confidence limits of mean difference in conjunctival hyperemia measurement
- - - - - The 95% limit of agreement of conjunctival hyperemia measurement

the two images of the same eye were shown in Table 3. The Spearman correlation coefficient (rs) values of 0.988, 0.992 and 0.995 for blood vessel area, strong red area and weak red area, respectively. (Table 3)

In comparison with the Spearman correlation coefficients obtained from the software repeatability testing, we found that the Spearman correlation coefficients obtained from the software reproducibility were higher in all three areas.

Bland-Altman plots of the mean difference conjunctival hyperemia percentage in each area of two images on each eye was shown in Figure 8. The mean percentage of conjunctival blood vessel area differences were 0.051%; 95% CI: -0.161-0.262%; 95% LoA: -0.611-0.713%. The mean percentage of conjunctival strong red area differences were 0.111%; 95% CI: -0.235-0.456%; 95% LoA: -0.972-1.192%. The mean percentage of conjunctival weak red area differences were -0.124%; 95% CI: -0.646%-0.398% and 95% LoA: -1.757% -1.509%.

The intraclass correlation coefficient of clinical grading from ten ophthalmologists was 0.969 (95% CI: 0.952-0.981). The correlation of ten clinical graders and automated grading in each area was tested the Spearman correlation coefficient (rs). (Table 4). The correlation of ten clinical graders and automated grading in the summation of blood vessel and strong red area, as well as in the strong red area alone, as depicted in the graph. (Figure 9)

Table 4. The correlation of clinical grading and various automated grading

	rs
Clinical grading & blood vessel area	0.462
Clinical grading & strong red area	0.752
Clinical grading & weak red area	0.374
Clinical grading & vessel + strong red area	0.824
Clinical grading & vessel + weak red area	0.273
Clinical grading & strong + weak red area	0.401
Clinical grading & vessel + strong red + weak red area	0.574

rs, Spearman correlation coefficient

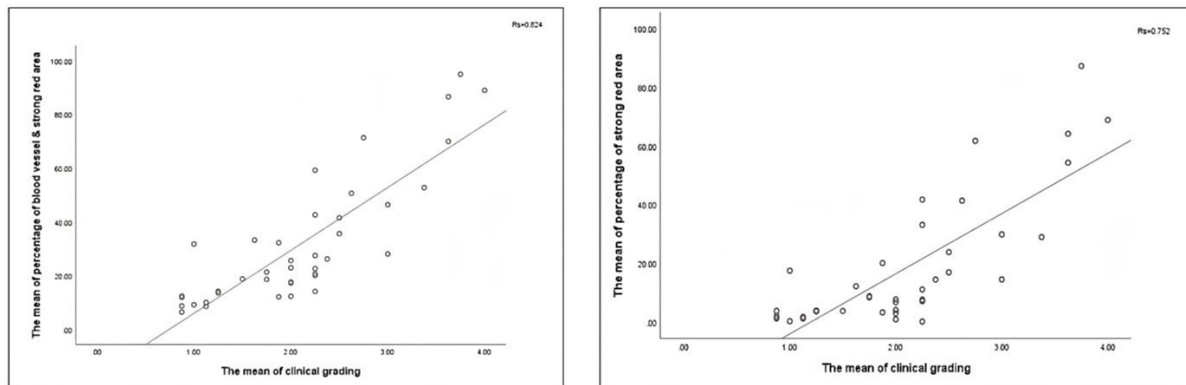


Figure 9. The correlation of ten clinical graders and automated grading.

DISCUSSION

The results of the present study show that our newly developed conjunctival hyperemia analysis software can automatically perform quantitative assessment of conjunctival hyperemia in the percentage of three areas in ROI: blood vessel area, strong red area and weak red area. The percentage of conjunctival hyperemia after application of 10% phenylephrine ophthalmic solutions was significantly reduced in all area which resulted from vasoconstriction effect of phenylephrine. While a self-controlled design comparing pre- and post-medication outcomes could have provided additional strength, we instead employed phenylephrine blanching as a standardized reference to account for vascular variability. This served as an internal benchmark, though it may not fully reflect the long-term effects of anti-glaucoma therapy. The results showed very high repeatability and reproducibility. The clinical grading had the best correlation with the summation of blood vessel and strong red area, followed by the strong red area. The previous studies only focused on blood vessel (6-17, 30) but we here have extended this to blood vessel, strong red area and weak red area. Currently, several objective conjunctival grading systems were developed to improve accuracy in clinical assessment. These grading systems were developed using different software algorithm, such as edge detection, smoothing, color extraction, morphometry and thresholding (6-18, 30). The summary of software algorithm is shown in Table 5.

A primary objective of this study was to determine the repeatability and reproducibility of conjunctival hyperemia analysis software. The results

showed very high repeatability and reproducibility. To the best of our knowledge, there has been no grading software using the same software algorithm as ours. Local feature matching was used for the automatic ROI registration between two images. This algorithm provides precise results in comparing the conjunctival hyperemic change in clinical practice, especially in patients with difficulty maintaining the eye in the same position during taking anterior segment image at every visit. The automatic ROI registration also improves software repeatability and reproducibility.

We found the correlation between clinical grading by ophthalmologist and automated grading scale by our software show the best result with the summation of blood vessel and strong red area, which we can apply for our clinical practice.

Our method incorporates ROI registration and classification into multiple redness categories (blood vessel, strong red, weak red). This approach enhances precision but depends on high image quality and adequate computational resources. The limitations of our study include the fact that no controls were included, so a range of percentage of conjunctival hyperemia in normal subjects was not established. These limitations indicate the need for further investigation in a normal population. A limitation is the use of 10% phenylephrine as a reference, as it produces an unnaturally white appearance that may not represent physiologic baseline conjunctiva. This factor should be considered in interpreting the findings.

To improve the repeatability of the system, the automatic ROI selection should be developed. This work is ongoing. There are some failure cases of ROI registration such as blurred images. We

Table 5. Summary of software processing and algorithm in previous studies

Authors	Summary of software processing and algorithm					
	Image collection tool	Resolution of image (pixel)	Software algorithm	Region of interest	Grading scale	Automatic registration
Schulze MM, et al. (6)	NikonFS-3V	Not mentioned	Luminance/ chromaticity	Average redness	0-100	No
Yoneda T, et al. (8)	Canon	3888 × 2592	Thresholding	Blood vessel	10-point step	No
Wolffsohn JS (15)	Not mentioned	700 × 525	Edge detection	Average redness	%	No
Fieguth P, et al. (7)	Not mentioned	Not mentioned	Edge detection	Average redness	%	No
Peterson RC, et al. (30)	NaCV-53200, Yokohama	Not mentioned	Edge detection	Blood vessel, Average redness	0-100	No
Sumi T, et al. (19)	Topcon	3216 × 2136	Thresholding	Pixel coverage	%	No
Li M, et al. (23)	Haag-streit BX900, Topcon SLD2, Kanghua SL-7E	512 × 768	Deep learning-based segmentation/ classification (mask distillation)	Blood vessel, Average redness	1-4	No
Ostheimer P, et al. (22)	Occyo GmbH	Not mentioned	color-intensity-based formula	Average redness	-0.5-1.0	No
The present study	Topcon	3264 × 2448	Thresholding/ Morphological top-hat transformation/Local features	Blood vessel, Average redness in 3 scale	%	Yes

suggest that the quality of the image should be assessed before input to the grading system.

To improve the correlation of the system to the clinical grading by ophthalmologist, more experiments should be conducted to identify the optimal threshold for area classification.

The experiments presented in this paper demonstrated the robustness of the system to variation of the eye gazing directions, scales of the eye in the image, orientation of the eyes, the directions of light sources. Further investigation should be done for evaluating the robustness across different light sources or image resolution.

CONCLUSION

This pilot study validates an automated conjunctival hyperemia analysis software that delivers objective, repeatable, and reproducible results. The proposed software provides an automated, quantitative, and objective assessment of conjunctival hyperemia with excellent repeatability and reproducibility, including strong correlation with clinical grading by ophthalmologists.

This pilot validation also demonstrates that the algorithm's use of automatic ROI registration between two images gives precise results for tracking conjunctival hyperemic changes in clinical practice. The use of our software may help improve disease monitoring in various ocular conditions.

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CONFLICTS OF INTEREST

The authors have no conflicts of interest to report

AUTHOR CONTRIBUTIONS

L.H.: conceptualization, methodology, investigation, data curation, formal analysis, and writing – original draft and writing – review & editing; K.Path.: supervision and writing – review & editing; N.T.: supervision and writing – review & editing; W.C.: supervision and writing – review & editing; S.A.: supervision and writing – review & editing; P.K.: data curation, investigation, and writing – review & editing; N.P.: data curation and writing – review & editing; T.E. o'brien: supervision, formal analysis, and writing – review & editing; K.Pat.: conceptualization, methodology, investigation, supervision, writing – original draft, and writing – review & editing.

All authors approved the final version of the manuscript and agreed to be accountable for all aspects of the work.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request

INSTITUTIONAL REVIEW BOARD STATEMENT:

The study protocol was approved by Research Ethics Committee, Faculty of Medicine, Chiang Mai University (Certificate of Approval No. 452/2015; Study Code: OPT-2558-03328; Research ID: 3328; approval date: October 26, 2015). The study was conducted in accordance with the Declaration of Helsinki and applicable institutional ethical guidelines.

INFORMED CONSENT STATEMENT:

Informed consent was obtained from all subjects involved in the study.

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