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Measurement variability of the bulbar conjunctival microvasculature in healthy subjects using functional slit lamp biomicroscopy (FSLB)

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Abstract

The goal was to determine the variability of the quantitative measurement of the bulbar conjunctival microvascular morphology and hemodynamics by testing the repeatability and variation during office hours. Functional slit-lamp biomicroscopy (FSLB) was used to image the bulbar conjunctival microvasculature, including the vessel diameter, blood flow velocity/rate and fractal dimensions of the microvascular network. The temporal side of the bulbar conjunctiva in 20 healthy subjects was imaged. The subject was imaged at 9AM to test the measurement repeatability by two independent graders. The intraclass correlation coefficient (ICC) and coefficient of repeatability (CoR) were calculated. These same subjects were then imaged every two hours from 9AM to 5PM to test the variation during office hours. Custom software was used to semi-automatically process all measurements. The CoR% and ICC values between two graders for measuring the vessel diameter were 4.87% and 0.989, respectively. For the axial blood flow velocity, the CoR% and ICC were 11.49% and 0.997, respectively. From 9AM to 5PM, There were no significant variations in the vessel diameter and hemodynamics ($P > 0.05$). Whereas, the fractal dimensions of the non-invasive microvascular perfusion maps (nMPMs) were significantly increased at 3PM and 5PM compared with the baseline obtained at 9AM ($P < 0.05$). FSLB appears to be capable of measuring vessel diameter, blood flow velocity and fractal dimension of the

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microvascular network in the bulbar conjunctiva. Slight variations over office hours were observed in the microvascular network, while the blood flow velocity remained stable.

Keywords

bulbar conjunctival microvascular morphology; hemodynamics; functional slit lamp biomicroscopy (FSLB); repeatability; diurnal variation during office hours

Introduction

The semi-transparent conjunctiva and white sclera readily allow the visualization and quantitative analysis of the conjunctival microvascular morphology and hemodynamics. The microvasculature of the bulbar conjunctiva has been the *in vivo* real-time study site for both ocular and systemic microvascular function in physiological and pathological conditions, such as dry eye (Rodriguez et al., 2013), contact lens wear, (Jiang et al., 2014; Cheung et al., 2012) diabetes (To et al., 2011; Cheung et al., 2009) and sickle cell disease (Wanek et al., 2013; Cheung et al., 2001). Distinct vasculopathies on the bulbar conjunctiva have been identified in these conditions (Jung et al., 2013; Wolf et al., 1990; Jung et al., 1983; Cheung et al., 2012; Wanek et al., 2013; Jiang et al., 2014; Rodriguez et al., 2013; To et al., 2011; Cheung et al., 2009; Cheung et al., 2001). Several techniques have been developed to examine the conjunctival blood flow *in vivo*, including modified scanning laser Ophthalmoscopy (Duench et al., 2007), adapted slit-lamp biomicroscopy digital imaging (Wanek et al., 2013; Shahidi et al., 2010a; Koutsiaris et al., 2010a), slit-lamp stereomicroscope (Jung et al., 1983) and computer-assisted intravital microscopy (CAIM) (To et al., 2011; Cheung et al., 2009; Cheung et al., 2001; Cheung et al., 2012). The adapted slit-lamp bio-microscopy digital imaging (Koutsiaris et al., 2010; Shahidi et al., 2010) and CAIM (To et al., 2011; Cheung et al., 2009; Cheung et al., 2001; Cheung et al., 2012) were modified from traditional slit-lamp biomicroscopes using complicated optics and can only image the conjunctiva with a fixed field of view. The adapted slit-lamp bio-microscopy (Koutsiaris et al., 2010; Shahidi et al., 2010b) has a small field of view ($\sim 1 \text{ mm}^2$) and the CAIM has a relatively larger field of view ($\sim 8 \text{ mm}^2$). None of these systems could image both the small and the large fields due to the limited adaptation. The laser Doppler flowmetry (Ohtani, 1996) and the orthogonal polarized spectral imaging (Schaser et al., 2003) require the contact of the eye. The modified scanning laser ophthalmoscope (Duench et al., 2007b) only provides arbitrary units of the blood flow. While these devices mentioned above can measure the conjunctival blood velocity and flow, none of these systems can create non-invasive microvascular perfusion maps (nMPMs), either due to the limitation of the field of view or the lack of the image processing algorithm for the nMPMs. We have developed a functional slit lamp biomicroscope (FSLB), in which a digital camera was adapted to reach extremely high magnifications at a high imaging speed (Jiang et al., 2014). Our preliminary data demonstrated the feasibility for imaging the microvasculature in the human eye (Jiang et al., 2014). The goal of this study was to determine the measurement variability of the quantitative analysis of the bulbar conjunctival microvascular morphology and hemodynamics through FSLB by determining the measurement repeatability and the variation during office hours.

Materials and Methods

The FSLB has been described previously in detail (Jiang et al., 2014). Briefly, a digital camera was attached to a slit-lamp. The inherent Movie Crop Function in the camera adds a $\sim 7.5\times$ magnification to the basis of the slit-lamp optical system, resulting in extremely high magnifications (up to $\sim 210\times$). This simple and easy approach provides high spatial resolution, high magnification and high speed (Jiang et al., 2014). To measure the blood flow velocity and flow rate, custom software has been developed based on the space-time imaging (STI) methods (Shahidi et al., 2010; Deneux et al., 2012) for quantifying microvascular hemodynamics. An image field of $0.94\times 0.70\text{ mm}^2$ ($1.47\text{ }\mu\text{m}$ per pixel, $\sim 210\times$) was used to image the blood flow. In a video model using the Movie Crop Function, the International Standards Organization (ISO) was set to 400, and the shutter speed (SS) was set to 1/60. An average image from 30 frames of the video was used for the vessel diameter segmentation (Jiang et al., 2014). The full width at the half maximum (FWHM) was used to obtain the vessel diameter (Jiang et al., 2014). After the vessel walls were identified, the vessel center lines were marked, and a space-time image was created. The grader manually outlined the prominent bands, corresponding to the movement of red blood cell clusters or the space between clusters. The slope of the band was the measurement of the axial blood flow velocity (Jiang et al., 2014). The values of the axial blood flow velocity (V_a , mm/s), cross-sectional blood flow velocity (V_s , mm/s), vessel diameter (D , μm) and blood flow rate (Q , pl/s) were obtained (Jiang et al., 2014).

To obtain the bulbar conjunctiva's microvascular network, the camera was set to a still image shot model with an ISO of 500 and a SS of 1/15. The magnification was $\sim 22\times$ optical magnification with an image size of $5,184\times 3,456$ pixels. A green filter was used to capture the temporal conjunctiva field of $15.74\times 10.50\text{ mm}^2$. Using custom-developed software (Jiang et al., 2014), the microvascular network was automatically segmented, and fractal analysis was performed using a commercially available software program (BenoitTM, TruSoft Inc, St. Petersburg, FL, USA). The monofractal (Dbox) and multifractal (D0) values were obtained to evaluate the vessel density and complexity of the non-invasive microvascular perfusion maps (nMPMs).

Five video clips on the temporal bulbar conjunctiva were recorded from multiple locations within an area of $5\times 5\text{ mm}^2$, which was approximately 1 mm beyond the limbus on the temporal bulbar conjunctiva. It typically took about one minute on each imaging location with a total of 5 minutes for imaging one eye. A green filter was used in the illumination path. A light spot on the ocular surface was set to about $2\times 2\text{ mm}^2$. The study subject did not have heating sensation. Five venules of the right eye were measured from these video clips, and the same venules were repeatedly located and measured in different imaging sessions. The average V_a , V_s , D and Q values were used. The large field of view mentioned above was used to acquire the microvascular network on the bulbar conjunctiva. Two still images were used to process the nMPMs, and the results of the fractal analysis were averaged from these two images.

This study was approved by the institutional review board for human research at the University of Miami. Each subject signed a consent form and was treated according to the

tenets of the Declaration of Helsinki. Twenty normal healthy subjects were recruited in this study, including 11 males and 9 females with a mean age of 35.2 ± 5.9 years (range 26.8 to 50.3) who were imaged with the FSLB at 9 AM. Two expert graders (SW and WY) processed the same video clips and outlined the blood flow bands in the STI to test the measurement's repeatability. At the same visit, the same group of subjects was imaged every two hours from 9 AM to 5 PM to observe the measurement variations during office hours.

All of the data were analyzed using the Statistical Package for the Social Sciences software (ver. 17, SPSS Inc., Chicago, IL, USA). The intraclass correlation coefficient (ICC) and coefficient of repeatability (CoR) were calculated to evaluate the measurement repeatability (Bland and Altman, 1986; Bartko, 1966). The ICC indicates the proportion of variability between the two different measurements compared with the overall variability. The CoR was defined as twice the standard deviation of the difference between the two measurements for the same subject by one grader. The CoR% was defined as the percentage value of the CoR divided by the overall mean measurements (Bland and Altman, 1986; Bartko, 1966). The 95% limits of agreement (LoA) were calculated as the mean $\pm$ 1.96 standard deviation of the difference (Bland and Altman, 1986; Hamilton and Lewis, 2010). A narrow 95% LoA represents good repeatability. Bland and Altman plots illustrated the repeatability between the results from the same grader and two graders (Hamilton and Lewis, 2010). Repeated measures ANOVA (Re-ANOVA) was used to test the statistical significance among the changes at the different time points and Fisher LSD post-hoc tests were used to determine pair-wise differences. Differences with $P < .05$ were defined to be statistically significant.

Results

The results of the measurement repeatability are listed in Table 1. The CoR% and ICC values for measuring the vessel diameter between two graders were 4.87% and 0.989, respectively. For the axial blood velocity, the CoR% and ICC were 11.49% and 0.997, respectively. The 95% LoAs of the intraclass repeatability and the Bland and Altman plots are illustrated in Fig. 1.

There were no significant differences during the test hours in the hemodynamic measurements for office hour variation (Re-ANOVA, $P > 0.05$, Fig.2, Table 2). The Va was 0.62 ± 0.31 mm/s at 9 AM compared to 0.63 ± 0.25 mm/s at 5 PM ($F = 0.14$, $P = 0.97$). The D was 21.05 ± 2.79 μ m at 5 PM compared to 21.68 ± 2.47 μ m at 9 AM ($F = 0.33$, $P = 0.86$). By visual inspection of the raw images, the microvascular network of the bulbar conjunctiva appeared not to have obvious changes during the office hours. There was no obvious evidence of increased redness (vessel injection) in the afternoon. However, the increased density of the network was clearly evident in the nMPMs and the skeletonized images obtained in the late afternoon (Fig. 3). Fractal analyses showed that the microvascular network density and complexity became more intensive in the late afternoon (Fig. 2 and 3, Table 2). Fractal dimensions of the microvascular network at 3 PM and 5 PM revealed significant increases compared with those obtained at 9 AM for both the Dbox ($F = 2.51$, $P = 0.048$) and D0 ($F = 3.14$, $P = 0.019$) measurements.

Discussion

FSLB is based on a traditional slit-lamp biomicroscope approach using an easy and simple adaptation that can be conveniently and widely used for the non-invasive imaging of the conjunctival microvasculature. This device will likely broaden the clinical applications of the classic optical apparatus, compared to the adaptation using add-on optics and sophisticated video acquisition equipment developed previously (Wanek et al., 2013; Shahidi et al., 2010; Koutsiaris et al., 2010; To et al., 2011; Cheung et al., 2009; Cheung et al., 2001; Cheung et al., 2012). FSLB can be easily translated into routine clinical practice and is more suitable for conducting clinical trials and routine patient care. In addition, FSLB can provide a quantitative analysis of the bulbar conjunctival microvascular morphology through photography and of the microvascular circulation through videography. Although imaging modalities including FSLB have been used to determine the conjunctival microvascular changes in various clinical conditions (Cheung et al., 2009; Wanek et al., 2013; Jiang et al., 2014), the measurement repeatability and variation of the conjunctival microvascular morphology and hemodynamics have remained untested. These unknown variables may limit their wider use in patient care. Furthermore, they may obstruct study designs and sample size calculations if the measured parameters are used as the endpoints of clinical trials. For example, if the blood flow velocity and microvascular network are used as the endpoints for evaluating the treatments of dry eye syndrome, a chronic ocular surface inflammatory disease, the sample size calculation will be partly based on the repeatability.

The range of the normal bulbar conjunctival blood flow velocity was 0.2 to 2.0 mm/sec, as previously reported (Cheung et al., 2001; Duench et al., 2007; Shahidi et al., 2010; Jiang et al., 2014; Koutsiaris et al., 2007). In the present study, the baseline blood flow velocity in the vessels of the conjunctiva was approximately 0.6 mm/sec. The wide range of the blood flow velocity reported in the previous studies may be due to the wide variation of the human blood flow velocity on the bulbar conjunctiva and the measurement errors. Although many aspects such as hardware configurations, vessel sample size and image process may introduce measurement errors, graders' interpretation may be one of the major sources for measurement errors. The method for semi-automatically measuring blood flow velocity is often dependent on the determination of the slope in the time space image as we used in the present study and other studies (Shahidi et al., 2010; Wanek et al., 2013; Jiang et al., 2014). The measured repeatability of the axial blood flow velocity in the current study was approximately 10% of the CoR% value between two graders and the ICC was more than 0.9 for the blood flow velocity. The repeatability could be interpreted as good, because the variation between two graders appeared to be much smaller than the differences existed in diseased eyes compared to normal healthy subjects. Wanek et al. used a modified slit-lamp to measure blood flow velocity on human bulbar conjunctiva in two different types of Sickle Cell diseases (Wanek et al., 2013). The difference between these two biophysical variations of hemoglobin (Hb) S and Hb C was about 24%. We used the FSLB to image the blood flow velocity in the eye after 6 hours of contact lens wear and found the change was up to 47% (Jiang et al., 2014). As a rule of thumb, the method with the good repeatability of measuring the blood flow velocity using FSLB could be sensitive enough to detect more than 10%

alternation from baseline or controls. Further calculation of the sample size will determine the detection power for specific study designs.

Information on the office hour variation of the microvascular circulation may be helpful in interpreting research data because the human hemodynamics varies diurnally (Duench et al., 2007). Although diurnal bulbar conjunctival variation in blood flow has been reported (Duench et al., 2007), to the best of our knowledge, this is the first study investigating the office hour variation of both blood flow velocity and fractal dimension of the microvascular network. We focused on the changes in the conjunctival microvasculature during office hours because the majority of clinical studies and patient care are conducted during this period of time. The alteration in the conjunctival blood flow velocity during office hours found in the present study was similar to that reported by Duench et al (Duench et al., 2007). By using a modified Heidelberg Retinal Flowmeter (HRF, Heidelberg Engineering, Heidelberg, Germany), Duench et al. found the blood flow velocity was highest upon waking, and lowest at the mid-morning. After that, a slight increase was evident in the afternoon. However, they did not mention if the increase during the office hours was statistically significant (Duench et al., 2007). In the present study, we did not find the office hour variations in the vessel diameter and axial blood velocity. In contrast, there were significant increases of fractal dimensions of the nMPMs in the late afternoon, which may indicate the higher sensitivity of the fractal analysis compared to the other measured parameters. Because the vessel diameter did not show significant changes over the office hours, the increased fractal dimensions (increase in vessel network density and complexity) of the nMPMs may be due to the re-opening of vessels rather than the vasodilation (increased diameter) in the afternoon. The analysis of nMPMs may represent the global changes of the microvascular network, which may be more sensitive to detect slight changes. Whereas the microcirculation measured as the blood flow velocity may not share the same rhythmic characteristics of the microvascular network. Therefore, fractal analysis of microvascular network might be more sensitive in studying ocular surface diseases, such as dry eye syndrome. This viewpoint is warranted for further studies with enlarged sample size and multiple diseases which might affect the conjunctival microvasculature. In the present study, reproducibility of the measurement in healthy subjects was studied. In pathologically altered bulbar conjunctivas, different sectors might show major differences in microvascular network and higher measurement variation may result in a much lower reproducibility. Further studies are needed to test the suitability in studying eyes with diseases such as dry eye syndrome.

In conclusion, FSLB appears to be capable of measuring vessel diameter, blood flow velocity and fractal dimension of the microvascular network in the bulbar conjunctiva. Slight variations over office hours were observed in the microvascular network, while the blood flow velocity remained stable.

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Highlights

- The measurement variability of the bulbar conjunctival microvasculature was tested.
- The measurement repeatability and variation during office hours were determined.
- Good repeatability of the measurement was apparent.
- There were no significant variations in the vessel diameter and hemodynamics.
- The density of the microvascular network was increased in the afternoon.

Figure 1. Bland-Altman plots of the interclass repeatability for FSLB imaging processing
 (Top left) Bland-Altman plots of the interclass differences in the axial blood flow velocity (V_a) measurements. (Top right) Bland-Altman plots of the interclass differences in the cross-sectional blood flow velocity (V_s) measurements. (Bottom left) Bland-Altman plots of the interclass differences in the vessel diameter (D) measurements. (Bottom right) Bland-Altman plots of the interclass differences in the blood flow rate (Q) measurements.

Figure 2. FSLB measurement changes of the variation on the bulbar conjunctival vasculature (Top) Diurnal variations of the axial blood flow velocity (Va) and cross-sectional blood flow velocity (Vs). (Middle) Diurnal variations of the vessel diameter (D) and blood flow rate (Q). (Bottom) Diurnal variations of the monofractal (Dbox) and multifractal (D0) values were obtained by fractal analysis. The Dbox and D0 measurements at 3 PM and 5 PM showed statistical increases compared with those obtained at 9 AM (marked as asterisk, $P < .05$). Bars = Standard Errors.

Figure 3. Variations in the bulbar conjunctival microvascular network measured by fractal analysis

(Top left) Raw conjunctiva image obtained by FSLB at 9 AM. (Second left) Raw conjunctiva image obtained by FSLB at 11 AM. (Third left) Raw conjunctiva image obtained by FSLB at 1 PM. (Forth left) Raw conjunctiva image obtained by FSLB at 3 PM. (Bottom left) Raw conjunctiva image obtained by FSLB at 5 PM. (Top middle) Image of segmented non-invasive microvascular perfusion maps (nMPMs) from the raw image obtained at 9 AM. (Second middle) Image of segmented nMPMs from the raw image obtained at 11 AM. (Third middle) Image of segmented nMPMs from the raw image obtained at 1 PM. (Forth middle) Image of segmented nMPMs from the raw image obtained at 3 PM. (Bottom middle) Image of segmented nMPMs from the raw image obtained at 5 PM. (Top right) Inverted skeletonized image used for fractal analysis at 9 AM. (Second right) Inverted skeletonized image used for the fractal analysis at 11 AM. (Third right) Inverted skeletonized image used for the fractal analysis at 1 PM. (Forth right) Inverted

skeletonized image used for the fractal analysis at 3 PM. (Bottom right) Inverted skeletonized image used for the fractal analysis at 5 PM. The red dashed lines show the cropping areas for the segmentations. The monofractal (D_{box}) and multifractal (D_0) values are shown with the corresponding images. Bars = 3 mm.

Table 1
The intraclass repeatability for measuring microvascular measurements of human bulbar conjunctiva (n = 20 subjects)

	M1	M2	Dif	CoR%	ICC	95% LoA
D (µm)	21.56 ± 2.44	21.65 ± 2.58	0.09 ± 0.53	4.87	0.989	-0.94 to 1.12
Va (mm/s)	0.62 ± 0.31	0.62 ± 0.32	0.00 ± 0.04	11.49	0.997	-0.07 to 0.07
Vs (mm/s)	0.44 ± 0.22	0.44 ± 0.22	0.00 ± 0.03	11.73	0.997	-0.05 to 0.05
Q (pl/s)	165.61 ± 86.62	166.83 ± 91.00	1.22 ± 8.43	10.14	0.998	-15.30 to 17.74

M1: the first measurement; M2: the second measurement. Dif, the difference between two measurements. CoR%, the percentage of coefficient of repeatability. ICC, intraclass, interclass and intersession correlation coefficient. 95% LoA, 95% limits of agreement. D, blood vessel diameter. Va, axial blood flow velocity. Vs, crossed section blood flow velocity. Q, flow rate.

Table 2
FSLB parameters changes of diurnal variation on bulbar conjunctival vasculature during office hours (n = 20 subjects)

	9 AM	11 AM	1 PM	3 PM	5 PM
D (µm)	21.68 ± 2.47	21.03 ± 3.13	21.51 ± 2.81	21.13 ± 3.02	21.05 ± 2.79
Va (mm/s)	0.62 ± 0.31	0.63 ± 0.23	0.61 ± 0.27	0.62 ± 0.26	0.63 ± 0.25
Vs (mm/s)	0.44 ± 0.22	0.45 ± 0.17	0.43 ± 0.18	0.43 ± 0.18	0.44 ± 0.18
Q (pl/s)	166.20 ± 85.78	162.37 ± 76.86	162.79 ± 86.23	159.01 ± 77.91	158.03 ± 69.57
Dbox	1.61 ± 0.06	1.62 ± 0.06	1.62 ± 0.05	1.64 ± 0.04 *	1.64 ± 0.04 *
D0	1.68 ± 0.04	1.69 ± 0.05	1.69 ± 0.04	1.70 ± 0.02 *	1.70 ± 0.04 *

D, blood vessel diameter. Va, axial blood flow velocity. Vs, crossed section blood flow velocity. Q, flow rate. Dbox, monofractal value. D0, multifractal value.

* , Fisher LSD post-hoc test $P < 0.05$.