Assessment of conjunctival goblet cell density using laser scanning confocal microscopy versus impression cytology

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ABSTRACT

Purpose: To determine the association between conjunctival goblet cell density (GCD) assessed using in vivo laser scanning confocal microscopy and conjunctival impression cytology in a healthy population.

Methods: Ninety (90) healthy participants undertook a validated 5-item dry eye questionnaire, non-invasive tear film break-up time measurement, ocular surface fluorescein staining and phenol red thread test. These tests where undertaken to diagnose and exclude participants with dry eye. The nasal bulbar conjunctiva was imaged using laser scanning confocal microscopy (LSCM). Conjunctival impression cytology (CIC) was performed in the same region a few minutes later. Conjunctival goblet cell density was calculated as cells/mm^2.

Results: There was a strong positive correlation of conjunctival GCD between LSCM and CIC *(*ρ=0.66). Conjunctival goblet cell density was 475 ± 41 cells/mm^2 and 466 ± 51 cells/mm^2 measured by LSCM and CIC, respectively.

Conclusions: The strong association between in vivo and in vitro cellular analysis for measuring conjunctival GCD suggests that the more invasive CIC can be replaced by the less invasive LSCM in research and clinical practice.

Key words: Conjunctiva, Goblet cells, Laser scanning confocal microscopy, Impression cytology

1. Introduction

Conjunctival goblet cells are known to release mucin granules onto the ocular surface and contribute to the production of the mucin of the tear film. The mucin phase is a thin, highly hydrated glycoprotein layer that covers the corneal and conjunctival epithelium over the glycocalyx, which is a mucopolysacharide component of low molecular weight [1]. The term *mucin* refers to glycoproteins manly produced by the goblet cells, the stratified cells from the corneal and conjunctival epithelium, as well as the lacrimal gland.

Genetic studies have identified 17 types of mucin in the human epithelium and they have been classified according to their function and origin – gel forming or secretory and membrane-associated. On the ocular surface, mucins expressed by the goblet cells (MUC5AC) are known to be gel forming/or secretory. Small structured mucins are also secreted by the lacrimal gland (MUC7) [2]. A decreased number of gel forming or secretory goblet cells is common in any type of dry eye, which is described as a multifactorial disorder that causes damage to the ocular surface [3].

The term ‘dry eye’ is difficult to define because, regardless of the numerous causes, including contact lens wear, the associated clinical manifestations vary greatly in intensity, even over time in the same patient. Dry eye symptomatology may not correspond with the signs observed by the practitioner. Subjective symptoms combined with assessment of objective evidence forms the basis of diagnosis. Various studies have shown disagreement between dry eye symptomatology and the results of corresponding clinical tests [4-6], with only 57% of symptomatic subjects presenting clinical signs of dry eye [4,6,7]. This finding has been attributed to the aetiology and

pathophysiology of dry eye [8]. As a result, a single objective test without subjective symptoms is not sufficient for a diagnosis of dry eye [3]. Therefore, to establish an association between these two diagnostic tools in healthy individuals, is important to firstly determine the absence of dry eye because this condition of the ocular surface has been shown to have an affect on goblet cell counts [4,9-13]

To date two approaches have been used to assess goblet cells in the anterior eye: in vitro cell analysis obtained from conjunctival impression cytology (CIC) and in vivo laser scanning confocal microscopy (LSCM) [11,14-20]. CIC is a mildly invasive technique of cell removal from the conjunctiva that is used for examination under a light microscope. Conjunctival cells obtained using CIC can also be analysed using flow cytometry and polymerase chain reaction techniques, which allow the amount of mucin produced by goblet cells to be quantified [20-22].

The most common method used to report goblet cell density (GCD) is the number of cells per unit area (mm²) [23-26]. Previous studies have suggest that the average GCD in healthy participants is about 427 ± 376 cells/mm² using the CIC technique on interpalpebral sites of the exposed bulbar conjunctiva [27].

Few reports have been published on LSCM assessment of GCD in healthy participants. Two reports have shown the average GCD from four cardinal points of the bulbar conjunctiva (nasal, superior, temporal and inferior) to be 111 ± 58 cells /mm² [24] and 432 ± 72 cells/mm² [28]. One report indicated an average of 260 cells/mm² determined from only one site (nasal bulbar conjunctiva) [29]. However, reductions of GCD have been found in participants with ocular surface disorders such chemical burns (136 ± 79 cells/mm²) [14] and Sjögren dry eye syndrome (332 ± 137 cells/mm²) [11].

Impression cytology is considered as the ‘gold standard’ technique for assessing cell morphology of the ocular surface. The scale system developed by Nelson and co-workers [30] reflects metaplastic changes to epithelial cells as well as changes in the number of goblet cells using CIC. This scale has been used to identify cells on the ocular surface using CIC and LSCM techniques in eyes treated with both preserved and preservative-free glaucoma therapies [4,21]. A positive correlation of GCD using LSCM and CIC has also been demonstrated in people with Sjögren syndrome ($\rho = 0.908$; $P < 0.05$) [11] and chemical burns ($\rho = 0.946$; $P = 0.000$) [14].

LSCM allows non-invasive in vivo evaluation of the human conjunctiva at a cellular level with magnification of approximately 600X and a field of view of 400 μm^2 [24]. The technique allows the capture of en face monochrome images of conjunctival cell layers.

This study reports, for the first time, a correlation analysis between the gold standard CIC technique and the new, non-invasive technique of LSCM in a healthy population. The CIC technique has been widely used for the past three decades to report GCD; however, limitations of this technique, mainly relating to its invasive nature, have been raised previously by many authors. It is therefore important to understand the utility of the less-invasive LSCM compared to the current standard of GC assessment, namely CIC. Demonstration of a correlation between these two techniques will serve to validate LSCM as a viable alternative procedure to assessing GCD in human populations.

2. Methods

2.1. Research design and participants

This was a cross-sectional study of GCD measured using in vivo LSCM and in vitro CIC. A total of 90 participants (44 women, 46 men; age 30.8 ± 8.5 years) were enrolled in the study after meeting inclusion/exclusion criteria. Individuals were not eligible if they had a history of contact lens wear for at least 6 months, current pregnancy, ocular trauma or surgery, ocular surface dysfunction, current classification as symptomatic for dry eye (DE) based on answers to the DEQ-5 dry eye questionnaire [33], current or long-term use of topical ocular medication, or ocular or systemic disease that may affect the conjunctiva. The study was approved by the Queensland University of Technology human research ethics committee and was conducted in accordance to the tenets of the Declaration of Helsinki.

All participants completed the DEQ-5 questionnaire [33] and underwent an ocular surface and dry eye examination following guidelines from the International Dry Eye Workshop 2007 [3].

Non-invasive breakup time (NIBUT) was recorded using a digital timing device as the average of three measurements in both eyes using keratometer mires (KM-1 Takagi Seiko Co Ltd, Nagano-ken, Japan).

The degree of ocular surface staining with fluorescein was graded from 0–4 according to the validated Efron grading scale system [34].

A phenol red thread (PRT) test (Tianjin Jingming New Technological Development Co., Ltd, China) was placed in the lower conjunctival sac on the temporal side of each

eye for 20 seconds without anesthetic with both eyes open [35] and the length of thread that became moist with tears and consequently turned yellow was measured against the scale on the test package. The length of wetting of the thread from the two eyes were averaged to give a single value for each participant.

All examinations were performed in the morning by the same examiner. Since the goblet cell distribution is apparently random using LSCM throughout the bulbar conjunctiva tissue [24], we assume that this is a representative and reliable approach that roughly correspond to the CIC technique. Hence we adopted the following sampling approach.

2.2. In vivo laser scanning confocal microscopy

Conjunctival LSCM was performed using the Heidelberg Retinal Tomograph (HRT3) equipped with a Rostock Corneal Module (Heidelberg Engineering GmbH, Heidelberg, Germany). One eye (the eye preferred by the participant) was examined. The eye was anaesthetized with 0.4% oxybuprocaine hydrochloride (Chauvin Pharmaceuticals Ltd, UK). To optimise the quality of CIC specimens collected following LSCM, no drop of ocular gel was used between the ocular surface and the front of the TomoCap (diameter 12 mm). The participant was instructed to direct their gaze in the opposite direction of the region of measurement (nasal bulbar conjunctiva). The centre of the surface of the TomoCap was positioned on the conjunctiva about 2 to 4 mm from the limbus.

Images were captured from the superficial layers of the conjunctiva; specifically, the focal plane of the instrument was gradually moved into the conjunctival epithelium between 10 to 44 μm until goblet cells could be visualised [36]. Goblet cells at the nasal

bulbar conjunctiva were scanned while moving the applanating lens approximately 2 to 4 mm from the limbal area at 9 different locations (approximating a 3 x 3 grid) and at approximately 3 different depths.

2.2.1. Validation of Image Analysis Approach for LSCM

A preliminary validation study was performed with 10 healthy volunteers. The aim was to determine the number of random goblet cells images obtained by LSCM to achieve an acceptable level of data variance in the measurements of GCD at each examination in the main study.

A sequence of approximately 30 image frames was captured. The variance of every possible combination of 3 to 30 images of goblet cells was plotted against the number of images taken, to determine the point at which variability was optimised and became relatively constant (i.e. the point at which additional repeated measures would not have resulted in an appreciable reduction in variability) . This analysis revealed that a minimum of 11 images were necessary to determine the average of GCD at each examination. This resulted in a variance of the standard deviation of approximately ±40 cells/mm^2. Quantification of cells was conducted using the manual cell count mode of the Heidelberg Eye Explorer software (Heidelberg Engineering GmbH, Heidelberg, Germany).

2.3. Conjunctival impression cytology

A few minutes after performing LSCM, the same eye was anaesthetized again and the centre of a Biopore membrane (Millicell cell culture inserts; Millipore Corp, Cork, Ireland, United Kingdom) was gently applied to the nasal bulbar conjunctival surface at approximately 2 to 4 mm from the limbus. The sample was allowed to air dry and then immersed in 95% methanol for fixation using a well culture plate sample holder. The sample was then refrigerated at -4 ºC for no more than 24 hours. To verify the location of the impression and the integrity of the exposed bulbar conjunctiva, a slit lamp examination with fluorescein was conducted under cobalt blue illumination with a yellow Boston filter.

The staining procedure was performed using Giemsa stain according to the following guidelines from the manufacturer (Sigma-Aldrich): Millicell inserts with more than 60% of cellular material across the field of the filter were assessed. The same well culture plate sample holder was used to retain the specimens during staining. The specimen was allowed to air dry at room temperature, the Giemsa stain was diluted 1:20 with deionized water and the specimen was immersed in the diluted Giemsa solution for 30 minutes. The sample was rinsed with tap water prior to examination.

A Leica DM2500 microscope (Leica Microsystems) was used to visualize the specimen collected; this system had a magnification of x200 and field of view of 640 x 480 μm^2. Approximately 10 images were captured from each sample by scanning in X and Y directions. Morphological identification of goblet cells using Giemsa stain was undertaken according to the image selection criteria described below.

2.3.1. Validation of Image Analysis Approach for CIC

The same statistical approach performed previously for assessing LSCM was used to validate CIC. The mean GCD for each specimen was determined by averaging cell counts obtained from five best quality images of non-disrupted cell material selected from the 10 captures images. This number of images yielded a variance of a standard deviation of approximately ±160 cells/mm^2. ImageJ software was used to facilitate counting of goblet cells and the number of cells per square mm was determined with the aid of a scale bar.

2.4. Image selection criteria for LSCM and CIC

Suitable images for cell count that contained abundant goblet cells [23] where randomly selected and were non-overlapping by more than 20%. High quality images were selected from LSCM scans that included goblet cells identified according to their size, shape and reflectivity, i.e. 25-30 μm in diameter [16,37], hyper-reflective [38], bigger than surrounding cells [39], round to oval [40] in shape and sometimes with a visible nucleus [11] (Figure 1A).

Acceptable images from CIC were those with no-disrupted cell material that contained goblet cells approximately 25-30μm in diameter. The cells had a pale membrane with defined borders and a visible nucleus localised centrally, although sometimes eccentrically in bigger cells (approximately 30 μm) [41]. Goblet cells were easily differentiated from surrounding cells because of their balloon-like appearance and cell size [27] (Figure 1B).

2.5. Statistical analysis

The association between CIC and LSCM was assessed using Spearman correlation and bootstrapped confidence intervals (95%). To analyse the agreement between measurements on the same participant, a regression approach for non-uniform differences was carried out using the Bland-Altman technique with linear regression and 95% limits of agreements [42]. Global values of GCD were used for this analysis (the average of 5 and 11 images for CIC and LSCM, respectively). SPSS for Windows version 16 (SPSS Sciences, Chicago, IL) was used for this statistical analysis.

3. Results

The Spearman's rho correlation revealed a statistically significant relationship between GCD assessed with CIC and LSCM (ρ= 0.66, 95% CI: 0.52, 0.77). The GCD assessed using LSCM was found to be significantly higher than that assessed using CIC (475 ± 41 cells/mm² and 466 ± 51 cells/mm², respectively; paired t = 2.26, p = 0.026). The mean difference between the two measurements was 9 cells/mm².

A Bland-Altman plot of the GCD values obtained using the two methods is shown in Figure 2. This plot shows the relation between differences in GCD vs. mean GCD. On the graph, the middle line represents the linear regression. The upper and lower lines represent the 95% Limits of Agreement. Regression analysis revealed an R^2 of 0.49 ($p<0.001$). The downward slope of the regression line indicates that, for higher mean

CGD values, a higher value was assigned to GCD as assessed with LSCM and a reduced spread of data is associated with lower GCD values obtained with CIC.

The results of our assessment of ocular surface integrity and dry eye assessment of the participants in this experiment are summarised in Table 1. The 90 participants were asymptomatic for dry eye based on results of the DEQ-5 (scores of < 7 points) and all the participants passed the ocular surface staining with scores of ≤ 2 points using the validate Efron grading scale. Only 6 participants failed the PRT test with scores of < 10mm/20s and 23 participants failed NIBUT with scores of > 10s of tear break.

4. Discussion

Here we report, for the first time, a strong association between CIC and LSCM for the assessment of GCD in *healthy* participants. This finding is consistent with previous reports that examined the correlation of GCD measurements using these techniques. These studies from the literature have positively correlated GCD measurements assessed with CIC and LSCM in patients with *pathology*, such as chemical burns on conjunctiva (ρ=0.929) [14] and Sjögren syndrome (ρ=0.908) [11]. The reason why Le and co-workers [14] and Hong and co-workers [11] reported higher correlations between CIC and LSCM in diseased eyes than we reported based on healthy eyes is unclear.

Similarly to these previous results, readings from CIC were slightly lower than those made from LSCM ($p = 0.026$), as shown in Figure 3. This phenomenon could be attributed to (a) the improbability of all cells in the sample region to attach to the filter at the time of peeling from bulbar conjunctiva when performing CIC, and (b) the

inability of CIC to sample cells at deeper layers of the conjunctival epithelium, unlike LSCM which can scan cells at different depths of the epithelium.

The distribution of the GCD values in the Bland-Altman plot indicate that the higher the GCD average, the greater the difference between GCD values obtained with LSCM and CIC. The reason for this difference profile is unclear.

Systematic errors related to sampling techniques are the source of variations between invasive and non-invasive techniques. For example, staining methods can vary using the CIC technique according to the filter used to collect the cells. Conventional cellulose acetate filters allow the observation of cells under a light microscope using coloured stains. For immunofluorescence staining, however, the filter must have specific properties such as mixed cellulose esters and larger pore size. A few reports in the literature have mentioned that different filter types can improve sample consistency and cell attachment [41,43]. However, in some studies using conventional cellulose acetate filters, greater applied pressure was applied to the conjunctiva for longer periods of time during sample collection in order to obtain the same outcomes as those obtained with mixed cellulose esters.

There appears to have been no validated approach to the number of images acquired in previous studies when attempting to correlate GCD assessed using LSCM and CIC. One study used an average of 3 images from each of the cardinal points of the bulbar conjunctiva (nasal, superior, temporal and inferior) using LSCM. The same study used an average of 3 consecutive images from only two sites of the interpalpebral conjunctiva (nasal and temporal) when performing CIC [14]. Another study using LSCM captured images from the superior bulbar conjunctiva in the Z-axis and averaged

4 images for the total GCD. These measurements were correlated with an average of 3 consecutive images obtained from only two sites of the interpalpebral conjunctiva (nasal and temporal) using CIC [11]. In our study, a statistically validated approach was used to determine an acceptable level of accuracy in the measurements of GCD at each examination.

In healthy individuals, GCD values from covered conjunctiva (upper and lower) have been reported to be significantly higher than those from the exposed regions (nasal and temporal) [27]. However, values from the exposed conjunctiva vary greatly from study to study. Using CIC, reports of mean GCD mean values from nasal bulbar conjunctiva range from 65 to 1108 cells/mm² [13, 44-47]. The reason why these studies show such large differences in GCD values may be due to differences in sampling techniques, such as differences in the number of images used to report an average GCD value, the level of magnification used to image cells, sampling area analysed, staining procedures and sample collection techniques.

Using LSCM, only one value has been reported of GCD in healthy participants, which was from the nasal area (262 ± 116) [29]. In the present study, the average GCD using LSCM and CIC were 475 ± 41 cells/mm² and 466 ± 51 cells/mm², respectively. The difference in these values for healthy participants could be attributed to the validated sampling approach and the larger number of images selected used here to determine GCD. As well, we adopted an image selection criteria that required an abundant number of goblet cells to be present in images selected for analysis [23].

Currently, assessment and quantification of GCD from images obtained by in vitro CIC is mostly based on counts from superficial cells that easily adhere to the filter acetate.

These procedures of sample collection can result in harvesting more or less cells depending on pressure applied to the filter and time of contact between the ocular surface and the filter acetate.

The level of magnification and field of view used when performing microscopy during manual cell counting can impact GCD estimates determine GCD. A level of magnification of 200x was used in this study because this magnification has been demonstrated to introduce less variability in GC counts compared to 100x and 400x [23].

Given the demonstrated association between GCD measurements using CIC and LSCM, researchers and clinicians may prefer to use LSCM for assessing GCD. LSCM has the advantage of being reiterative and non-invasive and, with demonstrated repeatable quantitative intersession measurements of cell density using cell count software [44]. Conversely, CIC is invasive (involving tissue removal), with no evidence of repeated measure capability in the literature. Further, repeated measurements cannot be made at the same location or region of tissue unless a period of time is allowed for tissue regrowth.

Images obtained using LSCM can be assessed immediately, whereas a time-consuming process of histochemical staining of CIC samples is required before cell counts can be made. The disadvantage of LSCM is the initial cost of the instrumentation, although CIC is also expensive when the costs of materials and reagents is factored in as well as the time necessary for a technician to prepare, stain and analyse the tissue samples.

In summary, we have shown that GCD assessed using CIC and LSCM are positively correlated, meaning that either technique can be used to obtain valid results. Estimates

of GCD using LSCM can be predicted from CIC and the two methods agree. LSCM is relatively a new approach for the assessment and quantification of goblet cells in a non-invasive and reiterative manner, and is less time consuming than CIC.

References

[1] Tiffany JM, Bron AJ. Role of tears in maintaining corneal integrity. Trans Ophthalmol Soc UK 1978;98:335-8.

[2] Gipson IK. Distribution of mucins at the ocular surface. Exp Eye Res 2004;78:379-88.

[3] Report of the 2007 International Dry Eye WorkShop (DEWS). Ocul Surf 2007;5:65-204.

[4] Bjerrum KB. Test and symptoms in keratoconjunctivitis sicca and their correlation. Acta Ophthalmol Scand 1996;74:436-441.

[5] Hay EM, Thomas E, Pal B, Hajeer A, Chambers H, Silman AJ. Weak association between subjective symptoms of and objective testing for dry eyes and dry mouth: results from a population based study. Ann Rheum Dis 1998;57: 20-24.

[6] Schein OD, Munoz B, Tielsch JM, Bandeen-Roche K, West S. Prevalence of dry eye among the elderly. Am J Ophthalmol 1997;124:723-728.

[7] Pflugfelder SC. Tear fluid influence on the ocular surface. Adv Exp Med Biol 1998;438:611-617.

[8] McCarty CA, Bansal AK, Livingston PM, Stanislavsky YL, Taylor HR. The epidemiology of dry eye in Melbourne, Australia. Ophthalmology 1998;105:1114-1119

[9] Nelson JD, Wright JC. Conjunctival goblet cell densities in ocular surface disease. Arch Ophthalmol 1984;102:1049-1051.

[10] Wang Y, Dogru M, Matsumoto Y, Ward SK, Ayako I, Hu Y, Okada N, Ogawa Y, Shimazaki J, Tsubota K. The impact of nasal conjunctivochalasis on tear functions and ocular surface findings. Am J Ophthalmol 2007;144:930-937.

[11] Hong J, Zhu W, Zhuang H, Xu J, Sun X, Le Q, Li G, Wang Y. In vivo confocal microscopy of conjunctival goblet cells in patients with Sjogren's syndrome dry eye. Br J Ophthalmol 2010;94:1454-8.

[12] Rivas L, Alvarez M, Rodriguez J, Murube J. Ophthalmological tests in patients with keratoconjunctivitis sicca with and without association of primary Sjogren's syndrome. Ger J Ophthalmol 1995;4:306-310.

[13] Rivas L, Oroza MA, Perez-Esteban A, Murube-del-Castillo J. Topographical distribution of ocular surface cells by the use of impression cytology. Acta Ophthalmol 1991;69:371-376.

[14] Le Q, Wang W, Hong J, Sun X, Zheng T, Zhu W, Xu J. An in vivo confocal microscopy and impression cytology analysis of goblet cells in patients with chemical burns. Invest Ophthalmol Vis Sci 2010;51:1397-1400.

[15] Mastropasqua L, Agnifili L, Fasanella V, Curcio C, Ciabattoni C, Mastropasqua R, Toto L, Ciancaglini M. Conjunctival goblet cells density and preservative-free tafluprost therapy for glaucoma: an in vivo confocal microscopy and impression cytology study. Acta Ophthalmol 2013;91:397-405.

[16] Kobayashi A, Yoshita T, Sugiyama K. In vivo findings of the bulbar/palpebral conjunctiva and presumed meibomian glands by laser scanning confocal microscopy. Cornea 2005;24:985-8.

[17] Kojima T, Matsumoto Y, Dogru M, Tsubota K. The application of in vivo laser scanning confocal microscopy as a tool of conjunctival in vivo cytology in the diagnosis of dry eye ocular surface disease. Mol Vis 2010;16:2457-64.

[18] Kojima T, Matsumoto Y, Ibrahim OM, Sato EA, Dogru M, Tsubota K. In vivo evaluation of superior limbic keratoconjunctivitis using laser scanning confocal microscopy and conjunctival impression cytology. Invest Ophthalmol Vis Sci 2010;51:3986-92.

[19] Wakamatsu TH, Okada N, Kojima T, Matsumoto Y, Ibrahim OM, Dogru M, Adan ES, Fukagawa K, Katakami C, Tsubota K. Evaluation of conjunctival inflammatory status by confocal scanning laser microscopy and conjunctival brush cytology in patients with atopic keratoconjunctivitis (AKC). Mol Vis 2009;15:1611-9.

[20] Pisella PJ, Malet F, Lejeune S, Brignole F, Debbasch C, Bara J, Rat P, Colin J, Baudouin C. Ocular surface changes induced by contact lens wear. Cornea 2001;20:820-5.

[21] Hori Y, Argüeso P, Spurr-Michaud S, Gipson IK. Mucins and contact lens wear. Cornea 2006;25:176-81.

[22] Corrales RM, Galarreta D, Herreras JM, Saez V, Arranz I, González MJ, Mayo A, Calonge M, Chaves FJ. Conjunctival mucin mRNA expression in contact lens wear. Optom Vis Sci 2009;86:1051-8.

[23] Doughty MJ. Sampling area selection for the assessment of goblet cell density from conjunctival impression cytology specimens. Eye Contact Lens 2012;38:122-9.

[24] Efron N, Al-Dossari M, Pritchard N. In vivo confocal microscopy of the bulbar conjunctiva. Clin Exp Optom 2009;37:335-44.

[25] Zhu W, Hong J, Zheng T, Le Q, Xu J, Sun X. Age-related changes of human conjunctiva on in vivo confocal microscopy. Br J of Ophthalmol 2010;94:1448-53.

[26] Wei A, Hong J, Sun X, Xu J. Evaluation of age-related changes in human palpebral conjunctiva and meibomian glands by in vivo confocal microscopy. Cornea 2011;30:1007-12.

[27] Doughty MJ. Goblet cells of the normal human bulbar conjunctiva and their assessment by impression cytology sampling. Ocul Surf 2012;10:149-69.

[28] Zhu WQ, Xu JJ, Sun XH, Zheng TY, Le QH. Normal human bulbar conjunctiva on confocal microscopy in vivo. Zhonghua Yan Ke Za Zhi 2009;45:344-9.

[29] Efron N, Al-Dossari M, Pritchard N. Confocal microscopy of the bulbar conjunctiva in contact lens wear. Cornea 2010;29:43-52.

[30] Nelson JD. Impression cytology. Cornea 1988;7:71-81.

[31] Ciancaglini M, Carpineto P, Agnifili L, Nubile M, Fasanella V, Lanzini M, Calienno R, Mastropasqua L. An in vivo confocal microscopy and impression cytology analysis of preserved and unpreserved levobunolol-induced conjunctival changes. Eur J Ophthalmol 2008;18:400-7.

[32] Efron N. Contact lens-induced changes in the anterior eye as observed in vivo with the confocal microscope. Prog Ret Eye Res 2007;26:398-436.

[33] Chalmers RL, Begley CG, Caffery B. Validation of the 5-Item Dry Eye Questionnaire (DEQ-5): Discrimination across self-assessed severity and aqueous tear deficient dry eye diagnoses. Cont Lens Ant Eye 2010;33:55-60.

[34] Efron N, Morgan PB, Katsara SS. Validation of grading scales for contact lens complications. Ophthal Physiol Opt 2001;21:17-29.

[35] Blades KJ, Patel S. The dynamics of tear flow within a phenol red impregnated thread. Ophthal Physiol Opt 1996;16:409-15.

[36] Zhang X, Li Q, Liu B, Zhou H, Wang H, Zhang Z, Xiang M, Han Z, Zou H. In vivo cross-sectional observation and thickness measurement of bulbar conjunctiva using optical coherence tomography. Invest Ophthalmol Vis Sci 2011;52:7787-91.

[37] Zhivov A, Stachs O, Kraak R, Stave J, Guthoff RF. In vivo confocal microscopy of the ocular surface. Ocul Surf 2006;4:81-93.

[38] Messmer EM, Mackert MJ, Zapp DM, Kampik A. In vivo confocal microscopy of normal conjunctiva and conjunctivitis. Cornea 2006;25:781-8.

[39] Villani E, Beretta S, Galimberti D, Viola F, Ratiglia R. In vivo confocal microscopy of conjunctival roundish bright objects: young, older, and Sjögren subjects. Invest Ophthalmol Vis Sci 2011;52:4829-32.

[40] Pisella PJ, Malet F, Lejeune S, Brignole F, Debbasch C, Bara J, Rat P, Colin J, Baudouin C. Ocular surface changes induced by contact lens wear. Cornea 2001;20:820-5.

[41] Doughty MJ. Contact lens wear and the goblet cells of the human conjunctiva-A review. Cont Lens Ant Eye 2011;34:157-63.

[42] Bland JM, Altman DG. Measuring agreement in method comparison studies. Stat Methods Med Res 1999;8:135-60.

[43] Albietz J M. Conjunctival histologic findings of dry eye and non-dry eye contact lens wearing subjects. Eye Contact Lens 2001;27:35-40.

[44] Prabhasawat P, Tseng SCG. Impression cytology study of epithelial phenotype of ocular surface reconstructed by preserved human amniotic membrane. Arch Ophthalmol 1997;115: 1360-1367.

[45] Zhang B, Yao Y. Application of impression cytology in diagnosis of ocular surface diseases. Zhejiang Univ Sci 2002;31: 383-387.

[46] Kim C, Shin YJ, Kim NJ, Khwarg SI, Hwang J-M, Wee WR. Conjunctival epithelial changes induced by cilia in patients with epiblepharon or entropion. Am J Ophthalmol 2007;144: 564-569.

[47] Rummenie VT, Matsumoto Y, Dogru M, Wang Y, Hu Y, Ward SK, Igarashi A, Wakamatsu T, Ibrahim O, Goto E. Tear cytokine and ocular surface alterations following brief passive cigarette smoke exposure. Cytokine 2008;43: 200-208.

[44] Popper M, Morgado AM, Quadrado MJ, Van Best JA. Corneal cell density measurement in vivo by scanning slit confocal microscopy: method and validation. Ophthal Res 200;36:270-6.

Table 1
Ocular surface integrity and dry eye assessment of participants.

Statistic	DEQ-5	NIBUT (s)	OSS (0-4)	PRT (mm/20s)
Mean ± SD	3 ± 2	13 ± 6	0 ± 1	20 ± 8
Min-Max	0 - 8	4 - 30	0 - 2	6 - 40

DEQ-5, 5-Item Dry Eye Questionnaire; NIBUT, non-invasive tear break-up time; OSS, ocular surface staining; PRT, phenol red thread test

Figure Legends

Figure 1**.** Conjunctival goblet cells imaged using LSCM and CIC techniques. (A) LSCM shows goblet cells (green arrows) to be approximately 25-30 μm in diameter, hyper-reflective, larger than surrounding epithelial cells and round to oval in shape. Epithelial cells are smaller and darker (white arrows). A nucleus is visible in some goblet cells and epithelial cells. (B) goblet cells highlighted using Giemsa stain appear to be approximately 25-30μm in diameter with a pale cytoplasm and defined borders. Epithelial cells are smaller and darker. A nucleus is visible in some goblet cells and epithelial cells.

Figure 2. Relation between differences in GCD vs. mean GCD. The middle line is the linear regression and the upper and lower lines are the 95% Limits of Agreement. There are 180 data points that represent two values per participant. Each data point represents the average value of 5 and 11 images by CIC and LSCM, respectively.