Development of Feasible Methods to Image the Eyelid Margin Using In Vivo Confocal Microscopy

Nanyu Zhou, BMed, MAppSc(Optom), Katie Edwards, BAppSc(Optom), PhD, Luisa H. Colorado, BAppSc(Optom), PhD, and Katrina L. Schmid, BAppSc(Optom) (Hons), PhD

**Purpose:** To develop a feasible method to image eyelid margin structures using in vivo confocal microscopy (IVCM) for use in clinical research. Second, to assess the association between IVCM and meibography parameters.

**Methods:** IVCM was performed on the central upper eyelid margin of 13 healthy participants (31 ± 5 years). Overall morphology montages (1600 × 1600 μm) were created of 3 participants. Single frames (400 × 400 μm) of 10 participants were imaged to determine the feasibility of measuring eyelid features. Meibography was performed with EASYTEARview+ in the same 10 participants. Imaged software was used to quantify image structures.

**Results:** In the montages, structures of rete ridges, meibomian gland openings, and the lid wiper region were observed. The maximum possible montage size, using multiple single frames, was approximately 5200 × 1500 × 150 μm in the X, Y, and Z directions, respectively. The mean number, density, area, perimeter, and shortest and longest diameters of the rete ridges of the 9 nonoverlapped frames were 12 ± 2/frame, 73 ± 5/mm², 2504 ± 403 μm², 250 ± 33 μm, 40 ± 6 μm, and 84 ± 13 μm, respectively. Sampling analysis determined at least 5 nonoverlapped frames were necessary to accurately represent the parameters of the ridges. The mean areas of 3 meibomian gland openings were 785 ± 784 μm², 1036 ± 963 μm², 950 ± 1071 μm², 848 ± 954 μm², 737 ± 831 μm², and from 30 μm to 130 μm at 20-μm depth intervals, respectively. No significant association between IVCM and meibography parameters (P = 0.53) was found.

**Conclusions:** Imaging rete ridges with IVCM should include at least 5 nonoverlapping single frames in the upper eyelid margin. At least 3 openings imaged between 30 and 130 μm at 20-μm depth intervals are recommended to determine the opening area.

**Key Words:** in vivo confocal microscopy, lid margin imaging, meibomian gland openings, meibography, rete ridges

Meibomian gland dysfunction (MGD) is the most common cause of dry eye disease. MGD is characterized by functional abnormalities of the meibomian glands (MGs) and altered secretion of the meibum from the glands. It affects both the ability to express the gland meibum and secretion quality. The 2 main techniques to image the lid structures in MGD are low magnification meibography and high magnification in vivo confocal microscopy (IVCM). Meibography imaging uses infrared light to illuminate the MG by using imaging devices, such as slit lamp, infrared video-camera, keratography, or a mobile pen-shaped meibography system. Different types of optical coherence tomography are also used to scan MG, and then generate infrared MG images using different methods. The low magnification techniques image the overall structure and morphology of the glands located in the tarsal plate in vivo.

IVCM uses high magnification to obtain eyelid margin structures in ocular research. Although different studies have generally imaged the same structures in MGD research, there is some confusion as to what the observed structures are. Previous studies have mistaken called the structures with an irregular shape and white border in the eyelid margin “acinar units.” A recent study using histology in cadaveric human tissue has since clarified that the “acinar units” are in fact rete ridges, structures in the tissue of the eyelid margin.

According to previous investigations using IVCM technique, there was significant within-patient variability in the rete ridges size, shape, and surrounding tissue. No standard protocol has been established for imaging eyelid structures using IVCM, with studies using arbitrary numbers of images. The high magnification that IVCM allows provides cellular level imaging of structures; however, such high magnification also means that it is difficult to know the exact location or structure that is being imaged. Consequently, previous MGD studies have not imaged consistently and have not compared the same lid locations. A standardized IVCM imaging protocol of the eyelid margin is needed to address this clinical need.

The aim of this study was to develop a feasible sampling method to quantify parameters of the eyelid margin and to assess the association between the parameters of MG as imaged with IVCM and meibography using EASYTEARview+.

**METHODS**

This was an observational, cross-sectional clinical study, conducted at the Institute for Health and Biomedical Innovation, Queensland University of Technology, Australia.
The research was conducted in accordance with the tenets of the Declaration of Helsinki and was approved by the University’s Human Research Ethics Committee. Written informed consent was obtained from all participants.

**Participants**

Thirteen healthy participants, aged 25 to 40 years, were recruited, including 9 women and 4 men. None of the participants reported any dry eye history at the time of examination. None had existing ocular or systemic disease, were pregnant, or contact lens wearers. The upper eyelid margin of the right eye was imaged.

**Methodologies**

**In Vivo Confocal Microscopy**

IVCM was performed by using the Heidelberg Retina Tomograph 3 with Rostock Cornea Module (Heidelberg Engineering, Dossenheim, Germany). Topical anesthesia was obtained using 0.4% oxybuprocaine applied to the conjunctival fornix. The top eyelid was everted, and the participant was positioned using the head and chin rest. The objective of the microscope was covered with a polymethylmethacrylate cap (Tomo-Cap; Heidelberg Engineering GmbH, Dossenheim, Germany). GenTeal Gel (Alcon) was used as a coupling agent between the microscope and cap.

Two assessments were performed to characterize the anatomical structures of the lid; first, the overall morphology of the eyelid margin by using IVCM montages (1600 × 1600 μm); second, optimizing image sampling techniques for IVCM of rete ridges parameters by using single frames (400 × 400 μm). Both assessments were from the center upper eyelid margin. A separate assessment was performed with the aim to evaluate the depth of MG openings in the central eyelid margin.

**Overall Morphology of the Eyelid Margin**

To explore the overall morphology of the eyelid margin, a noncommercial composite mode of the Heidelberg software was used. The mode uses a tracking imaging system to create montages in a frame of 1600 × 1600 μm. Montages of the center upper eyelid margin of 3 participants between 26 and 37 years of age (1 woman and 2 men) were created. It is important to capture as large an area of the tissue as possible to understand the morphology and then be able to use a validated sampling technique as explained below.

**Image Sample Size for Rete Ridges Assessment**

To determine the number of images required to reliably characterize the physiology of the rete ridges, single frames (400 × 400 μm) were imaged (X, Y, and Z scans applied) in the center upper eyelid margin of 10 participants between 25 and 40 years of age (8 women and 2 men). For each participant, single frames of rete ridges were analyzed for number, density, area, perimeter, and shortest and longest diameters using ImageJ. Images with clear visible rete ridge units were used for the analysis in 9 randomized, nonoverlapping single frames.

Analysis was carried out to determine the minimum number of single frames needed to reliably represent the parameters of the rete ridges (density, area, perimeter, and shortest and longest diameters) on the central upper eyelid margin. In brief, a standard deviation analysis was performed using random selection of 2, 3, 4, 5, 6, 7, 8, and 9 frames for FIGURE 1. The procedures of analyzing meibography image using ImageJ. The original image was filtered to enhance the contrast. The analysis inner eyelid area was chosen by “selection brush tool” in the ImageJ software. The MG area was selected by adjusting the threshold.
each individual. Then the average value of the SDs of 10 individuals was plotted against the number of frames selected for the analysis.

**Meibomian Gland Opening Depths**

For each of the 10 participants, 3 MG openings were imaged, and the size of the openings was measured using ImageJ. To determine the imaging depths, Z scan was applied on a random opening at random depth. Clear sharp images were obtained between approximately 30 to 130 μm; therefore, the imaging depths of openings were 30 to 130 μm at 20 μm-depth intervals. This was a compromise between tracking a significant length of the gland and being fast enough to not lose the position of the gland in the image frame. An interval of 20 μm was chosen because single frames of MG openings are not easily tracked. A small movement of the participants leads to lost visualization of the openings. The complete scan, from 30 to 130 μm, has to be conducted efficiently and as quickly as possible.

**Meibography**

EASYTEARview+ (EASYTEAR S.R.L., Trento, Italy) was used to obtain illuminated meibography images. The participants were positioned using a head and chin rest. After the upper eyelid was everted, focus adjustments were made. Images were taken and recorded to the connected computer. Three images were taken on the right eye of each participant. Only the clearest image was selected and analyzed using ImageJ. The area occupied by the glands and the tortuosity of the glands were measured for the clearest image per participant.

The area of the total analysis inner upper eyelid surface and the area occupied by the glands were measured (Fig. 1). Meiboarea was calculated by using the following formula:

\[
\text{Meiboarea} = \frac{\text{the area occupied by the glands (mm}^2\text{)}}{\text{total analysis area of inner eyelid surface (mm}^2\text{)}}
\]

The tortuosity index evaluated by tracing the MG by using the “Freehand line” tool (Fig. 2). The straight distance of the ends of the yellow lines was measured by the “Straight Line” tool. The tortuosity of each gland was calculated by using the following formula:

\[
\text{Tortuosity} = \frac{\text{length of trace line (mm)}}{\text{length of straight line (mm)}}
\]

**Association of MG Parameters Between IVCM and Meibography**

Infrared meibography images of the MG of the upper lid were obtained using the EASYTEARview+ from the same 10 participants. The meibography and IVCM images were analyzed, and all the associations of MG parameters between IVCM and meibography imaging techniques were determined.

**Statistical Analysis**

Microsoft Excel 2013 (Microsoft Corp, Redmond, WA) and Statistical Package for the Social Sciences (version 23; IBM, Armonk, NY) were used for the analysis. The sampling analysis was conducted to determine how many frames of rete ridges were needed to represent the central eyelid margin. For each frame of IVCM, the number, the density, the average area, the average perimeter, and the shortest diameter and the longest.

![FIGURE 2. The tortuosity measurement of meibography using ImageJ. The original image was filtered to enhance the contrast. The visible glands were traced using the “freehand line” tool. The distance of ends of MG calculated by the “straight line” tool.](image)

![FIGURE 3. In vivo confocal microscopy montages (1600 × 1600 μm) from the upper lid margin of 3 participants. A, A 37-year-old man. B, A 26-year-old man. C, A 34-year-old woman.](image)
diameter of rete ridges were analyzed. A repeated measures ANOVA was conducted to determine whether the opening areas were significantly altered with depths. Spearman correlation was used to assess all the associations between measured anatomical characteristics from IVCM images and meibography images.

RESULTS

In Vivo Confocal Microscopy

Overall Morphology of the Eyelid Margin

In IVCM montages, structures of rete ridges, MG openings and the lid wiper region were observed from the top to the bottom (Fig. 3). These structures vary between individuals in size, shape, and density. For example, the rete ridges in image A are smaller, more roundish, and have a greater density than image C.

Image Sample Size for Rete Ridges Assessment

Using IVCM single frames, the maximum length of the X, Y, and Z scans over which clear images could be captured were approximately 5200, 1500, and 150 μm, respectively. In general, this scanning area included 9 nonoverlapped frames of rete ridges and 5 MG openings (Fig. 4). Six MG openings were found in a number of participants with shorter distances between openings and less curved lid margins.

The mean number, density, area, perimeter, and shortest diameter of the rete ridges of 9 nonoverlapped frames were 12 ± 2/frame, 73 ± 5/mm², 2504 ± 403 μm² and 250 ± 33 μm, 40 ± 6 μm, and 84 ± 13 μm, respectively (Table 1).

The sampling analysis showed that the average of at least 5 nonoverlapped frames of rete ridges were necessary to represent a valid value of density, area, perimeter, and shortest and longest diameters of rete ridges located on the central eyelid margin of each individual. These frames should be taken in the area between eyelash follicles and MG openings (refer to Figure 3). The average value of the standard deviations of 10 individuals was plotted against the number of frames selected for the analysis. The standard deviation becomes stable at around 5 frames (Fig. 5). Increasing the numbers of frames further had no effect on the variation in the measures.

Meibomian Gland Opening Depths

The lid area that was clearly imaged contained 5 MG openings, and thus, an attempt at imaging 5 opening at fixed depths was conducted. It took a long time (20–30 minutes depending on the participants) to find the 5 different openings and image them at fixed depths. Considering that it has taken approximately 15 minutes for imaging the rete ridges and in consideration of time limitations and the tolerance of the participants, 3 different MG openings were considered acceptable for imaging at set depths per person (Fig. 6). In most individuals, a clear image of the MG openings appeared at approximately 30-μm depth and became unclear at approximately 130 μm. In a few participants, the image became unclear at approximately 90 or 110 μm.

The mean areas of 3 meibomian openings were 785 ± 784 μm², 1036 ± 963 μm², 950 ± 1071 μm², 848 ± 954 μm², 737 ± 831 μm², 735 ± 743 μm², and from 30 μm to 130 μm at 20-μm depth intervals, respectively. Figure 7 showed a box-whisker plot of opening area versus depth. A repeated measures ANOVA analysis showed the areas significantly varied with depths (F = 4.430, P = 0.008).

Meibography

The average of the meiboarea and tortuosity for the 10 participants were 0.50 ± 0.04 and 1.13 ± 0.09, respectively. The participant results are shown in Table 2.

Association Between Imaging Techniques

Meibography image analysis indicated the MG occupied 42% to 54% of the area of inner eyelid area in the 10 participants. Spearman correlation showed there was no significant association between measured characteristics of IVCM images and meibography images in the 10 participants (Table 3). The highest correlation between measures occurred between the shortest diameter of the rete ridges and meiboarea; however, this relationship was not significant (Fig. 8).

| TABLE 1. Participants’ Characteristics and Rete Ridges Parameters |
|---------------------------------------------------------------|
| Participants’ characteristics |  |
| Participants, N | 10 |
| Age, yrs | 31 ± 5 |
| Gender, F/M | 8/2 |
| Rete ridges parameters |  |
| Number per frame | 12 ± 2 |
| Density, mm² | 73 ± 5 |
| Area, μm² | 2504 ± 403 |
| Perimeter, μm | 250 ± 33 |
| Shortest diameter, μm | 40 ± 6 |
| Longest diameter, μm | 84 ± 13 |
DISCUSSION

Here is described a feasible method to image lid margin structures using IVCM for use in clinical research. High magnification upper eyelid margin imaging with IVCM should include at least the average of 5 nonoverlapping single frames of rete ridges area. In consideration of the participants expose to the 20 to 30 minutes evaluation to assess MG openings depth, the average of at least 3 MG openings at 20 μm-depth intervals between 30 and 130 μm is recommended. For some participants who are tolerant and cooperative during the scanning, 5 MG openings can be assessed. No significant associations were found between IVCM assessments of rete ridges parameters and MG openings area and the meiboarea and tortuosity assessed with meibography. This is likely because these are not actually part of the same structures.  

There has been controversy over what structures are imaged during IVCM assessment of the eyelid margin; this is because of the high magnification, allowing imaging of only small parts of the tissue at a time. Initially IVCM images with winding cellular structures in the eyelid margin were identified as MG. Later, many published studies showed images reporting to be of parts of the MG or the MG “acinar units.” A study comparing histological sections of human eyelid margin to IVCM images have identified these structures are in fact rete ridges. This was based on both size, shape, distribution and location of MG, and also the fact that IVCM with 670 nm laser wavelength can only penetrate approximately 100 μm into the tissue. Therefore, it is impossible to image MG, which are located at approximately 500 μm depth from the surface at the eyelid margin. 

FIGURE 5. The average value of the standard deviations of rete ridges parameters of a random selection of frames analyzed of 10 individuals versus the number of frames.
montages of the lid margin produced here further confirm the structures that are possible to image during IVCM are rete ridges, MG openings and the lid wiper region.

MG travel down and up the length of both lids, and as such are also located beneath the palpebral conjunctiva on the everted eyelid; however, our attempts to image the MG through the lid tissue at this position were not successful. According to previous histology studies, the distance between the palpebral conjunctiva and MG is approximately 200 to 300 μm.28–31 With 100 μm penetrating depth of IVCM, successfully imaging MG through the palpebral conjunctiva is unlikely.

Moreover, the shape of the rete ridges of the lid margin is comparable with the rete ridges seen in dermal papillae. Rete ridges, also known as rete pegs or rete processes, are the epithelial extensions that project into the underlying connective tissue in both skin and mucous membranes.32 Figure 9 shows a confocal image of rete ridges in the lid margin and a confocal image of rete ridges in a finger of the same participant. The similarity of the 2 images supports the conclusions that these winding cellular structures are rete ridges.

Although potentially not part of the MG system, the location of the ridges is close to the glands. In previous studies, these structures were imaged with IVCM and called “acinar units.” Some of these studies have also shown an association with MGD signs and symptoms and the structure of the rete ridges.3,14,15,33 Therefore, the morphology of the rete ridges may reflect the health of the glands function, although they are not themselves part of the glands, and they should still be included as a clinical variable in the studies of MGD, and hence, the development of a protocol to measure them in this study.

**FIGURE 6.** Three different MG openings of the central upper eyelid margin from 1 participant. Each opening was imaged from 30 to 130 μm at 20-μm depth intervals.

**FIGURE 7.** A box-whisker plot of opening area versus opening depth of 10 participants.
TABLE 2. The Meiboarea and Tortuosity Results of Each Participant

| Participant ID | Meiboarea | Tortuosity |
|----------------|-----------|------------|
| 1              | 0.53      | 1.07       |
| 2              | 0.42      | 1.17       |
| 3              | 0.46      | 1.07       |
| 4              | 0.53      | 1.05       |
| 5              | 0.51      | 1.16       |
| 6              | 0.54      | 1.08       |
| 7              | 0.52      | 1.09       |
| 8              | 0.51      | 1.11       |
| 9              | 0.53      | 1.35       |
| 10             | 0.46      | 1.12       |

In the past, arbitrary protocols have been used for imaging eyelid structures using IVCM. The images of the “acinar units” in these studies showed dramatic differences in shape, size, and pattern. Some of the images resembled the red ellipse of Figure 3A, whereas others were similar to the green box of Figure 3A, and some images showed similarities to the green box of B in Figure 3. These differences in appearance have been attributed to disease status, whereas in reality, based on the results of the current study and the histology study, different structures were being imaged.

It is very difficult to have confidence in the exact sampling location and orientation when imaging in a small field of view with IVCM on the lid margin. The structures of rete ridges and the lid wiper also have similarities in appearance. For example, the structures of rete ridges and the structures of the lid wiper in Figure 3C are very similar. It is easy to mistakenly confuse the lid wiper structures as rete ridges when IVCM can only show 400 × 400 μm frames during the imaging process.

TABLE 3. Spearman Correlation (r) Between Measured Characteristics of MG Openings and Rete Ridges Assessed With IVCM and Meiboarea and Tortuosity of Meibography Images in 10 Participants (all P > 0.05)

| IVCM                      | Meibography       |
|---------------------------|-------------------|
| Rete ridges parameters    |                   |
| Number per frame          | -0.53             | 0.52          |
| Density, mm²              | -0.53             | 0.52          |
| Area, μm²                 | 0.59              | -0.32         |
| Perimeter, μm             | -0.05             | 0.06          |
| Shortest diameter, μm     | 0.63              | -0.32         |
| Longest diameter, μm      | 0.04              | -0.10         |
| Meibomian gland opening area, μm² |         |
| 30 μm                     | -0.25             | -0.26         |
| 50 μm                     | -0.14             | -0.31         |
| 70 μm                     | -0.18             | -0.21         |
| 90 μm                     | -0.02             | -0.19         |
| 110 μm                    | -0.07             | -0.18         |
| 130 μm                    | 0.14              | 0.03          |

We presume that this error may have been made in other studies. We hope that the standard IVCM imaging protocol of the eyelid margin described here will provide clarity and consistency in future clinical studies of MGD. The rete ridges should be imaged between eyelashes and MG openings in the future studies. The distinctive architecture of the gland openings should also be used as a landmark to guide sampling location.

The MG openings were imaged between 30 μm and 130 μm with 20 μm-depths intervals in our study. We found that the image first blurred at different depths for individual participants. Generally, clear images could be obtained from 30 to 70 μm in all participants. In addition, according to Figure 7, the biggest average size of the openings is at 50 μm, with the gland narrowing at the top and bottom. The gland opening may assume this goblet shape, a characteristic found in many glands. Histology has also showed a goblet shape of the MG openings. This supports our hypothesis. However, it is possible that this shape is an artifact of the sampling technique, where pressure is exerted on a straight tube, and it folds into a goblet shape. In addition, the MG opening can be obstructed by the shed epidermis inside the lumen.

We acknowledge that inclusion of 3 MG gland openings at 6 different depths (18 images per participant) is too onerous for most clinical studies. Future studies are needed to further investigate the depth-dependent change in shape of the gland openings, particularly to determine which depth may best reveal pathological or aging changes. Until these changes are better understood, if only one image of opening can be acquired in clinical studies, it is essential to specify a constant depth in the protocol to minimize the variation in measures. Sampling at around 50 to 70 μm depth is recommended because it was at this depth that we could also obtain the clearest images of rete ridges.

The structure of the lid wiper assessed with IVCM has been reported to be papillae, whereas another study recognized it as continuing rete ridges. In our study, the appearance of the lid wiper structure was similar to the rete ridges in some participants, but different in others. During the imaging process, a very fine flow movement was observed in the lid wiper structure, but not in the rete ridges. Therefore, we believe the rete ridges are different from the lid wiper structures.
Digital analysis of meibography was reported in previous studies. These studies calculated the percentage of MG to total eyelid image by area. The MG area as a portion of the total eyelid area for individuals with a meiboscore of 0, 1, 2, and 3 were 51.9% ± 5.7%, 47.7% ± 6.0%, 32.0% ± 4.4%, and 16.7% ± 6.4%, respectively. Similar results were found here, the MG occupied 42% to 54% of inner upper eyelid area in the 10 participants indicating that MG atrophy had commenced even in this young adult asymptotic group. Previous study also reported that meibomian gland tortuosity was altered in the progression of MGD. The average tortuosity of healthy participants was 1.31 in the previous study. In our study, the average tortuosity was 1.13 ± 0.09 in the 10 healthy participants. A further study with a bigger cohort of both healthy and MGD is required for a better understanding of the role of MG tortuosity in MGD.

Spearman correlation showed that there was no significant association between the measured characteristics of rete ridges parameters and the area of MG openings assessed with IVCM and the area and tortuosity of the MG assessed from the meibography images. Future longitudinal studies should determine the temporal relationship between these 2 measures to potentially identify an early marker for MGD. In conclusion, this study has clearly identified the upper lid margin structures and their locations relative to each other. Future clinical research studies that image upper eyelid margin structures with IVCM should include at least 5 nonoverlapping single frames of rete ridges and at least 3 MG openings at 20-μm depth intervals between 30 and 130 μm. Further work is needed to confirm the correlations between in vivo and histology imaging to confirm the identity of some features, as well as studies in individuals with MGD to determine which structures are most sensitive to alteration in disease, and therefore, may be the best markers for disease.

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