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A CONFOCAL MICROSCOPY STUDY OF THE ANTERIOR CORNEAL MOSAIC IN THE SUB-BASAL NERVE PLEXUS

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Abstract: *In vivo confocal microscopy of the corneal sub-basal nerve plexus and assessment of its morphology promises to provide a novel, highly sensitive tool for early diagnosis and therapy monitoring of diabetic peripheral neuropathy. Ridge-like tissue deformations in the region of the sub-basal nerve plexus, induced by pressure upon the corneal surface, can prevent the imaging of sub-basal nerves over the entire field of view and impede the assessment of nerve morphology. We present an investigation of the distribution of the deformation heights, yielding a mean value of 28.9 µm in our data. Given the depth of focus of the used confocal microscope of 6-7 µm, our results strongly suggest the need for three-dimensional imaging methods for the reliable assessment of sub-basal nerve morphology.*

Keywords: *cornea, sub-basal nerve plexus, anterior corneal mosaic, confocal microscopy, image processing*

Introduction

Since the introduction of in vivo confocal microscopy of the cornea, the corneal sub-basal nerve plexus (SNP) has received a great amount of attention. It consists of a network of small peripheral nerve fibres and nerve fibre bundles (diameters < 5 µm) and is located immediately at the anterior surface of the cornea's basal epithelial membrane. Due to its general two-dimensional arrangement parallel to the ocular surface, the SNP lends itself ideally to the imaging by in vivo confocal microscopy. A multitude of different studies of corneal sub-basal nerve structures has been conducted and published, assessing morphological changes in ocular (e.g. keratoconus, dry eye, contact lens wear) and systemic conditions and diseases (e.g. diabetes, Fabry's disease) and after corneal surgery. Recent studies point toward the potential of sub-basal nerve morphology as a sensitive surrogate marker for early diagnosis and monitoring of progression or therapy success in diabetic peripheral neuropathy [1].

The reliable assessment of morphological parameters of the SNP, however, depends on the quality and first and foremost on the general visibility of the imaged nerve structures. Especially in the use of confocal laser scanning microscopy (CLSM) – the most commonly used technology for in vivo imaging of the SNP because of its high lateral resolution and very low depth of focus – ridge-like deformations occurring in the region of the SNP and the neighbouring tissues can prevent the imaging of sub-basal nerve fibres over the entire field of view (Fig. 1).

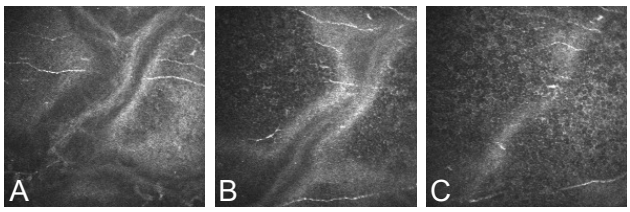

Figure 1: CLSM images of ridge-like deformation in the region of the SNP; focus plane at SNP level (A), at 19 µm anterior to SNP level (B) and at 38 µm anterior to SNP level (C).

These deformations are one expression of a phenomenon termed the anterior corneal mosaic (ACM) by Bron in 1968 [2], who was the first to systematically examine it. The first description of the ACM ridges in in vivo corneal confocal microscopy dates back to 2006 by Kobayashi et al. [3] who later proved that the observed ridges in fact align with the ACM visible in slit lamp images of the cornea and are therefore closely connected to each other [4]. They also described fibrilar structures (which they termed K-structures after Kobayashi) beneath the ridges which they identify as collagen fibre bundles in the anterior stroma. Finally, our group published an image processing technique – using software algorithms specifically designed to the imaging technology – for reconstructing two-dimensional images of the SNP from CLSM volume scans [5]. These SNP reconstruction images show the sub-basal nerve structures over the entire image area despite the presence of ACM ridges in the imaged volume.

The mentioned image processing technique has since been used extensively with volume scans taken from 151 subjects. We here present an analysis of the measured heights of the ACM ridges.

Methods

The image data used herein was acquired from 151 subjects during a study investigating nerve alterations in early stages of diabetes.

In vivo confocal microscopy was performed using an HRT II CLSM system in conjunction with the RCM objective module (both Heidelberg Engineering GmbH, Heidelberg, Germany). Using a modified volume scan operating mode which continually shifts the focus plane of the microscope back and forth, a number of image stacks (with an axial image distance of ca. 0.5 µm) were acquired for each subject, each stack representing a partial volume of the subject's cornea.

Care was taken that each image stack extends over the entire height of present ACM ridges. A stack size of 96 images (scan depth: ca. 48 µm) was chosen for ridge heights of less than 48 µm, and 120 images (ca. 60 µm) otherwise.
All acquired image stacks were subsequently processed to correct motion artefacts, reconstruct the imaged volume and compute a depth map of the SNP. The depth map indicates the location of the SNP (or rather, the surface of Bowman's membrane, on top of which the SNP is located) throughout the volume. The height of the ACM ridges in a specific partial volume is then simply given by the difference between the maximum and the minimum value of the depth map.
To see if the ACM ridges intensify with increasing examination time, a longitudinal analysis of the calculated ACM ridge heights of each subject was performed by computing a linear regression of the height values of the subject over the total examination time of about 10 minutes. Only subjects with at least 10 image stacks from a single examination session were included in this analysis.

Results

The number of single stacks analysed per subject varied between 1 and 55 with a mean ± standard deviation of 28.9±12.6. Altogether 4362 single image stacks have been included in the analysis. Fig. 2 shows the distribution of the calculated ACM ridge heights. The values ranged from 1.5 µm to 58.5 µm, with a mean ± standard deviation of 26.7±9.1 µm.

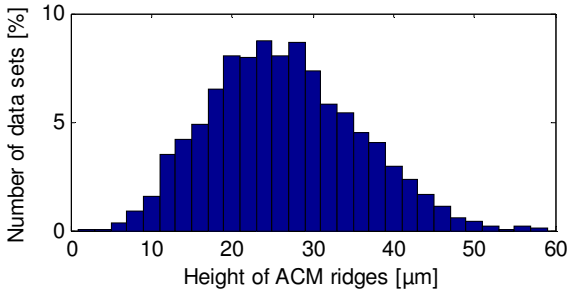

Figure 2: Distribution of ACM ridge heights

10 subjects were excluded from the longitudinal analysis of ACM ridge progression because less than 10 image stacks were present for them. The linear regression of the calculated ridge heights of the remaining 141 subjects yielded a mean slope of the regression lines of 0.037 µm per consecutive stack.

Discussion

The results of the longitudinal analysis do not suggest any significant increase of ridge height over the time of a single examination session. In fact in 44.6% of the analysed subjects the slope of the regression line was negative, so no coherent tissue reaction to a prolonged CLSM examination could be observed.
Given the low depth of focus of the HRT II (6-7 µm), the average ridge height of 28.9 µm found in this study strongly suggests the need for three-dimensional imaging when assessing the corneal sub-basal nerves by CLSM (Fig. 1).
According to Bron's systematic examination of the ACM, pressure applied upon the cornea is the most important causal factor for the development of the ridge-like deformations in the region of the SNP [2]. It might therefore be argued that by taking care to minimize the pressure applied upon the cornea by the microscope, the physician could also prevent the formation of the ACM ridges or at least keep them to a level at which they don't interfere too much with imaging the sub-basal nerve structures. A very careful examination procedure may explain why the presence of ACM ridges is almost never mentioned in published corneal confocal microscopy studies of the SNP.
Several arguments however lead us to the conclusion that our previously published three-dimensional imaging and image processing approach is still necessary for reliable morphological nerve fibre assessment, especially considering the scenario of a fully automated imaging process, without an operator selecting an appropriate region or focal depth.
Firstly, keeping the contact pressure at a constant, low level for the entire examination, without losing the optical coupling between cornea and microscope, is a difficult task even for an experienced operator. Secondly, even very small ridges that don't pose any difficulties for the human observer do complicate automatic nerve segmentation methods. And thirdly, the automatic (and therefore objective) reconstruction of two-dimensional SNP images from an imaged volume may have advantages even in the complete absence of ACM deformations by eliminating the need for any subjective identification of the exact depth of the SNP layer, which to our belief is crucial for the reliable assessment of very small nerve fibres. To examine the last point we are currently conducting a study to compare morphological nerve parameters gained from a single CLSM image out of a volume scan against those gained from the SNP reconstruction image generated from the scan.

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