Real-time mapping of the subepithelial nerve plexus by in vivo confocal laser scanning microscopy

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Title

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Abstract

Aim*:* To produce two-dimensional reconstruction maps of the subepithelial nerve plexus (SEP) in living cornea by in vivo laser scanning confocal microscopy in real time.

Methods: In vivo confocal laser scanning microscopy (Heidelberg Retinal Tomograph II in conjunction with the Rostock Cornea Module) was performed on normal eyes (n=6) and eyes after LASIK (n=4). Source data (frame rate 30Hz) were used to create large-scale maps of the scanned area in Automatic Real Time composite mode. The algorithm aligns single live images onto the previously mapped composite image using landmark feature-based image processing.

Results: Real-time mapping of the SEP was performed on a large-scale area up to 3.2 mm x 3.2 mm (3072 x 3072 pixels) both in healthy subjects and in post-LASIK patients. Two-dimensional structures of the SEP were imaged in all 10 eyes. Mapping quality as well as acquisition time were dependent on subject compliance and examiner experience.

Conclusion: The described method permits real-time in vivo mapping of the SEP, thus providing the necessary basis for statistically robust conclusions concerning morphometric plexus alterations.

Introduction

In vivo confocal laser scanning microscopy (CLSM) is a powerful tool for analysing living corneal tissues in laboratory animals[1] and humans.[2-5] One of the principal limitations of this method is the small field of view for imaging, which is an obstacle to the statistically robust evaluation of findings in a large-scale area (fig 1).

Recently, several research groups have published reports of two-dimensional reconstruction mapping of stained corneal whole mounts[6] as well as of the living human cornea[7-10]. In addition, our own group has published results of in vivo three-dimensional reconstruction of the ocular surface and cornea.[11-13] The composite procedure in all these studies was performed off-line, i.e. mapping required manual external software support for image post-processing. Imaging artefacts (for example, applanation-induced tissue folds, epithelial compression, and movement) generate problems in image rendering. To date these artefacts have been associated with reduced image quality and have necessitated time-consuming off-line processing.

We present here an experimental on-line mapping approach based on in vivo CLSM with the Heidelberg Retinal Tomograph II (HRTII) (Heidelberg Engineering GmbH, Heidelberg, Germany) in conjunction with the Rostock Cornea Module (RCM). This combination allows on-line scanning, processing and mapping of the living human cornea. The study focuses on the subepithelial nerve plexus (SEP), one of the most interesting and dynamically changing structures of the cornea.

Materials and methods

In vivo CLSM was performed with the HRTII/RCM equipped with a water contact objective (Zeiss, 63x/0.95W, 670 nm, ∞/0, Jena, Germany), as described elsewhere.[14] Microscopy of the area is manual: the investigator has to move the objective in the x-y axis in order to depict the structures of interest and manually control the quality of the image. For better handling and image quality the patients were asked to gaze at the microscope's fixation light, which was moved manually to the opposite side away from the region of interest in order to map the maximal corneal surface.

CLSM source data (frame rate 30Hz, 384x384 pixels, 400x400 µm) were used to create large-scale maps of the scanned area by selecting the Automatic Real Time (ART) composite mode. The algorithm is based on an affine transformation of six parameters (2 x 2 transformation matrix and translation in x and y directions). In an initial step the six transformation parameters between the current live image and all images in the composite reference image pool are calculated using features inside the current live image and the reference image. If the reference image pool is empty, the actual live image is added to the pool in centre position. The affine transformation parameters are calculated using a least squares fit of the overdetermined equation system derived from live image and reference image coordinates. The actual live image can be mapped to various images inside the reference pool; therefore all determined sets of transformation parameters will be combined and averaged to a parameter set.

Live images with correlations and an overlap <75% are added to the reference image data pool. Images with ≥75% overlap as well those without correlations to the reference image data pool are rejected. Finally, the actual live image is inserted into the ART composite image on the basis of the transformation parameters by pixel-by-pixel averaging and decreasing the weighting towards the image edge. Processing of the next image is then started. The maximal ART composite image size using source images is 3072x3072 pixels (3.2x3.2 mm) and corresponds to 8x8 non-overlapping images.

In total, ten human corneas were investigated (six from healthy volunteers, aged 22-35 years, two females and four males; four from patients who had undergone LASIK, aged 30-44 years, two females and two males, 6 months postoperatively). The study was performed at the Helios Klinikum, Erfurt, Germany once approval had been obtained from the Ethics Committee of the Thuringia State Medical Board, Jena, Germany. The study was explained in detail to the patients, and informed written consent was obtained before any investigative procedures were conducted. Image acquisition was performed in ART composite mode at

the level of the SEP in the central cornea in healthy volunteers and in the central as well in the temporal and nasal flap areas in the LASIK group.

Results

CLSM in ART composite mode permitted real-time mapping of the living cornea. The acquisition time for a single composite image was up to 3 minutes. The quality and size of the composite image were considerably influenced by subject compliance and examiner experience (AZ). Real-time mapping of the SEP was performed on a large-scale area up to 3200x3200 µm (3072x3072 pixels) in all 6 healthy volunteers (fig 2).

Mapping of regenerated SEP structures was achieved in all LASIK patients (n=4). Representative images obtained 6 months postoperatively show the SEP in the central flap zone (fig 3A), and the cut zone with epithelial cell ingrowth and nerves growing outside and inside the flap area (fig 3B). In some cases several corneal layers are present in one mapping image due to the focus shift during microscopy.

Discussion

Since Li et al.[15] presented their on-line three-dimensional confocal imaging system based on a tandem scanning confocal microscope in 2000, there have been no further attempts to design another on-line imaging system. The aim of the present article is to demonstrate a novel approach to real-time mapping of corneal structures using in vivo CLSM.

One of the main problems of mapping is image quality and the associated problems of off-line processing: up to 50% of off-line data are not suitable for processing due to artefacts.[13] The present algorithm aligns only live images of sufficient quality and permits quality control in real time. In some cases different corneal layers are presented in one mapping image: this is analogous to the oblique imaging technique in section mode described elsewhere.[13] Moreover, in vivo confocal microscopes currently used in ophthalmology depict only a very small area (contact mode: HRTII/RCM 400x400 µm, i.e. 0.160 mm^2 [13] (fig 1); Confoscan 4 /Nidek 460x345 µm (www.nidek-intl.com/cs.html), i.e. 0.159 mm^2), and consequently the reproducible investigation and quantification of identical areas is virtually impossible. The real-time method described here enables mapping of corneal areas up to 3200x3200 µm and opens up new horizons for in vivo tissue analyses in real time. The established quantification parameters of the SEP, e.g. number and length of sub-basal nerves, nerve density and tortuosity,[16] can now be measured in a statistically robust manner. Our system also permits follow-up studies of the SEP, which is a highly dynamic structure known to be characterised by continuous pattern changes.[17]

Nevertheless, the quality and size of the composite image were considerably influenced by subject compliance. To overcome these difficulties it is possible to reduce the mapping image to 1536x1536 pixels (1.6x1.6 mm) and thus to shorten microscopy time. It is also advisable to accumulate sufficient practice with the single-image mode before switching to mapping mode. However, a general examiner with an average experience will not have any problems with averagely compliant patients.

Patel et al.[8] used in vivo CLSM for off-line mapping of SEP in keratoconus (mean mapped area: 6.60±0.7 mm horizontally and 5.91±0.72 mm vertically; duration of investigation: 40 minutes, including 20 minutes for microscopy). Wide-field montages were post-processed using external software with a mean of 402±57 images. Similarly, Yokogawa et al.[10] produced two-dimensional reconstruction maps of normal corneal K-structures (mean mapped area: 5.88±0.5 mm horizontally and 3.51±1.37 mm vertically; 10 minutes for microscopy, 677±211 images). Our system permits an area of 3.2x3.2 mm to be mapped with a total of 64 theoretically non-overlapping single 400x400 µm images. Given that the

total CLSM time for a single composite image is less than 3 minutes with no post-processing, the advantages of the on-line system are apparent.

The method presented here permits real-time in vivo mapping of the subepithelial nerve plexus, and thus provides an essential basis for statistically robust conclusions regarding morphometric plexus changes. Our on-line system can be used for large-scale in vivo mapping of a number of two-dimensional structures following imaging with the HRTII/RCM confocal laser scanning microscope. Potential clinical applications include analysis of corneal nerve degeneration and regeneration, of surgical outcomes (e.g. corneal grafting, refractive surgery), and of contact lens wear or ocular surface disease.

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Competing interests

None.

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Figures and Legends

Figure 1

Sample CLSM image (400x400 µm; depth: 57 µm) of SEP in a healthy volunteer (image scaled to corneal diameter).

Figure 2

Composite CLSM image of SEP in a normal human cornea: image size (A) 1.6 x 1.6 mm (depth: 60 µm) and (B) 3.2 x 3.2 mm (depth: 62 µm) (images scaled to corneal diameter).

Figure 3

Composite CLSM images of the human cornea 6 months after LASIK (images scaled to corneal diameter):

A Central cornea with regenerated SEP nerve fibre structures; some keratocyte nuclei are visible in peripheral upper areas of the image due to the focal plane shift during image acquisition (image size: 3.2 x 3.2 mm; depth: 58 µm).

B Cut zone showing epithelial cell ingrowth (*), and SEP (arrows) both outside and inside the flap area (image size: 1.6 x 1.6 mm; depth: 61 µm).

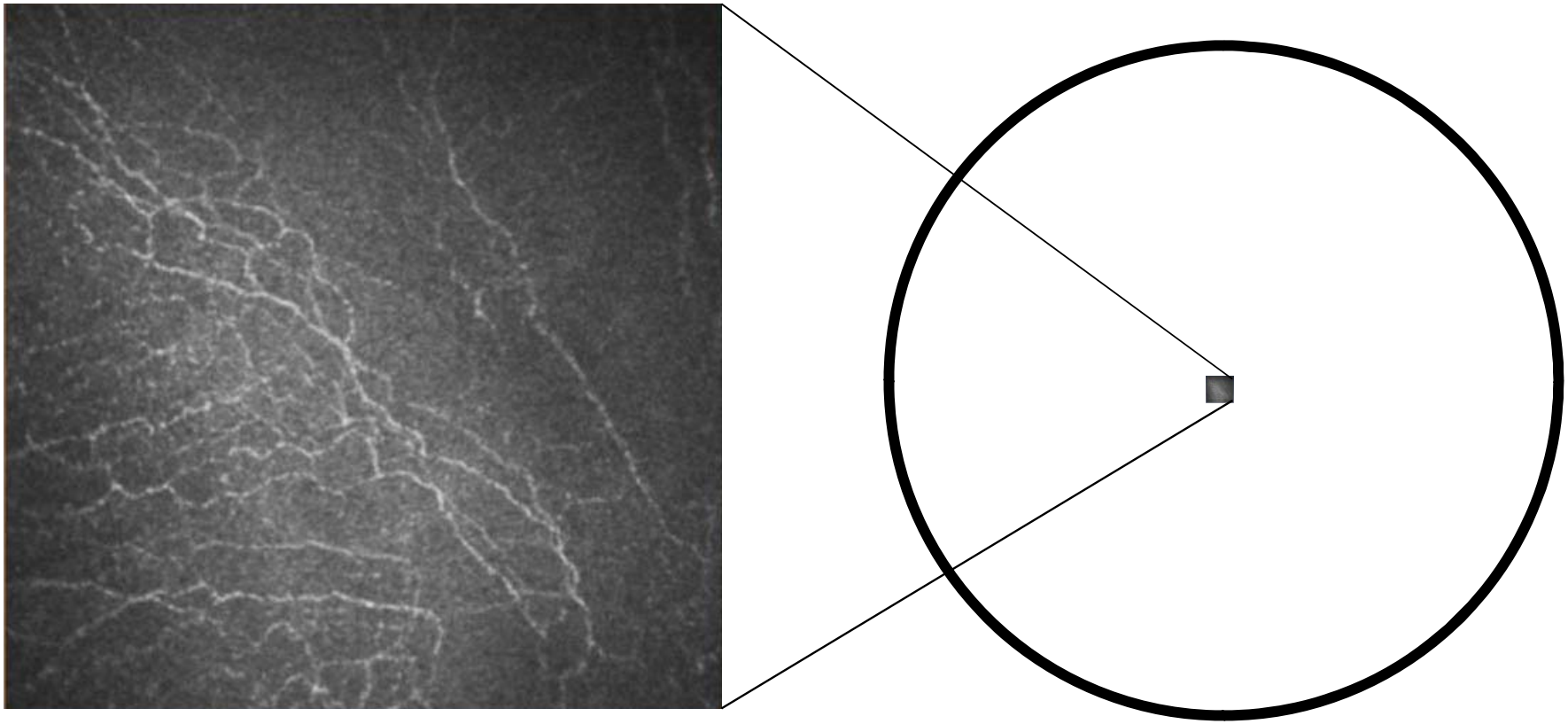

Fig. 1

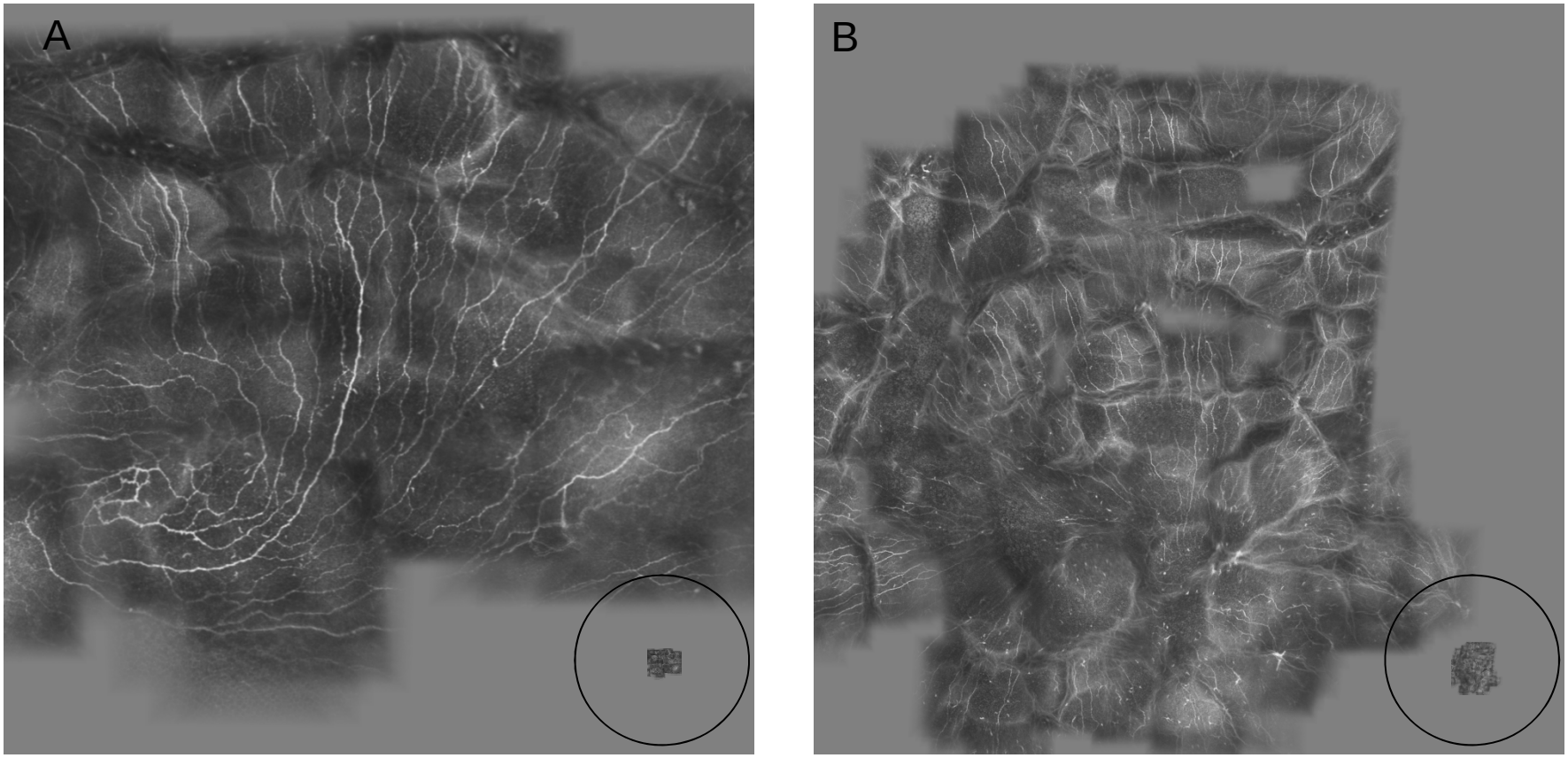

Fig. 2

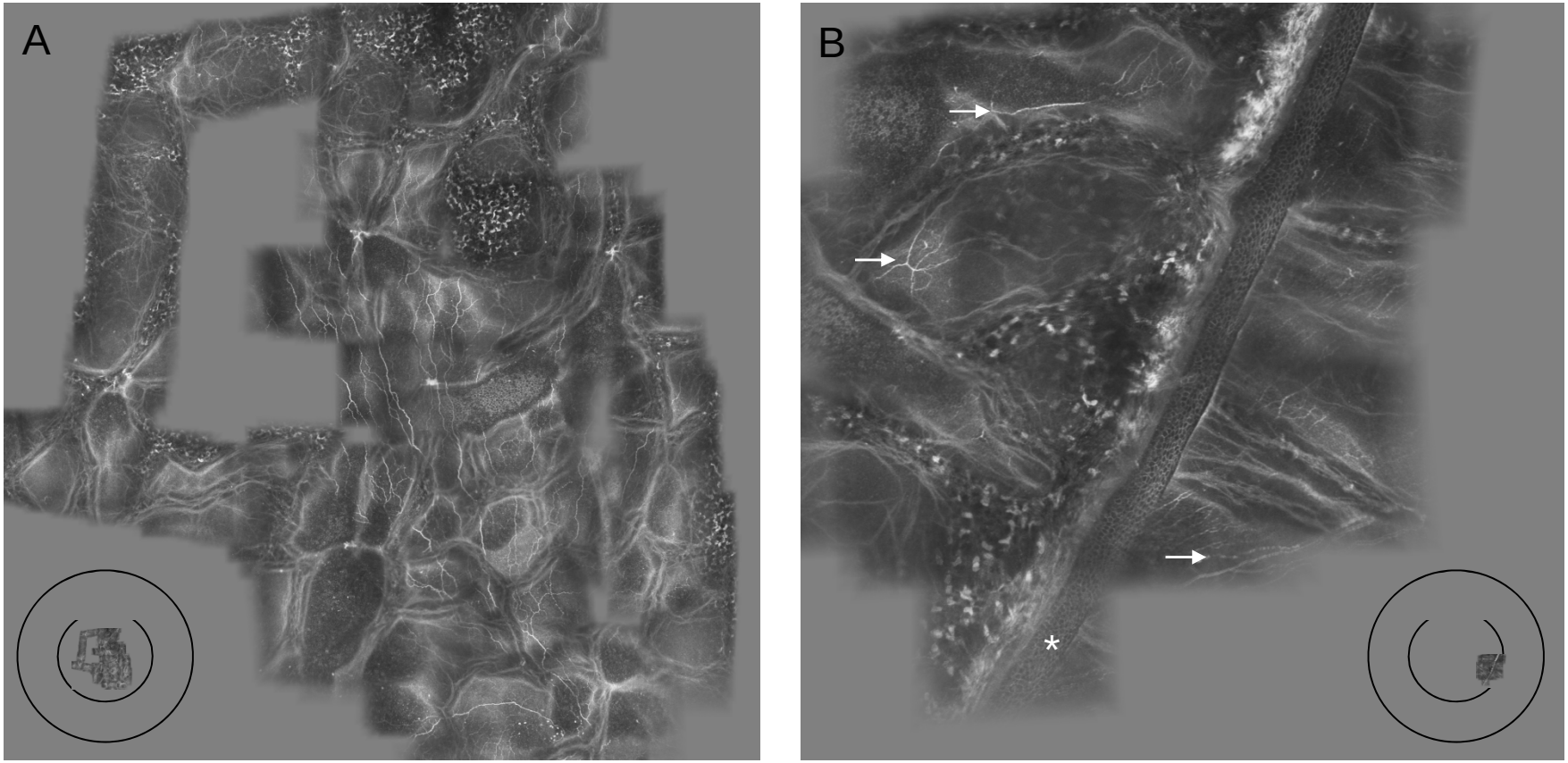

Fig. 3