Keratocyte Dimensions in The Human Cornea by *IN VIVO* AND *EX VIVO* Confocal Microscopy

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Abstract - Purpose: ***To analyze and evaluate the dimensions of human stromal keratocytes imaged by laser scanning in vivo and ex vivo confocal microscopy.***

Methods: ***50 eyes of 50 subjects of differing ages, with clinically healthy corneas, were examined by real time in vivo confocal microscopy. Five anterior and 5 posterior frames were carefully selected and for each frame 5 distinctive structures, corresponding to the stromal keratocytes, were measured. Additionally, 10 central corneal specimens of age-matched subjects, labelled with Cell Tracker (CT) were prepared by cryosection and additional labelling with DAPI. Subsequently, two channels - ex vivo confocal images, corresponding to the nuclei (DAPI) and the cell body (CT) were obtained. The dimensions were measured using measurement software and analyzed statistically.***

Results: ***The mean ± standard deviation for the longest diameters, for in vivo, ex vivo DAPI and ex vivo (CT) were measured to be 22.80 ± 4.36 μm, 17.33 ± 3.44 μm and 26.44 ± 7.97 μm for the younger age group (mean ± SD years, n=25) and 23.42 ± 4.00 μm, 16.44 ± 2.7 μm, 23.62 ± 6.82 μm for the older age group (mean ± SD years, n=25). Statistical analysis revealed significant difference between ex vivo (DAPI) measurements and in vivo measurements for both age groups (p<0.0001). However, no statistical difference was noted between in vivo and ex vivo (CT) measurements (p=0.02 for group 1 and p=0.3 for group 2).***

Conclusions: ***This study clearly demonstrates that the dimensions of the structures observed throughout the corneal stroma correspond best with the ex vivo dimensions of the entire keratocyte body rather than the nucleus alone. The long accepted concept that in vivo confocal microscopy allows visualisation of the keratocyte nuclei should be reconsidered.***

Keywords: corneal stroma, confocal microscopy, keratocytes, keratocyte nuclei, keratocyte diameter.

I. INTRODUCTION

The precise role of corneal stromal keratocytes is still not completely established, however, it has been proven that keratocytes are responsible for collagen production, matrix synthesis and turnover, and corneal stromal repair after disease or injury. (1,2) The majority of studies evaluating the keratocyte network, have used animal models and a variety of *ex vivo* microscopic techniques. (3-6) Introduction of *in vivo* confocal microscopy into clinical practice by Cavanagh et al. (1989) made possible not only real time, dynamic, observation of the living human keratocytes, but also various qualitative and quantitative analyses. (7-12)

The pioneers of *in vivo* confocal microscopy hypothesized that the bright elements observed throughout the corneal stroma were keratocyte nuclei. (8) This hypothesis was probably based, at least in part, on published *ex vivo* studies that clearly distinguished at least three sub-populations of keratocytes with complex cytoplasmic morphology not identifiable by *in vivo* confocal microscopy. (1, 3) These morphological *ex vivo / in vivo* disparity is probably due to the significantly lower resolution of the latter technique, which prevents visualisation of the fine keratocyte processes in normal corneas. (13) Furthermore, the earlier *in vivo* confocal microscopes had lower resolution and image contrast in comparison with the most recent laser scanning in vivo technology.

On the basis of the *ex vivo* characteristics of human keratocytes, we believe that at least three cellular elements should be observed in the keratocyte population. (1) These elements are: the nucleus – corresponding to the central, thickest part of the cell, clearly identifiable *ex vivo* when stained with various nucleic acid stains such as 4'6-diamino-2-phenylindole, dihydrochloride (DAPI); the keratocyte body – the cytoplasm of the cell body around the nucleus, i.e. the major body of the cell excluding the processes, which may be stained with Cell Tracker (CT) fluorescent probe; the cell processes – diversely shaped, fine elements responsible for the inter-keratocyte communication, also identified by Cell Tracker but not identifiable by *in vivo* confocal microscopy. (1, 14) On the basis of this appreciation of keratocyte structure, the oval/round particles observed by *in vivo* confocal microscopy may be either cell nuclei or "cell bodies".

We suggest the hypothesis that *in vivo* confocal microscopy might actually identify the entire cell body (excluding the processes) rather than just the cell nuclei *per se*. To test this hypothesis we performed morphometric analysis of the bright particles (keratocytes) observed *in*

vivo and correlated these measurements to the selective measurement of the keratocyte cell bodies stained by Cell Tracker and cell nuclei stained with DAPI, using *ex vivo* confocal microscopy. We believe these studies challenge the previously accepted hypothesis that *in vivo* confocal microscopy of the normal cornea visualizes only keratocyte nuclei. (7, 8, 15-21).

II. METHODS

In vivo experiment:

Subjects

The study was based on two separate age groups with clinically healthy corneas and no history of ocular or systemic disease affecting the cornea. Group 1 consisted of 25 young volunteers, mainly members of staff, 12 males and 13 females, with mean age of 28±6 years (range from 22 to 34) in all of whom both eyes were examined but 12 right and 13 left eyes were randomly selected for the purpose of this study. Group 2 consisted of 25 subjects (13 males and 12 females) with a mean age of 71±5 years (range from 65 to 75 years, undergoing assessment for cataract surgery) and included 13 right eyes and 12 left eyes. The confocal study was approved by the local ethics committee.

Following a detailed explanation and acquisition of written informed consent, *in vivo* confocal microscopy was performed, on both corneas of all subjects from. The exclusion criteria for both groups are listed in Table 1.

Confocal microscopy

Laser scanning in vivo confocal microscopy was performed with HRT II Rostock corneal module (Heidelberg Engineering GmBH, Dossenheim, Germany) and took up to 10 minutes per subject for both eyes. After explanation of the examination procedure and instillation of anaesthetic drops (Alcain, Alcon Inc), the confocal examination was commenced. The right eye was done first. The 400x lens was used for all examinations. All scans that were incomplete, associated with significant ocular movement, or were poorly centered were repeated.

The size of the images was 400 by 400 microns, with lateral resolution of 2 microns and slice thickness of 5 microns. The images were stored within the proprietary database.

Image selection and measurements

For each complete *in vivo* confocal examination, following evaluation of the images along the z-axis, the single pass with maximum number of high-quality, evenly distributed and sequential frames was selected. For each cornea, 3 anterior, 4 middle and 3 posterior frames were selected. The first frame was chosen to be immediately behind Bowman's layer, and the last frame just in front of Descemet's membrane. The rest of the frames were distributed within 40 μm between each other.

Subsequently one experienced examiner selected ten images per cornea (CNG), and two independent experienced analysers (MNR and DIG) did the measurements using analysis 3.1 (Soft Imaging System, Münster, Germany) software. For each selected frame, five bright, well delineated stromal particles (keratocyte bodies/ nuclei) were identified. For each particle the longest diameter and the middle perpendicular diameter were measured.

Ex vivo confocal microscopy:

Tissue selection and labelling

This component of the study was conducted in 2001 with the assistance of the research staff and corneal research tissue from the New Zealand National Eye Bank. Ten corneas of ten subjects were divided into Group 1 (n=5) with mean age of 23±8 years and Group 2 (n=5) with a mean age of 76±9 years. For each group the central corneal specimens (within 1.5 mm of the corneal apex) were processed. Tissue processing was done by the senior author (CNG).

All tissue was obtained within 24-72 hours post-mortem and was immediately divided into small blocks (4 mm^2 enface surface area) and incubated with Cell Tracker (CMFDA, Molecular Probes, Eugene, OR) for 12 hours ($+4^0$ C), following a standard protocol provided by the manufacturer. Subsequently, the tissue was fixed in 2.5% paraformaldehyde and freeze-stored in 20% DMSO in transport media at -80^0C. The frozen tissue was washed with PBS and processed by coronary (parallel to the epithelial surface) cryosectioning on a semi-automatic cryostat (50μm thickness). For each sample 14-17 sections were obtained. The samples were subsequently labelled with 4'6-diamino-2-phenylindole, dihydrochloride (DAPI, Molecular Probes, Eugene, OR) following the manufacturers' protocol.

The *ex vivo* in situ imaging was conducted using a Leica TCS SP2 confocal laser scanning microscope fitted with a Leica RXE upright microscope (Leica Microsystems). For all acquisitions a 40x immersion lens was used. The excitation profiles of Cell Tracker (492 nm) and DAPI (350 nm) required application of Argon and UV lasers respectively. After appropriate hardware and software adjustment the best extended focus (series of 10 images over 10 μm) image for each section was collected starting with the first section after the basal epithelium to the last section in front of the endothelium. Between 10 and 14 extended focus images (10 μm thick) were obtained for each corneal sample. Three of the samples were evaluated as slightly skewed in respect to the horizontal plane but the images were obtained from the most parallel area. Ten

scans (3 anterior, 4 middle and 3 posterior) per each cornea were selected using the aforementioned criteria. Selected series were processed (proprietary Leica software) to produce extended focus images comparable to the 10 μm section from *in vivo* confocal evaluation and were saved into a hard disc drive ready for morphometric analysis.

The automatic calliper tool for arbitrary distances incorporated into the analysis 3.1 (Soft Imaging System, Münster, Germany) software was used to measure the longest and the mid-peripheral diameter of the cells labelled with Cell Tracker and the nuclei labelled with DAPI. Five distinctive structures were measured per each section. All measurements were automatically saved into an excel table (Microsoft office 98).

Statistical methods

Continuous normally distributed data were analyzed using a mixed models approach to repeated measures. Missing random data were imputed using a maximum likelihood approach and the main effects of age and layer and their interaction effect tested. All tests were two-tailed and a 5% significance level was maintained throughout.

III. RESULTS

Summary results from the morphometric analysis are presented in Tables 2, 3 and 4. The mean ± standard deviation for the long and mid-peripheral diameters, for both age groups and for *in vivo*, *ex vivo* DAPI and *ex vivo* Cell Tracker (CT) were calculated (Table 2). Furthermore the overall median was also estimated (Table 3). On the basis of the *ex vivo* measurements, nucleo-cytoplasmic index was calculated for the long and mid-perpendicular diameter and for the assumed cell area (Table 4). Further analysis was performed for different corneal layers including the anterior 3 layers up to a maximum depth of 150 μm, the posterior 3 layers (maximum distance of 150 μm) and the 4 mid-stromal layers (spread over mid corneal thickness of 200 μm). The morphometric parameters of the nuclei stained with DAPI were statistically different from the identical measurement of the particles imaged *in vivo* ($p<0.001$) regardless of the age group or position, however, statistical difference between *in vivo* and *ex vivo* CT was encountered only for the long diameter of the posterior layers of the young age group. Overall, the study results demonstrated a better correlation between the "particles" *in vivo* and the cell bodies *ex vivo* for both age groups.

IV. DISCUSSION

This study was developed from the hypothesis that the "particles" observed by *in vivo* confocal microscopy of the corneal stroma appeared too large to represent keratocyte nuclei alone. Interestingly, as previously discussed in the literature, for the different layers of the normal cornea one can observe only the cell borders (endothelium and basal epithelium) or the cell borders and nucleus within the cell borders (wing cells).(8, 13, 22) These observations might logically suggest that the relatively bright, homogenous particles observed throughout the stroma are simply cell nuclei. (8, 13, 22) However, as most of the researchers using specular or *in vivo* confocal microscopy have noted, the technology is based on optical principles and differentiation of two sub-cellular elements depends on the difference of their refractive index. (23, 24) Furthermore, the appearance of the image is highly dependent on the position of the imaged structure in relation to the illumination beam, corresponding to the angle of incidence. (23-25) In this regard, keratocyte cytoplasm may have a refractive index similar to the extracellular matrix and in such an environment *in vivo* confocal microscopy would only distinguish keratocyte nuclei. However, keratocyte processes directed to neighboring structures may occasionally be observed. These processes cannot be connected directly to the nucleus, yet on the other hand cells expressing such processes do not appear to be of a different size compared to the neighboring cells/nuclei. These phenomena directed our studies towards undertaking a precise morphometric analysis in order to correlate cell/nuclei dimensions *ex vivo* with our *in vivo* observations of the human corneal stroma.

In the era of stereology, when the cornea is increasingly analyzed as a three-dimensional structure, various semi-quantitative or more accurate quantitative studies have attempted to evaluate the keratocyte density in healthy and diseased corneas. (10, 11, 26, 27) It therefore becomes increasingly important to define the exact nature of these stromal particles imaged by *in vivo* confocal microscopy. Indeed, when estimating the volume density of the cellular component and residual volume of the surrounding matrix, the nature of the particles is of paramount importance in order to establish precise correlation with the *ex vivo* data. (5) Furthermore, in pathological conditions the size and shape of the stromal "particles" may present significant variability (13, 28, 29) and interpretation of such alterations might differ significantly if they affect the entire cell body or only the nucleus *per se*.

The main purpose of this study is to clarify the nature of the particles observed throughout the stroma by *in vivo* confocal microscopy. The results are convincing that the particles observed are generally larger than the size of the normal keratocyte nucleus *ex vivo*. This conclusion, however, requires careful considerations of potential tissue processing artifacts. In the current studies the soeciment was stored in iso-osmotic standard transport media with 0.5% dextran prior to fixation. However, as noted in Table 2, the measurements for the young and old subjects were significantly different with regards to the size of the cell body, but not the cell nuclei. In addition, because the

nucleus per se is less prone to oedema (due to membrane permeability and water content) the difference for the nucleus parameters is smaller at a mean of 6.5%. On the other hand tissue processing similar to our methodology has been shown to cause 5-6% tissue shrinkage. (10, 19) That may also contribute to the wider standard deviation for the *ex vivo* measurements.

The x-y resolution of a confocal microscope must also be taken into consideration. All in vivo confocal microscopes exhibit better x-y than z-resolution. Therefore, the structures observed may be not the entire cell bodies, but only their parts thicker than 1 μm. Although, this is only an assumption that cannot be confirmed at the current stage of *in vivo* confocal technology, an elegant experimental study by Doughty et al provides support for this hypothesis with respect to keratocyte dimensions assessed by electron microscopy. (6) Firstly, Doughty et al encountered disparity of up to 42% for the cell length on coronal and transverse sections. Secondly, these researchers measured the z-thickness of the nucleus to be 1.01±0.44μm. Therefore, some of the nuclei *per se* would not be observed *in vivo* and on this basis measured cellular density would be lower for the living cornea. However, in the published literature there appears to be an excellent agreement between keratocyte density as assessed by *in vivo* and *ex vivo* microscopy techniques. (10, 19)

Interestingly, Prydal et al analyzed the stromal particles observed by *in vivo* confocal microscopy, referred to them as "cells", applied a computer algorithm for area calculation and then estimated the variation of cell size from 77.7 to 210.6 μm^2 (151.1μm^2±29.8) (22), which is in the range of our empirically calculated cell area. (22) Although our results are similar, our estimation was based on the hypothesis that every irregular object may be represented as an ellipse. If we assume that the longer diameter of the object is the longer diameter of the particle of interest, we have to estimate the middle perpendicular diameter of the presumed ellipse. If the middle perpendicular diameter of the particle is measured, the error will depend on the shape of the particles and for ellipse, comma, boomerang, clover and heart shape, the calculated area will be reasonably accurate. For some shapes such as triangular there will be an overestimation and for quadrangular shapes there will be an underestimation. But for the entire population of 1250 largely ellipsoid cells that were assessed, such minor variations should not influence the cell areas.

Finally, we are aware that some individual variability may bias our results especially for the *ex vivo* data, where only 10 corneas were analyzed. However, obtaining healthy central corneal tissue for research is very difficult and therefore our *ex vivo* human sample is of relatively modest size. That is the reason why we used a prior obtained data from fail instead of repeating the ex vivo experiment.

The pioneers of *in vivo* confocal microscopy provided us with a revolutionary tool for microstructural analysis of the living cornea. Today, corneal researchers are still hampered by some limitations with regards to resolution, image contrast and analytical instruments for *in vivo* microstructural analysis. Although our hypothesis that the stromal particles observed by *in vivo* confocal microscopy are cells not nuclei, may have minimal value for the *in vivo* observations per se, analysis of the *in vivo* confocal results in the future will be significantly biased by this new morphological interpretation. We also suggest that the terms cells or keratocytes, instead of keratocyte nuclei are more appropriate when addressing the stromal particles imaged by *in vivo* confocal microscopy.

V. ACKNOWLEDGEMENTS

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Table-1: Exclusion criteria for the subjects listed for analysis of keratocyte morphology.

1. History of: - contact lens wear - anterior segment disease - ocular trauma - ocular surgery - glaucoma - diabetes mellitus - systemic disease with corneal involvement - use of ocular medications - use of systemic medications, affecting the cornea
2. Clinical evidence of: - corneal degenerations - corneal dystrophies (including subclinical) - recurrent erosion syndrome

- dry eye disease
- corneal residuals from ocular trauma
- previous surgery
- elevated IOP
- corneal signs of systemic disease
- corneal deposits of systemic drugs

Table-2: The overall mean keratocyte dimension ± standard deviation (10 layers per cornea) measured *in vivo* and *ex vivo* (Cell Tracker (CT) and DAPI), for both age groups. Group 1 comprises 25 subjects (28±6 years) *in vivo* and 5 corneas *ex vivo* (23±8 years). Group 2 comprises 25 subjects (71±5 years) *in vivo* and 5 corneas *ex vivo* (76±9 years).

Group and type examination		Longest diameter (mean ±SD) µm	Mid-perpendicular diameter (mean ±SD) µm	Mean cell area (mean ±SD) μm^2
Group	*In vivo*	22.80 ± 4.36	9.99 ± 2.76	211.00 ± 9.95
	Ex vivo DAPI	17.33 ± 3.44	7.96 ± 1.76	125.52 ± 5.31
	Ex vivo CT	26.44 ± 7.97	11.23 ± 3.54	278.48 ± 26.00
Group	*In vivo*	23.42 ± 4.00	10.87 ± 2.64	230.75 ± 8.65
	Ex vivo DAPI	16.44 ± 2.7	7.42 ± 2.51	111.73 ± 5.33
	Ex vivo CT	23.62 ± 6.82	8.82 ± 2.59	206.52 ± 17.38

Table-3: The median keratocyte dimensions measured *in vivo* (n=1250) and *ex vivo* marked by Cell Tracker (n=250) and DAPI (n=250), for both age groups. Group 1 comprises 25 subjects (28±6 years) *in vivo* and 5 corneas *ex vivo* (23±8 years). Group 2 comprises 25 subjects (71±5 years) *in vivo* and 5 corneas *ex vivo* (76±9 years).

Group and type examination		Longest diameter median µm	Mid-perpendicular diameter median µm	Mean cell area median μm^2
Group 1	*In vivo*	22.40	9.90	204.75
	Ex vivo DAPI	17.19	8.04	124.92
	Ex vivo CT	25.07	10.50	248.30
Group 2	*In vivo*	23.20	9.90	215.01
	Ex vivo DAPI	16.21	8.04	115.40
	Ex vivo CT	21.98	10.55	207.67

Table-4: *Ex vivo* nucleo-cytoplasmic (N/C) ratio for two age groups. Group 1 comprises of 5 corneas (23±8 years) and Group 2 includes 5 corneas (76±9 years).

Group and type examination		Longest diameter	Mid-perpendicular Diameter	Mean area
Group	*Ex vivo* DAPI	17.33 ± 3.44 µm	7.96 ± 1.76µm	125.52 ± 5.31μm^2
	Ex vivo CT	26.44 ± 7.97µm	11.23 ± 3.54µm	278.48 ± 26.00 μm^2
	N/C ratio	66 %	70 %	45%
Group	*Ex vivo* DAPI	16.44 ± 2.7 µm	7.42 ± 2.51 µm	111.73 ± 5.33 μm^2
	Ex vivo CT	23.62 ± 6.82 µm	8.82 ± 2.59 µm	206.52 ± 17.38 μm^2
	N/C ratio	70 %	84 %	54 %

Table-5: Analysis of the morphometric parameters of the structures observed by *in vivo* and *ex vivo* confocal microscopy (CT and DAPI), divided into 3 layers: anterior post-Bowman's stromal layers (n=3 within 150 μm), mid-stromal layers (n=4 within 200 μm) and posterior pre-Decemet's layers (n=3 within 150 μm).

Group and type examination		Longest diameter			Mid-perpendicular diameter		
		Anterior	Posterior	Middle	Anterior	Posterior	Middle
Group 1	*In vivo* (1)	24.103	21.120	23.032	10.445	9.180	10.249
	Ex vivo DAPI (2)	17.256	17.227	17.457	7.790	8.248	7.882
	Ex vivo CT (3)	26.157	25.300	27.513	11.183	11.593	10.999
	1 vs 2 vs 3 P<0.001	1 vs 2	1 vs 2	1 vs 2 1vs 3	1 vs 2	1 vs 2	1 vs 2
Group 2	*In vivo*	23.999	23.066	23.244	10.707	10.877	10.980
	Ex vivo DAPI	16.148	16.896	16.320	6.942	8.231	7.160
	Ex vivo CT	23.388	22.802	24.407	8.734	8.538	9.109
	1 vs 2 vs 3 P<0.001	1 vs 2	1 vs 2	1 vs 2	1 vs 2	1 vs 2 1vs 3	1 vs 2

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