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Two-photon optical microscopy imaging of endothelial keratoplasty grafts

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Abstract
Purpose To investigate the microstructure of endothelial keratoplasty grafts using two-photon optical microscopy.
Methods Six endothelial keratoplasty grafts obtained from human donor corneoscleral tissues and prepared by submerged hydrodissection technique were imaged by two-photon optical microscopy. In each graft, two liquid bubbles were created in order to investigate the presence of a conserved cleavage plane regardless of the volume of posterior stroma that remained attached to Descemet's membrane (DM); the first bubble (bubble A) was generated under DM and the second bubble (bubble B) injection was done in order to obtain a layer of deep stroma that kept the two bubbles separated. Six human donor corneoscleral tissues were used as controls. Second harmonic generation and two-photon emitted fluorescence signals were collected from each specimen.
Results Dissection of stroma occurred along the posterior collagen lamellae at variable distance from DM, which ranged between 3 and 16 μm in bubble A and between 23 and 41 μm in bubble B. The residual stroma included, anteriorly, bands of collagen lamellae, and thin bundles of stromal collagen fibrils, posteriorly, which were tightly intertwining with the underlying DM. There was no anatomically distinct plane of separation between these pre-Descemetic stromal collagen bundles and the overlying collagen lamellae with this hydrodissection technique.
Conclusions Two-photon optical microscopy provided label-free high-resolution imaging of endothelial keratoplasty grafts, showing that the most posterior stroma changes organization at approximately 10 μm above the DM. The pre-Descemetic stromal collagen fibrils form an intertwined complex with DM, which cannot be separated using hydrodissection.

Keywords Endothelial keratoplasty · Two-photon optical microscopy · Descemet's membrane

Introduction

During the last decade, several improvements have been done for the selective replacement of diseased endothelium together with Descemet's membrane (DM) either with or without corneal stroma attached [1]. Descemet's membrane endothelial keratoplasty (DMEK) was developed to only replace the diseased endothelium with a healthy donor DM and endothelial layer. Several techniques for DMEK lenticule preparation from a donor cornea have been developed, such as DM peeling, pneumatic dissection, and hydrodissection of DM, each offering specific advantages but mostly depending on personnel skill [2–15]. Manual peeling of DM with two forceps has been shown to provide the cleavage plane consistently located between interfacial matrix of the Descemet's membrane and posterior stromal collagen lamellae [1]. A comparison

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between forceps dissection and pneumatic dissection revealed no advantages of the pneumatic over the manual technique [16]; in addition, remnants of stromal collagen attached to DM have been reported in most cases after air injection [14, 15, 17].

The submerged hydro-separation (SubHyS) technique has been previously shown to be highly effective for the separation of the stroma and DM [2–4]. In previous case series, complete detachment of the Descemet endothelial graft has been achieved in almost all cases with only one injection of liquid bubble. Several advantages of standardized SubHyS method performed in the eye bank over previous techniques have been found, such as the endothelium is not dried, the diameter of the Descemet endothelial grafts is large, and the integrity and numerosity of endothelial cells can be evaluated prior to shipping the grafts to the surgery room.

In previous studies [2–4], these authors have analyzed the separation of the stroma from DM after SubHys technique using light and scanning electron microscopy. Although a clear separation between the stroma and DM has been found in several samples, high-magnification analysis of Descemet endothelial grafts using electron microscopy has shown remnants of stromal collagen attached to DM. On the other hand, sample preparation for electron microscopy can introduce imaging artefacts of grafts' microstructures. Two-photon optical microscopy can be an alternative to these traditional methods, since it provides intrinsic tissue optical sectioning capabilities combined with deep penetration and the multimodality signals approach [18, 19]. In this study, we investigated the microstructure of endothelial keratoplasty grafts after SubHys technique using two-photon optical microscopy. The technology has been already shown to offer valuable information on the microstructure of human cornea close to in-vivo conditions by generation and analysis of two-photon emitted fluorescence (TPEF) and second harmonic generation (SHG) signals [20–22]. The advantages of two-photon optical microscopy over electron microscopy include molecular specificity and no requirements for fixation; in addition, the technique can image tissues up to 1 mm depth.

The aim of this study was to investigate the ultrastructure of endothelial keratoplasty grafts after SubHys technique in human donor corneas using two-photon microscopy. We were interested to understand whether this novel microscopy technique was able to resolve the presence of a conserved cleavage plane (i.e., an acellular pre-Descemetic layer) regardless of the volume of posterior stroma that remained attached to DM. A two liquid bubble injection strategy was performed in order to generate the cleavage along varying depths of the deep stroma and to allow clear visualization of the bubbles' wall by two-photon optical microscopy. To the best of our knowledge, this is the first study reporting the results of two-photon optical microscopy imaging of the deep stroma and DM in human corneal tissues and endothelial keratoplasty grafts.

Materials and methods

Twelve eye bank donor corneoscleral tissues, from different donors, which did not meet requirements for transplantation, were retrieved from the Veneto Eye Bank Foundation (Venezia Zelarino, Italy). Written informed consent from the next of kin was obtained for the use of samples in research. All human tissues were used in compliance with the guidelines of the Declaration of Helsinki for research involving the use of human tissues. The experimental protocol was approved by the National Research Council (CNR) research ethics and bioethics advisory committee. Donors did not have history of corneal pathologies, eye surgery, or any major systemic diseases.

Hydroseparation technique

Six corneoscleral tissues underwent SubHyS technique for endothelial keratoplasty graft preparation; in addition, six corneoscleral tissues were used as controls.

The advantage of SubHyS procedure over pneumatic technique is that the bubble formation is fully controlled by the operator. The SubHyS procedure was carried out under sterile conditions. Each corneal tissue was completely submerged in a sterile small petri-plate containing around 10–15 ml of sterile organ culture (OC) medium. Each tissue was held at the sclera with stainless steel toothed forceps. As per our original procedure [3, 4], a 25-gauge needle (bent with bevel up) connected to a 1 ml syringe filled with OC medium was inserted into the peripheral cornea. The needle was initiated through the sclera near the Schwalbe's line at the scleral spur, which was used as reference. The needle was then moved radially beneath the endothelium towards the central cornea. Injection was performed when the bevel was completely inside the cornea. Based on previous experience [3, 4], we developed the two-injection strategy in order to create the cleavage of the two bubbles at varying tissue depths close to DM. Specifically, one of the bubbles was created from one end of the cornea, and the other bubble was created from the opposite end of the tissue to generate a double bubble (Fig. 1a and b). The first bubble (bubble A, which represents the standardized procedure in the eye bank) [3, 4] was generated under DM and its formation was stopped halfway; the second bubble (bubble B) injection was done in order to obtain a layer of deep stroma overlying DM that kept the two bubbles separated. The use of the SubHyS procedure was valuable to keep the two bubbles separated and anatomically intact for subsequent optical coherence tomography and two-photon microscopy imaging.

Optical coherence tomography imaging

After the double bubble preparation, each donor was imaged by anterior segment optical coherence tomography (AS-OCT;

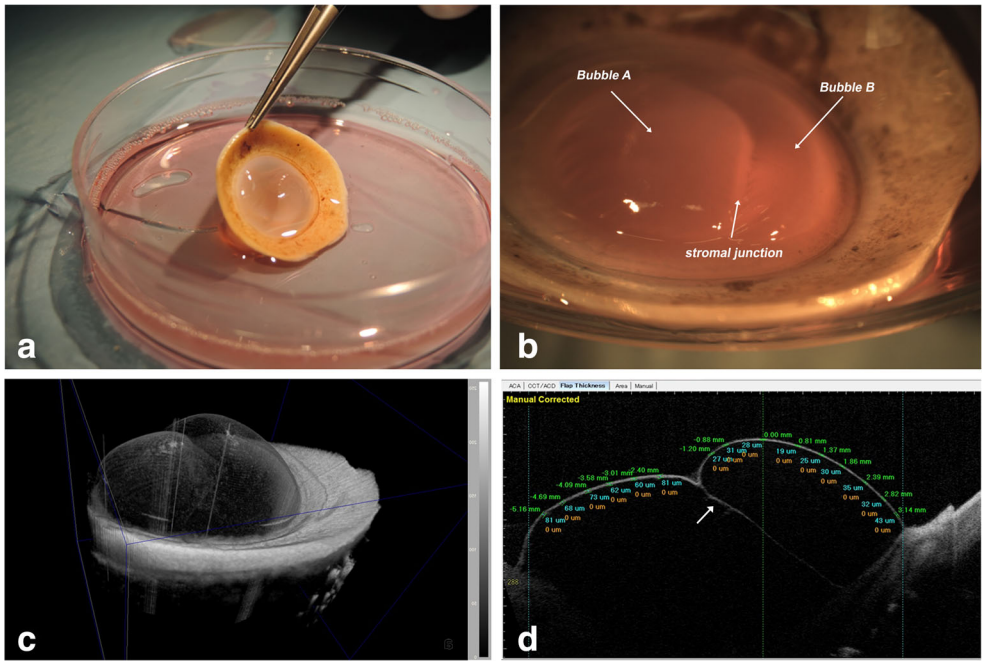

Fig. 1 Descemet's membrane (DM) endothelial keratoplasty graft preparation using submerged hydro-separation method and two-injection strategy. **a**, **b** The main bubble (bubble A) was formed when the injection site was between the stroma and DM without undergoing any stromal filling ("standard" plane of cleavage in SubHys technique in the eye bank); the bubble formation was stopped halfway [3, 4]. The secondary bubble (bubble B) was generated in the posterior stroma in order to obtain a layer of stroma that kept the two bubbles separated. **c**, **d** 3D and 2D OCT image reconstruction of the graft showing the thickness profile of the two detached bubbles. **d** The double bubble strategy was used in order to obtain a layer of stroma that kept the two bubbles separated (*arrow*), and thus to investigate the presence of an anatomically distinct pre-Descemetic acellular layer by two-photon optical microscopy

CasiaSS-1000, Tomey Corporation, Nagoya, Japan) in order to measure the graft thickness and the edges of the two bubbles. In each donor, the graft thickness of each detached bubble was measured over five locations on the 2D cross-sectional images using the proprietary AS-OCT software. After AS-OCT imaging, donor grafts were fixed in 4% paraformaldehyde (PFA) and shipped to the laboratory for two-photon imaging; fixation was used in order to avoid tissue swelling of thin endothelial keratoplasty grafts during shipping. In addition, fresh donor corneal tissues were shipped to the laboratory in vials including dextran-enriched culture medium, and used as controls. The use of controls was valuable to confirm that fixation did not change the ultrastructure of the posterior corneal stroma, DM, and endothelium.

Two-photon optical microscopy imaging

All corneal specimens were imaged using a commercial two-photon microscope (Leica DM6000CS, Leica Microsystems GmbH, Wetzlar, Germany). Each donor tissue was placed on a glass slide under an upright microscope and illuminated with a pulse width of 140 f. (measured at the sample plane) at 80 MHz of repetition rate generated by a Ti: Sapphire laser (VISION II, Coherent, Santa Clara, CA, USA) tuned to 810 nm. The laser power was attenuated by an electro-optical-modulator (EOM) and then coupled into the Leica SP8-Spectral Scan-Head (Leica Microsystems GmbH, Wetzlar, Germany), where it passes through the *x*–*y* scanning mechanism before being focused by a water immersion objective (HCX IRAPO L 25X/0.95W, 2.5 mm working distance, Leica Microsystems GmbH, Wetzlar, Germany). SHG and TPEF signals were collected in forward and backward direction by a pair of nondescan detectors (NDDs) respectively. The forward scatter signal that passed through the sample was collected with the use of a short pass filter ($\lambda < 680$ nm, SP680) and a 10nm FWHM band pass filter centered at 405nm (FF01-405/10-25, Semrock Inc., Rochester, NY, USA) positioned in front of the transmission NDD unit in order to collect the SHG signal. The reflected light coming from the sample was filtered by an IR filter SP680 and encountered a dichroic beam splitter (Di02R405-25x36,Semrock Inc., Rochester, NY, USA). The reflected light paths from the dichroic were filtered by 525/50 and entered the reflection NDD unit.

Each sample was mounted downward (i.e., endothelium up) with the corneal surface parallel to the scanning plane and was scanned with a 1-μm step size in the *z*-axis, extending from the endothelium to the posterior stroma up to 150 μm above the DM. Images with 1024 × 1024 pixels resolution were recorded on multiple locations in the central region of the grafts' bubbles and the posterior stroma of control tissues. Image processing, analysis, and visualization were carried out

using proprietary Leica software and an image processing package (Image J, NIH, www.imagej.nih.gov/ij/) using custom-written macros.

Results

The mean donor age of endothelial keratoplasty grafts was 65.2 ± 11.7 years; the post-mortem interval was 11.4 ± 7.2 h and the mean endothelial cell density (ECD) was 1,933 ± 103 cells/mm [2]. In control tissues, the mean donor age was 67.2 ± 5.9 years with post-mortem interval of 16.9 ± 5.9 h and mean ECD of 2,066 ± 196 cells/mm [2]. Two bubbles were obtained in all sclerocorneal tissues; the bubbles were of mixed type, and bubble B showed the largest diameter in all cases, as planned (Table 1).

Endothelial keratoplasty grafts — AS-OCT imaging

Three-dimensional AS-OCT imaging showed the presence of two bubbles in all samples (Fig. 1c). The cleavage was near the corneal periphery in both bubbles, and the two bubbles were separated by a thin layer of stroma. The thickness of bubble A was smaller than bubble B, as expected (Fig. 1d). The thickness profile of the two bubbles was almost constant in all cases, with differences ≤13 μm between the center and periphery of each bubble's wall (Table 2). The average thickness of bubbles A and B was 27 ± 5 μm and 60 ± 5 μm respectively. OCT imaging did not allow discriminating between the components of the bubble walls due to limited spatial resolution (axial 10 μm, lateral 30 μm).

Endothelial keratoplasty grafts — two-photon optical microscopy imaging

Stromal collagen fibrils attached to DM were found in both bubbles of each sample. In bubbles A, the thickness of residual stroma attached to DM ranged between 3 and 16 μm (mean, 8.8 ± 4.7 μm); in bubbles B, it was on average 23–41 μm (mean, 34.3 ± 5.7 μm). In all cases, the residual stroma was composed by bands of regularly overlapping collagen lamellae and, within 2 μm from DM, by short bundles of collagen fibrils, which were intertwining with the underlying DM (Fig. 2). The difference between bubbles A and B was represented by the presence of more collagen lamellae in the latter as the distance from DM increased. Descemet's membrane was composed of tiny collagen fibrils regularly arranged to form a mesh, as typically seen in basement membranes. The average DM thickness was 10 ± 2 μm. In bubbles A, hydrodissection always occurred along the deepest collagen lamellae overlying the pre-Descemetic collagen bundles (Figs. 3 and 4), which were intact in all cases; the mean bubble A wall thickness was 29 ± 6 μm. In bubbles B, dissection occurred between the posterior stromal collagen lamellae at variable distance from DM along the periphery and center of each bubble (Table 1). The most posterior keratocytes were visualized at a distance of 11–15 μm from the inner endothelium surface (i.e., 2–7 μm above DM). The cells were interspersed with the posterior stromal collagen lamellae. The mean bubble B wall thickness was 44 ± 10 μm. The mean corneal endothelium thickness was 11 ± 2 μm.

Table 1 Diameter (mm) of both detached bubbles in each corneal graft after SubHys technique. Measurements were done by using commercial AS-OCT device

Graft	Bubble A	Bubble B
142935	2.75	3.10
142577	4.67	10.27
142617	3.71	7.35
150381	1.91	7.38
150320	4.80	4.96
150395	3.95	4.31
Mean ± SD (mm)	3.63 ± 1.12	6.23 ± 2.61

Control tissues — two-photon optical microscopy imaging

In control tissues, the posterior stroma revealed a depth-dependent structural anisotropy. Descemet membrane was composed of collagen fibril bundles crossing each other at regular angles (60–90°) to form a mesh; on average, DM thickness was 9 ± 2 μm. In all cases, short bundles of pre-Descemetic stromal collagen (thickness: 2 μm) were tightly interdigitating with DM. The overlying stromal collagen resembled the regular arrangement of lamellae as the distance from DM increased, becoming clear ≥10 μm above the DM. The collagen fibrils lying at different planes were changing their arrangement and organization as a function of depth, with no discontinuities in the ultrastructure of posterior stroma (Fig. 5). No anatomically distinct acellular layer above DM was found in any case. In the central region of the posterior stroma, the keratocytes were resolved 2 μm above DM (electronic supplementary multimedia file 1). In fresh control tissues, the endothelium thickness was on average 10 ± 2 μm.

Discussion

Two-photon optical microscopy was reliable for investigating the microstructure of endothelial keratoplasty grafts obtained by liquid dissection in human donor tissues. The technique provided label-free high-resolution imaging of donor grafts, showing that the most posterior stroma changes organization at approximately 10 μm above the DM. Neither anatomically distinct acellular pre-Descemetic layer nor any reproducible

Table 2 Thickness (μm; mean ± SD) profile of both detached bubbles in each corneal graft after SubHys technique. Measurements were done by using commercial AS-OCT device. Points 1 and 5 correspond to the periphery of each bubble's wall; points 2, 3, and 4 represent the central points of the bubble walls

Graft	142935		142577		142617		150381		150320		150395	
Corneal location	Bubble A	Bubble B	Bubble A	Bubble B	Bubble A	Bubble B	Bubble A	Bubble B	Bubble A	Bubble B	Bubble A	Bubble B
1	34	60	28	65	28	67	26	68	28	64	28	67
2	28	61	25	57	31	64	25	64	26	63	20	63
3	31	50	19	54	24	54	24	63	23	52	19	62
4	27	51	27	63	18	53	20	59	34	57	28	53
5	35	62	31	64	34	58	32	61	30	65	32	56
Mean ± SD (μm)	31 ± 4	57 ± 6	26 ± 4	61 ± 5	27 ± 6	59 ± 6	25 ± 4	63 ± 3	28 ± 4	60 ± 6	25 ± 6	60 ± 6

plane of dissection between DM and the most posterior corneal stroma was found after SubHys technique. The double bubble strategy using hydrodissection was valuable to create two liquid bubbles of different thickness and to allow us to investigate the presence of a conserved cleavage plane (i.e., an acellular pre-Descemetic layer), regardless of the volume of posterior stroma that remained attached to DM. When the bubble was created at the standard plane of cleavage (i.e., bubble A in this study) [3, 4], the average stromal collagen attached to DM was 8.8 ± 4.7 μm. In all grafts, dissection was found along the regularly arranged collagen lamellae overlying the pre-Descemetic stroma. These pre-Descemetic collagen fibril bundles (on average 2 μm in thickness) were tightly packed and were firmly intertwining with DM. Although the pre-Descemetic stroma and DM were microstructurally distinct, they formed a structural–functional unit, which could not be separated by liquid dissection.

Stromal keratocytes were found between the dissected collagen lamellae of the deep stroma overlying the pre-Descemetic collagen fibril bundles at 2–9 μm from DM. The graft thickness and appearance was not altered by fixation since there was no difference between measurements (i.e., thickness of pre-Descemetic collagen stroma, DM, and endothelium) collected by grafts, which were fixed in 4% PFA, and fresh donor tissues, which were used as controls.

Different optical microscopy methodologies provided comparable results on the histology of the stroma and DM in endothelial keratoplasty corneal grafts, which were prepared by pneumatic dissection or hydrodissection [13–17]. These studies have shown that stromal collagen fibrils are still

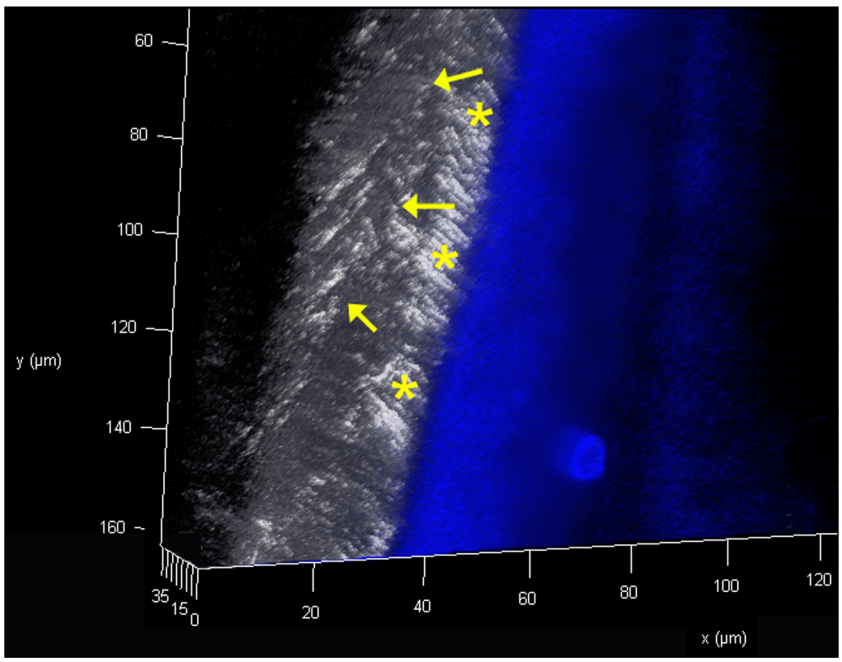

Fig. 2 Two-photon image reconstruction of the peripheral wall of bubble Ain tissue 142577. After SubHys technique, dissection occurs along the most posterior collagen lamellae, leaving intact the pre-Descemetic collagen fibrils. These fibrils are arranging in vertical branches and form an intertwined complex (*arrows*) with Descemet's membrane (*asterisks*). *Bars* show micrometers

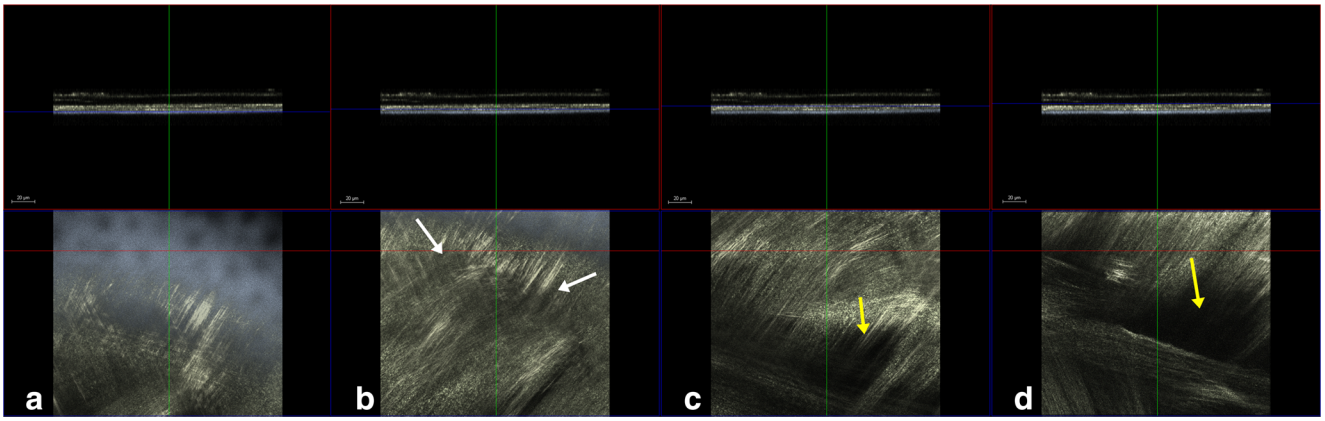

Fig. 3 *Upper row*: two-photon cross-sectional image reconstruction of the central wall of bubble Ain tissue 150381. *Bottom row*: two-photon image Z-stack of the same graft. Cellular structures are *blue* and collagen is *white* (false colors). **a**, **b**) Collagen fibrils forming Descemet's membrane (DM) and pre-Descemetic stroma are intertwining with each other (*white arrows*), thus representing a structural unit that cannot be separated by SubHys technique. **c**, **d** Hydro-separation occurs along the stromal collagen lamellae (*yellow arrows*). *Scale bars*: 20 μm

attached to DM after graft preparation. In 2013, Dua et al. [13] have claimed the identification of an acellular pre-Descemetic layer (i.e., Dua's layer) of 10 ± 3 μm thickness constituted by tightly packed collagen bundles, whose fibrils were thinner (21 ± 2 nm vs 24 ± 2 nm) than overlying collagen fibrils of the deepest stroma. In 2002, Hirano et al. [15] used transmission electron microscopy (TEM) to analyze seven specimens obtained by deep anterior lamellar keratoplasty (DALK). The authors evidenced an irregular mixing of collagen fibrils between the corneal stroma and DM. In 2010, Jafarinasab et al. [17] collected DM and recipient stroma taken from three eyes (age 22–35 years) which had DALK with the big-bubble technique, and used light microscopy and TEM for imaging those grafts. The thickness of DM ranged between 9 μm and 13μm and the residual corneal stroma thickness ranged between 6 μm and 12 μm. The residual stroma consisted of collagen lamellae and keratocytes. Recently, Scholtzer-Schrehardt et al. [14] performed TEM and immunohistochemistry analysis on ten endothelial keratoplasty grafts obtained by pneumatic dissection. The authors showed that the dissection did not occur at a single reproducible stromal plane but resulted in great variation in wall thickness along the graft. The wall of the bubble after air injection was composed of a thin stromal sheet varying in thickness from 4.5 μm to 27.5 μm (mean, 14.9 ± 6.5 μm), including the endothelium, DM, and stroma. The thickness of the wall was thinner in the central portion (mean, 8.3 ± 2.9 μm) than in the peripheral parts (mean, 22.6 ± 3.4 μm) of the bubble. Accordingly, the stromal sheet separated

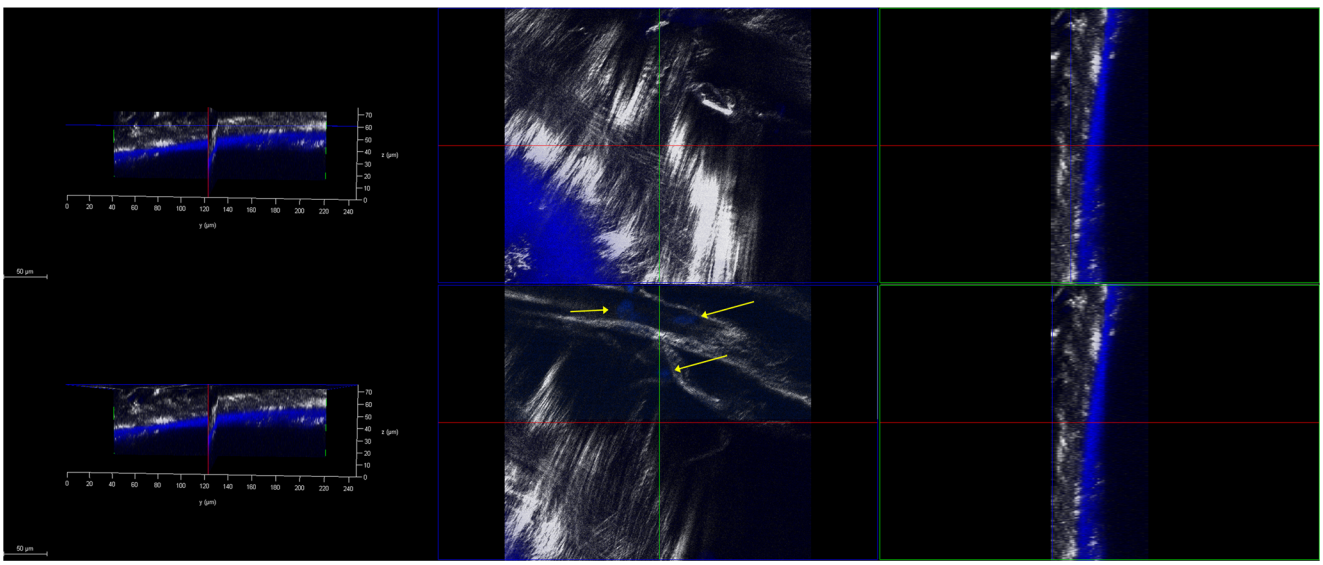

Fig. 4 Two-photon cross-sectional image reconstruction of the central wall of bubble A in tissue 142617. Cellular structures are *blue* and collagen is *white* (false colors). *Upper row*: the dissection plane with SubHys technique occurs along the collagen lamellae overlying the pre-Descemetic collagen fibril bundles. *Bottom row*: stromal keratocytes (*yellow arrows* shows the cell nuclei) are lying between the interrupted collagen lamellae, here at a distance of 9 μm from DM. *Scale bars*: 50 μm

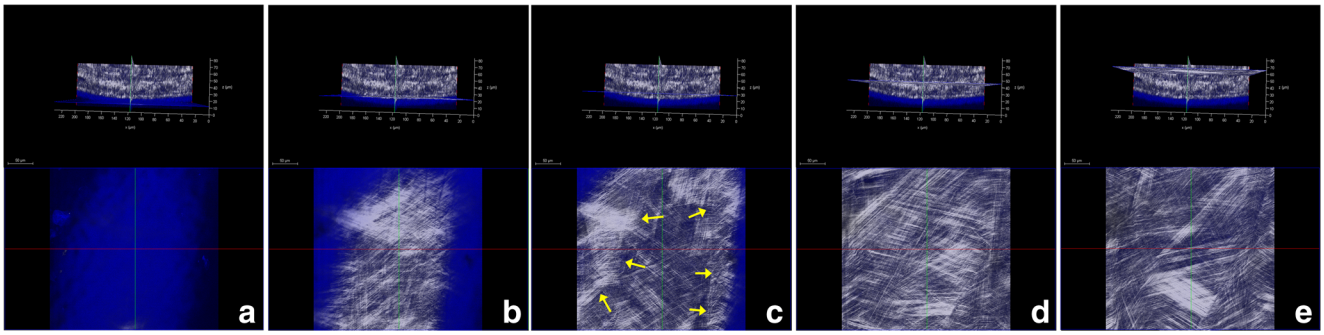

Fig. 5 *Upper row*: 60 μm Z-stack image reconstruction in a fresh donor corneal tissue specimen used as control. Cellular structures are shown in *blue* and collagen is *white* (false colors). *Bottom row*: multimodal two-photon en face imaging of the posterior region of the cornea showing the corneal endothelium (**a**), Descemet's membrane (DM; **b**), pre-Descemetic collagen fibril bundles (**c**) and posterior collagen lamellae (**d** and **e**) respectively. The most posterior stroma of the human cornea shows a highly depth-dependent 3D architecture; the collagen fibrils, which are arranged in wide lamellae crossing each other at 60–90° degrees through the posterior stroma, change their organization at approximately 10 μm from the DM. The pre-Descemetic collagen fibrils are arranged in tightly packed bundles, which are intertwining with underlying DM (**c**, *yellow arrows*). *Scale bars*: 50 μm

by air injection was composed anteriorly by a variable number of stromal collagen lamellae and posteriorly by the pre-Descemetic interwoven collagen fibrils extending in DM. TEM analysis has shown that the stromal keratocytes reside at variable distance, ranging from 1.5 μm to 18 μm from the central to peripheral cornea, from DM. The present findings on the cellular and extra-cellular components of the deepest human corneal stroma using two-photon optical microscopy imaging are consistent with previous TEM studies, however with the advantage of providing conditions closer to the in-vivo situation with no or minimal tissue processing and marker-free three-dimensional tissue reconstruction.

The human corneal stroma shows highly anisotropic structural organization and arrangement of stromal collagen fibrils even in the most posterior stroma. The collagen lamellae shows a grid-like structure, crossing each other at almost vertical angles (60°–90°) up to 10 μm above DM; the deepest stromal collagen lamellae, lying anteriorly to DM, are thinner than overlying lamellae and form a branch network with DM collagen fibers. The meshwork of interwoven stromal collagen fibrils entering into DM has been previously observed in human corneas and termed "Bowman's-like" zone [23]. The current evidence that bundles of collagen fibrils (i.e., the pre-Descemetic stromal collagen) are firmly attached to DM and that cannot be separated by either pneumatic or hydrodissection techniques reinforces previous hypothesis on the existence of different cohesive strength between the posterior stromal collagen lamellae (low) and the Descemet-pre-Descemetic collagen unit (high). From current understanding, either the pneumatic or liquid dissection are generated along the most posterior collagen lamellae, which offer the least resistance to non-physiologic increase of pressure, overlying the pre-Descemetic collagen fibril bundles. A few laboratory studies have investigated the local stiffness of distinct spatial regions of the human cornea, including DM and posterior stroma [19, 24, 25]. Last et al. [25] used AFM to measure the elastic modulus of different layers of the human cornea, including DM. The average elastic modulus of DM (50 ± 18 KPa) was 7 times stiffer than the anterior basement membrane (7.50 ± 4.2 KPa). The difference in elastic modulus of DM and anterior basement membrane has been related to the differences in structural organization, composition, and function between these basement membranes. Abrams et al. [26] used AFM to image the topography of DM and anterior basement membranes. The topographic measurements revealed smaller pore sizes in DM, which creates a more compact structure. This important structural difference is consistent with the observed differences in elastic modulus. Indeed, the anterior basement membrane supports epithelial cells that are continually renewed via migration and proliferation from the peripherally located limbal stem cells, while DM supports endothelial cells that are static and functional throughout the life of an individual.

In conclusion, two-photon optical microscopy was valuable to resolve the ultrastructure of the deep stroma and DM both in endothelial keratoplasty grafts and human donor sclerocorneal tissues. There was no anatomically distinct plane of separation between the pre-Descemetic stromal collagen fibril bundles and the overlying collagen lamellae, as resolved by two-photon optical microscopy (1 μm axial resolution, 0.6 μm lateral resolution). The most posterior stromal collagen in the human cornea forms a stiff fibrillary network with the underlying DM, which cannot be separated by hydrodissection.

Compliance with ethical standards

Ethical statement This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest All authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers'

bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge, or beliefs) in the subject matter or materials discussed in this manuscript.

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References

1. Kruse FE, Schlötzer-Schrehardt U, Tourtas T (2014) Optimizing outcomes with Descemet's membrane endothelial keratoplasty. Curr Opin Ophthalmol 25(4):325–334
2. Ruzza A, Parekh M, Salvalaio G, Ferrari S, Camposampiero D, Amoureux MC, Busin M, Ponzin D (2015) Bubble technique for DMEK tissue preparation in the eye bank: air or liquid? Acta Ophthalmol 93(2):e129–e134
3. Parekh M, Ruzza A, Salvalaio G, Ferrari S, Camposampiero D, Busin M, Ponzin D (2014) Descemet membrane endothelial keratoplasty lenticule preparation from donor corneas using a standardized and validated submerged hydro separation method. Am J Ophthalmol 158(2):277–285
4. Salvalaio G, Parekh M, Ruzza A, Ferrari S, Camposampiero D, Ponzin D (2014) DMEK lenticule preparation from donor corneas using a novel 'SubHyS' technique followed by anterior corneal dissection. Br J Ophthalmol 98(8):1120–1125
5. Parekh M, Salvalaio G, Ruzza A, Camposampiero D, Griffoni C, Zampini A, Ponzin D, Ferrari S (2013) Posterior lamellar graft preparation: a prospective review from an eye bank on current and future aspects. J Ophthalmol 2013:769860
6. Jardine GJ, Holiman JD, Galloway JD, Stoeger CG, Chamberlain WD (2015) Eye Bank-prepared femtosecond laser-assisted automated Descemet membrane endothelial grafts. Cornea 34(7):838–843
7. Brissette A, Conlon R, Teichman JC, Yeung S, Ziai S, Baig K (2015) Evaluation of a new technique for preparation of endothelial grafts for Descemet membrane endothelial keratoplasty. Cornea 34(5):557–559
8. Muraine M, Gueudry J, He Z, Piselli S, Lefevre S, Toubeau D (2013) Novel technique for the preparation of corneal grafts for Descemet membrane endothelial keratoplasty. Am J Ophthalmol 156(5):851–859
9. Schlötzer-Schrehardt U, Bachmann BO, Tourtas T, Cursiefen C, Zenkel M, RösslerK KFE (2013) Reproducibility of graft preparations in Descemet's membrane endothelial keratoplasty. Ophthalmology 120(9):1769–1777
10. Groeneveld-van Beek EA, Lie JT, van der Wees J, Bruinsma M, Melles GR (2013) Standardized 'no-touch' donor tissue preparation for DALK and DMEK: harvesting undamaged anterior and posterior transplants from the same donor cornea. Acta Ophthalmol 91(2):145–150
11. Dapena I, Moutsouris K, Droutsas K, Ham L, van Dijk K, Melles GR (2011) Standardized "no-touch" technique for descemet membrane endothelial keratoplasty. Arch Ophthalmol 129(1):88–94
12. Kymionis GD, Yoo SH, Diakonis VF, Grentzelos MA, Naoumidi I, Pallikaris IG (2011) Automated donor tissue preparation for descemet membrane automated endothelial keratoplasty (DMAEK): an experimental study. Ophthalmic Surg Lasers Imaging 42(2):158–161
13. Dua HS, Faraj LA, Said DG, Gray T, Lowe J (2013) Human corneal anatomy redefined: a novel pre-Descemet's layer (Dua's layer). Ophthalmology 120(9):1778–1785
14. Schlötzer-Schrehardt U, Bachmann BO, Tourtas T, Torricelli AA, Singh A, González S, Mei H, Deng SX, Wilson SE, Kruse FE (2015) Ultrastructure of the posterior corneal stroma. Ophthalmology 122(4):693–699
15. Hirano K, Sugita J, Kobayashi M (2002) Separation of corneal stroma and Descemet's membrane during deep lamellar keratoplasty. Cornea 21(2):196–199
16. Yoeruek E, Bayyoud T, Hofmann J (2012) Comparison of pneumatic dissection and forceps dissection in Descemet membrane endothelial keratoplasty: histological and ultrastructural findings. Cornea 31:920–925
17. Jafarinasab MR, Rahmati-Kamel M, Kanavi MR, Feizi S (2010) Dissection plane in deep anterior lamellar keratoplasty using the big-bubble technique. Cornea 29(4):388–391
18. Lombardo M, Merino D, Loza-Alvarez P, Lombardo G (2015) Translational label-free nonlinear imaging biomarkers to classify the human corneal microstructure. Biomed Opt Express 6(8): 2803–2818
19. Labate C, Lombardo M, De Santo MP, Dias J, Ziebarth N, Lombardo G (2015) Multiscale investigation of the depth-dependent mechanical anisotropy of the human corneal stroma. Invest Ophthalmol Vis Sci 56:4053–4060
20. Winkler M, Chai D, Kriling S, Nien CJ, Brown DJ, Jester B, Juhasz T, Jester JV (2011) Nonlinear optical macroscopic assessment of 3-D corneal collagen organization and axial biomechanics. Invest Ophthalmol Vis Sci 2(12):8818–8827
21. Winkler M, Shoa G, Xie Y, Petsche SJ, Pinsky PM, Juhasz T, Brown DJ, Jester JV (2013) Three-dimensional distribution of transverse collagen fibers in the anterior human corneal stroma. Invest Ophthalmol Vis Sci 54(12):7293–7301
22. Morishige N, Takagi Y, Chikama T, Takahara A, Nishida T (2011) Three-Dimensional analysis of collagen lamellae in the anterior stroma of the human cornea visualized by second harmonic generation imaging microscopy. Invest Ophthalmol Vis Sci 16 52(2): 911–915
23. Binder PS, Rock ME, Schmidt KC, Anderson JA (1991) High-voltage electron microscopy of normal human cornea. Invest Ophthalmol Vis Sci 32:2234–2243
24. Dias JM, Ziebarth NM (2013) Anterior and posterior corneal stroma elasticity assessed using nanoindentation. Exp Eye Res 115:41–45
25. Last JA, Liliensiek SJ, Nealey PF, Murphy CJ (2009) Determining the mechanical properties of human corneal basement membranes with atomic force microscopy. J Struct Biol 167:19–24
26. Abrams GA, Schaus SS, Goodman SL, Nealey PF, Murphy CJ (2000) Nanoscale topography of the corneal epithelial basement membrane and Descemet's membrane of the human. Cornea 19(1):57–64