

Article

Imaging Fungal Hyphae and Infection-Induced Stromal Changes in Ex Vivo Porcine Corneas Using Line-Field Spectral Domain Optical Coherence Microscopy

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Abstract

Microbial keratitis requires rapid diagnosis to enable timely, pathogen-directed treatment. Corneal confocal microscopy (CCM) is used to quickly diagnose *acanthamoeba* and fungal keratitis, but it relies on undesirable contact objective lenses, and no supplier has maintained a stable market presence. Here, we present a preliminary investigation of the potential abilities of non-contact optical coherence microscopy (OCM) as an emerging alternative technology. Using an ex vivo porcine corneal infection model, we demonstrate that OCM can produce *en face* images, similar to CCM, as well as 3D visualizations of fungi hyphae in stromal tissue. Like CCM, bacterial pathogens were too small to directly identify in OCM images. Nevertheless, OCM detected that ex vivo infection induced systematic changes to the stroma that are consistent with the breakdown of keratocyte structure. These proof-of-concept results demonstrate the potential of non-contact OCM for imaging microbial keratitis and support further in vivo evaluation.

Keywords: optical coherence microscopy; keratitis; line-field spectral domain; *en face* projection; 3D visualization; tissue damage

1. Introduction

Microbial keratitis is a sight-threatening condition caused by an infection of the cornea by bacteria, viruses, fungi or protozoa [1–4]. Prompt treatment directed against the causative microorganism(s) is crucial to a successful outcome. Microbiological diagnosis requires taking a sample from the cornea that is then processed in a laboratory to detect, isolate and characterize the causative microorganism(s), followed by antimicrobial susceptibility tests [5,6]. This usually takes several days, and microorganisms are isolated in less than 50% of cases [4].

Given the diverse characteristics of causative pathogens in terms of size, shape, and tissue response, microscopic imaging holds promise in detecting fungi, bacteria, or protozoa, thus aiding in early treatment decisions. In vivo corneal confocal microscopy (CCM) has demonstrated efficacy in identifying fungal and *acanthamoeba* infections owing to their distinct features [7]. Although non-contact CCM has been used for ocular conditions, such as exfoliation syndrome [8], CCM for microbial keratitis typically necessitates an uncomfortable contact procedure requiring anesthesia. Furthermore, clinical CCM systems typically only provide *en face* 2D images rather than tomographic data to enable



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3D visualizations. Finally, all major established and proven product lines, Heidelberg's Rostov Corneal Module and Nidek ConfoScan, and more historically devices by Tanden Scanning Corp, [9,10], are, as of writing, discontinued and have not been replaced. This raises concerns of availability for long-term continued and expanded clinical CCM use.

Optical coherence microscopy (OCM) [11] is an alternative in vivo imaging technique that can match the lateral resolution of CCM, while providing better axial resolution and true tomographic 3D data without requiring physical contact [12]. Here, the term OCM describes an optical coherence tomography (OCT) [13,14] system with a high numerical aperture (NA) microscope objective to give high lateral resolution. This is usually, but not always (especially historically) [15], combined with high axial resolution. If the resolution in the axial and both lateral dimensions approaches 1 μm , then the term micro-OCT is a synonym [16]. The concept of OCM has existed since at least 1994 [17], but in recent years there has been renewed interest [12,18–20].

The drawback of the high NA objectives in the Fourier domain (i.e., excluding the sequential time domain, where focus can be kept at the depth being imaged) OCM systems, compared to OCT, is that the depth of field (DoF), the physical depth that is within a required resolution, is reduced significantly. Therefore, typically in OCM only a small slice of the Fourier domain volume has a high lateral resolution. In scanning point OCT/OCM systems, this compromise of the DoF, for a given resolution, can be reduced using Bessel beam optics [21,22]. However, the drawbacks of this are the inherent introduction of side lobes on the lateral point spread function, that the highest lateral resolution achievable for a given NA will be reduced, and the increased complexity and reduced off-the-shelf options for the optics. For full field systems [23] and with more practical difficulty LF systems [24], Digital Adaptive Optics (DAO) uses the optical phase information inherent within OCT/OCM volumes to allow the image volume to be digitally refocused in a manner closely related to digital holography. This extends the achievable DoF. The practical issue in this method is achieving phase stability across the whole volume. In this work we did not apply either of these methods, but we note their existence and future applicability to this field.

Standard anterior segment (AS) OCT itself does have some use in microbial keratitis, such as tracking the infection size and corneal edema to monitor treatment response [25], but it provides little diagnostic information when compared to CCM.

Although OCM is an actively expanding research area, to the authors' knowledge no published studies have explicitly evaluated its potential for rapid morphological identification and differentiation between bacterial, fungal and protozoal microbial keratitis. Imaging fungal structures with OCM has previously been demonstrated [26,27], but these were not performed in corneal tissue.

In this paper we built a $\sim 1\text{--}2\ \mu\text{m}$ isotropic resolution fully NIR (no emissions below 700 nm) LF-SD-OCM system suitable for ex vivo studies. We report direct OCM imaging of fungal hyphae in stromal corneal tissue. We also show that infection with a smaller bacteria pathogen causes measurable differences in OCM *en face* images compared to uninfected controls; these differences are visually consistent with stromal microstructure breakdown.

2. Materials and Methods

2.1. OCM Apparatus

Figure 1 depicts the LF-SD-OCM system constructed for this study, adopting similar design principles to two prior LF-SD-OCT systems [28,29] but incorporating significant modifications to achieve notably higher lateral resolution. A relatively inexpensive mode-locked supercontinuum light source (Whitelase Micro, Fianium, Southampton, UK) with standard collimated free space output was used. To achieve a resultant longer illumination

line at the sample, the beam was expanded with a reflective beam expander (BE06R/M, Thorlabs, Newton, NJ, USA) by a factor of 6. To remove unused wavelengths and achieve a more symmetrical effective raw spectrum, a custom bandpass filter and spectral shaping filter, designed and manufactured for this light source for prior systems [29,30], was used. This provides broadband light illumination over the spectral range of 700 to 1000 nm with a central wavelength of 850 nm. Per nomenclature defined in [31], the line-field generation is negative. A cylindrical lens ($F = 50$ mm, LJ1695RM-B, Thorlabs) was placed before the interferometer beam splitter and objective lenses after (in sample and reference arms). In the sample arm, after a single galvo mirror (GVS011/M, Thorlabs) to allow 3D collection, a standard OCT telecentric scan lens ($F = 54$ mm, LSM54-850, Thorlabs) was used to create an intermediate image plane. The (Michelson) interferometer beam splitter used was a 50:50 cube (BS014, Thorlabs).

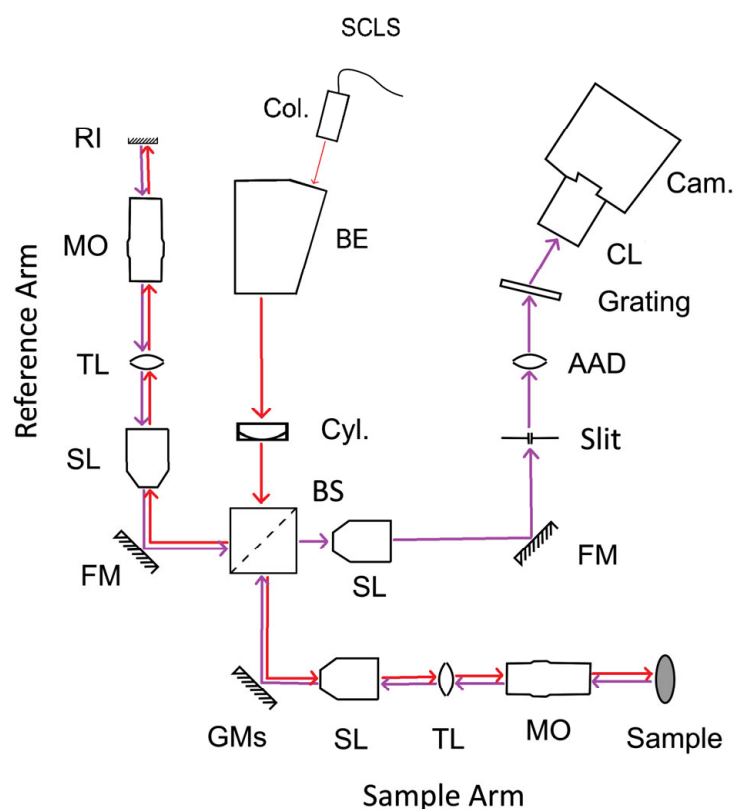


Figure 1. Diagram of LF-SD-OCM system constructed for this study. SCLS—supercontinuum light source (Fianium micro with continuously single mode fiber and Col.—collimator, free space output), BE—beam expander (BE06R/M, Thorlabs), Fil.—custom bandpass and spectral shaping filter, Cyl.—cylindrical lens (LJ1695RM-B, Thorlabs), BS—cube beam splitter (BS014, Thorlabs), GMs—galvo beam scanning mirrors (GVS011/M, Thorlabs), SL—scan lens (LSM54-850, Thorlabs), TL—tube lens (TTL200-B, Thorlabs), MO—microscope objectives (MY20X-824, Mitutoyo via Thorlabs), FM—folding mirror, RI—reference interface (flat side of plano-convex lens), Slit—custom $10\ \mu\text{m} \times 10\ \text{mm}$ (Thorlabs), AAD—air-spaced achromatic doublet (ACA254-75-B, Thorlabs), grating (VPH 600 L/mm @840 nm, Wasatch Photonics), CL—camera lens (MVL75M1, Navitar via Thorlabs), Cam.—sCMOS camera (Neo, Andor). Red lines show illumination paths, purple lines show imaging paths.

Up to this intermediate image plane, the design is the equivalent of prior LF-SD-OCT systems [28–30]. To convert this into an OCM system with the required lateral resolution, this image plane was translated onto the sample (illumination-object plane) with a conventional infinity correction $20\times$ magnification microscope design, consisting of

a 200 mm tube lens (TTL200-B, Thorlabs) and a 0.40 NA objective ($F = 10$ mm, MY20X-824, Mitutoyo via Thorlabs).

The reference arm of the interferometer is a Linnik arrangement, with scan lens, tube lens and microscope objective matching the sample arm. A static folding mirror replaces the galvo mirror, and optical alignment is identical apart from an adjusted path length mismatch to place the focal plane outside the zero path difference plane in the sample arm. This is because we did not use any “full-range” (direction of axial displacement) reconstruction method, used the outside volume, and need the focal plane to be within that outside volume. The interface used for the reference signal was a flat glass surface with a reflection coefficient of $\sim 4\%$ (-14 dB).

The LF-SD Low Coherence Interferometer (LCI) design was slitted. The collection arm of the interferometer consisted of another matched scan lens and a folding mirror to form an image plane on a $10\text{ }\mu\text{m} \times 10\text{ mm}$ slit (Custom order, Thorlabs) of the imaging spectrograph. This slit acts as a physical 1-D confocal gate. The custom imaging spectrograph was designed and constructed to maximize optical fidelity. The light that passed through the slit was collimated by an air-spaced achromatic doublet lens (ACA254-75-B, Thorlabs); then, the light passed through a holographic volume grating (VPH 600 L/mm @840 nm, Wasatch Photonics, Logan, UT, USA) as the spectrally dispersing component. A camera lens (MVL75M1, Navitar via Thorlabs) was the final optical element, imaging the LF spectrograms onto a sCMOS camera (2560×1540 , $6.5\text{ }\mu\text{m} \times 6.5\text{ }\mu\text{m}$, raw pixels, Neo, Andor, Belfast, UK). A 3D-printed partial enclosure of the custom imaging spectrograph was used to minimize stray light. An integration time of 3 ms was used for this ex vivo setup.

Raw interferometric data were streamed directly to an NVMe solid-state drive, enabling continuous acquisition at $42.478\text{ frames s}^{-1}$. Each frame consisted of 1540 (lateral) $\times 1280$ (axial) pixels, corresponding to an A-scan rate of $65.4\text{ kA-scans s}^{-1}$. Each volumetric dataset consisted of 1334 consecutive frames, resulting in a reconstructed volume of $1540 \times 1334 \times 1280$ voxels. The acquired volumes covered a lateral area of $500\text{ }\mu\text{m} \times 500\text{ }\mu\text{m}$ with sampling intervals of $0.325\text{ }\mu\text{m}$ and $0.375\text{ }\mu\text{m}$ in the line-field and scan dimensions, respectively. Axially, the full reconstructed depth of the volume in air was 1.29 mm. However, for any given application the usable volume depth is likely much smaller due to the limited DoF. Here, for the *Candida albicans* sample, for a single lateral position the thickness of the material where hyphae could be identified was $\sim 15\text{ }\mu\text{m}$ in corneal tissue. It should be noted that due to sample shape the focal plane is not flat, and the definition of usable depth is highly dependent on exact application.

A single unaveraged volume required 31.4 s to acquire. For the final dataset, frame density was increased by a factor of ten and incoherently averaged, resulting in a total acquisition time of 314 s per reconstructed volume.

The software and data processing used was an adapted version of that used in [28]. Numerical dispersion correction (NDC), with limited lateral position dependence, was applied to improve the axial resolution.

The axial FWHM resolution of this apparatus was measured to be $2.3\text{ }\mu\text{m}$ in air, so it was $1.7\text{ }\mu\text{m}$ in corneal tissue ($n_G = 1.387$ [32,33]). The lateral FWHM at the focal plane was estimated via a high-resolution USAF 1951 target by the standard method in [34], described and proven in [35], to be $1.6\text{ }\mu\text{m}$ (G9-E3) in the full-field dimension and $1.3\text{ }\mu\text{m}$ (G9-E5) in the confocal dimension. The calculated theoretical Abbe diffraction limit of the optics was $1.1\text{ }\mu\text{m}$ at the central wavelength. The absolute sensitivity was measured to be ~ 90 dB.

2.2. Ex Vivo Microbial Keratitis Model

The following experimental conditions were investigated, with each cornea treated as an independent biological sample: (i) uninfected controls ($n = 2$ corneas), compris-

ing one with epithelial scratches and one without epithelial scratches; (ii) *Pseudomonas aeruginosa* strain PA103-infected corneas ($\sim 1.86 \times 10^7$ CFUs per cornea; $n = 2$), comprising one scratched and one unscratched; and (iii) *Candida Albicans* SC5314-infected corneas (1.2×10^4 CFUs per cornea; $n = 4$). Of the *C. albicans*-infected corneas, two were cultured in the presence of 10% (v/v) horse serum to promote hyphal formation and two were cultured without horse serum to maintain predominantly yeast-form growth. Within each culture condition, one cornea was scratched and one was left unscratched. Epithelial scratches were intended to facilitate pathogen attachment and tissue infiltration.

Porcine eyes were obtained from a local abattoir within 6 h of slaughter. A multiple-stage washing (using sterile phosphate buffered saline (PBS) (Sigma, Burlington, MA, USA), sterile PBS containing 1% (v/v) penicillin/streptomycin and amphotericin B (P/S/AmpB) (Sigma) and 5% iodinated povidone/PBS (Ecolab, Northwich, UK)) and dissection process produced corneoscleral buttons. If required, three linear scratches were then made using a sterile 16-gauge needle. The posterior cavity of each corneoscleral button was filled with sterile LB agar, which was allowed to solidify to provide structural support. The buttons were subsequently embedded, epithelial side up, to the limbus in sterile LB agar within a 10 cm petri dish. Where required, the corneas were infected at this point, then submerged in DMEM-F12 media (Sigma) to prevent dehydration and resultant tissue degradation, and incubated overnight at 37 °C. Prior to imaging, the liquid medium was removed.

For each sample, three volumetric datasets were acquired at different locations across the cornea. Each volume was captured with 10× B-scan oversampling density to allow post-processing binned averaging.

2.3. Simple Segmentation Method

For the purpose of demonstrating that there was a measurable statistical difference in the OCM data between *Pseudomonas aeruginosa*-infected corneas and control corneas, a simple arbitrary threshold island segmentation of linear (to interference electric field) signal *en face* images was carried out. The Otsu method is a standard method to automatically calculate image threshold values. To get consistent isolated segmented areas to allow size comparison a $2 \times$ Otsu value was used as the thresholding value. After thresholding, each area of connected segmented pixels was found by a brute force algorithm, and the mean size of these areas was calculated for each *en face* images in a selected volume of stromal tissue. This method was sufficient, without further optimization, for the purpose of this study.

3. Results

3.1. *Candida Albicans*

Of the four *C. albicans*-infected corneas prepared, hyphal formation was only observed in the scratched cornea cultured in the presence of horse serum. This was expected, as epithelial scratching was used to facilitate infection, while horse serum was included to promote the yeast-to-hyphal transition. Substantial hyphae were detected in two of the three OCM volumes acquired under these conditions.

Figure 2 compares an OCM projection of *C. albicans*-infected stromal tissue with that of an uninfected control. *En face* fly-throughs of the corresponding OCM volumes are provided in Supplementary Materials S1 and S2, illustrating the three-dimensional spatial distribution of the hyphae within the cornea and the surrounding stromal architecture. Numerous filamentous structures consistent with fungal hyphae are visible within the infected stroma. This appearance corresponds to that reported in CCM images of fungal keratitis, including the examples presented by Bakken et al. [36]. However, in contrast to

CCM, all conventional SD-OCM setups inherently provide raw 3D tomographic image data of the pathogen structure.

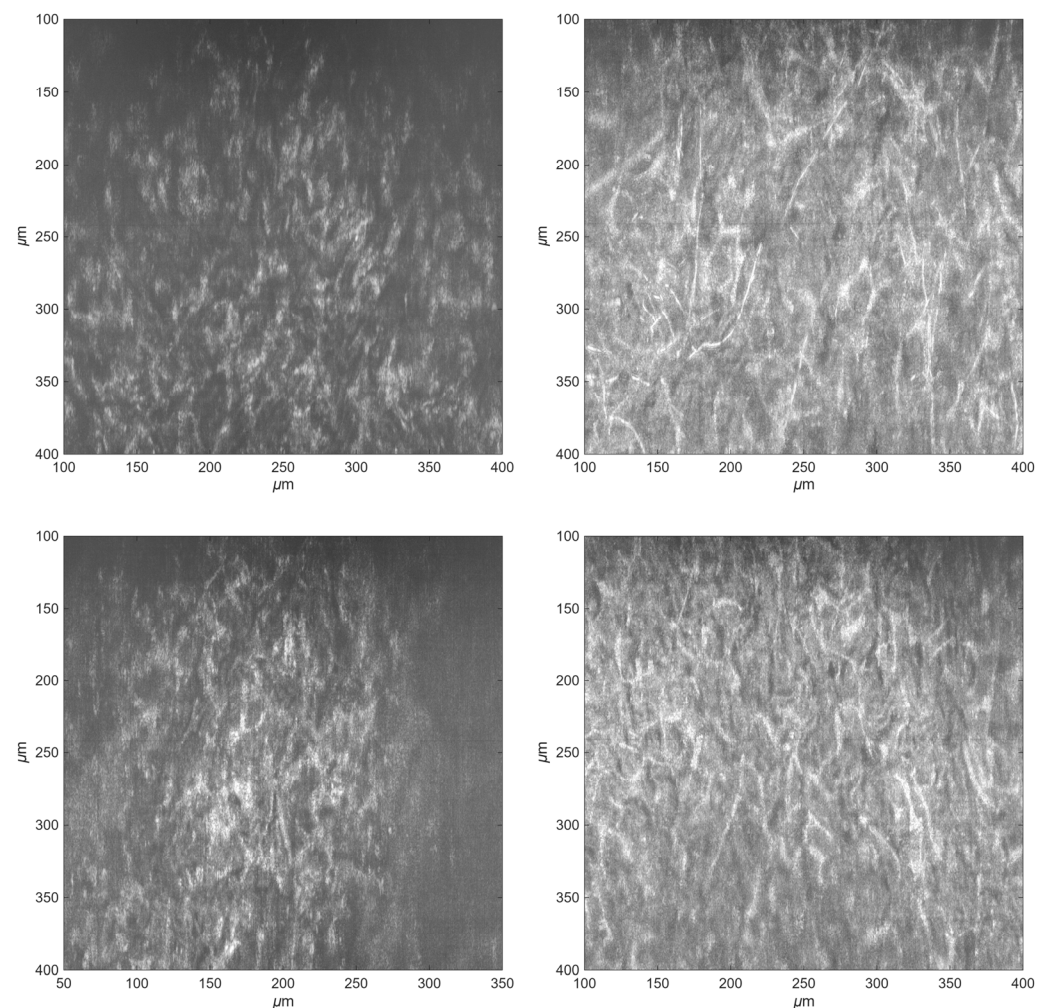


Figure 2. *En face* images generated by the maximum projection of raw OCM volume over an 8 μm ($n_G = 1.387$) thickness: (Left) control porcine cornea stroma ((top left) without and (bottom left) with corneal scratches); (Right) infected with *Candida albicans* (both of cornea with corneal scratches and serum to aid infection and hyphae formation). Grey scale mapping of signal is identical in both images. Supplementary Materials S1 and S2 show respective *en face* fly-through of the (top) two volumes with adjacent pixel maximum projection.

Despite identical image-processing and display settings, the infected stroma in Figure 2 appears significantly lighter than the uninfected control, with reduced definition of the normal keratocyte-associated stromal pattern. Similar non-specific infection-associated alterations in stromal appearance were also observed following *P. aeruginosa* infection.

Figure 3a shows the *en face* image of hyphae: for a single *en face* image the hyphae can drift above and below the axially resolved slice, showing only fragmented parts of the full length. However, if a continuous *en face* image equivalent to CCM is wanted, then a maximum projection over a larger depth range can be displayed. Figure 3b shows a cross-sectional image through the hyphae. It has a thickness of $\sim 2 \mu\text{m}$, matching the prior literature [37]; $\sim 2 \mu\text{m}$ is close to the imaging resolution, so further systematic quantification with the device would not be meaningful. Supplementary Materials S3 is a rotating 3D rendering of the hyphae tomograph data showing how OCM can visualize hyphae structures and how they relate to each other where they cross in 3D (Figure 3c,d show two

example frames). Three hyphae are present, with one sitting at a different alignment and passing a few microns above the others.

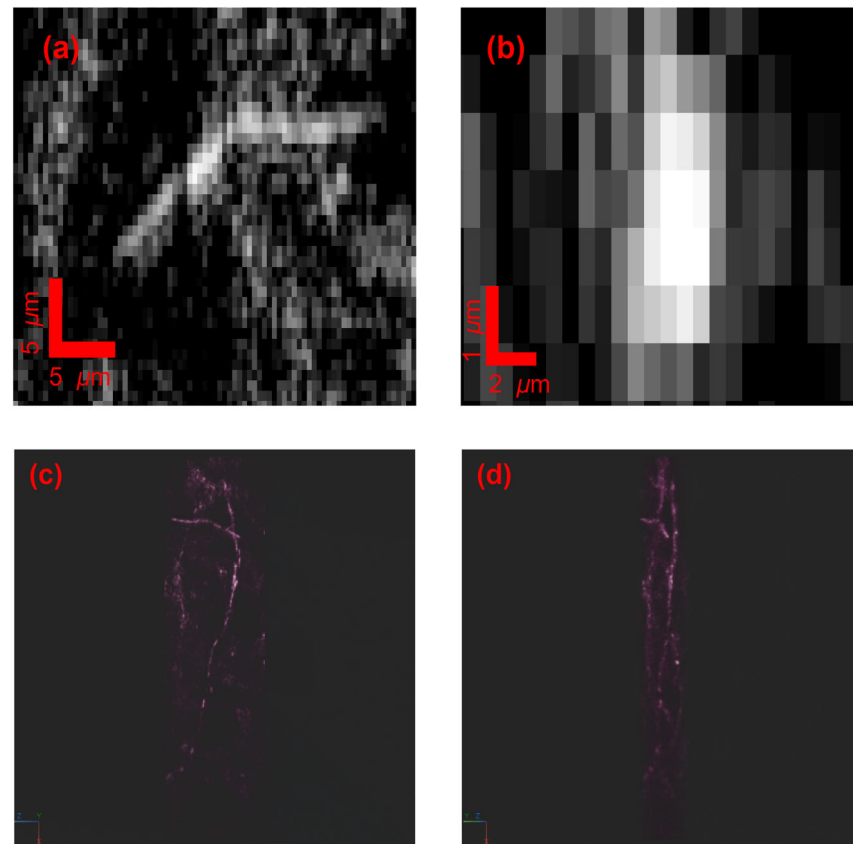


Figure 3. LF-SD-OCM visualization of fungal hyphae within a *Candida albicans*-infected porcine cornea. (a) *En face* OCM image showing a section of filamentous structures consistent with *C. albicans* hyphae within the cornea stroma. (b) Cross-sectional OCM image through one of these hyphae. Scale bars show indicated distances. (c,d) Representative frames ($\sim 37 \times 60 \mu\text{m}$) from a 3D rotating rendering of an OCM volume, illustrating adjacent hyphae structures with different orientations and axial positions (Supplementary Materials S3 (Candida.tif)).

3.2. Effects of a *Pseudomonas aeruginosa* Infection on Stromal Tissue

The imaging of individual *Pseudomonas aeruginosa* bacteria suspended in agar only gave speckly quasi-point signals (a null result, so not shown here). This is due to the bacteria's similar size to the system's 3D PSF. Therefore, the image signals from individual bacteria do not contain sufficient structural information to distinguish the bacteria from other potential scatters in stromal tissue.

However, infections lead to damage of the host tissue, and this damage is clearly noticeable in OCM (as shown here) and OCT [25] signals. The primary structural features of the stroma visible in OCT and OCM images are the keratocytes. Figure 4a,b show an *en face* and cross-sectional image of healthy keratocyte, respectively. Supplementary Materials S4 (with example frames shown in Figure 4c,d) shows a rotating animation of the tomographic image of the keratocyte, showing how OCM can visualize and measure its 3D structure. The dimensions of $30 \mu\text{m}$ lateral and $1 \mu\text{m}$ thick and the general shape matches what is expected from prior histology studies [38]. In OCT/OCM imaging they can be subjectively distinguished, as the keratocyte part is scattering while the mature lamella is effectively a transparent medium, similar to Bowman's layer and Descemet's membrane. This contrasts with what is seen commonly after histological processing, which leads to delamination between lamellae, making them very visible.

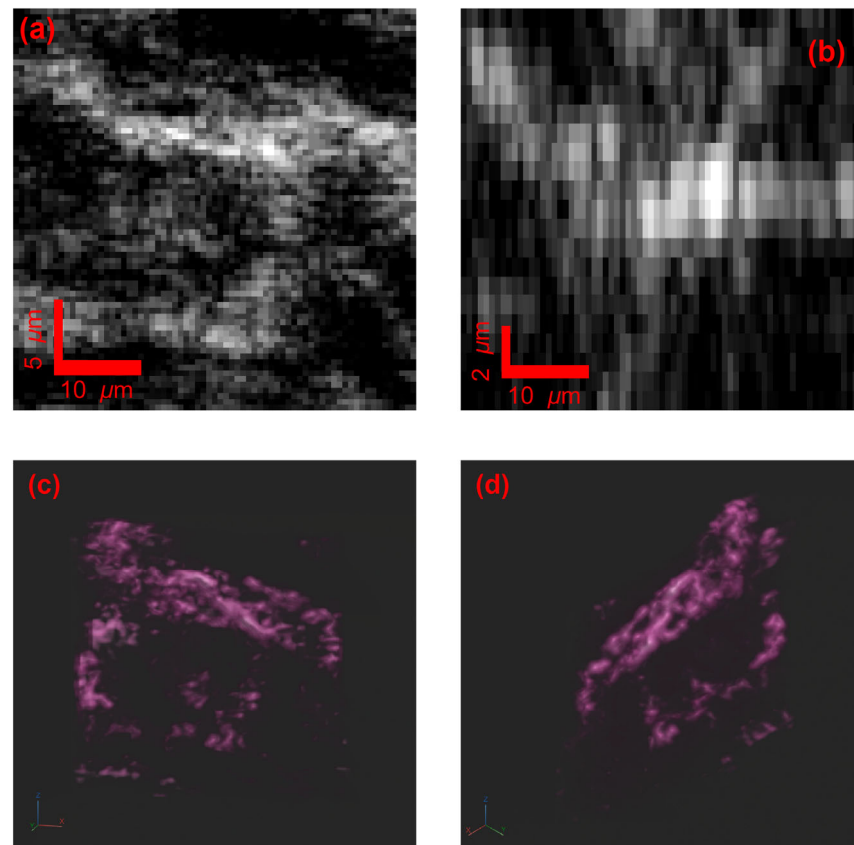


Figure 4. LF-SD-OCM visualization of a keratocyte within uninfected porcine corneal stroma. (a) *En face* and (b) cross-sectional image sections of a healthy keratocyte in the stroma of a control cornea. Scale bars show indicated distances. Supplementary Materials S4 (ControlCornea_1.gif) is the rotating 3D rendering of this keratocyte, whilst (c,d) shows its example frames.

Figure 5 shows *en face* images of the (a) control and (b) *Pseudomonas aeruginosa*-infected cornea stroma. Their significant structural difference is visually apparent. The keratocyte structure in the infected stroma has broken down in smaller segments. Though some of the scattering centers may be from the individual bacteria themselves, as already highlighted, unambiguous identification from the background is not straightforward. In an *in vivo* setting the situation would be further complicated, with the main “foreign” bodies visible likely to be leukocyte cells of the immune system [39], though the leukocyte level itself may provide clinically useful information.

Figure 5c,d show the corresponding threshold segmentations. From these segmentations, Figure 5e shows all the calculated mean segmentation sizes for all volumes as a function of depth. Visually, the infected and control corneas are differentiated, consistent with the differences in the example images shown, but differences between the scratched and unscratched methodology appear negligible. The start of the image depth range is visual where the calculated mean segmentation size is most consistent, so the mean values over a depth range of 174 to 188 (20 *en face* images) were taken for each volume. For these values, the mean $\pm$ standard deviation is 9.5 ± 1.3 (N = six volumes, over the two corneas) for the healthy controls and 6.2 ± 0.4 (N = six volumes, over the two corneas) for the infected corneas (arbitrary units, nominally *en face* pixels). The corresponding standard two-sample *t*-test from these values gave a *p* value of 0.00014, so the OCM image data of control and infected corneas is statistically well differentiated.

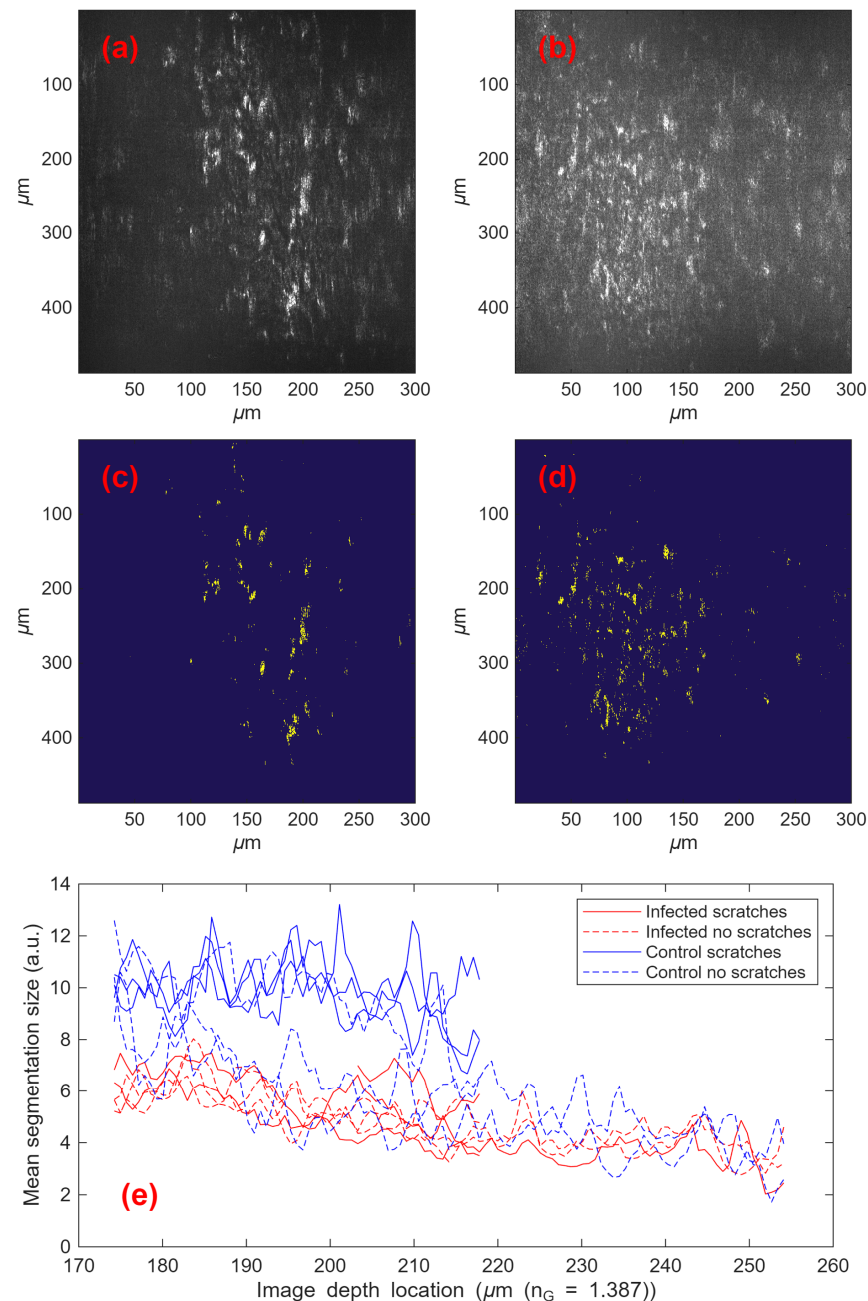


Figure 5. Comparison and segmentation of stromal OCM images from uninfected and *Pseudomonas aeruginosa*-infected porcine cornea. (a,b) Representative *en face* (at 174 μm depth) images of stromal tissues from an uninfected control (a) and a *P. aeruginosa*-infected (b) cornea displayed using identical image-processing and grey-scale settings. The infected stroma shows increased background scattering and reduced definition of the normal keratocyte-associated pattern. (c,d) Corresponding threshold segmentation for uninfected cornea (c) and *P. aeruginosa*-infected cornea (d). (e) All mean segmentation area sizes. Appendix A provides example *en face* images and segmentations for all 12 measured volumes.

4. Discussion and Future Work

This paper presents findings from previous unpublished proof-of-concept data evaluating LF-SD-OCM for imaging fungal hyphae and infection-associated stromal changes in ex vivo porcine corneas. To the best of our knowledge, the OCM images shown here represent the first published OCM visualization of fungal hyphae in stromal tissue. Their elongated, branching appearance and approximate dimensions are highly consistent with those reported in CCM images [36] and encountered in clinical practice. This supports

their interpretation as *C. albicans* hyphae. This is expected because the underlying physical causes of imaging resolution (light scattering and diffraction) limits are the same. Therefore, it is reasonable to extrapolate that OCM will also have the same diagnostic applicability to other pathogens that can be imaged with CCM, such as *acanthamoeba* and other fungal structures. This proof-of-concept data is therefore of interest to OCM researchers.

As this study was designed to establish whether OCM could visualize morphological features consistent with fungal hyphae in an ex vivo corneal model, rather than to evaluate clinical diagnostic performance, the findings should be interpreted within the limitations of this experimental design. Although four *C. albicans*-infected corneas were prepared, substantial hyphal formation was only observed in two of the volumes within the scratched horse-serum-treated cornea. This was consistent with the use of epithelial scratching to facilitate infection and horse serum to promote the yeast-to-hyphal transition. However, the small number of independent samples and the absence of external reference methods preclude the full assessment of diagnostic accuracy, sensitivity, specificity, or reproducibility. Definitive clinical validation will require larger, independently characterized datasets and correlation with established reference methods, including microbiological culture, PAS or GMS histopathology. Future prospective clinical studies with parallel CCM imaging will also be required.

Bacterial and viral infections generally lack easily identifiable morphological features at the spatial resolution of current CCM systems, and consequently this technique is not currently considered viable for their diagnosis [40]. Individual bacteria, such as *Pseudomonas aeruginosa*, are too small to be directly distinguished from other scatterers within the tissue when using red and infra-red light-based modalities, such as CCM and OCM. Bacteria such as *Staphylococceae*, which form characteristic clusters, may be more amenable to such visualization. However, the results presented here indicate that infection changes the optical properties of stromal tissue in both fungal and bacterial infections, leading to quantifiable differences between the infected and control stromal images acquired by OCM. This matches the differences seen in AS-OCT [25], but these OCM images visually show that these changes are consistent with breakdown/damage to the keratocyte structure. This data is therefore of interest to researchers in both OCM (high numerical aperture objectives) and AS-OCT (conventional low numerical aperture objectives) fields.

The optical apparatus was designed to give proof-of-concept OCM performance for ex vivo samples. The imaging path consisted of three separable systems in series: a microscope, a 1:1 imaging interferometer, and an imaging spectrograph. Although this apparatus was sufficient for acquiring ex vivo OCM data to demonstrate fundamental potential of the technique, we are not proposing it is a practical design for in vivo imaging. However, the LF-SD-OCM format employed here has recently been demonstrated for in vivo imaging by other groups [12,41], achieving comparable image quality. We have also been developing a compact and efficient LF-SD-OCM system, Eye-Screen [42,43], for in vivo imaging of sub-basal nerves. After completing the usability stage of the clinical study, we intend to secure funding and ethical approval for a limited clinical study in patients with suspected microbial keratitis to obtain corresponding in vivo data and evaluate potential clinical efficacy.

The principal technical characteristics of the LF-SD-OCM system used in this study are compared with those of the Heidelberg Retina Tomograph 3 with Rostock Cornea Module (HRT3), the most widely reported clinical CCM platform, in Table 1. The two systems provide broadly comparable lateral resolution, while the present OCM system offers improved axial resolution, a larger field of view, non-contact imaging, and inherently volumetric tomographic acquisition. However, the proof-of-concept LF-SD-OCM apparatus

used in this study has a substantially longer acquisition time and currently a more limited depth-of-field than the clinically established CCM system.

Table 1. Comparison of the principal technical characteristics of the present proof-of-concept LF-SD-OCM and the clinically established Heidelberg Retina Tomograph 3 with Rostock Cornea Module (HRT3-RCM) used for clinical corneal confocal microscopy.

	HRT3-RCM Corneal Confocal Microscope	Present Line-Field Spectral Domain Optical Coherence Microscope
Clinical Suitability	Established for clinical corneal imaging	Designed for proof-of-concept ex vivo and not suitable for in vivo clinical use in its current form.
Imaging Principle	Confocal reflectance microscopy	Spectral-domain optical coherence microscopy.
Corneal Contact	Contact imaging using a coupling gel and topical anesthesia	Non-contact imaging.
Light Source	670 nm red diode laser	700–1000nm broadband illumination.
Field of View	400 × 400 µm	500 × 500 µm.
Optical Resolution	~1 µm laterally; ~5 µm axially	~1.6 × 1.3 µm laterally; 1.7 µm axially.
Image Acquisition Format	Sequential single <i>en face</i> optical sections; depth stacks can be acquired by repositioning the focal plane	Volumetric tomographic acquisition providing <i>en face</i> , cross-sectional B-scans and 3D-reconstructions.
Image Acquisition Rate	Up to 30 frames per second	31.4 s per unaveraged volume and 314 s with tenfold acquisition and incoherent averaging. However, this is proof-of-concept hardware. Faster OCM systems suitable for in vivo clinical applications have been reported [12,41,43].
In-focus Depth	Thin optical sections, approximately corresponding to the axial resolution. The focal plane can be repositioned through the cornea	Approximately 15 µm usable depth of field (DoF) in the present configuration without using DoF extension methods and using a 0.4 NA objective. The focal region can be positioned at different corneal depths.
Main Technical Strengths	High lateral resolution; established clinical use; direct visualization of large pathogen structures	Non-contact acquisition; improved axial resolution; larger field of view; inherently volumetric three-dimensional data
Main Technical Limitations	Requires corneal contact; predominantly <i>en face</i> imaging; limited commercial availability of established systems	Long acquisition time and limited depth of field in the present implementation. Optimization required for clinical implementation.

The comparison should therefore be interpreted as a summary of the respective technical characteristics of the two modalities rather than as evidence of clinical superiority. Acquisition speed represents only one aspect of clinical utility, and any meaningful comparison between CCM and OCM would also need to consider image quality, field of view, diagnostic performance, operator dependence, patient tolerability and integration into the clinical workflow. Such a comparison would require a dedicated prospective clinical study and is beyond the scope of the present proof-of-concept ex vivo investigation.

For small pathogen (mainly bacterial and viral) infections that are not currently considered diagnosable with CCM, two immediate research questions emerge: (i) Do different pathogens produce sufficiently distinct patterns of tissue damage such that they can be differentiated based on characteristic OCM features? If so, this would lend itself to a straightforward brute force convolutional neural network (CNN) AI model approach. (ii) Using the current or similar segmentation statistical analysis, can it quantify the degree or state of infection in ex vivo tissue to monitor the longitudinal progression? If viable, how effective is it in monitoring in vivo microbial keratitis?

5. Conclusions

The ex vivo proof-of-concept data presented here shows that OCM can produce diagnostic images of fungi hyphae (*Candida albicans*) in corneal tissue, like CCM images in the literature. Although only *Candida albicans* was investigated experimentally in this study, the imaging principles of OCM suggest that similar capabilities may be achievable for other large pathogen classes, such as *Acanthamoeba*. However, further experimental studies will be required to validate this hypothesis.

Like CCM, direct visual differentiation of small bacteria (*Pseudomonas aeruginosa*) in tissue does not seem to be practical with OCM. However, the systematic structural changes in keratocytes induced by infection is clear in OCM data.

Given the lack of security and limited non-contact functionality of the CCM market, development of non-contact OCM devices for rapid microbial keratitis screening is an area of current research potential.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/photonics13090821/s1>, Supplementary Material S1: AVI video of an en face fly-through of part of a collected OCM image volume of control (uninfected) porcine stroma. Supplementary Material S2: AVI video of an en face fly-through of part of a collected OCM image volume of porcine stroma with *Candida albicans* hyphae present. Supplementary Material S3: GIF animation of rotating 3D projection of a section of OCM volume showing overlapping *Candida albicans* hyphae. Image captured in porcine stroma. Supplementary Material S4: GIF animation of rotating 3D projection of a section OCM volume showing intact keratocyte in control (uninfected) porcine stroma.

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Data Availability Statement: Due to the oversampling methodology used within this paper, the total raw data size for the total 24 volumes used is approximately 1.72 TB. Please contact Prof. Yao-Chun Shen, y.c.shen@liverpool.ac.uk, to if you require access to this data.

Conflicts of Interest: The authors declare no conflicts of interest.

Appendix A. Additional Figures

Following from Figure 5, this appendix provides an example *en face* image and segmentation for each measured volume of each cornea. Figures A1 and A2 are of the scratched corneas, infected with *Pseudomonas aeruginosa* and control respectively. Figures A3 and A4 are of the unscratched corneas, infected with *Pseudomonas aeruginosa* and control respectively.

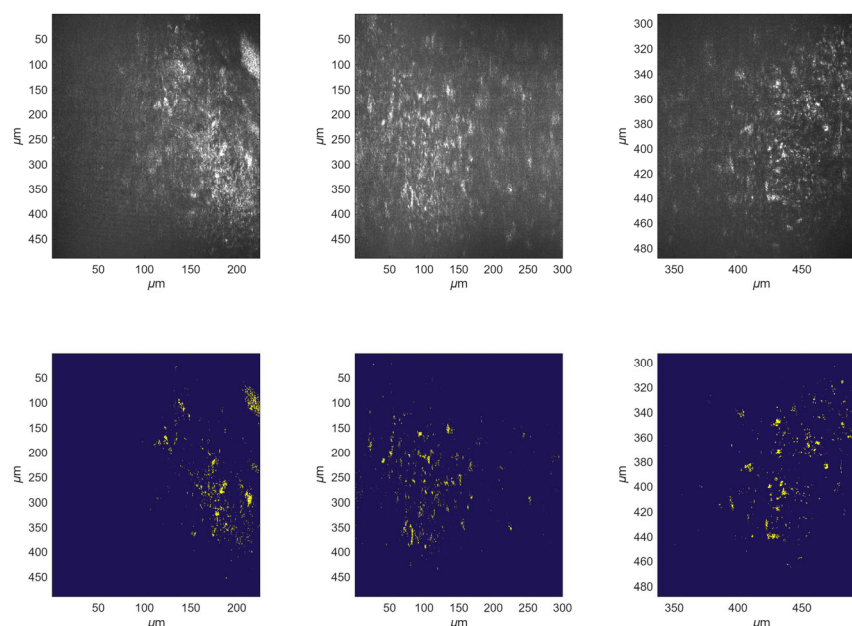


Figure A1. Cornea scratched and infected with *Pseudomonas aeruginosa*. (Top) *En face* images with (bottom) corresponding segmentations for each of the 3 lateral locations measured.

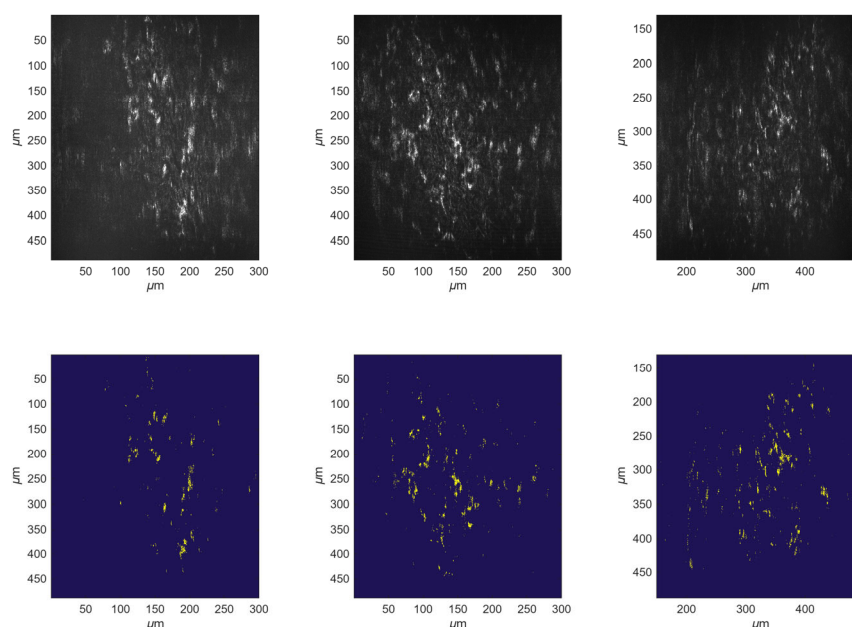


Figure A2. Control scratched cornea. (Top) *En face* images with (bottom) corresponding segmentations for each of the 3 lateral locations measured.

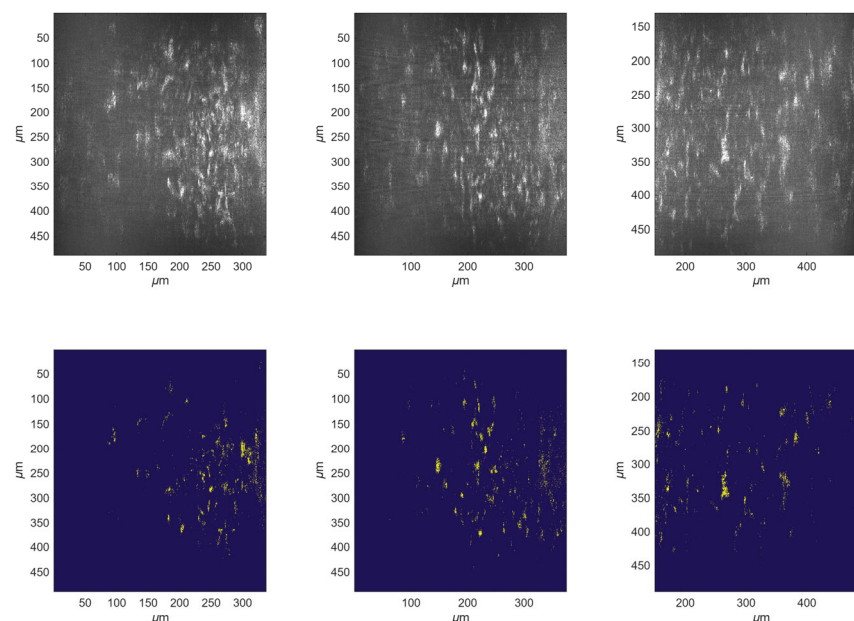


Figure A3. Non-scratched cornea infected with *Pseudomonas aeruginosa*. (Top) *En face* images with (bottom) corresponding segmentations for each of the 3 lateral locations measured.

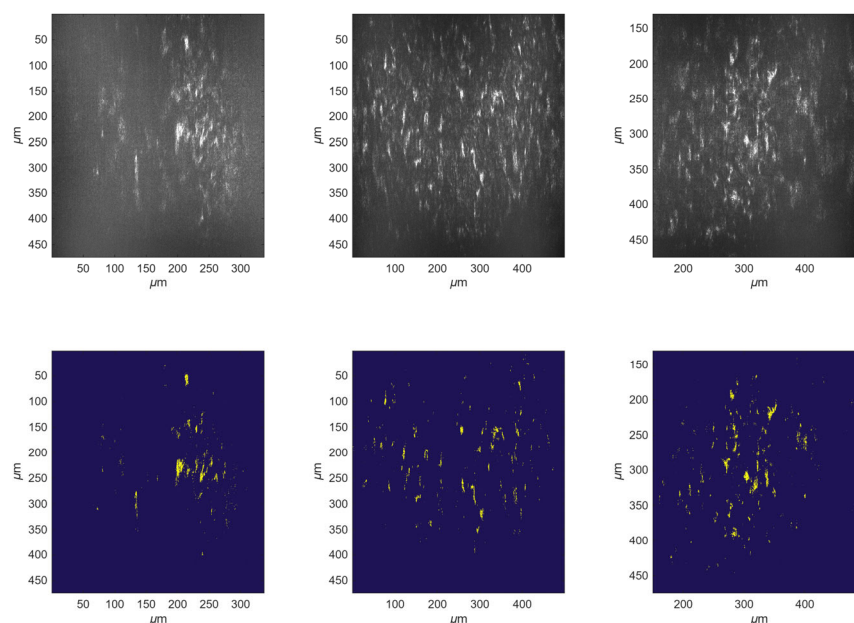


Figure A4. Control non-scratched cornea. (Top) *En face* images with (bottom) corresponding segmentations for each of the 3 lateral locations measured.

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