Multiwavelength confocal laser scanning microscopy of the cornea

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Abstract: Confocal reflectance microscopy has demonstrated the ability to produce in vivo images of corneal tissue with sufficient cellular resolution to diagnose a broad range of corneal conditions. To investigate the spectral behavior of corneal reflectance imaging, a modified laser ophthalmoscope was used. Imaging was performed in vivo on a human cornea as well as ex vivo on porcine and lamb corneae. Various corneal layers were imaged at the wavelengths 488 nm, 518 nm, and 815 nm and compared regarding image quality and differences in the depicted structures. Besides the wavelength- and depth-dependent scattering background, which impairs the image quality, a varying spectral reflectance of certain structures could be observed. Based on the obtained results, this paper emphasizes the importance of choosing the appropriate light source for corneal imaging. For the examination of the epithelial layers and the endothelium, shorter wavelengths should be preferred. In the remaining layers, longer wavelength light has the advantage of less scattering loss and a potentially higher subject compliance.

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1. Introduction

To date, clinical and scientific investigations regarding corneal confocal laser scanning microscopy are performed mainly with the combination of the Heidelberg Retina Tomograph (HRT), which is a confocal scanning laser ophthalmoscope (SLO), and the Rostock Cornea Module (RCM; both Heidelberg Engineering GmbH, Heidelberg, Germany) [1,2]. Hence, most images were captured using a red laser with a wavelength of 670 nm. Besides the HRT/RCM system, there is also the Confoscan (NIDEK, Gamagori, Japan), which is a slit-scanning confocal microscope using a white halogen lamp. Although the HRT/RCM system generally generates better corneal images due to the two-dimensional confocality, the Confoscan provides better endothelial images [3,4] even though it is only confocal in one direction. This fact already indicates the influence of different light sources on imaging and underlines the need for a wavelength-dependent study.

Currently, research is conducted on new corneal imaging devices that achieve cellular resolution. These devices may use other modalities, light sources, and thus different wavelengths than 670 nm. For example, asymmetric fundus retroillumination [5] and various optical coherence tomography (OCT) methods including spectral domain OCT [6], ultrahigh resolution OCT [7], Gabor-domain OCT [8], full-field OCT [9–12] and curved-field OCT [13] were investigated. These methods usually operate in the near-infrared spectrum [6–11,13], but also the visible spectrum [12]. In order to ensure comparability between the existing and new technologies, the spectral reflection dependence on different cellular corneal structures must be determined.

Although the absorption characteristic through the full cornea is well investigated [14], the spectrally dependent reflectance of different cellular structures is yet unknown. Due to
wavelength-dependent scattering, more light is scattered in all directions at shorter wavelengths [15], thus reducing the amount of detected light from the focused structure. This effect integrates with the depth. Consequently, the detector gain must be increased to obtain a sufficiently strong signal from structures at deeper layers. This, in turn, leads to a decreased signal-to-noise ratio and an amplification of unwanted, disruptive backscattering of optical surfaces and corneal layers outside the focus. Although the latter effect is quite small due to confocality, all of these effects could add up to a diffuse background that scales with the imaging depth. However, the optical resolution is inversely proportional to the wavelength, too [16]. As a result, longer wavelengths, which provide deeper penetration and less scattering, come at the expense of optical resolution. Besides these effects, reflectance images captured at different wavelengths may potentially reveal different cellular structures.

It is known, e.g. from dermatology, that the backscatter from the inter- and intracellular structures can vary with wavelength, size, geometry, and refractive index of the scattering structure [17,18]. Thus, by acquiring reflectance images from multiple wavelengths, it could be possible to extract wavelength-dependent information. Therefore, the aim of this work is to investigate the influence of different wavelengths on corneal imaging.

2. Materials and methods

To acquire images of the cornea at different wavelengths, the SPECTRALIS platform (Heidelberg Engineering GmbH, Heidelberg, Germany), a confocal scanning laser ophthalmoscope with three distinct laser wavelengths (blue – 488 nm, green – 518 nm, near-infrared – 815 nm), was used in conjunction with a custom-built objective module. The objective module is a redesigned version of the RCM and was previously presented as RCM 2.0 in combination with the HRT [19] as well as with the SPECTRALIS [20]. The RCM 2.0 was adapted to the SLO to shift the focal plane from the retina to the cornea and to increase the in-plane resolution. Furthermore, a piezo actuator was implemented to control the focal plane within the cornea. For measurements, a sterile cap was used to contact the cornea. To reduce surface reflections, a carbomer gel (Vidisic; Bausch & Lomb / Dr. Mann Pharma, Berlin, Germany) was applied as an immersion medium between the objective lens and contact cap as well as between the cap and the cornea. Since this gel is a tear substitute, it provides the same refractive index change between ex vivo and in vivo measurements. The new design of the RCM 2.0 enabled a fixed position of the contact cap during focal plane changes. Nevertheless, it was still possible to adjust the position of the contact cap before tissue imaging. This is important in order to capture the largest possible cornea stack.

The combination of SPECTRALIS and RCM 2.0 is shown in Fig. 1(A). Additionally, Fig. 1(B) shows a simplified schematic of a confocal SLO with three laser diodes of different wavelengths. These are directed on the same optical path with dichroic mirrors and their back reflected signals can be detected sequentially with a detector.

The obtained magnification and thus the field of view (FOV) depends on the scan angle of the SLO, the distance between the optics of RCM 2.0 and SLO, as well as slightly on the selected wavelength. Exemplifying this, Fig. 2 compares images taken with the HRT and SPECTRALIS, each in conjunction with the RCM 2.0. While the HRT captured images demonstrate a FOV of $350 \times 350 \mu m^2$, the images taken with the SPECTRALIS have a FOV of $710 \times 710 \mu m^2$ at the near-infrared wavelength of 850 nm. This difference in FOV is mainly caused by the larger scan angle of the SPECTRALIS with 30° compared to the HRT with 15°. The magnification of every optical setup and wavelength used was measured. Consequently, all images were scaled accordingly. Since the objective lens (Achromplan 63x/0.95 W; Zeiss, Jena, Germany) is not optimized for the wavelengths of the SPECTRALIS platform, its images show a central back reflection. While the size of the back reflection depends on the distance between RCM 2.0 and SPECTRALIS as well as the selected wavelength, the intensity of the back reflection furthermore depends on the illuminance and detector gain. In the example of Fig. 3, the intensity of the back...
reflection is clearly visible for 488 nm as well as 518 nm and is vanishingly low at 815 nm. With the RCM 2.0, a radiant power of 62.8 µW, 43.3 µW, and 15.2 µW at 488 nm, 518 nm, and 815 nm respectively was measured at maximum laser power. This results in a corneal irradiance of less than 0.014 W/cm² and a retinal irradiance of less than 0.003 W/cm². These values are below the limits of Group 1 ophthalmic instruments of the safety standard DIN EN ISO 15004-2. The actual radiant power used in the in vivo measurements was 26.6 µW, 22.2 µW, and 15.2 µW at 488 nm, 518 nm, and 815 nm, respectively.

In vivo measurements were performed on four subjects. Since one subject already underwent numerous eye examinations of different kinds in the past, confirming a healthy eye, the images presented in this paper are from this 51-year-old male subject. However, the other three subjects claimed to have no eye problems and are assumed to have healthy eyes as well. The study was conducted in accordance with the Declaration of Helsinki and it was explained in detail to the subjects. Informed consent was obtained before any investigative procedures were conducted. In addition, the endothelium of the human subject was imaged with a specular microscope (EM-4000; Tomey GmbH, Nürnberg, Germany) and compared to the confocal images. Finally, ex vivo measurements were performed on several porcine and lamb eyes. In the case of the human cornea, images of the epithelium, subbasal nerve plexus (SNP), stroma, and endothelium were recorded and compared at all three wavelengths. Due to eye movements, the images at the three different wavelengths could not be captured at the same lateral position in the cornea. Images of the porcine and lamb eyes were taken from the epithelium, stroma, and endothelium at the blue and near-infrared wavelengths less than 12 h after enucleation and compared to the in vivo images of the human cornea.

For a direct comparison between images captured at different wavelengths, it is important to adjust the histogram to the full intensity scale. Otherwise misleading conclusions may be drawn. In the case of the in vivo measurements, subimages showing the relevant structures were selected,
Fig. 2. Comparison of the HRT + RCM 2.0 and SPECTRALIS + RCM 2.0 combinations showing superficial cells of a human cornea.

Fig. 3. Comparison of in vivo human cornea images of superficial epithelial cells acquired at three distinct wavelengths as indicated. The histograms of the subimages in the yellow dashed squares were adjusted to the full intensity range for a consistent comparison of the image quality.

the histograms were stretched so that the entire intensity range was used for the cell structures and finally presented as an inset in the original image. In the case of the ex vivo figures, only the histogram stretched subimages are presented.

No auto-brightness functionality was used for all image acquisition and the detector gain was changed manually if necessary. Since the incorporated scanning laser ophthalmoscope is a commercial device, some wavelength-dependent parameters such as the spectral transmittance of the optics or the quantum efficiency of the detector are unknown and hence, comparisons of
signal strengths between wavelengths are difficult to obtain. Therefore, the focus of the study was a dedicated view on the visibility of different corneal structures at the respective wavelengths. Then it was compared whether these corneal structures look similar or different at the specific wavelengths.

3. Results

3.1. In vivo measurements of a human cornea

In general, imaging could be performed at all investigated wavelengths and species. Depending on the wavelength, there are not only differences in the image quality but also in the reflectance of certain structures.

Figure 3 shows the cell borders, cytoplasm, and nuclei of the human superficial epithelial cells, which appear more prominent using the blue or green compared to the near-infrared wavelength. Particularly the blue and green wavelengths reveal certain superficial cells in which the cytoplasm reflects much more than others. Most cell nuclei are visible at the blue wavelength. Especially in the area around the central back reflection, many nuclei are revealed that are invisible at the other two wavelengths. At 815 nm, the cytoplasm and cell borders of the superficial cells have a rather uniform, homogeneous reflectance. This makes it more difficult to distinguish adjacent cells. However, slightly deeper in the epithelium, the wing cells in Fig. 4 show no discernible differences between the three wavelengths. Cell borders are brighter than the cytoplasm. None of the wavelengths reveals cell nuclei.

![Fig. 4.](image)

**Fig. 4.** Comparison of in vivo human cornea images of epithelium acquired at three distinct wavelengths as indicated. The histograms of the subimages in the yellow dashed squares were adjusted to the full intensity range for a consistent comparison of the image quality.

As Fig. 5 and Fig. 6 demonstrate, the influence of scattering at lower wavelengths increases at deeper layers, such as the SNP and the anterior stroma. In both cases, the images at 488 nm and 518 nm have a stronger diffuse background than at 815 nm. Nevertheless, at all wavelengths, nerve fibers and keratocyte nuclei are imaged clearly and sharply.

No diffuse background is visible in the endothelium in Fig. 7, because the relatively highly reflective cell structures are more widespread. Still, an intensity loss due to the scattering was present, so that the detector gain had to be increased. Also, the wavelength-dependent endothelial reflectance is very different compared to the previous corneal layers. The hexagonal cell structure
Fig. 5. Comparison of *in vivo* human cornea images of the subbasal nerve plexus acquired with three distinct wavelengths as indicated. The histograms of the subimages in the yellow dashed squares were adjusted to the full intensity range for a consistent comparison of the image quality.

Fig. 6. Comparison of *in vivo* human cornea images of anterior stroma acquired at three distinct wavelengths as indicated. The histograms of the subimages in the yellow dashed squares were adjusted to the full intensity range for a consistent comparison of the image quality.

can be resolved at the blue and green wavelengths, but not using the near-infrared light. Contrary to the epithelial cells, the cell borders of the endothelium appear to be darker than the cytoplasm. While at 488 nm and 518 nm the endothelial structure is equally well visible, at 815 nm cells can only be seen sporadically. In addition, some endothelial cell nuclei appear as bright spots at the blue and green wavelengths.
Fig. 7. Comparison of in vivo human cornea images of endothelium acquired at three distinct wavelengths as indicated. The histograms of the subimages in the yellow dashed squares were adjusted to the full intensity range for a consistent comparison of the image quality.

Figure 8 compares specular and confocal microscopy of the endothelium. The latter shows sharper cell structures at the blue and green wavelengths.

Fig. 8. Comparison of in vivo human cornea images of endothelium captured with specular and confocal microscopy at three different wavelengths. The histogram of each image is stretched for better comparison.

3.2. Ex vivo measurements of porcine and lamb corneae

Since the previous results at the blue and green wavelengths revealed no substantial difference, in vivo images of the human cornea are compared in the following with ex vivo images of porcine
and lamb corneae only at 488 nm and 815 nm. Interestingly, the superficial cells of animal corneae in Fig. 9 show different behavior than in the human images. In the case of the porcine cornea, cell nuclei reflect similarly at both wavelengths. In the case of the lamb cornea, the nuclei reflect somewhat more strongly at 815 nm and thus show the opposite behavior compared to the human cornea. Furthermore, the cytoplasm of the porcine and lamb cornea reflects at the near-infrared wavelength with a different structure, in the latter also considerably stronger. However, the cell borders of all three species are best visible at 488 nm. Please note, differences in backscattering characteristics may also be induced by the post mortem time in case of the animal corneae.

**Fig. 9.** *In vivo* (human) and *ex vivo* (porcine, lamb) images of superficial epithelial cells acquired at blue and near-infrared wavelengths.

**Fig. 10.** *In vivo* (human) and *ex vivo* (porcine, lamb) images of stroma acquired at blue and near-infrared wavelengths.
Figure 10 illustrates that the scattering in the stroma is noticeable for all species at the blue wavelength. In the animal corneae, especially in the porcine, the cell density of keratocytes seems to be lower than in the human cornea. This might be related to the advanced age of the animals [21,22]. While at 815 nm keratocyte nuclei and stromal nerve of the porcine cornea are more visible, keratocyte nuclei of the lamb cornea are only weakly visible at both wavelengths and the visibility of the stromal nerve is rather similar.

The endothelium in Fig. 11 also shows substantial differences between 488 nm and 815 nm in the porcine and lamb cornea. In the human and porcine cornea, some endothelial cell nuclei appear as bright spots at 488 nm. In the lamb cornea these nuclei are not visible. While at 815 nm the entire endothelial structure including nuclei is almost invisible in the human cornea, it is visible in case of the porcine and lamb cornea and the nuclei appear darkened.

![Fig. 11. In vivo (human) and ex vivo (porcine, lamb) images of endothelium acquired at blue and near-infrared wavelengths.](image)

4. Discussion and conclusions

This work presents for the first time an investigation of the wavelength-dependent reflectance of corneal tissues. The presented images show that most differences in image quality can be attributed to a depth- and wavelength-dependent scattering background. Tissue and cell characteristic backscattering patterns (intra- and extracellular) are little to not wavelength specific. From the subbasal nerve plexus onwards, the negative effects in the form of a diffuse background become apparent at shorter wavelengths. This effect increases for shorter wavelengths the deeper the imaging is performed in the cornea. However, this diffuse background is most pronounced in the stroma among all species. In the endothelium, this effect is only noticeable in images of the porcine cornea. It is assumed that this is due to the advanced time after the eye was enucleated and therefore it is generally more opaque. Furthermore, in the case of the human and lamb cornea, the visible structures in the endothelium are more prominent at the shorter wavelengths than at the near-infrared.

No effect of scattering is observed in the epithelium. This is due to the fact that scattering background is depth-dependent and the epithelium is the top layer of the cornea. The images of the wing cells presented in this manuscript do not reveal differences in imaging quality or structure at the three selected wavelengths. Superficial cells, however, show structural differences
depending on the wavelength and on the investigated species. In the human cornea, the nuclei and cytoplasm are most reflective at the short wavelengths. The mature superficial cells, which are about to desquamate into the tear film, exhibit a stronger reflection because they have more microvilli than the younger cells [23]. In the lamb cornea, on the other hand, the opposite behavior was observed. This leads to the assumption that not every mammal eye is suitable for comparison to the human one. It is supposed that the desquamating cells change their protein pattern variously depending on the species, which leads to differences in reflectance [24,25]. Nevertheless, further investigations are needed to confirm this statement. Despite the aforementioned differences, in all three species, it is shown that the superficial epithelial cell borders are best defined at shorter wavelengths and thus individual cells are best distinguished.

In comparison to other imaging techniques, it is apparent that, for example, OCT methods using a NIR light source [6–11,13] are capable to capture images of the SNP and stroma with almost similar quality as the results presented here. However, the epithelial images do not show cell structures as clearly as the results obtained by corneal confocal microscopy in this paper. Since the previously referenced methods are non-contact methods, images of superficial epithelial cells are currently impossible due to the strong surface reflection. Interestingly, although the endothelial cells are clearly visible in the NIR with OCT methods we were barely able to image the human endothelium using 815 nm light. When using OCT in the visible spectrum [12], clear cell structures are visible in all corneal layers of the rat eye. Since a water immersion objective was used, superficial cells were imaged, too [12]. The presented corneal confocal microscopy at shorter wavelengths, provides endothelial images with clear cell structures, offering an alternative to the specular microscopy, which is still the current gold standard for endothelial imaging. In addition to the wavelength, the scattering geometry also has an influence on imaging, as shown by the asymmetric fundus retroillumination [5]. While backward scattering occurs in the confocal microscopy applied in this paper, retroillumination utilizes the spatially dependent transmittance revealing the cell structures. Nerve fibers in the SNP and stroma as well as the endothelium are visible at a large FOV. Additionally, the surface layers of the epithelium can be imaged despite the non-contact method. Nevertheless, the epithelial and endothelial images of retroillumination do not show the same level of detail as the images obtained with corneal confocal microscopy.

This paper demonstrates the importance of choosing the right wavelength or wavelength spectrum for confocal laser scanning microscopy of certain corneal structures. In doing so, not only the depth-dependent and wavelength-dependent scattering background should be considered, but also the behavior in the respective layer. Especially when developing new devices that use either a broadband light source or several wavelengths simultaneously, this should be decided depending on the area of application. If the main focus is on e.g. ocular surface diseases, shorter wavelengths in the visible spectrum should be preferred. For imaging the entire cornea, the wavelength should be shifted so far into the red that the scattering no longer has a negative influence on image quality. In case only the endothelium is going to be considered, also shorter wavelengths are more suitable. The optimal solution would be a multimodal instrument, which provides several wavelengths to choose from, depending on the examined corneal layer.

A second rationale for wavelength-dependent imaging is as follows. Backscattering from inter- and intracellular structures can vary with wavelength. In the translation, by acquiring reflectance images from multiple wavelengths it could be possible to extract wavelength-dependent information that could correlate to tissue and cell properties. For example, Wang et al. were able to characterize lymphocytes in skin [26] at 785, 810, and 850 nm. Surprisingly, our corneal imaging over the large wavelength range of nearly 350 nm shows only minor variations in the tissue-dependent backscattering patterns. Primarily, this implies that the choice of wavelength is of secondary importance, with the exception of the endothelium and epithelium. Whether multiwavelength imaging offers further diagnostic values, especially on the diseased corneae, is subject to future research.
One effect that has not yet been discussed is the effect of wavelength on the subject’s compliance. Taken into consideration that light from the visible spectrum could distract or glare the subject, an impairment of the in vivo examination is conceivable. Following this aspect, near-infrared light has the advantage that it is invisible to the human eye and should avoid glare induced eye movements.

The presented results were generated with only three distinct wavelengths. Thus, no continuous wavelength dependence of the image quality or depicted corneal structures is provided. Despite this, they do demonstrate the importance of choosing the right light source to obtain the best image quality in the different layers of the cornea. In order to further expand this research, additional wavelengths, broadband light sources, or tunable lasers could be included in such a study. In addition, more subjects with a known eye health status should be examined in order to confirm the reproducibility of the results shown here and to exclude any subject dependent differences. However, it should be noted that the ex vivo measurements were performed on several porcine and lamb eyes, and no differences were found within one species.

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