Confocal microscopy vs. Two-photon microscopy – Imaging of ocular surface pathologies

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ABSTRACT

Minimal-invasive imaging of ocular surface pathologies aims at securing clinical diagnosis without the necessity of actual tissue probing. For this matter confocal microscopy with the Cornea Module, mounted on a laser scanning microscope, is in daily use in ophthalmic practise. Two-photon microscopy is a new optical technique that enables high resolution imaging and functional analysis of living tissues based on tissue autofluorescence with minimal phototoxic damage. This study was set up to compare the potential of two-photon microscopy to the established Cornea Module.

Different ocular surface pathologies such as pterygia, papillomae, nevi and cysts were investigated using the Cornea Module for confocal microscopy in-vivo. The pathologies were excised, stored in tissue culture media and immediately investigated by two-photon microscopy without further fixation. After imaging, the specimens were sent for definite histopathological assessment.

Cornea Module and two-photon microscopy both generated high resolution images of the investigated tissues. At wavelengths of 710-730 nm two-photon microscopy exclusively revealed cellular structures whereas collagen fibrils were specifically demonstrated by second harmonic generation. Measurements of fluorescent lifetimes (FLIM) enabled the highly specific display of e. g. goblet cells or erythrocytes within capillaries. FLIM also enabled to demarcate nevus-cell clusters from epithelial cells.

At the settings used, two-photon microscopy reaches higher resolutions than the Cornea Module and has the option of tissue specific signals by wavelengths tuning and fluorescence lifetime imaging which give additional information about the tissue. The Cornea Module allows intravital real-time imaging with less technical effort that leads to the visualization of dynamic processes such as blood flow. The parallel detection of two-photon excited autofluorescence together with confocal imaging could expand the possibilities of minimal-invasive investigation of the ocular surface towards functional analysis at higher resolutions.

Keywords: Two-photon microscopy, confocal microscopy, in-vivo microcopy, ophthalmology

1. INTRODUCTION

Optical imaging of ocular surface pathologies is crucial for the clinical diagnosis of ophthalmological diseases. Slit-lamp bio-microscopy is well established for the diagnosis of ocular pathologies of the anterior and posterior parts of the eye. The introduction of reflection and fluorescence imaging of the retina opened further possibilities in the clinical diagnosis of retinal disorders. Other technologies based on laser scanning were able to improve the lateral and depth resolution. However, the limited aperture of the eye prevented a real microscopic imaging, therefore in contrast to optical coherence tomography (OCT), laser scanning microscopy is not able to discriminate the different retinal layers with high resolution. Instead OCT, which improves the depth resolution to less than 10 µm, is able to give retinal images with equal longitudinal and transverse resolution[1, 2]. Imaging the ocular surface is not limited by NA restrictions and is possible with subcellular resolution. Confocal microscopy based on reflected near-infrared light successfully has

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demonstrated the high resolution visualization of epithelial structures of cornea, sclera and conjunctiva, as well as fibroblasts und nerve fibers in the transparent cornea[3]. Also the corneal endothelial cells were resolved by these technique. The contrast in confocal reflection microscopy is generated by reflections at interfaces of tissue and cellular structures due to variations of the index of refraction. Hence reflection microscopy gives only morphological information, which is not specific to the composition or functional state of the tissue.

Fluorescence microscopy is able to provide more specific informations. Selectively binding fluorescence dye or antibody-dye conjugates can highlight specific tissue structures with high contrast. Dyes with fluorescence properties, which are sensitive to the molecular environment, can deliver functional information on Ca^{2+} concentration, membrane fluidity, cell viability or other parameters. The intrinsic tissue fluorescence, which is caused by collagen, elastin, NAD(P)H, and flavins can give specific structural or even functional information. Especially for clinical in-vivo application the use of autofluorescence is very appealing, since up to now there are only a few fluorescence dyes approved, that are not very specific.

Autofluorescence can not be imaged by confocal microscopy in scattering tissues. The UV/blue excitation light is strongly attenuated by the tissue, the emission is much weaker than that of fluorescence dyes, and photobleaching as well as phototoxicity limit illumination time and intensity. Modern technology allows to create high repetition rate laser pulses with peak radiances of a few hundred TW/cm^2 in the focus of high NA objectives. In the NIR range from 700 nm to 900 nm, the "optical window" of the tissue, the light can propagate to considerable depth without linear absorption. In the focus the photon density may reach a level at which two photons couple to an energy state and get simultaneously absorbed[4, 5]. The combined energy of two infrared photons is able to excite UVA and blue absorbing molecules including endogenous chromophores, which give rise to the tissue autofluorescence. Being limited to the Rayleigh length of the focal region, where the irradiance is high enough for 2-photon absorption, the 2-photon microscopy provides inherent three dimensional sectioning without the use of a pin-hole. This is a way to detect the tissue autofluorescence depth resolved in scattering tissue down to 200 µm[6]. Additionally photobleaching and photodamage are limited to the focal region[7]. Therefore 2-photon excited fluorescence microscopy is the only method to image tissue autofluorescence in thick living tissues. Longitudinal and transverse resolutions better than a micrometer are achieved[8]. Under 2-photon imaging conditions besides autofluorescence, second harmonic generation (SHG) in non-centro symmetric molecular structures can be imaged. A non-linear interaction with high electrical fields leads to the generation of light with doubled frequency or half the wavelength. Especially collagen is an efficient source of SHG signals, which strength may exceed that of the autofluorescence[9]. Collagen structures of ocular tissues could be visualized with high contrast by SHG imaging[10].

These unique properties suggest the use of 2-photon microscopy in clinical diagnosis. Recently the CE-marked device DermaInspect was introduced for skin imaging[11-13]. Possible applications include tumor diagnosis, visualization of skin aging and quantification of the up-take of chemical substances or nanoparticles. A further promising field for 2-photon microscopy is the imaging of the outer ocular surfaces of cornea, sclera, and conjunctiva.

In order to assess image quality and pin-point possible clinical applications we used the DermaInspect to image different pathologies of outer ocular surfaces after they were excised, and compared the 2-photon images with in-vivo images, that were taken prior to surgery with a commercial available device for confocal imaging (Cornea Module with the Heidelberg Engineering HRT II), which is in daily use in ophthalmic practice. Aim of this study was to compare the potential of two-photon microscopy and confocal microscopy for imaging ocular structures and find possible diagnostic applications.

2. MATERIAL AND METHODS

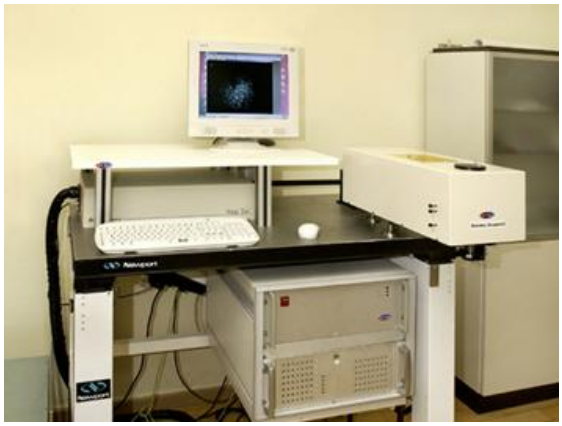
Different ocular surface pathologies were investigated in vivo by the Cornea Module in combination with the HRT II retinal imager (Heidelberg Engineering, Heidelberg, Germany) (Fig. 1a). After surgical excision the pathologies were stored in tissue culture media and immediately investigated by the 2-photon tomograph DermaInspect (JenLab GmbH, Neuengönna, Germany) without further fixation (Fig. 1b). The DermaInspect consisted of a solid-state, mode-locked 80 MHz titanium:sapphire laser (MaiTai, Spectra Physics, Darmstadt, Germany) with a tuning range of 710-920 nm, a mean laser output of >900 mW at 800 nm, and a 75 fs pulse width. Tissue autofluorescence was imaged by exciting the samples at 720 nm. A wavelength of 826 nm was used to measure the second harmonic generation (SHG) of the sample. The device contained a computer-controlled beam attenuator, a shutter, and a two axis galvoscanner. A 40x objective

with NA 1.3 and 140 µm working distance (Plan-Neofluar 40X, 1.3 oil, Zeiss, Göttingen, Germany), which was focused by a piezodriven holder, was used in this study. Larger scale motion of the sample in x- and y-directions were performed by computer-controlled stepper-motors (Owis GmbH, Staufen, Germany). The autofluorescences was detected by a standard photomultiplier module (H7732, Hamamatsu, Herrsching, Germany), after passing though a beam splitter (Chroma 640 DCSPXR, AHF analysentechnik AG, Tübingen) and a short-pass filter (BG39, Schott, Mainz, Germany), resulting in a detection bandwidth from 360 to 590 nm. To detect SHG, the same photomultiplier with a 413 nm band pass filter (Amko, Tornesch, Germany) was used.

Fluorescence lifetime imaging (FLIM) was performed by time correlated single photon counting[14]. Start signals were generated from a fast photomultiplier module (PMH-100-0, Becker & Hickl, Berlin, Germany) with a transient time spread of approximately 180 ps, that detected the fluorescence photons emitted by the tissue. The stop signal was provided by a fast photodiode that measured the excitation pulses. The timing between both signals was measured by a PC based single-photon counting board (SPC 830, Becker & Hickl, Berlin, Germany). The board was synchronized with the scanning of the excitation beam and 256 x 256 spatially resolved autofluorescence decay curves were measured in the image field. Curve fitting with a single exponential decay curve including a deconvolution with the time response of the system (SPCImage 2.6, Becker & Hickl, Berlin, Germany) was used to calculate a mean fluorescence lifetime for each pixel, which was displayed in color-coded images.

After imaging, the specimens were sent for definite histopathological assessment. The tissue was fixed, sliced in 10 µm sections and stained with HE (haematoxyline eosin). The pathologies included such as pterygia, papillomae, nevi and cysts. Informed consent was obtained prior the procedure from each patient.

a)



b)

Fig. 1: a) HRT II with Cornea Module. b) DermaInspect with femtosecond laser

3. RESULTS AND DISCUSSION

Due to the working principle and the intended use the Cornea Module and the DermaInspect have different technical characteristics (Table 1). To carry out confocal imaging with reflectance contrast, a relatively inexpensive diode laser is sufficient, whereas the DermaInspect uses a tunable femtosecond laser to generate autofluorescence, SHG, and fluorescence lifetime images. Both systems use different commercially available microscope objectives. The 63x, NA 0.8 water immersion objective of the Cornea Module allowed a diffraction-limited resolution of 0.4 µm laterally and 1 µm longitudinally. The field of view could be varied between 150 µm and 400 µm. An oil immersion 40x objective with NA 1.3 was used in the DermaInspect providing 0.2 µm x 0.5 µm resolution. The field of view could be varied between 20 µm and 230 µm. The Cornea Module achieves video rate imaging with up to 30 frames per second. Due to the lower autofluorescence signals the maximal imaging speed of the DermaInspect was limited to 1 frame per second at

quite restricted image quality. For high quality images as shown in Figures 1 to 4 an exposure time of 30 seconds was necessary. Both devices acquire single images and images stacks with variable z-spacing. In addition, the Cornea Module has the option to generate videos of dynamic tissue changes.

Table 1: Comparision of the technical specifications of the HRT with Cornea Module and the DermaInspect

	Cornea Module+HRTII (Heidelberg Egineering)	**DermaInspect (Jenlab)**
Technical background and working principle	Confocal microscope Diode laser 670nm Reflection contrast	Two-photon microscope Femtosecond laser 710-900 nm Autofluorescence, SHG, FLIM
Specifications	In-vivo human patients NA: 0.9 water immersion Resolution: 0.43 µm x 2 µm, 1-30 frames/sec Field of view 150 – 400 µm Single images, stacks, video	In-vivo human skin, ex-vivo samples NA: 1.3 Oil immersion Resolution: 0.23 µm x 0.55 µm, 0.02-1 frames/sec Field of view 20-230 µm Single images, stacks, SHG, FLIM

The pratical resolution of both systems, Cornea Module and DermaInspect, was assessed with images from corneal epithelial cells. Both devices provided subcellular resolution. The Cornea Module was able to resolve the individual cells with bright cell borders and dark nuclei (Fig. 2). Structures in the cytoplasm were barely resolved. The DermaInspect imaging provided a significant higher resolution, which allowed to identify even single cell organelles. Under 2-photon excitation the cytoplasm appears as a granular structure. Nuclei and actual cell borders remained dark. In epithelial cells

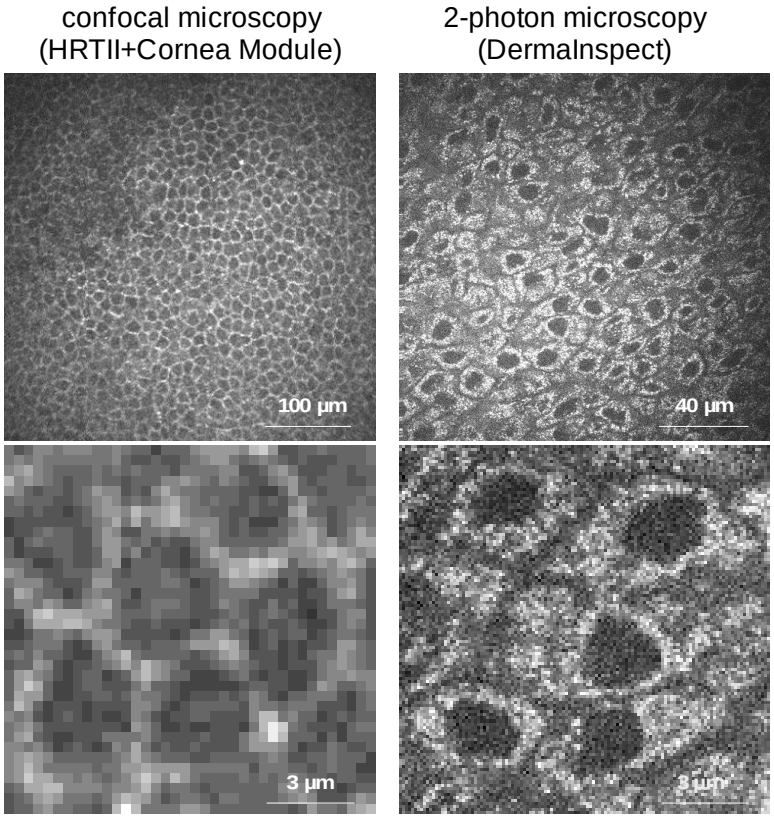


Fig. 2: Epithelial cells of the cornea imaged with confocal microscopy (HRTII+Cornea Module) and 2-photon microscopy (DermaInspect). The lower images show a magnified view.

a prominent source of the autofluorescence in the cytoplasm is NAD(P)H, which is mainly localized in the mitochondria. Due to the higher resolution and the higher contrast 2-photon images contained more information on the cell borders and the size of the nuclei as confocal images.

The differences in resolution were larger as expected from differences in the NA. In confocal microscopy the diffraction limited resolution is only achieved with aberration free optics and the smallest pin-holes size. Often the pin-hole for the rejection of out of focus light is significantly larger to increase the brightness of the images, which reduces the actual resolution[15]. In contrast, the resolution of 2-photon imaging usually reaches the diffraction limit. Additionally there is a gain in resolution of a square root of two for 2-photon microscopy due to the non-linear absorption process.

The spatial distribution of intracellular autofluorescence can distinguish different types of cells. In Figure 3 a Pterygium, that is a strand of conjunctiva growing over the sclera and cornea, was imaged. By 2-photon imaging two different types of cells were easily identified. Epithelial cells had a strong fluorescing cytoplasm and a darker nucleus. A second cell type with a large internal dark area and small fluorescing rim was identified by comparative histology as goblet cells. The goblet cells were also visible in confocal microscopy, but with a much lower contrast against the epithelial cell. 2-photon excited FLIM even was able to increased the contrast between both cell types (Fig. 3e), because the mucous within the Goblet cells was characterized by a significantly longer fluorescence lifetime than the surrounding cells.

In our experiments a wavelength of 720 nm efficiently excited the tissue autofluorescence which the DermaInspect detected efficiently between 380 nm and 590 nm. The SHG signal at 360 nm was strongly attenuated since at that wavelength the sensitivity of the detection is only 5% of the maximal sensitivity at 450 nm. At excitation wavelengths below 800 nm tissue autofluorescence is very weak due to the low absorption of the relevant chromophores and therefore SHG was dominant. Fluorescence in photodamaged tissue and macrophages, which can be excited below 800 nm may interfere with the SHG signal. Using a band pass filter at exactly half the excitation wavelength, the residual autofluorescence can be completely suppressed.

Figure 4 shows confocal, autofluorescence, and SHG images of an ocular papilloma with a central vessel. The cellular

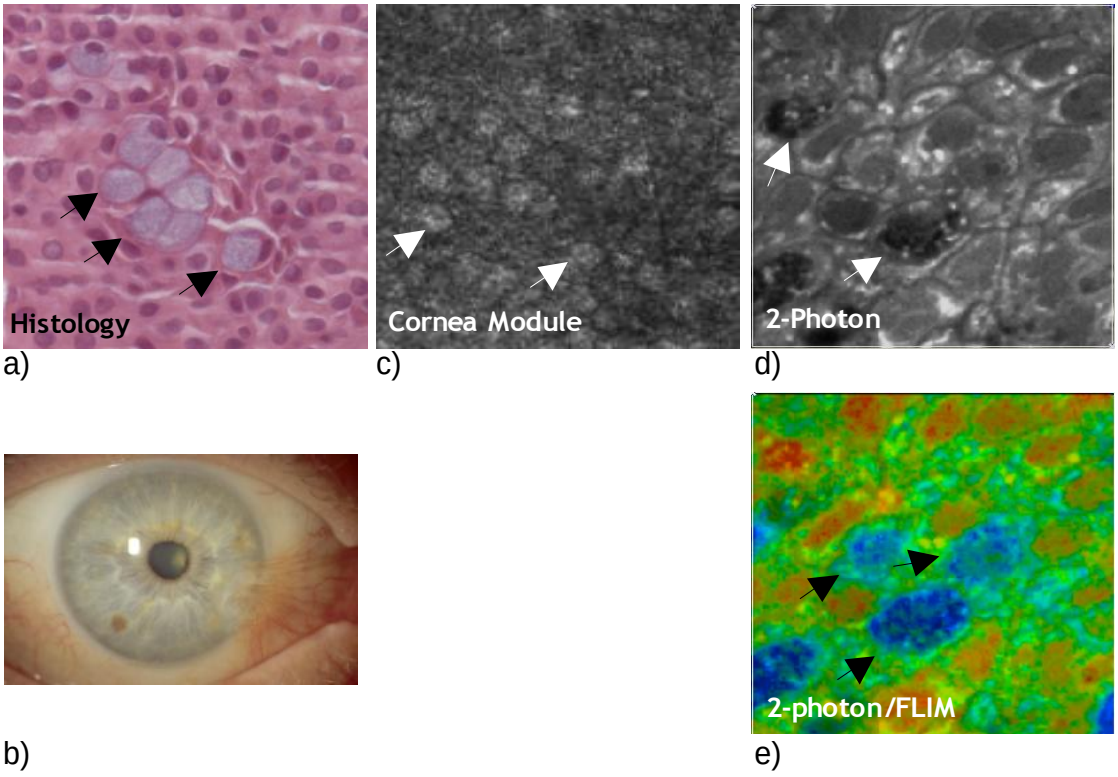


Fig. 3: Images of a Pterygium. a) Histology, b) macroscopic image, c) confocal image, d) 2-photon excited autofluorescence, e) fluorescence life time image. Arrows show goblet cells in the corresponding images.

structures are visible in the confocal (Fig. 4c) and the autofluorescence image (Fig. 4e). As in Figures 2 and 3, the cell border and the nucleus are visible much clearer in the 2-photon images. The central part of the image shows the blood vessel, which is characterized by low signal region with inhomogeneous appearance in both imaging modalities. SHG imaging is able to highlight the vessel wall with high resolution (Fig. 4d). The false color composite image shows the exact co-localization between SHG and autofluorescence image. SHG was also successfully used to visualize collagen fibrils in connective tissue in pterygium.

In contrast to one-photon excitation, 2-photon excitation reveals erythrocytes in blood vessels, that have a significant autofluorescence with extremely short fluorescence lifetime. Hence FLIM is able to visualize erythrocytes with very good contrast. Autofluorescence of Melanin was recorded in ocular naevi and melanoma. A bright, point like fluorescence pattern in epithelial cells was observed in pigmented lesion. A clear distinction from mitochodria or lysosomal fluorescence was possible by FLIM. The fluorescence lifetime of melanosomes was shorter than the time resolution of our system, which was a few hundred picoseconds with our detector

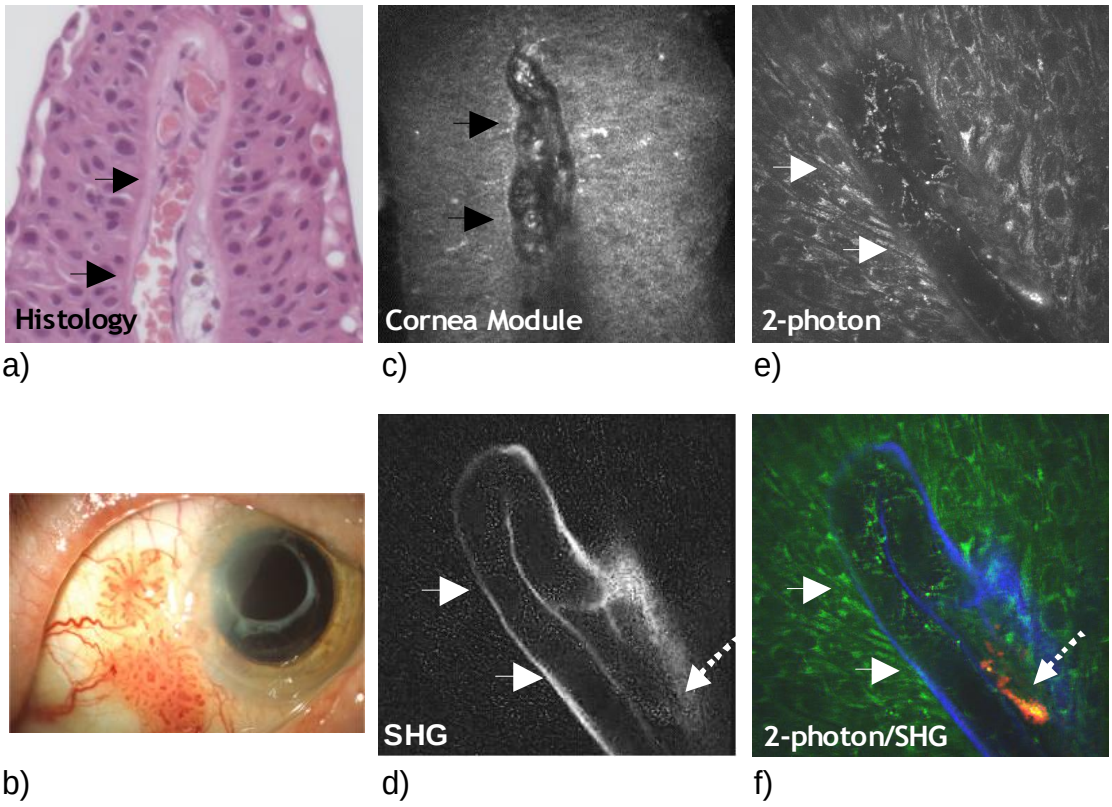


Fig. 4: Images of an ocular papilloma. a) Histology, b) macroscopic image, c) confocal image, d) second harmonic image, e) 2-photon excited autofluorescence, f) composite image autofluorescence/SHG. Arrows show blood vessels, dotted arrows macrophages

4. CONCLUSIONS

This comparative study shows that 2-photon excited autofluorescence is able to visualize healthy and pathological structures of ocular tissue surfaces with higher resolution and better contrast than a commercial confocal in-vivo microscope. Without the need of external fluorescing dyes all tissue structures which were visualized by the Corneal Module were also seen in three dimensions by 2-photon microscopy. In highly scattering tissues the depth of imaging was comparable with both systems. In contrary, confocal microscopy can work also at lower NA, which makes it more suitable for the imaging deeper structures in cornea, anterior chamber or the lens.

The images of confocal microscopy are only based on one parameter, the local reflection. Besides fluorescence intensity, 2-photon imaging is able to use three more parameters: emission spectrum, excitation spectrum and fluorescence lifetime. This enables a unique characterization of tissue components like mitochondria, hemoglobin, collagen or melanin. We demonstrated spectral and fluorescence lifetime based identification of vascular structures and pigmented lesions. The possibility to measure unique fluorescence signatures paves the way for an automatic identification of tissue structures, which is difficult in case it has to be based solemnly on morphological features.

Autofluorescence based FLIM has the potential to differentiate cell type or even stages of cell development. The intracellular autofluorescence can monitor NAD(P)H metabolism[16]. In this context we have demonstrated previously, that the dependence of the fluorescence lifetime on the excitation wavelength can discriminate different brain tissues and different tumor cells lines [17, 18].

The main disadvantage of 2-photon against confocal microscopy - beside from the expensive equipment needed - is a slow imaging speed. With one scanning beam, which is used in the DermaInspect, image acquisition time is limited by excitation threshold for tissue damage, which is only a factor of two from the excitation intensity we used. Possible solutions to this problem are either combining 2-photon with confocal imaging, so that spectrally resolved 2-photon imaging can be restricted to certain regions of interest or to use parallel scanning with multiple beams[19]. Our next step will be to integrate 2-photon imaging capabilities into the Cornea Module/HRT II for in-vivo imaging.

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REFERENCES

1. D. Huang, E. Swanson, C. Lin, J. S. Schuman, W. G. Stinson, W. Chang, M. R. Hee, T. Flotte, K. Gregory, C. A. Puliafito, and J. G. Fujimoto, "Optical Coherence Tomography," *Science* 254, 1178 (1991).
2. M. E. van Velthoven, D. J. Faber, F. D. Verbraak, T. G. van Leeuwen, and M. D. de Smet, "Recent developments in optical coherence tomography for imaging the retina," *Prog. Retin. Eye Res.* 26, 57-77 (2007).
3. R. Guthoff, J. Stave, and C. Baudouin, *Atlas of Confocal Laser Scanning In-vivo Microscopy in Ophthalmology* (Springer, Berlin, 2006).
4. W. Denk, J. H. Strickler, and W. W. Webb, "Two-photon laser scanning fluorescence microscopy," *Science (Washington, D. C., 1883-)* 248, 73-76 (1990).
5. F. Helmchen, and W. Denk, "Deep tissue two-photon microscopy," *Nature Methods* 2, 932-940 (2005).
6. D. W. Piston, "Imaging living cells and tissues by two-photon excitation microscopy," *Trends Cell Biol.* 9, 66-69 (1999).
7. M. Rubart, "Two-photon microscopy of cells and tissue," *Circ. Res.* 95, 1154-1166 (2004).
8. C. Y. Dong, K. Koenig, and P. So, "Characterizing point spread functions of two-photon fluorescence microscopy in turbid medium," *J. Biomed. Opt.* 8, 450-459 (2003).
9. P. J. Campagnola, A. C. Millard, M. Terasaki, P. E. Hoppe, C. J. Malone, and W. A. Mohler, "Three-Dimensional High-Resolution Second-Harmonic Generation Imaging of Endogenous Structural Proteins in Biological Tissues," *Biophys. J.* 81, 493–508 (2002).

10. S. W. Teng, H. Y. Tan, J. L. Peng, H. H. Lin, K. H. Kim, W. Lo, Y. Sun, W. C. Lin, S. J. Lin, S. H. Jee, P. T. So, and C. Y. Dong, "Multiphoton autofluorescence and second-harmonic generation imaging of the ex vivo porcine eye," *Invest. Ophthalmol. Vis. Sci*. 47, 1216-1224 (2006).
11. K. König, and I. Riemann, "High-resolution multiphoton tomography of human skin with subcellular spatial resolution and picosecond time resolution," *J. Biomed. Opt.* 8, 432-439 (2003).
12. F. Stracke, B. Weiss, C. M. Lehr, K. Konig, U. F. Schaefer, and M. Schneider, "Multiphoton microscopy for the investigation of dermal penetration of nanoparticle-borne drugs," *J. Invest. Dermatol*. 126, 2224-2233 (2006).
13. M. J. Koehler, K. König, P. Elsner, R. Bückle, and M. Kaatz, "In vivo assessment of human skin aging by multiphoton laser scanning tomography " *Opt. Lett.* 31, 2879-2881 (2006).
14. W. Becker, *Advanced Time-Correlated Single Photon Counting Techniques* (Springer, Berlin, Heidelberg, New York, 2005).
15. G. Cox, and C. J. Sheppard, "Practical limits of resolution in confocal and non-linear microscopy," *Microsc. Res. Tech.* 63, 18-22 (2004).
16. D. W. Piston, B. R. Masters, and W. W. Webb, "Three-dimensionally resolved NAD(P)H cellular metabolic redox imaging of the in situ cornea with two-photon excitation laser scanning microscopy," *J. Microsc.* 178 (Pt 1), 20-27 (1995).
17. S. R. Kantelhardt, J. Leppert, J. Krajewski, N. Petkus, E. Reusche, V. M. Tronnier, G. Hüttmann, and A. Giese, "Imaging of brain and brain tumor specimens by time-resolved multiphoton excitation microscopy ex vivo," *Neuro-oncol* 9, 103-112 (2007).
18. J. Leppert, J. Krajewski, S. R. Kantelhardt, S. Schlaffer, N. Petkus, E. Reusche, G. Hüttmann, and A. Giese, "Multiphoton excitation of autofluorescence for microscopy of glioma tissue," *Neurosurgery* 58, 759-767, discussion 759-767 (2006).
19. T. Nielsen, M. Fricke, D. Hellweg, and P. Andresen, "High efficiency beam splitter for multifocal multiphoton microscopy," *J. Microsc.* 201, 368-376 (2001).