Interpretation of Measurements of dynamic Fluorescence of the Eye

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ABSTRACT

First pathological alterations occur at cellular level, most in metabolism. An indirect estimation of metabolic activity in cells is measurement of microcirculation. Measurements of tissue autofluorescence are potentially suited for direct investigation of cellular metabolism. Besides redox pairs of co-enzymes (NADH-NAD, FADH2-FAD) several other fluorophores are excited in tissue. In addition, a number of anatomical structures are simultaneously excited, when investigating the eye-ground. In this study, spectral and time resolved comparison was performed between purified substances, single ocular structures and in vivo measurements of the time-resolved autofluorescence at the human eye. In human eyes, the ageing pigment lipofuscin covers other fluorophores at the fundus in long – wave visible range. Applying lifetime measurements, weakly emitting fluorophores can be detected, when the lifetimes are different from the strongly emitting fluorophore. For this, the autofluorescence was excited at 468 nm and detected in two spectral ranges (500 nm-560 nm, 560 nm-700 nm). In tri-exponential fitting, the short lifetime corresponds to retinal pigment epithelium, the mean lifetime corresponds probably to neural retina and the long lifetime is caused by fluorescence of connective tissue.

Keywords: fluorescence lifetime, autofluorescence, eye, excitation spectrum, emission spectrum, ocular structures, NADH, FAD

1.INTRODUCTION

First pathological alterations occur at cellular level, most in metabolism. An indirect estimation of metabolic activity in cells is measurement of microcirculation. Such investigations are concentrated on blood flow and oxygen saturation. Although these data are important, information on cellular processes are limited. In case of increased resistance of diffusion, dissolved oxygen can not penetrate from vessels or capillaries into cells. Despite lack of oxygen for energy metabolism in mitochondria, the venous oxygen saturation can be increased, pointing at an oversupply of oxygen in the tissue[1] .
The detection of autofluorescence of endogenous fluorophores can overcome this limitation. Chance (1976)[2] has found a three orders increased sensitivity of changes in fluorescence of redox pairs NADH- NAD (reduced and oxidized nicotinaminde adenine dinucleotide), FADH2-FAD (reduced flavin adenine dinucleotide) on oxygen concentration than measurement of oxygen saturation. These redox processes act in the basic processes of citric acid cycle and oxidative phosphorylation inside the cells. Between both redox pairs is a different behaviour: the reduced for NADH is high fluorescent in relative lack of oxygen, whereas the oxydized form FAD fluoresces when sufficient oxygen is available. Metabolic by- or end-products exhibit also autofluorescence. Specific components of tissue have also specific fluorescence properties.

In ophthalmology, the non-invasive investigation of endogenous fluorescence is of special interest at the eye ground. The autofluorescence of cornea and lens as well as of the fundus can be detected. Especially the fundus can be considered as a window into the body.
Besides the effect of redox pairs of coenzymes, the ageing pigment lipofuscin accumulates in age-related macular degeneration. Advanced glycation end-products (AGE) are increased in diabetes mellitus. Converting collagen 3 in collagen1 is discussed in developing of glaucoma. Collagens and elastin are involved in sclerotic processes.

So, several fluorophores are expected in ocular tissue: NADH, FAD, lipofuscin, AGE, collagen, elastin, amino acide tryptophan and tyrosine, components of hem synthesis pyridoxal oxalate, protoporphyrin IX etc.. For detection of these fluorophores there are different conditions in the anterior or posterior part of the eye. Considering the fundus, the limited spectral transmission of the ocular media must be taken into account. The fundus can not be excited for wavelengths below 400 nm and at 450 nm the transmission is only 50 %. The excitation and emission spectra of these substances were considered in relation to the ocular transmission[3]. In this study, the excitation of NADH was found unlikely at the fundus. The effective excitation spectra as product of ocular transmission and excitation spectrum of a pure substance are determined by lipofuscin for wavelengths longer than 500 nm, but FAD, AGE and connective tissue can be excited at the fundus in the shorter spectral range, too. Discriminating these fluorophores at the fundus, the detection of fluorescence is appropriate in a two spectral ranges 500 nm – 560 nm and 560 nm – 700 nm. Best distinction of fluorophores at the eye ground can be realised by combination of fluorescence lifetime measurements with excitation at different wavelengths and detection of fluorescence in at least two spectral ranges.

Based on the HRA (Heidelberg Engineering, Heidelberg, Germany), a laser scanner ophthalmoscope was developed for fluorescence lifetime measurements at the eye. Diode lasers (LASOS , Jena, Germany), emitting at 446 nm or 468 nm are used for excitation. The dynamic fundus fluorescence was detected in two spectral ranges by MCP-PMT (R3809U50, Hamamatsu, Gersching, Germany) in connection with a board for time correlated single photon counting (SPC 150, Becker/Hickl, Berlin, Germany). For compensating eye movements during the required measuring time, infrared fundus images (820 nm) were detected simultaneously with the autofluorescence. In the system, there is an automatic on line image registration of the contrast - rich IR images. The parameters of image transformation are used in reconstruction of dynamic fluorescence images. So, each in FIFO mode saved photon can be accumulated in the right time channel of the corresponding pixel.

2.INTERPRETATION OF FLUORESCENCE

Let us consider the whole eye as object of investigation. In this case, the excitation and emission spectra are of interest of all ocular structures. It can be assumed that several fluorophores form both spectra. Here, we investigate predominantly the evidence of NADH or FAD in ocular structures. For these investigations, the cornea, lens, neural retina, pigment epithelium, choroid, sclera vitreous, and aqueous humour were separated from porcine eyes about one hour after slaughtering the animals. Inserting these tissues in cuvettes, the absorption spectra were measured in a Lambda 2 UV/VIS spectrometer (Perkin Elmer). Excitation and emission spectra were detected in a LS 5 fluorescence spectrometer (Perkin Elmer). In addition to spectral measurements, the dynamic fluorescence was measured by the first version of the fluorescence lifetime laser scanner ophthalmoscope[4]. The excitation in these lifetime measurements was performed by a diode laser LDH 440 (Picoquant, Berlin, Germany) emitting light at 446 nm. The dynamic fluorescence was detected between 510 nm and 700 nm.
Looking for NADH, the fluorescence of all ocular tissue was measured at the emission maximum of NADH at 460 nm and the excitation wavelength was chosen between 300 nm and 430 nm. The presented spectra are the mean of measurements at ten tissue samples. As shown in Fig. 1, the characteristic excitation maximum of NADH around 350 nm was found in cornea, lens, neural retina. No local excitation maximum was detectable in BBB. Next, the tissue samples were excited at 350 nm and the fluorescence spectra were measured between 400 nm and 700 nm. The expected fluorescence maximum of NADH at 460 nm was detectable at least as local maximum for neural retina, lens, cornea (Fig. 2).

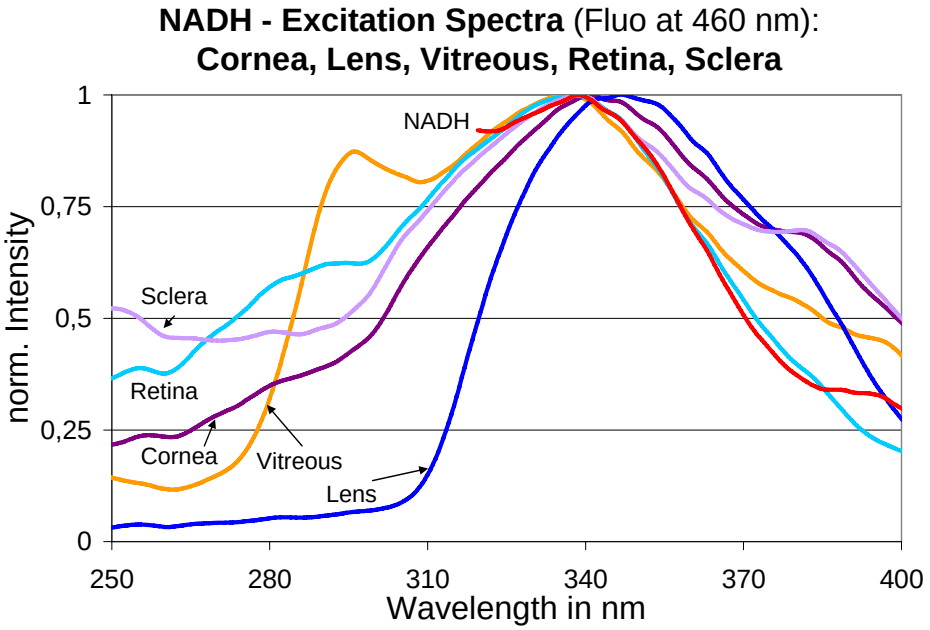

Fig. 1: Detection of NADH in excitation spectra of ocular tissues, changes of fluorescence detected at 460 nm

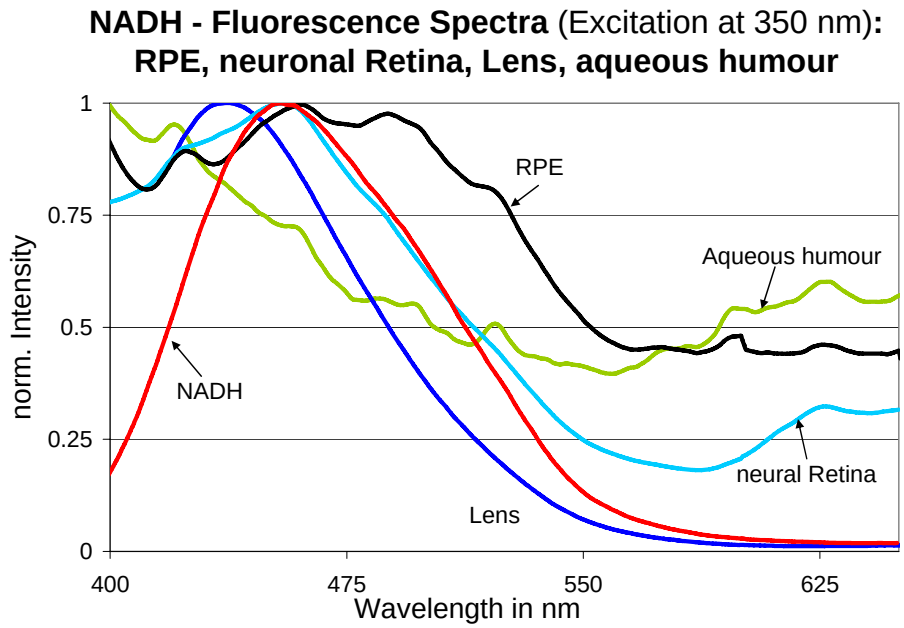

Fig. 2: Detection of NADH in fluorescence spectra of ocular tissues, excitation at 350 nm fluorescence detected at 460 nm

Looking for FAD, the excitation spectra of ocular tissue were determined measuring changes in the fluorescence intensity at 530 nm, when spectrally excited from 300 nm to 500 nm. The excitation maxima of FAD at 370 nm and 460 nm were detectable in the weak fluorescence of RPE, choroid and sclera (Fig. 3).

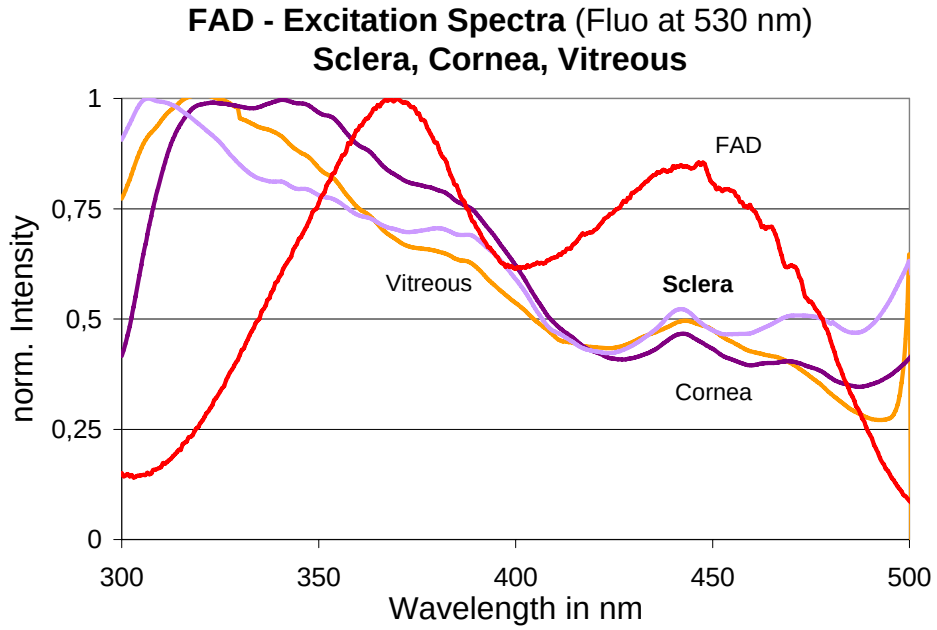

Fig. 3: Detection of FAD in excitation spectra of cornea, sclera, and vitreous, changes of fluorescence detected at 530 nm

In the excitation spectrum of the neural retina (Fig. 4), there is an overlap of several fluorophores and no clear local maxima can be found, corresponding to FAD. Such maxima are also missing in the excitation spectra of lens and cornea. Taking into account the transmission of the ocular media, the expected fluorescence of FAD was excited at 446 nm. A clear local fluorescence maximum around 530 nm was detectable in sclera, RPE, choroid, and also in neural retina (Fig. 5).
That means, the characteristic excitation maximum of FAD was really covered by other fluorophores in neural retina.
Measuring the autofluorescence of the fundus, the incoming light excites also the cornea, lens, and vitreous. The influences of anterior part of the eye can be suppressed by the principle of aperture separation or by the confocal imaging principle to a certain degree. Remaining contributions are caused by multiple scattering, illuminating the fundus by fluorescence light e.g. of the lens, or by direct measurement of fluorescence light of the anterior eye. The influence of these contributions was estimated[5]. Detecting the fluorescence above 600 nm, the fluorescence of the lens is further suppressed. No clear discrimination is possible according to excitation or fluorescence spectra for other ocular tissue.

For that reason, the fluorescence lifetime was determined of all ocular tissue in bi-exponential approximation. In histograms of lifetime Tau 1 (Fig. 6), the shortest lifetime was detectable in RPE, neural retina, and vitreous.

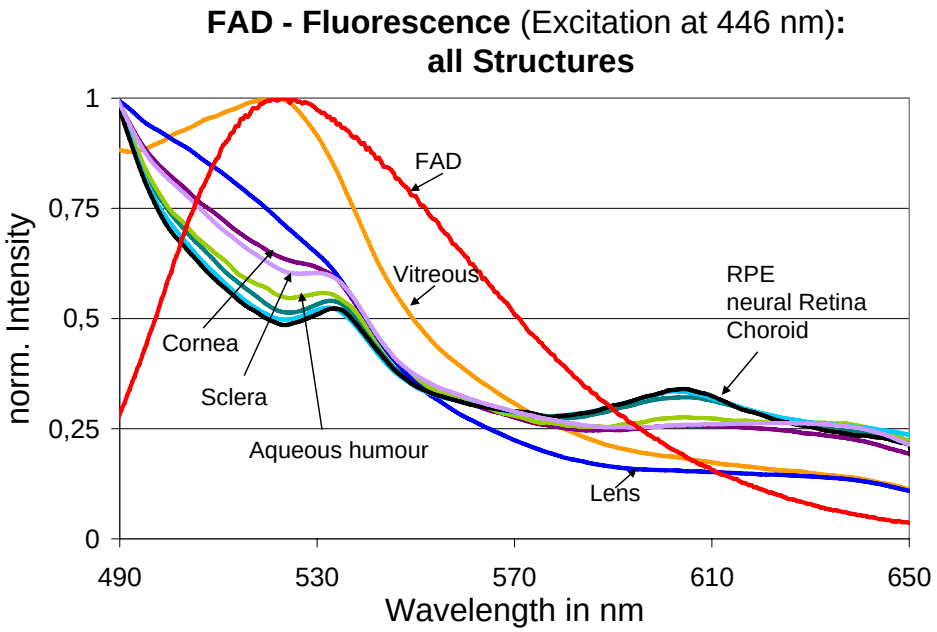

Fig. 4: Detection of FAD in excitation spectra of fundus structures (neural retina, RPE, and choroid), changes of fluorescence detected at 530 nm

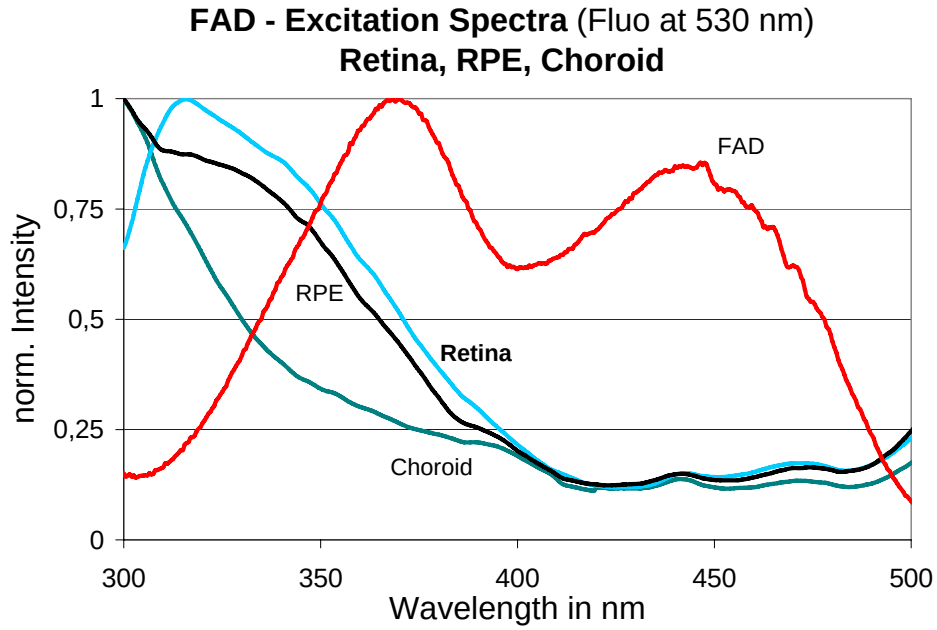

Fig. 5: Detection of FAD in Fluorescence spectra of ocular tissue, excitation at 446 nm

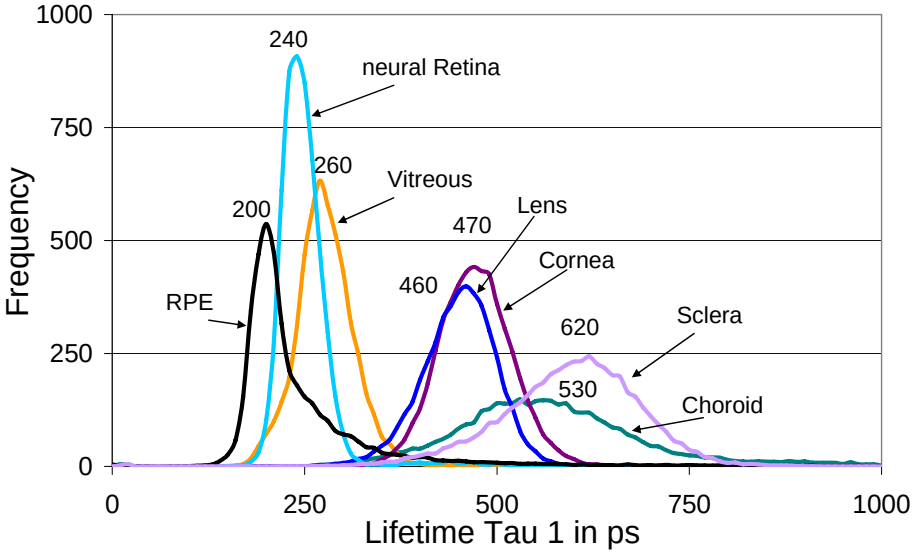

Fig. 6: Histogram of lifetime Tau 1 in ocular tissue by bi-exponential fit, excitation at 446 nm

Nearly same histograms were detected from lens and cornea as well as from sclera and choroid. RPE and neural retina can clearly be discriminated from lens and cornea as well as from sclera and cornea in histograms of lifetime Tau 2 (Fig. 7).

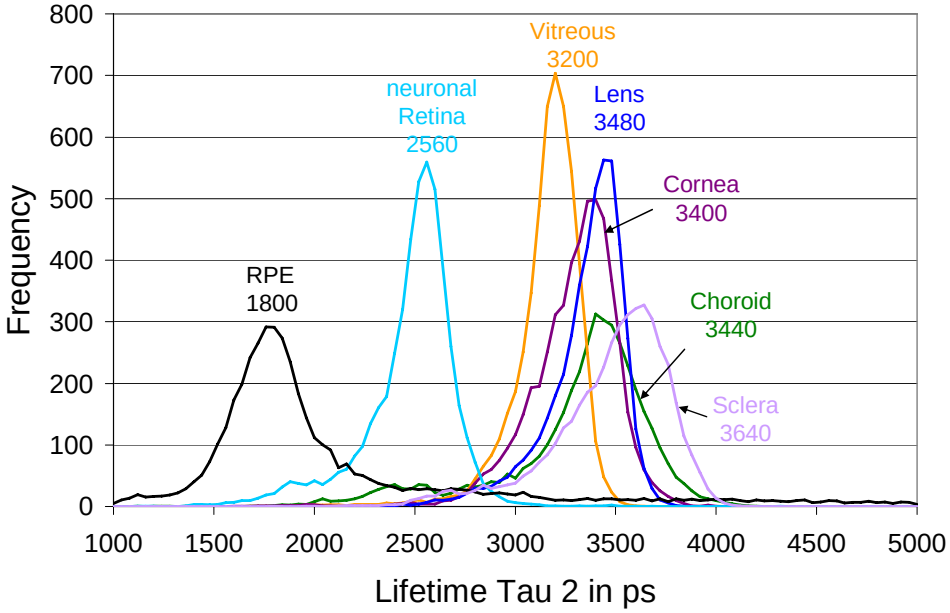

Fig. 7: Histogram of lifetime Tau 2 in ocular tissue by bi-exponential fit, excitation at 446 nm

The best discrimination between RPE and neuronal retina can be realized in histograms of relative contribution Q1 to fluorescence (Fig. 8). Qi is defined according to equation (1):

$$Q_i = \frac{a_i \cdot \tau_i}{\sum_j a_j \cdot \tau_j}$$

The contribution of the short component to the entire fluorescence is 72 % in RPE and 42 % in neural retina. Q1 is nearly equal in the other collagen containing tissue.

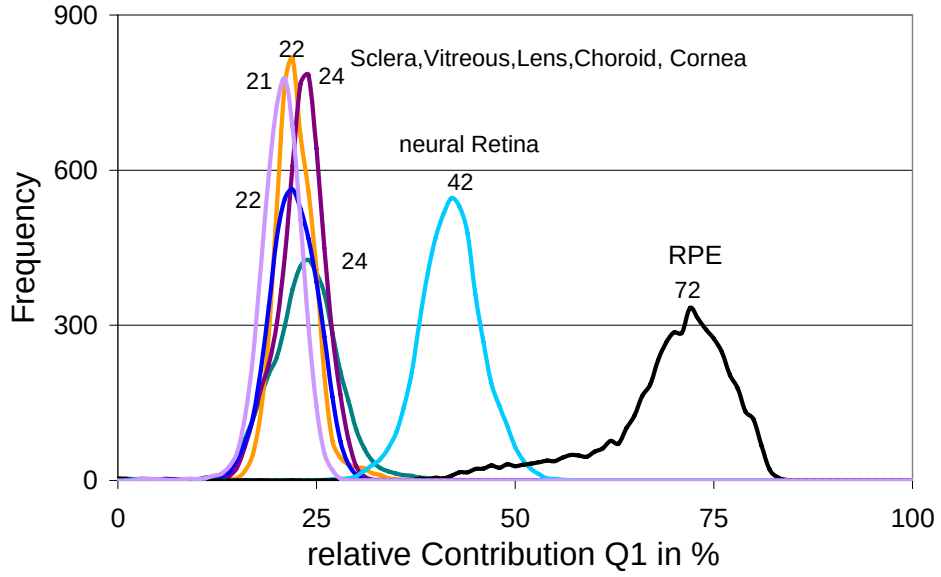

Fig. 8: Histogram of relative contribution Q1 for discrimination between RPE and neural retina

As pairs of Tau 1 and Tau 2 were determined for each pixel in the investigated tissue sample, such lifetime clusters can be drawn for each kind of tissue in a diagram. As shown in Fig. 9, the clusters of RPE and neural retina are located at the shortest values of Tau 1 and Tau 2.
The lifetime cluster of the other tissues move to longer lifetimes Tau 1 and Tau 2, ending with the lifetime cluster of sclera and choroid. The lifetime histograms in Fig. 6 and 7 can be considered as projection of these clusters at the Tau 1 or Tau 2 axis.

According this cluster diagram, a certain interpretation is possible of cluster diagrams of the living human fundus. As shown in Fig. 10, most data points are located at short lifetimes Tau 1 and Tau 2, but some points are located at longer lifetimes Tau 2 and covering a large range of lifetime Tau 1. In comparison with the anatomical structure of the eye ground, the fluorescence of the most pixels is determined by the short lifetime Tau 1 and Tau 2 in RPE and neural retina. The small number of pixels at longer lifetimes Tau 1 and Tau 2 originate from the optic disc. From this it can be concluded, that connective tissue, especially collagen, is the dominating fluorophore in the optic disc.

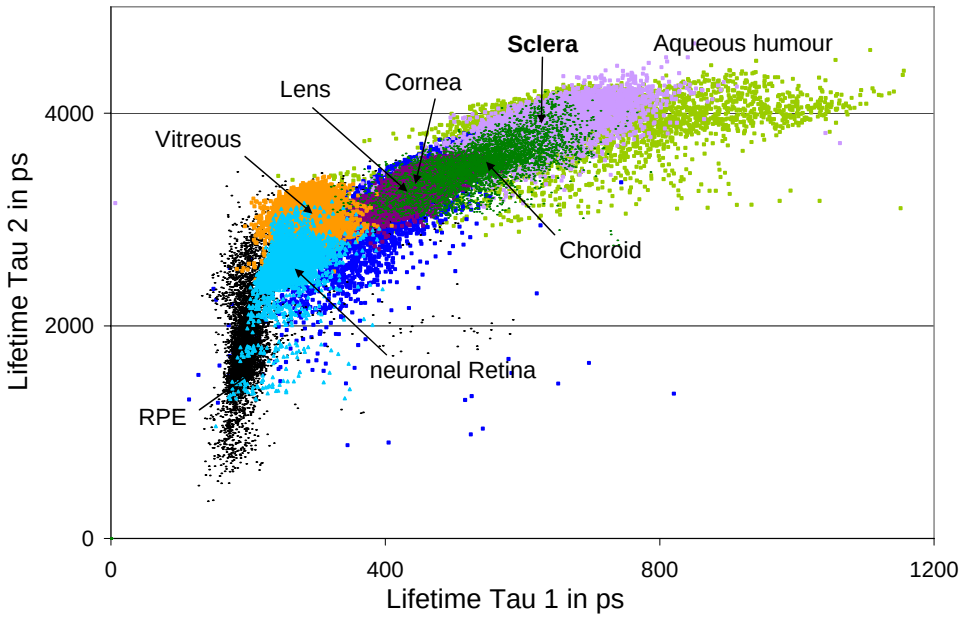

Fig. 9: Tau 1-Tau 2 Lifetime clusters of ocular tissue. The arrows point at the centres of each substance specific distribution.

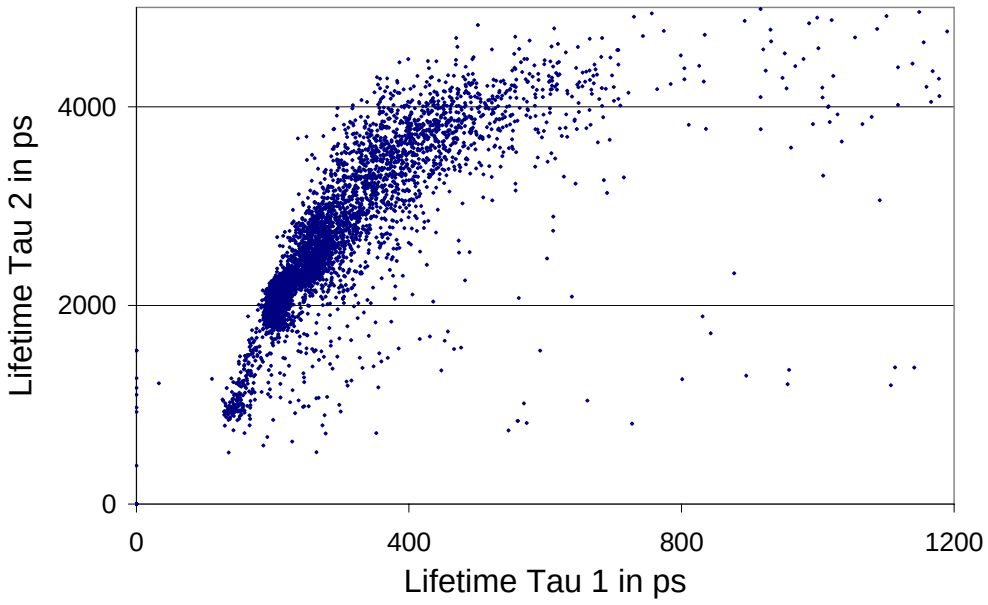

Fig. 10: Cluster of Lifetimes T2 – T1 of a healthy person (20 grd fluorescence image with optic disc)

The influence of fluorescence of the lens can also be studied in lifetime cluster diagrams. Switching to larger field stops in front of the confocal detector in the laser scanning system increases the contribution of lens

fluorescence. In this case, the short lifetimes of RPE and neural retina are covered by the longer lifetime of the lens. In vivo measured lifetime clusters are now wide distributed and moved to longer lifetimes.

Cluster diagrams of amplitudes a2 versus a1 permit a further discrimination of ocular structures. As shown in Fig. 11, there is a correlation between a2 and a1 for each kind of tissue. The gradient of each trend line is specific for each ocular structure. It is flat for RPE and steepest for aqueous humour.

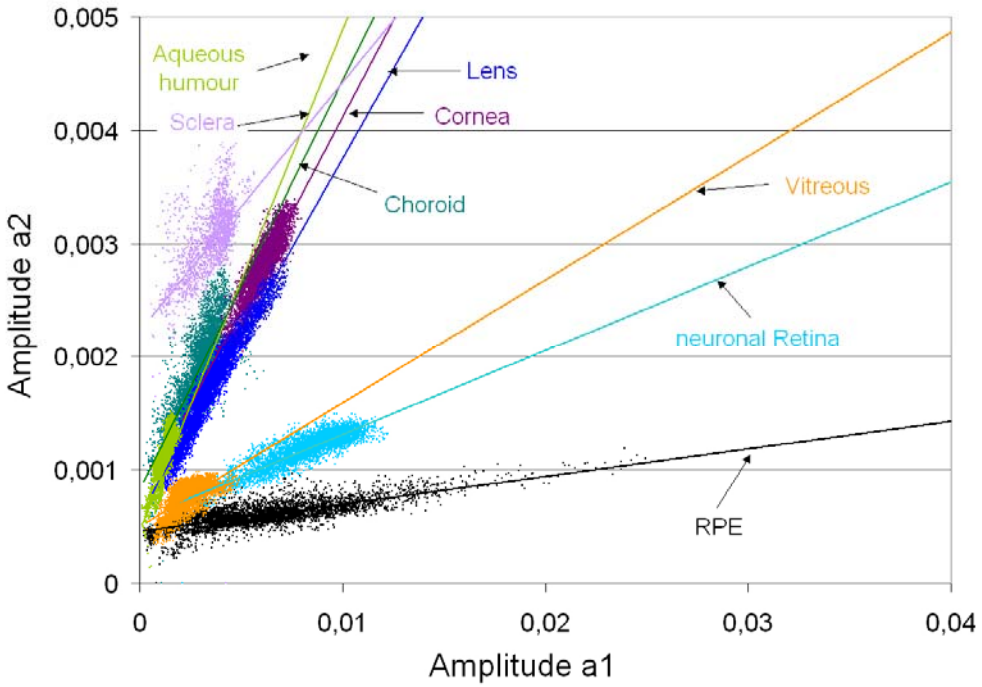

Fig. 11: Cluster of amplitudes: Trend lines for discrimination of ocular tissue

A certain relation exists between lifetime in tri-exponential approximation of dynamic fluorescence and anatomical structure of the eye ground. Let us consider the dynamic fluorescence which is measured in two spectral ranges. As demonstrated[3] , the fluorescence intensity of several substances is comparable in the short - wave spectral range (510 nm – 560 nm). In contrast, the fluorescence in the long - wave range (560 nm – 700 nm) is dominated by lipofuscin in RPE. In this way, we get two different sets of lifetime images from the same subject. In the human eye, there are locations, having specific anatomical structure. In the optic disc, there is no RPE and in the macula, there is no nerve fibre layer, but only photo – receptors. In the extended range outside of optic disc and macula, the anatomical structure consists of the nerve fibre layer, receptors, RPE, Bruch´s membrane, choroid, and sclera. In a section through the optic disc in the lifetime image, the amplitude a1 is decreased down to zero. That means, the short component Tau 1 might originate from RPE (Fig. 12).

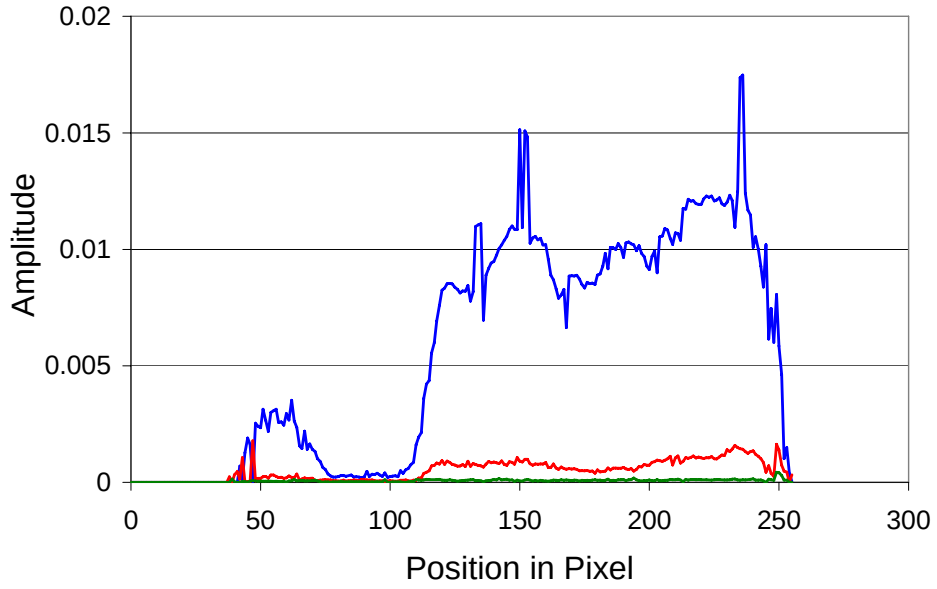

Fig. 12: Profile of amplitudes in a section through the optic disc (tri – exponential fit, short – wave channel)

In the optical section through the macula in the lifetime image of the short – wave detection channel, the lifetime Tau 2 decreases to zero exactly in the range where no nerve fibre layer anatomically consists. For that reason, the lifetime Tau 2 in tri – exponential fitting might correspond to the nerve layer (Fig. 13). Equal values of lifetime in macula and also in optic disc mean, that a bi – exponential fit is sufficient.

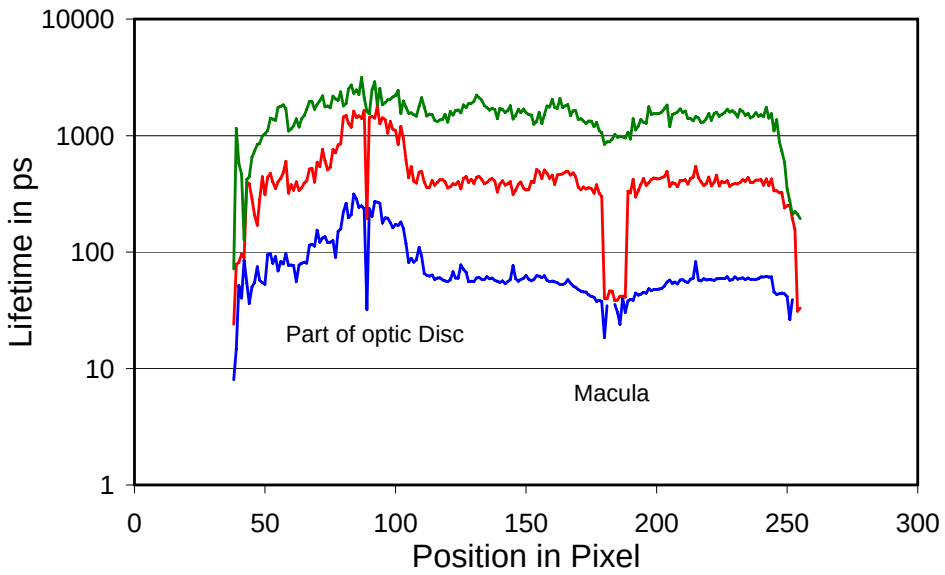

Fig. 13: Profile of lifetimes in a section through the macula (tri – exponential fit, short – wave channel)

In optical sections of lifetime images in the long –wave emitting range, the amplitude a1 also goes down to zero in the optic disc, but in the macular section the Lifetime Tau 2 is not altered in the macula (Fig. 14). As a tri – exponential fitting results in better reduced Chi2 than a bi – exponential model function, the correspondence between liftimes Tau i and anatomical structures is probably different in the considered spectral ranges.

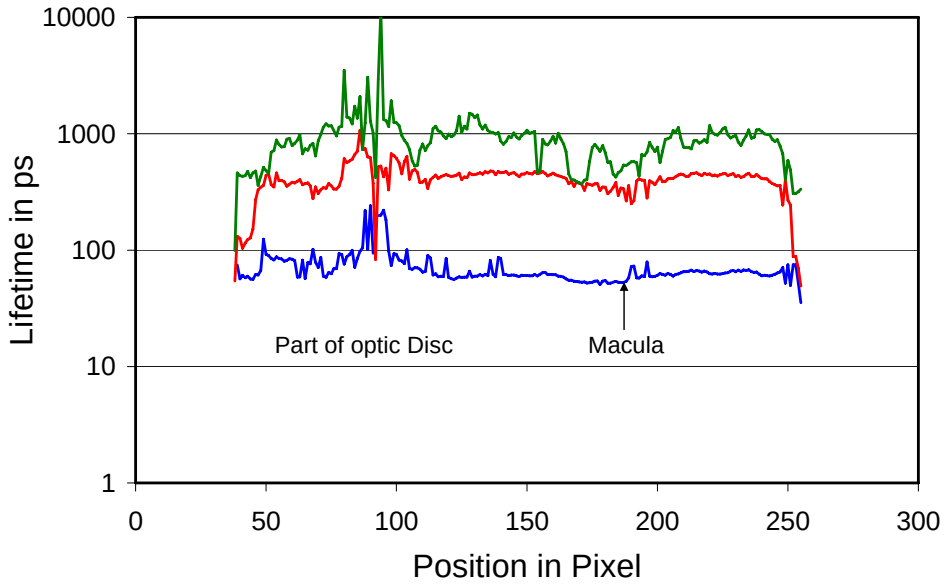

Fig. 14: Profile of lifetimes in a section through the macula (tri – exponential fit, short – wave channel)

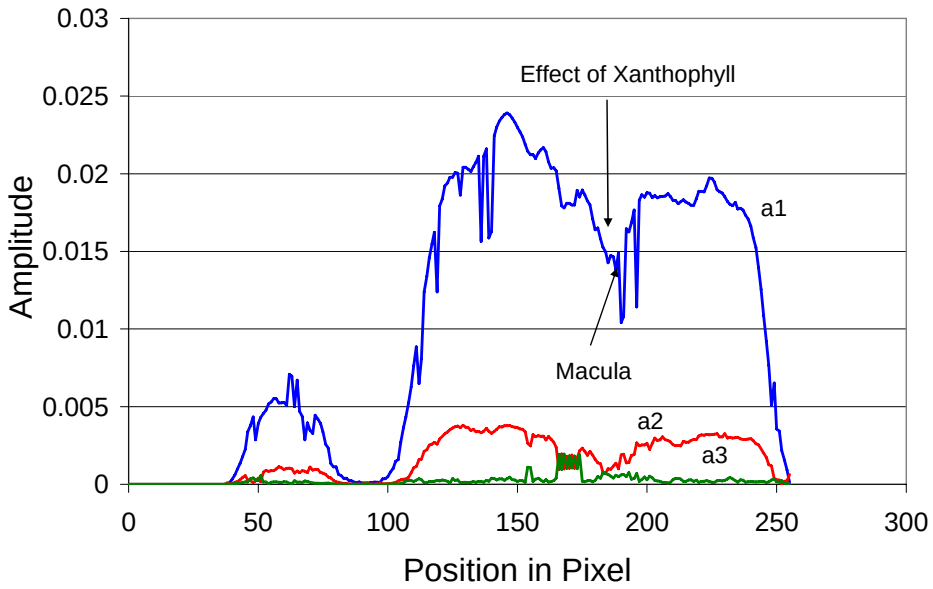

Fig. 15: Profile of amplitudes in a section through the macula (tri – exponential fit, long – wave channel)

The fluorescence intensity, expressed as amplitude, is influenced by the absorption of excitation or emission light by substances located in front of the fluorophore under investigation.. The dominating fluorophore lipofuscin in the long – wave emission range is located in retinal pigment epithelium. As consequence, the amplitude a1 is reduced by the absorption of xanthophyll in the macula. Very low values of a1 are detected when the optical section crosses retinal vessels and no retinal pigment epithelium can be excited (Fig. 15).

3.CONCLUSIONS

Fluorescence lifetime measurements, combined with selective excitation wavelengths and detection is at least two different spectral ranges, can result in information on metabolic processes. As the autofluorescence is always a sum signal, originating from different endogenous fluorophores, a careful interpretation is required. Comparison with expected purified substances and isolated ocular structures of porcine eyes result in information for optimising the developed lifetime ophthalmoscope. Comparison of retinal pigment epithelium between porcine and human eyes results in large difference in the content of lipofuscin, which is dominating in older human eyes. For interpretation of spectral changes in ageing eyes light exposure studies were performed on cultures of retinal pigment epithelium[6]. In clinical evaluation of lifetime measurements, a comparison is essential with results of known functional and morphological diagnostic methods.

REFERENCES

[1] Schweitzer D, Lasch A, van der Vorst S et al. „Änderung der retinalen Sauerstoffsättigung bei Gesunden und in frühen Stadien der diabetischen Retinopathie nach Atmung von 100% Sauerstoff“ Klein Monatsbl Augenheilkd 224, 402-410, 2007-07-19

[2] Chance B „Pyridine nucleotide as an indicator of the oxygen requirement for enrgy-linked function of mitochondria“ Circ Res 38 (5 Suppl 1), I31-I38, 1976

[3] Schweitzer D, Schenke S, Hammer M et al. „Towards metabolic Mapping of the Human Retina“ Microsc. Res. Tech. 70, 410 - 419, 2007

[4] Schweitzer D, Hammer M, Schweitzer F et al. „In vivo measurement of time-resolved autofluorescence at the human fundus“ J Biomed Opt 9, 1214-1222, 2004

[5] Schweitzer D, Hammer M, Schweitzer F „Grenzen der konfokalen laser Scanner Technik bei Messungen der zeitaufgelöstenAutofluoreszenz am Augenhintergrund“ Biomed Tech 50, 263-267, 2005

[6] Hammer M, Richter S, Guehrs K H and Schweitzer D. „Retinal pigment epithelium cell damage by A2-E and its photo-derivatives” Mol Vis. 12, 1348-1354, 2006