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In vivo autofluorescence lifetime imaging at the fundus of the human eye

Dietrich Schweitzer, Martin Hammer, Frank Schweitzer, Stefan Schenke
Experimental Ophthalmology, University of Jena, Bachstr. 18, D-07743 Germany
e-mail:Dietrich.Schweitzer@Med.Uni-Jena.DE

Eckhard Birckner
Physical Chemistry, University of Jena, Lessingstr.10, D-07743 Germany
Eckhard.Birckner@uni-jena.de

Wolfgang Becker, Axel Bergmann
Becker & Hickl GmbH, Nahmitzer Damm 30, 12277 Berlin, Germany,
becker@becker-hickl.com, bergmann@becker-hickl.com

ABSTRACT

Changes in cellular metabolism are assumed as first sign in fundus diseases, e.g. in age-related macular degeneration. Measurements of autofluorescence have the potential to detect such alterations. The required discrimination of fluorophores can not be reached in fundus images with a high lateral resolution, investigating emission spectra under different excitation. Time correlated single photon counting is optimal for determination of substance – specific lifetime under conditions of extremely weak fluorescence intensity. Analysing spectral properties of expected fundus substances, an increased sensitivity can be reached by combination of selected excitation or emission with lifetime measurement. As demonstrated in lifetime histograms of 40° fundus images, several fluorophores are excited by 446 nm, but predominantly lipofuscin by 468 nm excitation. Detection of lifetime in two emission ranges 500 nm to 560 nm and 560 nm to 700 nm improves further the discrimination of fluorophores.

Keywords: Autofluorescence, fluorescence lifetime, time correlated single photon counting, FAD, AGE, A2E, eye, emission spectrum, fluorescence spectrum

1. INTRODUCTION

Metabolic alterations are assumed as first step in several diseases. Such changes might be reversible, before new anatomical structures are formed. For investigation of the fundus, methods are requires which enables the detection of metabolic parameters with a high 2-dimensional resolution. Measurements of oxygen saturation and of blood flow in retinal vessels result in supply and in consumption of oxygen. On the other hand, multifocal ERG is an objective diagnostic method of receptor function. The tissue autofluorescence contains information about metabolism at cellular level. Also in the fundus tissue, several fluorophores can be expected like age-pigment lipofuscin, advanced glycation endproducts (AGE), connective tissue (collagens, elastin). Of special interest is the fluorescence of redox pairs NADH-NAD, and $FADH_2$-FAD. In the first pair, the reduced form NADH of nicodinamidedinucleotide exhibits a high fluorescence in case of lack of dissolved oxygen and the oxidised form NAD is non-fluorescent. In the second redox pair, the oxidised form FAD of flavindinucleotide fluoresce in case of sufficient availability of oxygen, like other flavins, and the reduced form is non-fluorescent under room conditions. This different behaviour can be used for studies of metabolism in mitochondria. In addition to the fundus fluorescence, in the lens there are also strong emitting fluorophores e.g. tryptophan.
The detection of fundus autofluorescence using a laser scanning ophthalmoscope[1] is a first step for investigation of local differences in fluorescence intensity, which is successfully applied in investigation of progress in age-

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related macular degeneration. In basic investigations[2] it was shown, that the fundus fluorescence is dominated by lipofuscin.

2. EXCITATION AND FLUORESCENCE SPECTRA OF FLUOROPHORES, EXPECTED AT THE FUNDUS

For more detailed studies, a discrimination of fundus fluorophores is required in fundus images with a high lateral resolution. In principle, fluorophores can be distinguished by the specific parameters excitation spectrum, emission spectrum , and fluorescence lifetime in the excited stage.
Under condition of the living eye, the transmission of ocular media enables spectral investigation of the fundus only in the range between 400 nm and 900 nm[3,4]. As the different excitation maxima of most intrinsic fluorophores are lower than 400 nm, a clear discrimination of fluorophores is not possible, based on differences in excitation spectra.
The dominating fluorophore at the fundus is lipofuscin, consisting of 10 components[5]. The component 8 was identified as A2E and can be synthesised[6]. Fig. 1 shows the excitation spectrum of A2E, when the fluorescence was detected at 660 nm and the emission spectrum for excitation at 446 nm. This figure contains the transmission of the ocular media, calculated for a 20 year old person[4]. The transmission of the ocular media decreases with age.

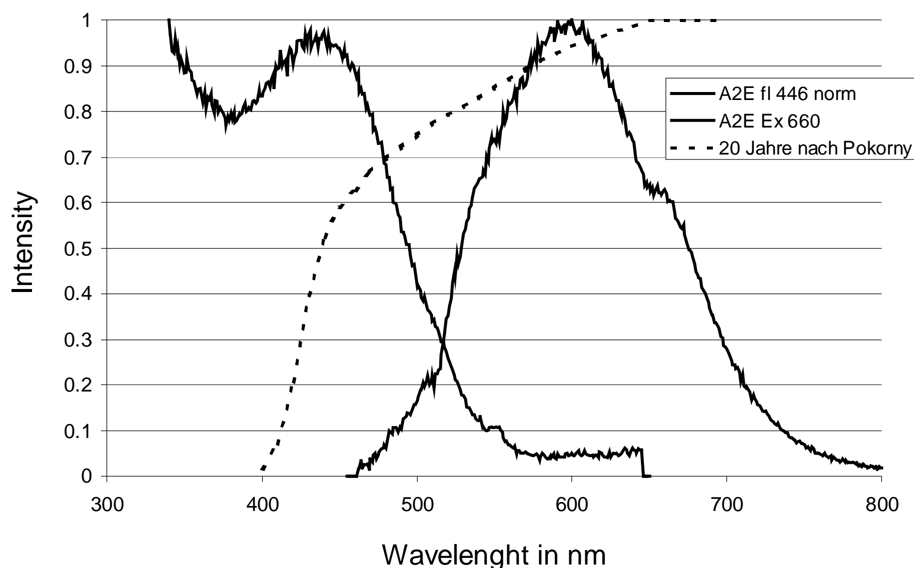

Figure 1: Excitation – and fluorescence spectrum of A2E as well as spectral transmission of ocular media of a 20 year old subject

Another substance, which might be excited at the living fundus, is the coenzyme FAD, which is important for evaluation of metabolic state in mitochondria. As shown in Fig. 2, there are 2 excitation maxima around 370 nm and 446 nm, when the fluorescence was measured at 524 nm. An excitation at 350 nm results in a two-humped fluorescence spectrum having maxima around 440 nm and 519 nm. Excitation at 446 nm or 470 nm results in same fluorescence spectrum with maximum at 524 nm.

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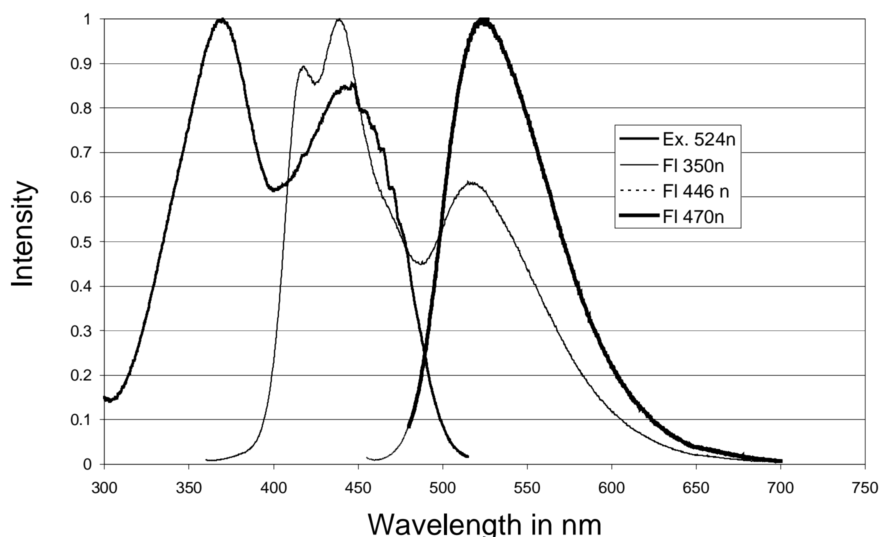

Fig. 2: Excitation and fluorescence spectra of FAD

There is also a chance for excitation of AGEs at the fundus. Excitation and fluorescence spectra of AGE, which is a mixture of several fluorophores, are given in Fig. 3.

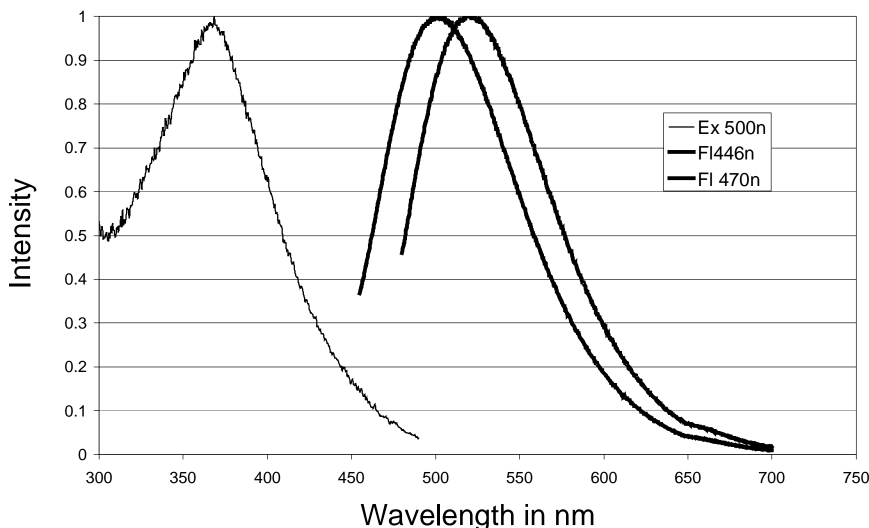

Fig. 3: Excitation and fluorescence spectra of AGE

Measuring the fluorescence at 500 nm, the maximum of excitation is at 370 nm. The spectral shape might allow an excitation of AGE also above 400 nm. The shift of fluorescence maximum from 502 nm to 523 nm in excitation at 446 nm and at 470 nm point to the excitation of several fluorophores in AGE.

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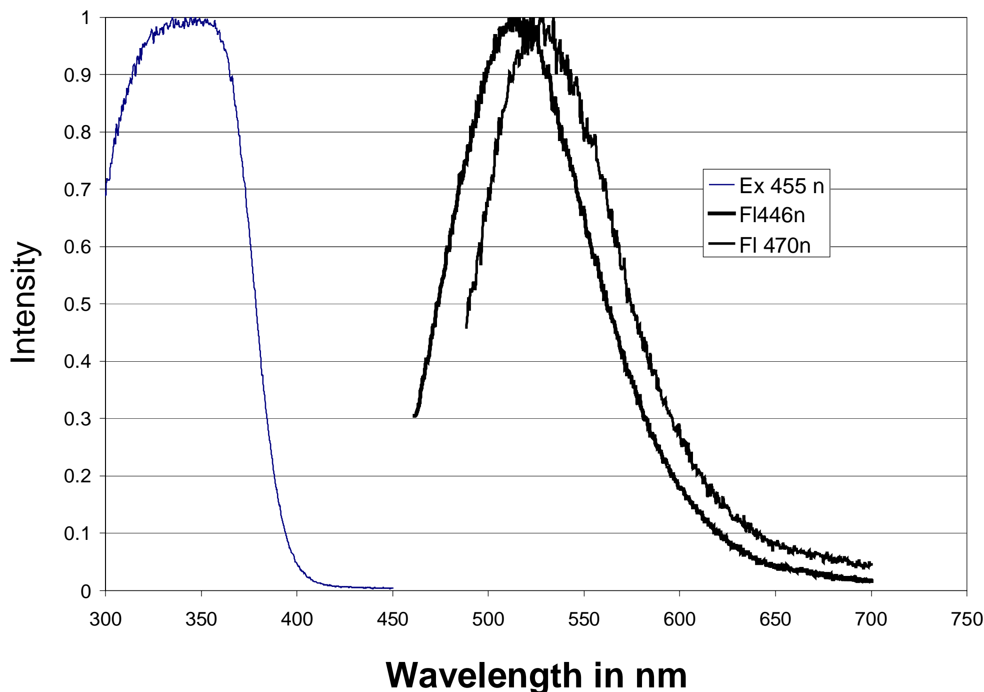

Fig. 4: Excitation and fluorescence spectra of NADH

Study of excitation and fluorescence spectra of NADH results in a broad maximum of excitation at 340 nm. Unfortunately, the excitation for wavelengths > 400 nm is so small, that measurement of NADH is unlikely at the living fundus, but not at the anterior part of the eye. The normalised fluorescence spectrum by 446 nm excitation has its maximum at 514 nm and for 470 nm excitation at 528 nm.

The excitation maximum of connective tissue is below 300 nm, but the shape is quite broad. Especially for collagen 2, an excitation might be possible by wavelengths > 400 nm. The fluorescence maximum changes for excitation at 400 nm, 446 nm and 470 nm from 455 nm, to 507 nm, and 527 nm. There is no large difference in the fluorescence spectra between collagenes and elastin.

Considering the "effective" excitation spectra of A2E, FAD, and AGE, as product of extinction spectra and ocular transmission in Fig. 5, the extinction maxima are located between 440 nm and 450 nm. Starting from about 500 nm to higher wavelengths, only A2E will be excited, whereas other fluorophores are excited additionally by shorter wavelengths. So, a certain discrimination of fundus fluorophores can be realised by changes in the excitation wavelength.

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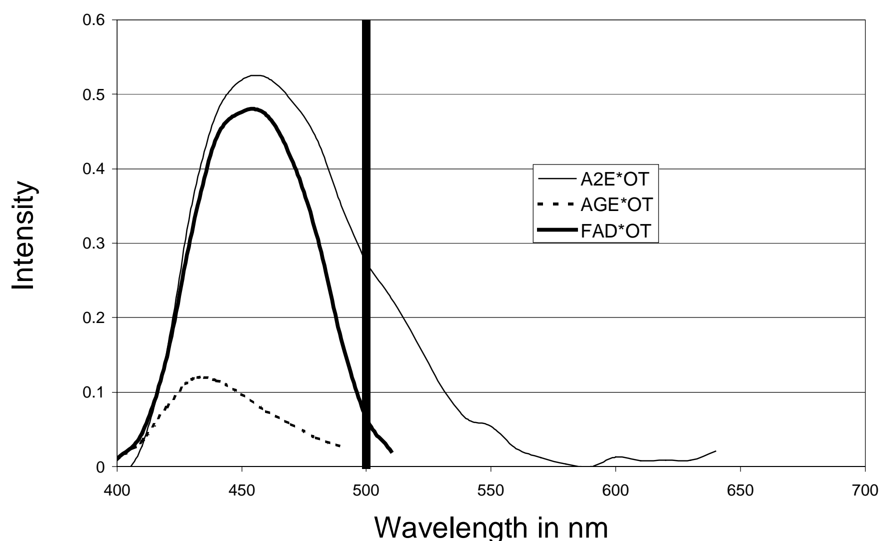

Fig. 5: Extinction spectra of A2E, FAD, and AGE, evaluated by ocular transmission. Predominantly A2E will be excited by wavelengths > 500 nm.

To improve furthermore the discrimination of fluorophores, also the spectral range of fluorescence can be selected. If the fundus fluorescence is excited by 446 nm and the fluorescence will be detected between 500 nm and 700 nm, the fluorescence in the range between 500 nm and 560 nm is be dominated by FAD and AGE and the fluorescence above 560 nm is determined by A2E or other components of lipofuscin.

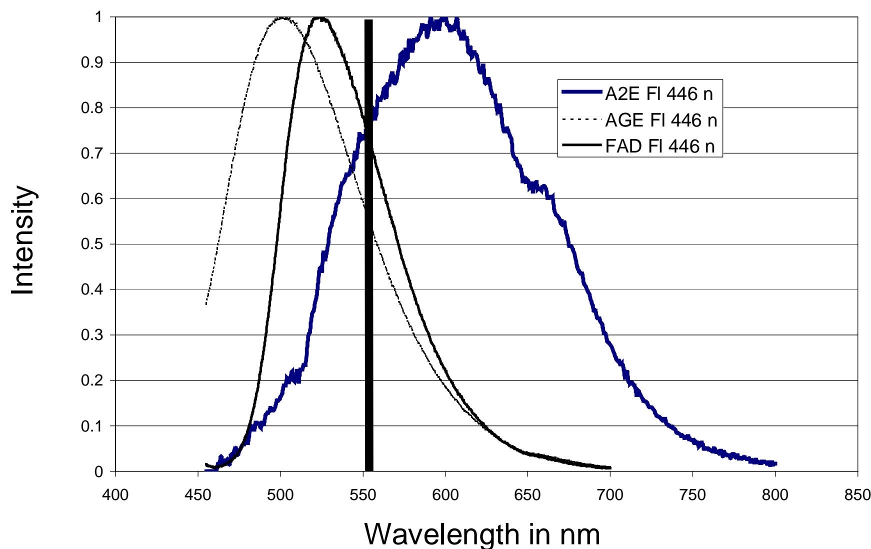

Fig. 6: Selection of emission ranges. The emission lower than 560 nm is dominated by FAD and AGE and for higher wavelengths by A2E.

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3. SET UP FOR IN VIVO FLUORESCENCE LIFETIME MEASUREMENTS

These differences in excitation and emission spectra can be used for optimisation of the experimental set up for fluorescence lifetime imaging (Fig. 7), described in detail in [7]. As shown in Fig. 7, a laser diode (LDH 440 and LDH 470, Picoquant, Berlin, Germany) excites the fundus via a laser scanning ophthalmoscope (cSLO, Carl Zeiss, Oberkochen, Germany) by pulses of 100 ps FWHM and a repetition rate of 40 MHz. The dynamic fluorescence is detected in single photon counting technique using a MCP-PMT (HAM-R3809U-50, Hamamatsu, Herrsching, Germany) in connection with a SPC 730 board (Becker/Hickl, Berlin, Germany).

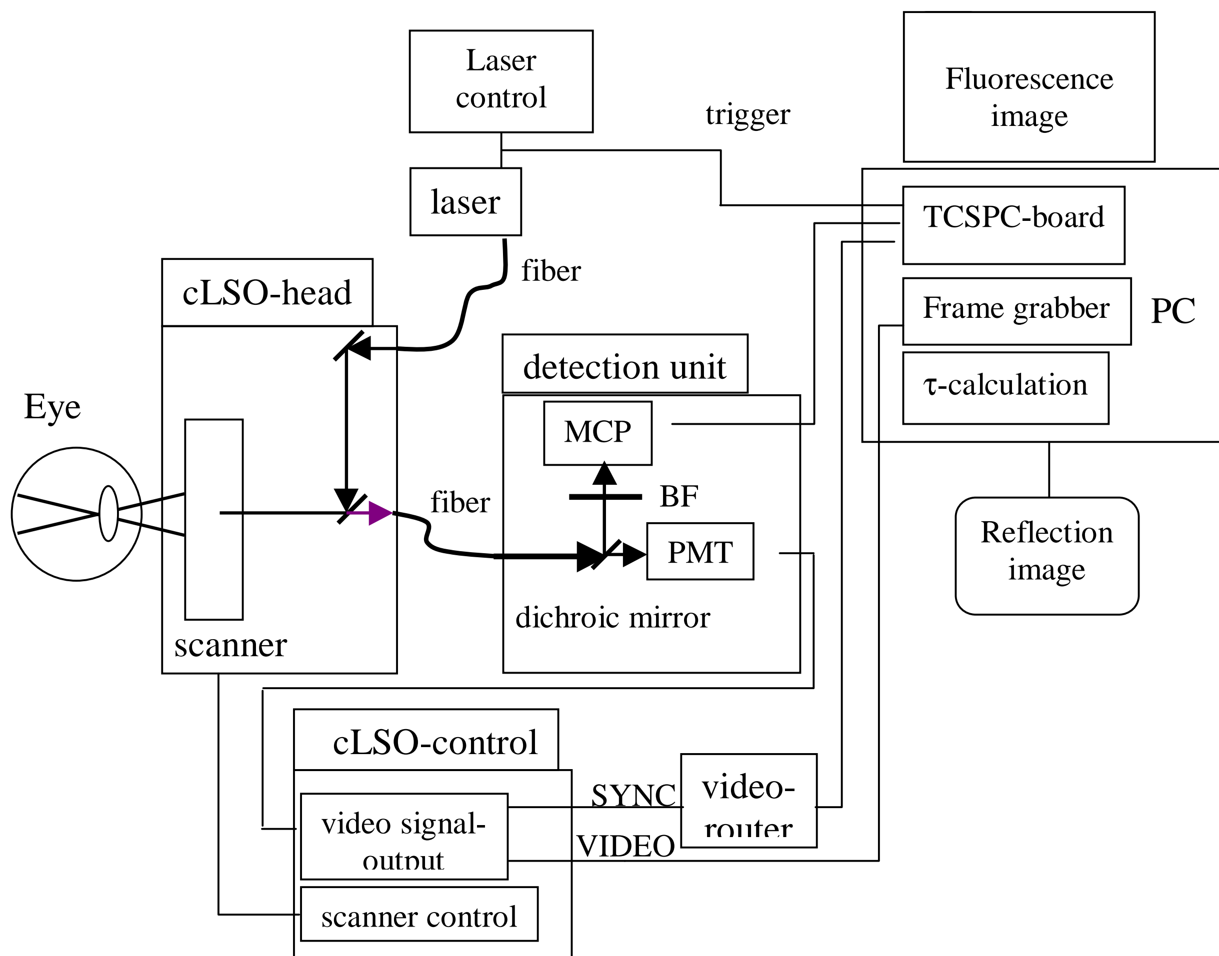

Figure. 7: Experimental set up for fluorescence lifetime measurements of the living human fundus. The dynamic fluorescence is measured in time-correlated single photon counting technigue. The reflected excitation is used as reference image for automatic compensation of eye movements.

The dynamic autofluorescence was measured in 40° fundus images. Simultaneously with the dynamic autofluorescence, fundus images of the reflected excitation light were measured. For accumulation of a sufficient number of photons at each image pixel, series of images are acquired. Any eye movement between the images was automatically registered according to the reflectance images. Fig. 8 is the control menu for the measuring system “EyeScan”. The adjustment of the ophthalmoscope was preformed, controlling the reflectance image on the left side. In the middle, there is the corresponding image of the fluorescence intensity. On the right is a

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diagram, showing the decay of the fluorescence in the measuring interval of e.g. 25 ns. During the automatic affine image registration, all reflectance images are excluded having a blurred contrast.

The evaluation of dynamic fluorescence images was performed using the system SPCImage 2.7 (Becker/Hickl, Berlin, Germany). The decay of normalised fluorescence intensity was approximated tri-exponentially for each fundus pixel (80µm x 80µm).
Fig. 9 is the control menu of SPCImage 2.7. It shows left a fluorescence intensity image after registration of about 60 single images by an automatic affine algorithm (Heidelberg Engineering, Dossenheim, Germany). The excitation wavelength was 468 nm and the blocking filter was at 510 nm (HQ510 LP, LOT, Darmstadt, Germany). In the middle is the lifetime image of the shortest lifetime (Tau 1) according to tri-exponential approximation. Despite macula and optic disc appear dark in fluorescence intensity images, the lifetime Tau 1 is different. The shortest lifetime in the 40° lifetime image of a healthy subject was determined in the macular range (Tau 1 about 150 ps) whereas the longest lifetime Tau 1 was detected in the optic disc (Tau 1 > 250 ps). The most frequent value of Tau 1 was 190 ps. A corresponding histogram of Tau 1 is presented in the right image in the SPCImage menu. According to the selected degree of exponential function, the approximation of the measured dynamic fluorescence of each selected pixel as well as the residuals can be seen in the lower image. In the lower part on the right side, there are the calculated amplitudes and lifetimes. The shift between instrumental response and in vivo measurements is given and also the offset, caused by room light or by any radiation sources (IR diodes) inside the scanning system. The influence of incompletely blocked excitation light can be considered as scatter value.
As result of these calculations, images can be presented of the lifetime Tau1, Tau 2, and Tau 3, of the amplitudes a1, a2, and a3 and of Q1, Q2, and Q3 as relative contribution of the single components. These images allow a visual interpretation of dynamic autofluorescence. Images of reduced chi^2 as criterion for approximation give an impression of quality of the model fit at all fundus sites. Scattering images can be used as additional information in interpretation of lifetime images.

A more objective interpretation can be reached in diagrams of Tau 3, Tau 2 as function of Tau 1[7, 8]. The lifetime cluster of isolated fluorophores, of ocular tissue or of selected ranges of the fundus can be drawn in such diagrams, allowing to a certain degree a substance specific interpretation of in vivo lifetime measurements.

4. FUNDUS FLUORESCENCE LIFETIME AT DIFFERENT EXCITATION

The effect of different excitation at 446 nm and at 468 nm was demonstrated in diagrams, showing how often Tau 1, Tau 2, and Tau 3 were calculated in fundus images of healthy subjects. Left in fig. 10a is the histogram of Tau 1 when the fundus is excited by 446 nm, and in Fig. 10b the fundus is excited by 468 nm.

Corresponding histograms of Tau 2 are shown in Fig. 11a, b, and for Tau 3 in Fig 12 a, b.

Especially in Tau 1 histograms, having different values of Tau 1, it is clearly seen, that several fluorophores are excited by 446 nm laser wavelength. In contrast, excitation by 468 nm laser light results in a dominating single lifetime Tau 1 = 190 ps. This result is in correspondence with the interpretation of the calculated “effective” excitation spectra in Fig. 5.

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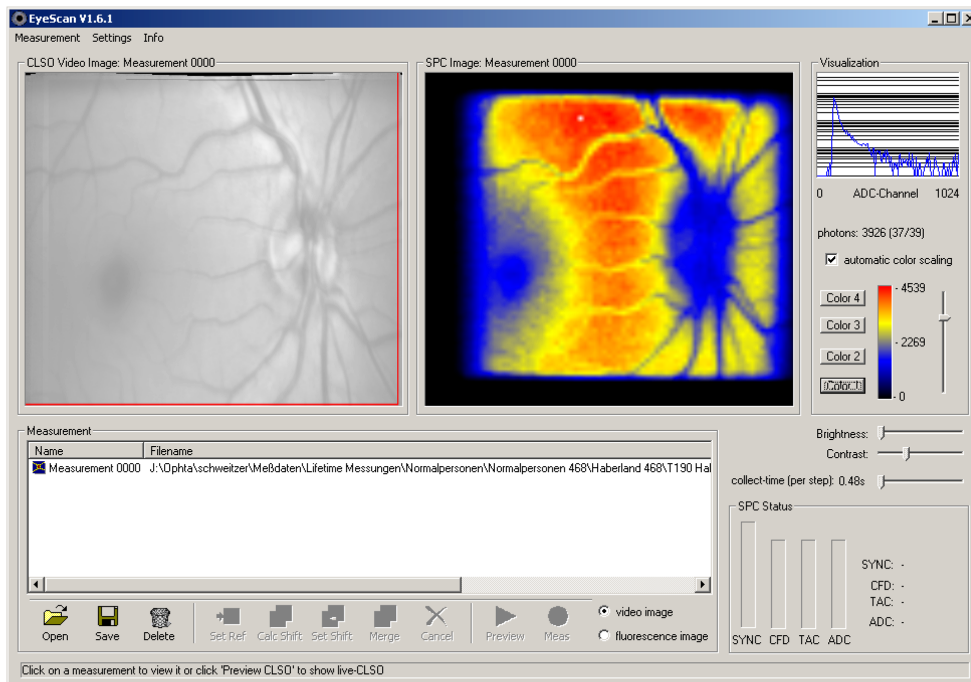

Figure 8: Control menu "EyeScan" of the Lifetime Mapper. Left – registered reflectance image, middle – image of fluorescence intensity, right – decay of fluorescence intensity at a single point, range 0 - 25 ns

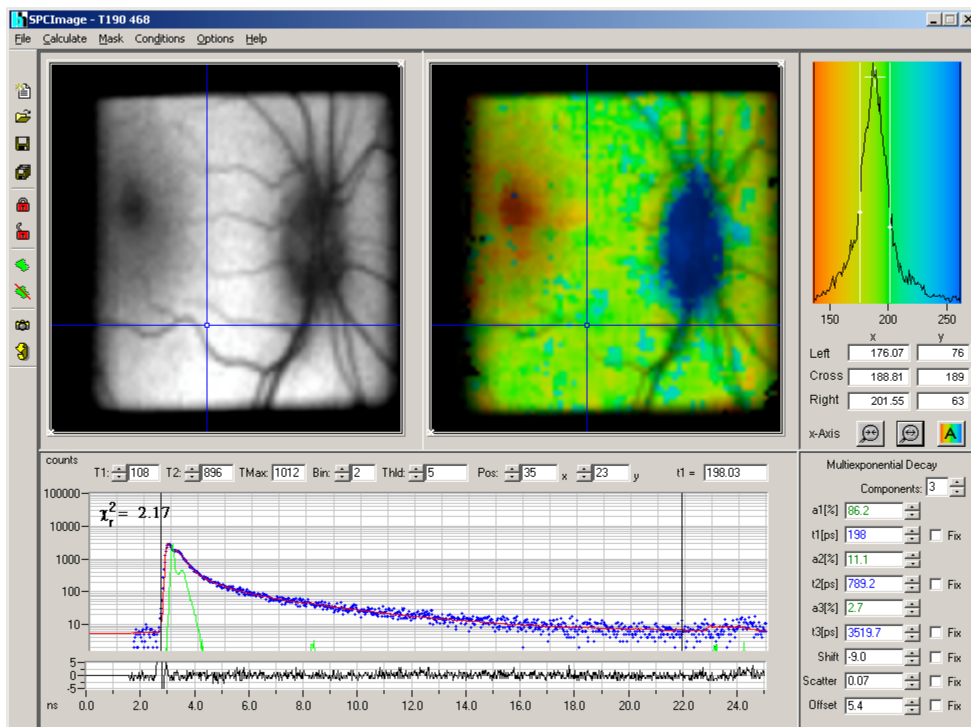

Figure 9: SPCImage – menu for calculation of lifetime images. Left – image of fluorescence intensity, middle- Image of lifetime Tau 1, right – histogram of Tau 1, below : left- measured photon distribution, instrumental response and Model function, right- amplitudes and lifetimes of the selected pixel

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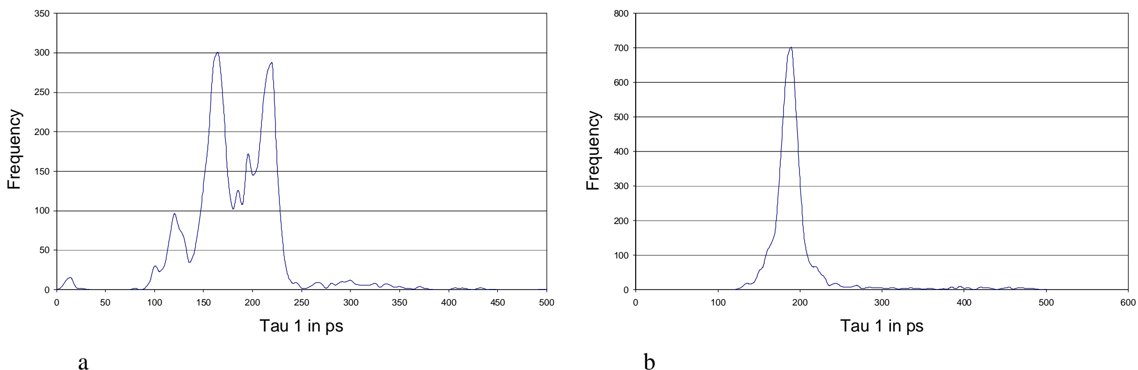
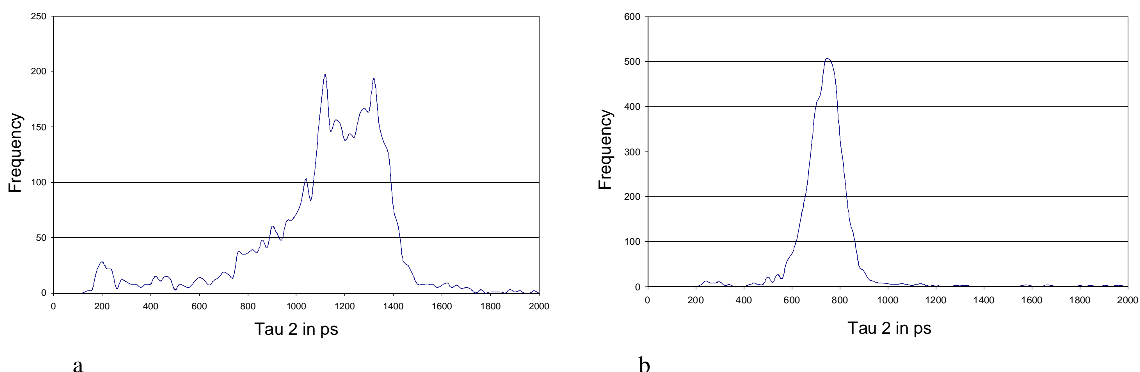


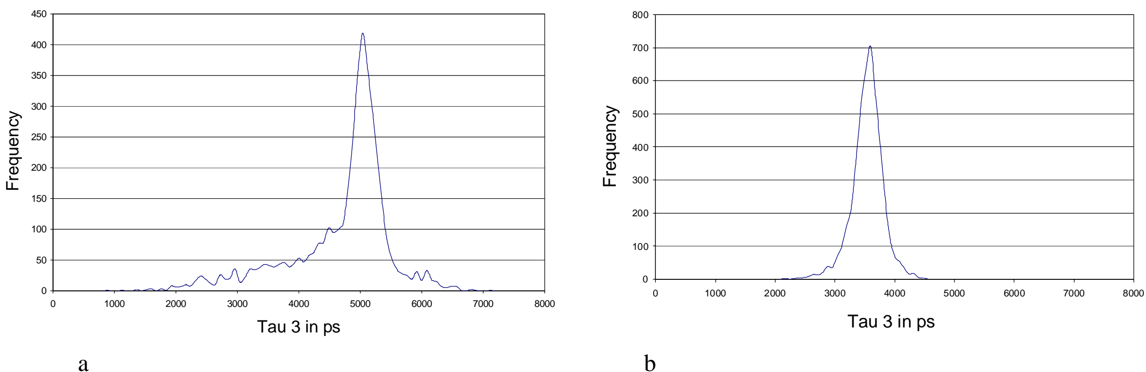
a b

Figure 10: Histogram of fluorescence lifetime Tau 1 in 40° fundus images of healthy subjects, a - excitation 446 nm, b- excitation 468 nm



a b

Figure 11: Histogram of fluorescence lifetime Tau 2 in 40 degree fundus images of healthy subjects, a - excitation 446 nm, b - excitation 468 nm



a b

Figure 12: Histogram of fluorescence lifetime Tau 3 in 40 degree fundus images of healthy subjects, a - excitation 446 nm, b - excitation 468 nm

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5. DISCUSSION

Discovering metabolic alteration in early age-related macular degeneration, detection of changes in relative contribution of fluorophores or of appearance of new substances are first helpful steps. Under the limiting conditions of the living human fundus (transmission of ocular media, maximal permissible exposure, measuring time), selection of excitation range or detection of fluorescence spectra does not result in a discrimination of fluorophores in fundus images. The measurement of dynamic fluorescence and calculation of fluorescence lifetime images exhibit information about substance specific lifetime. The detected fundus fluorescence is a sum of contribution of several fluorophores. Commonly, a direct correspondence can not be assumed between exponents of a model function and different fluorophores. Considering optical sections in fluorescence lifetime images, a certain correspondence exists between anatomical fundus layers and exponents of the model function[8]. The lifetime Tau 1 with the amplitude a1 of more than 90%, corresponds with the local distribution of the retinal pigment epithelium. A correspondence might also be possible between Tau 2 with a2 of about 10% and the neural retina. Furthermore, different values of Tau 3 in healthy subjects and patients wearing intraocular lenses point to the influence of lens fluorescence on calculation of the longest lifetime Tau 3. A solution for excluding this influence is the combination of the laser scanner principle with the principle of aperture division[9].

In this article, the excitation and fluorescence spectra of substances are investigated, expected at the fundus. Despite a clear discrimination of fundus fluorophores is not possible by selective excitation, changes in the wavelength result in different relative excitation of fluorophores. In experiments, excitation by 446 nm and 468 nm result in different histograms of Tau 1, Tau 2, and Tau 3 already in healthy subjects. Whereas an excitation by 446 nm results in several maxima of Tau 1 in the lifetime histogram and in a broad maximum of Tau 2, the excitation by 468 nm results in a single maximum of Tau 1 and a small maximum of Tau 2. According to “effective” excitation spectra, A2E or other components of lipofuscin are predominantly excited by 468 nm. A further improvement for selection of fundus fluorophores can be reached, measuring the dynamic fluorescence simultaneously in a shortwave range between 500 nm and 560 nm with a strong contribution of shortwave emitting fluorophores (FAD, AGE) and in a longwave range between 560 nm and 700 nm. In this case also an improved determination of lifetimes can be reached applying global fitting[11].

In relation to measurements of fluorescence spectra, lifetime measurements have different advantages. Lifetime measurements require only very low excitation intensity and can be performed 2-dimensionally with a sufficient lateral resolution. Fluorescence spectra are influenced by the absorption of layers in front of the emitting fluorophore. The lifetime is not changed by such absorbing layers. Especially, in investigating the macula, xanthophylls alters the fluorescence spectrum of the pigment epithelium. So, early macular changes in metabolism can hardly be determined by measurement of fluorescence spectrum. In intensity measurements, the influence of a weak emitting fluorophore is covered by a fluorophore with a strong emission. In contrast, lifetime measurements enables the discrimination of weak and of strong fluorophores, if the lifetimes are sufficient different. The fluorescence lifetime is not influenced by absorption of layers in front of the emitting fluorophore. As the fluorescence lifetime depends on parameters of the embedding matrix (pH, viscosity) an evaluation of the cellular milieu might be possible.

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