Autofluorescence lifetime measurements in images of the human ocular fundus

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ABSTRACT

Measurements of the autofluorescence at the fundus prove to be an important tool in early diagnosis and in discovering the pathomechanism, e. g., in age-related macular degeneration. In addition to the action of lipofuscin in the ageing process, flavines play an important role as prosthetic groups. As metabolic changes occur at cellular level, patient–specific optimized therapy should be possible according to endogenous fluorophores, before morphological alterations are manifest. As a first tool for the detection of dynamic autofluorescence, a laser scanner ophthalmoscope will be presented permitting lifetime measurements at the living human eye-ground under extremely weak detectable light.
Considering histograms of lifetimes after excitation at 457.8 nm and determined at the living human eye ground in parapapillary region, a lifetime $\tau \cong 1.38$ ns was calculated most frequently in the long-wave emission range (λ>550 nm). This points to the main contribution of lipofuscin. If the emission range is extended down to 515 nm, components with longer lifetimes are additionally detectable. Lifetime measurements at a human fundus specimen confirmed the lifetime of 1.38 ns in lipofuscin-rich pigment epithelium, whereas the mean lifetime of an intact fundus was 2.04 ns. A comparison of lifetimes before, during, and after breathing of 100% oxygen results in a quenching of the mean lifetime of 0.15 ns by oxygen.

Keywords: fluorescence, lifetime, mapping, human eye, laser scanning ophthalmoscope, time correlated single photon counting, lipofuscin, metabolism

1. INTRODUCTION:

Measurements of the autofluorescence at the fundus prove to be an important tool in early detection and in the discovering the pathomechanism in age-related macular degeneration. Investigations of Delori[1] and of von Rückmann[2] point to components of lipofuscin as dominant fluorophores. In addition to the action of lipofuscin in the ageing process, flavines play an important role as prosthetic groups in both the citric acid cycle and complex 1 and 2 in the respiratory chain[3]. As metabolic changes at the cellular level occur in diabetic retinopathy, a patient–specific optimized therapy should be possible according to fluorescence measurements. A trial for the detection of the autofluorescence of flavins was given by Teich[4].
In principle, different fluorophores can be distinguished according to excitation properties, emission spectra or by the lifetime of fluorescence after short time excitation.
These spectroscopic properties can be illustrated in the Jablonski energy-level diagram[5]. As depicted in Fig. 1, S_o, S_1, and S_2 describe ground, first, and second electronic states. Fluorophores can exist at each electronic energy level in vibrational energy levels (0,1,2). Electrons in the state S_o can reach the states S_1 or S_2 by absorption. According to the Boltzmann distribution, describing the relative number of molecules in the 0 and 1 vibrational state, most molecules will be in the lowest vibrational state at room temperature. After absorption of a sufficient large energy of photons λ, a molecule can reach the electronically excited stages S_1 or S_2. Molecules relax to the lowest vibrational level of S_1 within 10^{-12}s by internal conversion. The fluorescence emission from the lowest vibrational state of S_1 to the electronic ground state occurs within about 10^{-8}s. According to Lakowicz[5], an absorption spectrum reflects vibrational levels of the electronically excited state, whereas an emission spectrum reflects the vibrational levels of the electronic ground state. Absorption spectroscopy is not sensitive to molecular dynamics. In contrast, fluorescence spectroscopy is sensitive to all processes occurring during the lifetime in the excited state. In this way, influences of solvents, the viscosity of the embedding matrix or collision with quenchers can be measured.
Molecules in the first singlet state can undergo conversion to the first triplet state T_1 by intersystem crossing. As the spin of electrons is parallel in this state and in the ground state S_0, the transition from the first triplet state back to the ground state is forbidden. Consequently, the rate of such emission, called phosphorescence, is some orders smaller than those of fluorescence. Phosphorescence lifetimes are longer than 10^{-3}s. Quenching of phosphorescence of external fluorophores is used for measuring partial pressure of oxygen in living tissue, however, the application in humans is not permitted until now.

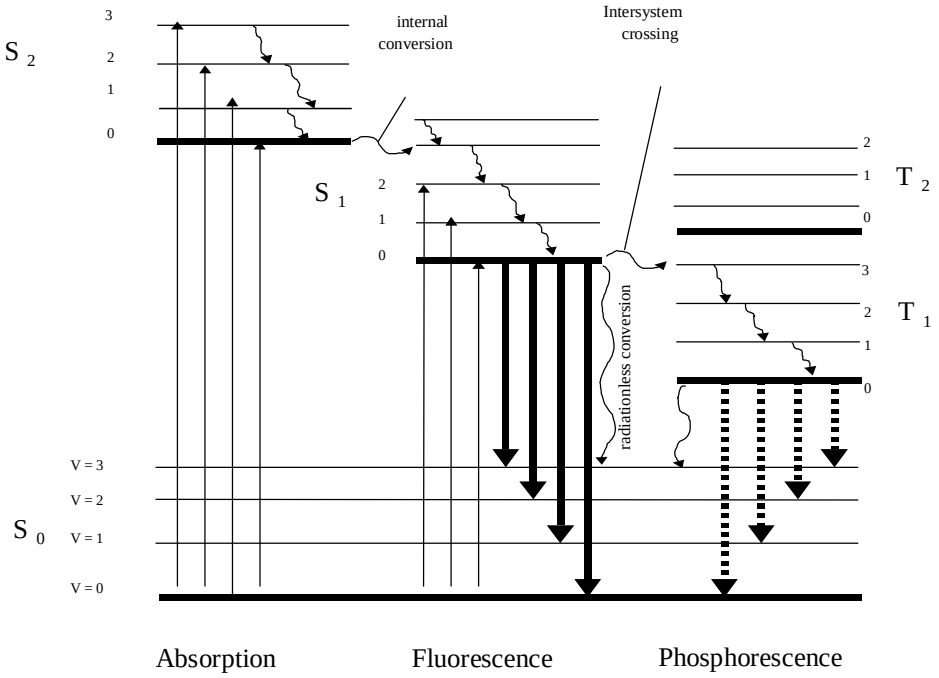


Fig. 1: Jablonski diagram for explanation of absorption, fluorescence, and phosphorescence

Caused by transmission of the ocular media (cornea, lens, chamber water, and vitreous), no excitation of the fundus is practically possible for wavelengths shorter than 400 nm[6]. Fig. 1 shows, e.g., excitation and emission spectra of lipofuscin[7] and of flavins[8].

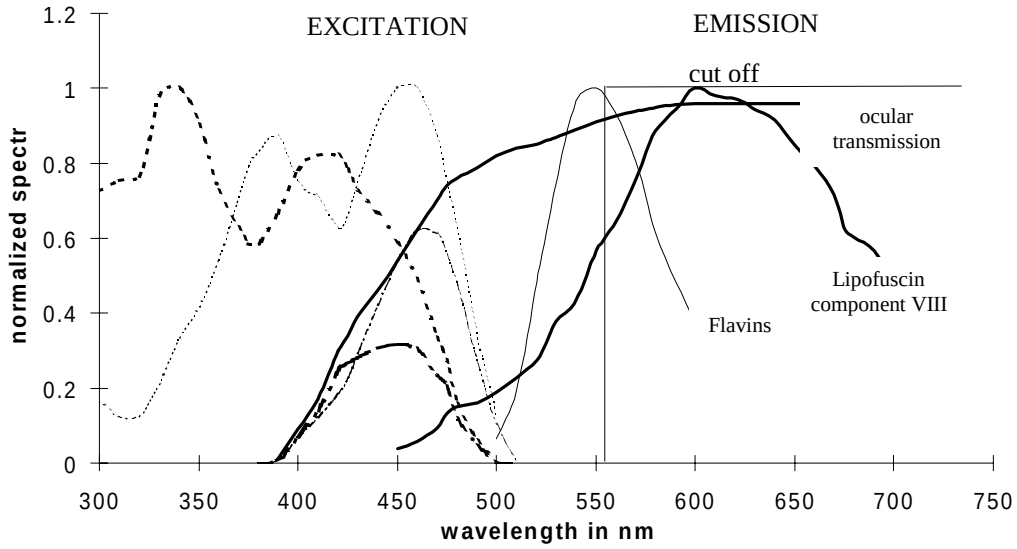


Fig. 2: Excitation spectra of lipofuscin[7] and of flavins[8]. For both fundus fluorophores, the excitation maxima are between 450 and 470 nm after transformation by ocular transmission

Only lipofuscin is excited at 320 nm. When the excitation spectra of both fluorophores are multiplied by the transmission spectrum of the ocular media, the resulting calculated excitation maxima are quite similar in the region of 450 nm up to 470 nm.

Consequently, in practical investigations of eye ground, more or less all fluorophores are excited simultaneously. As shown by Schweitzer et al.[9], different but overlapping fluorescence spectra are measurable at the fundus after excitation around 470 nm and around 510 nm.

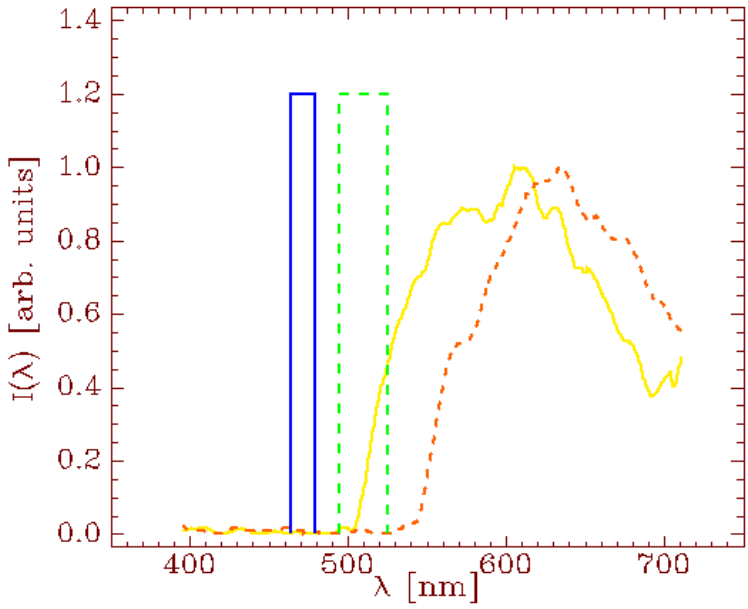
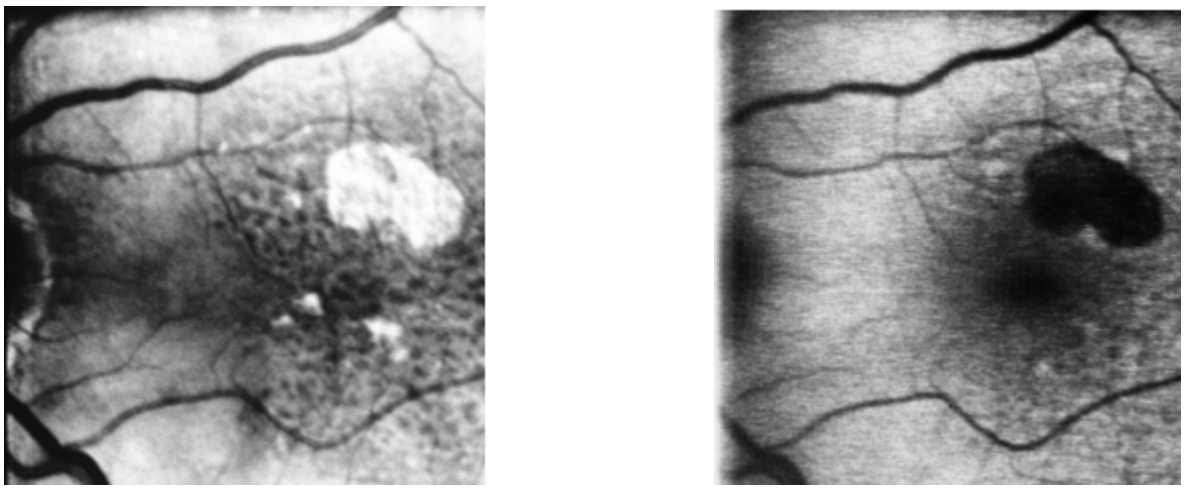


Fig. 3: Emission spectra of autofluorescence of a living human fundus. The shoulder and the maximum at 540 nm and at 580 nm at excitation by 470 nm might be caused by flavins. The emission spectrum (maximum at 630 nm) after excitation at 510 nm is mainly determined by lipofuscin.

Unfortunately, the reconstruction of fluorescence spectra requires a relative long measuring time for the collection of a sufficient high number of photons at each point of investigation. So a discrimination of fluorophores in a 2-dimensional field at the eye ground is practically impossible by applying different emission spectra. The 2-dimensional distribution of autofluorescence intensity can be determined by means of both laser scanner ophthalmoscopes or fundus cameras. As the fluorescence intensity is very weak, the blocking filters must suppress excitation light at least by a factor of 10^8. Otherwise, the reflected light might be misinterpreted as fluorescence light. Different illuminating techniques result in different autofluorescence images of the same eye ground. In the confocal laser scanner technique, the optic disc appears dark, however, in large field illumination, bright. The reason is that the optic disc consists of high scattering tissue and both excitation and emission light are scattered in the solid angle of 4π. The confocally detected light is under threshold of the detector and, therefore, the optic disc appears dark. In conventional fundus cameras, large field illumination excites tissue of optic disc from several directions. So a sufficient bright fluorescence can be detected. Unfortunately, both techniques do not permit discrimination of fluorophores.

A refinement of the clinical interpretation can be reached when images taken at different spectral ranges are superimposed by additive color mixing. In Fig. 4, the primary images are given for a color mixing of an infrared reflectance fundus image taken at 780 nm and of an autofluorescence image (λ_{ex}=488 nm, λ_{em}>530 nm). Both images are taken by means of a CLSO. If the intensity of the infrared image is red coded and green is attached to the fluorescence image in additive color mixing, a spot having the same intensity in IR and in fluorescence appears yellow. Pure IR intensity is red and pure autofluorescence is green.



a - infrared reflectance image at 780nm b - autofluorescence image (λ_{ex}=488nm)

Fig. 4: Infrared reflectance and autofluorescence images as primary information for additive colour mixing

A promising method is the evaluation of fluorescence lifetime[5], facilitating a discrimination of fluorophores in 2-dimensional images. The lifetime is determined by the structure of the ground stage of a specific fluorophore and is independent of the applied fluorescence intensity. That means, a very weak fluorophore can also be detected in aggregation to a fluorophor emitting with a strong fluorescence intensity, if the lifetimes of both fluorophores differ to a sufficient high degree. Measurements of fluorescence lifetime in age-related macular degeneration (AMD) are supported by the fact, that retinal pigment epithelial cells exhibit a lifetime of 0.12 ns after treatment by A2-E in addition to the lifetimes of untreated cells (0.35, 1.7 and 6.3 ns)[10]. A2-E is a pyridinium bis-retinoid, assumed to be a precursor of lipofuscin. Lipofuscin is a metabolic end product accumulating as an aging pigment during life. There is a permanent regeneration of a photoreceptor outer segments in the retina. The decomposition products of high active phagocytosis in retinal pigment epithelium diffuse to the choroid through Bruch's membrane. In age-related macular degeneration (AMD), the structure of the Bruch's membrane is changed, resulting in a decrease of diffusion. It leads to the accumulation of metabolic end products like lipofuscin in the pigment epithelium. Components of lipofuscin form free radicals which destroy cellular membranes. In addition, the diffusion of oxygen from choroid to photoreceptors is also reduced by the pathologically altered structure of Bruch's membrane. Both processes result in a lack of oxygen in retinal tissue. A compensation occurs partly by an increased consumption of oxygen in the retinal vessel system[9]. In progressive stages of the disease, new vessels grow from the choroid through Bruch's membrane and spread out under pigment epithelium. The diffusion distance will be increased from choroid to photoreceptors by this process for oxygen. So a positive feedback impairs the pathologic process.

Overviews of fluorophores in tissue and their spectrometric characterization are given by Richards-Kortum et al.[11] and by Wagniers et al.[8].

2. METHOD

Measurements of fluorescence lifetime are known from microscopic studies at single cells[12]. In principle, a 2-dimensional measurement of the lifetime is possible in time domain or in frequency domain. In time domain, the response to a short excitation pulse will be detected. In frequency domain, the sample will be excited by intensity-modulated light. Fluorescence light also shows modulation, but with a phase shift and the modulation is weaker. In this case, the lifetime will be calculated from the phase angle or the degree of demodulation[5]. For the decision, which principle would optimally fit the conditions of the in vivo measurement at the fundus, the expected number of detectable fluorescence photons was estimated. As the eye ground is the most sensible tissue for light, maximal permissible exposure is the decisive fact for choice of measuring principle. According to ANSI standard[13], the continuos wave criterion limits exposure under assumed conditions. Starting from a usually in laser scanning ophthalmoscopy applied mean excitation power of 400 µW in corneal plane, at a repetition rate of 80 MHz, each pulse has an energy of 5pJ. This excitation light will be diminished by transmission of the ocular media ($\tau_{ex} = 0.9$). For conversion of excitation into emitted light, a quantum yield of 100 nJ nm^{-1} sr^{-1}/J was assumed[1]. Furthermore, a wavelength range of 100 nm was considered for emission. From emitted fluorescence light, which illuminates the whole solid angle, only a small part will be detected, which falls in the solid angle of detection $\Omega = 0.02$ sr, determined by the ratio of effective aperture plane in the iris and the square of distance to the fundus. The emitted light has to penetrate ocular media and will also be diminished by $\tau_{em} = 0.9$. A further weakening occurs by transmission of ophthalmoscope, estimated as $\tau_{oph} = 0.3$, by transmission of the blocking filter $\tau_{cut} = 0.8$, and by the quantum yield of the detector $\Phi_{PMT} = 0.1$. Assuming all fluorescent light will be emitted at 600 nm, the ratio of the detectable energy of light, divided by the energy of 1 photon ($h \bullet \nu_{em}$) results in a number of detected photons. According to this calculation, only 0.06 fluorescence photons will be detected as response to the excitation pulse. That means, in a series of about 10 excitation pulses, only 1 fluorescence photon will be detectable. This value of detectable photons is much too less for the reconstruction of the modulation waveform of the emitted light. That means, a lifetime measurement of fundus autofluorescence in the frequency domain should not be possible. In contrast, the low probability for the detection of one single fluorescence photon is optimal for the application of the time-correlated single photon counting technique. In this technique, a transformation of lifetime in nanosecond time scale will be performed in a range of seconds.

SCHEME OF A LASER SCANNER OPHTHALMOSCOPE FOR THE 2 - DIMENSIONAL TIME - RESOLVED FLUOROMETRY

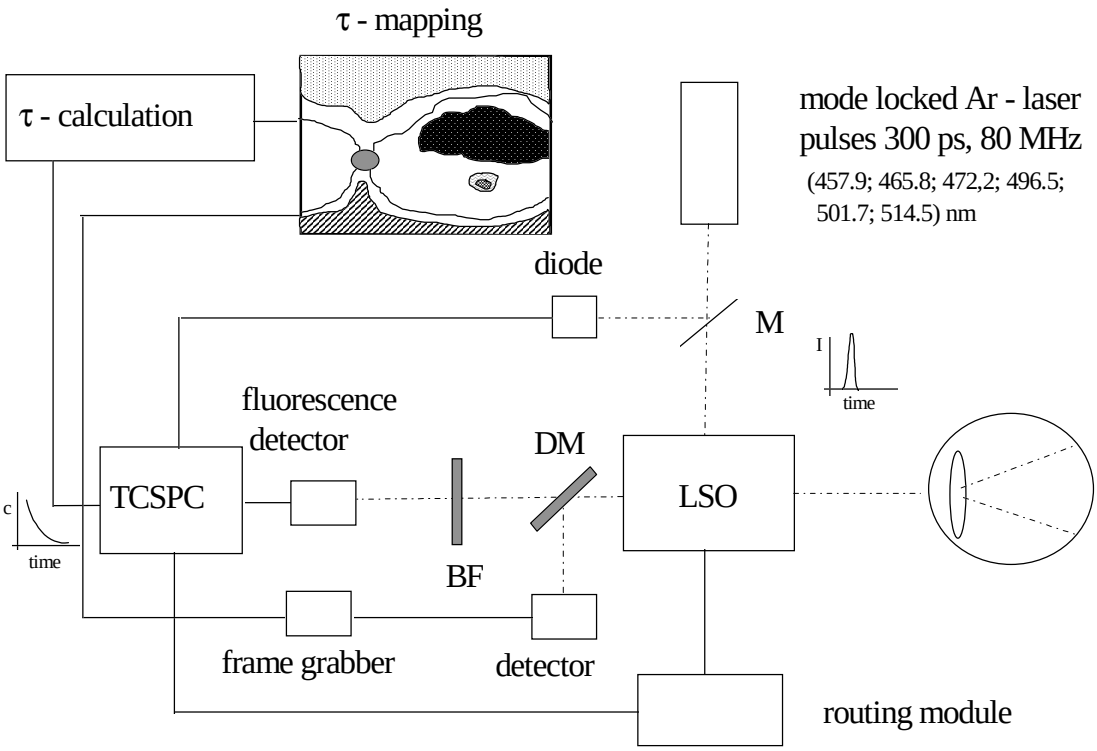


Fig. 5: Scheme of experimental set up according to Schweitzer[14]

According to these theoretical considerations, an experimental set up for the 2-dimensional measurement of the lifetime in a fundus image was developed (Fig. 5)[14]. The basic device in this arrangement is a conventional laser scanner ophthalmoscope (CLSO, Carl Zeiss, Germany). In this arrangement, the light source is an active mode-locked Ar^+ laser (ILA 190, LES Jena, Germany) delivering pulses of 300 ps duration after convolution by detector response at a repetition rate of about 80 MHz. Wavelengths are selectable between 457.9 and 514.5 nm. Excluded was the wavelength at 488 nm. No active mode locking was reached at this wavelength due to high internal magnification. The fluorescence light is detected by a photomultiplier (HAM-H 5783P-01, Hamamatsu, Japan) and registered in time-correlated single photon counting technique applying the SPC 536 unit (Becker & Hickl, Berlin, Germany). The spatial correspondence of the detected photons was realised by the routing module VRT-1 (Becker & Hickl, Berlin, Germany).
In function, a small part of each excitation pulse, reflected at the mirror M, is detected by a photodiode and starts a ramp of a time to amplitude converter in the unit for the time-correlated single photon counting. The main part of the excitation pulse hit the fundus via the CLSO. A dichroic mirror DM separates the reflected and the fluorescence light at the exit of CLSO. The filter BF blocks additionally reflected light. Only one photon must be detected by the photomultiplier for stopping the ramp in the time to amplitude converter. According to the reached amplitude, the content of the corresponding time channel will be added by one. As a result of the photon statistics, the content of time channels represents the time-dependent fluorescence after a sufficient number of excitations. In the scanning process, each fundus site will be excited by a series of pulses. During the acquisition time of some seconds, a series of fundus images will be detected. For compensation of eye movements, reflection images and fluorescence images are detected simultaneously. During the image registration of reflectance images, a movement vector is calculated. This vector is also used for the registration of the very weak fluorescence images. In this way, the allocation of fluorescence photons in single time channels to the originating location is reached. Based on the content of time channels, the lifetime of autofluorescence will be calculated for each fundus sites. The spatial resolution in a 20 degree field is 50µm•50µm. As shown by Köllner and Wolfrum[15], only about 300 photons are necessary for the calculation of a mono-exponential decay time with an error of less than 10%, applying the maximum-likelihood criterion.

3. RESULTS

3. 1 In vitro experiments

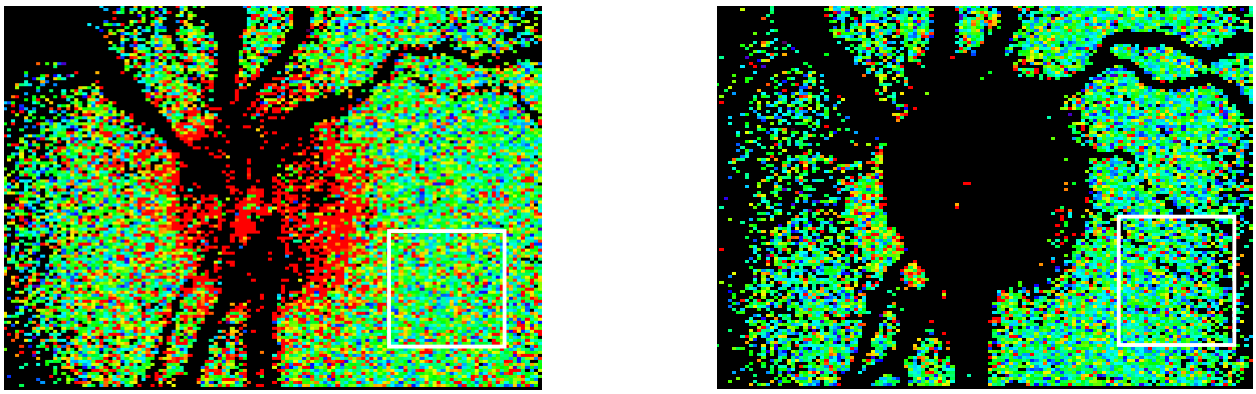
The function of the experimental set up was tested under conditions comparable to in vivo measurements at the fundus. For this purpose, chessboard-like structured samples of Rhodamin 6G and of Coumarin 522 were produced in spin-coating technique on glass plates. These fluorophores exhibit different excitation and emission spectra and also the fluorescence lifetimes are different. These samples were calibrated applying a streak-camera and integrating the fluorescence over a long time. The lifetime of Rhodamin 6G was determined as 2 ns and of Coumarin as 5 ns.
For demonstration of the effect of a lifetime image, samples of Rhodamin 6G and of Coumarin 522 were adjusted such that one fluorescent field of the first sample covers a fluorophore-free field in the other sample. Furthermore, samples were oblique to the confocal plane simulating different measurable fluorescence intensities. The excitation wavelength was selected such that equal fluorescence intensities were detected from both samples. Furthermore, the excitation power was adjusted so that only several hundred photons could be detected. These conditions corresponded well to in vivo measurements, where a limited number of photons will be detectable originating from simultaneously excited fluorophores. As demonstrated by Schweitzer et al.[14], the fields of Rhodamin 6 G and of Coumarin 522 are clearly distinguishable from each other in lifetime images. The calculated lifetimes correspond well with the calibration values of 2 ns and of 5 ns. As anticipated, no influence of fluorescence intensity was remarkable in the lifetime image. In contrast, no discrimination of fluorophores is possible in images of fluorescence intensity.

3.2 Investigation of the human ocular eye-ground

First investigations at the living human ocular fundus were started according to the Declaration of Helsinki[16]. At first, the influence of intra-retinal scattering on the widening of the excitation pulse was tested. For that purpose, the reflected light was time-resolved measured from the binned 20-degree fundus field in the oscilloscope mode. No significant broadening of the excitation pulse was detectable. Because of the decay time and jitters of the applied detector (HAM-H5783P-01, Hamamatsu, Japan), an evaluation of dynamic fluorescence was useful only at a time delay of 500 ps after the maximum fluorescence.

3.2.1 Autofluorescence in spectral ranges

Lifetime image of the living human ocular fundus were detected by 457,9 nm excitation wavelength and a mean power of only 30-40 µW. For the collection of some hundred photons at each image pixel, 5s integration time was necessary. As described by Schweitzer et al.[14], a lifetime of about 5 ns was found in papillary tissue and about 1.5 ns in para-papillary range.



a -515 nm < λ < 700 nm b -550<<λ < 700 nm

Fig. 6: Lifetime image of healthy fundus at excitation of 458 nm. a -emission range 515 nm <λ< 700 nm, b -emission range 550 nm<λ < 700 nm. Presented lifetime range 0.5 ns< τ < 2 ns. Fit threshold was 100 photons.

To get more substance-specific information, the lifetime of autofluorescence at healthy human fundus was measured in 2 spectral ranges (Fig. 6). Although a shortening of lifetime in long-wave emission range can be visually identified in colour coded images, an exact evaluation of lifetimes is difficult. Besides classes of lifetime at optic disc ($\tau \approx 5$ns) and in para-papillary range ($\tau \approx 1.5$ns), a high spatial variation of lifetime is remarkable in healthy tissue. A first step for the quantification of lifetime images is the evaluation of histograms of calculated lifetime within a regions of interest. It is clear that biological variation of lifetime is superimposed with uncertainty of lifetime-calculation from a small number of photons.

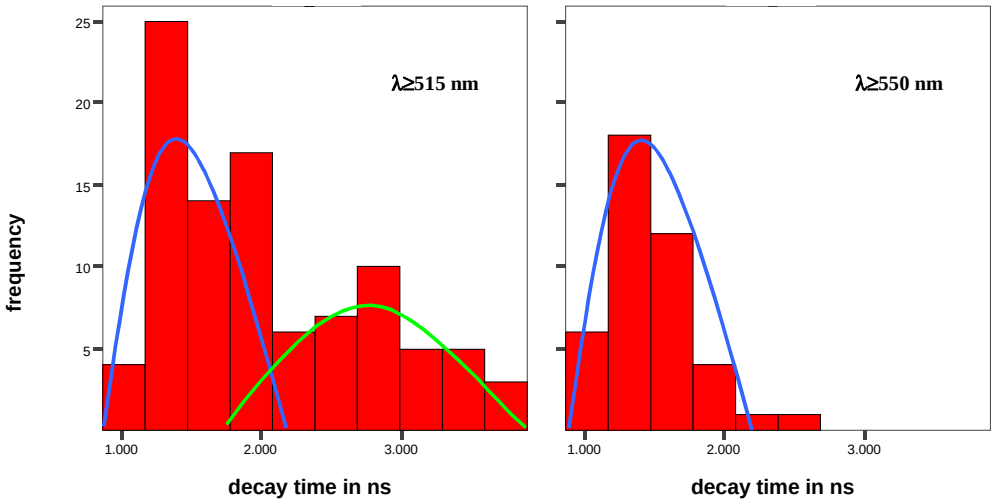


Fig. 7: Frequency of lifetimes in para-papillary range of a healthy fundus. Left: emission range 515 nm < λ_{em} < 700 nm, right: emission range 550 nm < λ_{em} < 700 nm

Derived from histograms, most frequent lifetimes around 1.38 ns are calculated in the para-papillary region, when fluorescence light in long-wave emission range is evaluated. Considering an extended emission range down to 515nm, contributions of longer lifetimes are remarkable. For a better understanding of the global consideration, distribution curves of the assumed fluorophores are additionally drawn. In a detailed investigation, an accumulation of lifetimes around 2 ns is also visible in the extended fluorescence range.

3.2.2 Investigation of fundus specimen

In a comparison experiment, a specimen of human fundus was investigated (Fig.8). In these images, the optic disc is located on the left. Removing the neuronal retina in the lower part, a cut through the macula was realized. Considering each pixel in the marked regions of interest, the frequency of lifetimes was determined again. When the neuronal retina is removed, autofluorescence originates predominantly from retinal pigment epithelium and the main fluorophor in pigment epithelium is lipofuscin[18]. As demonstrated in Fig. 9, histograms of lifetimes of both fundus parts differ considerably.

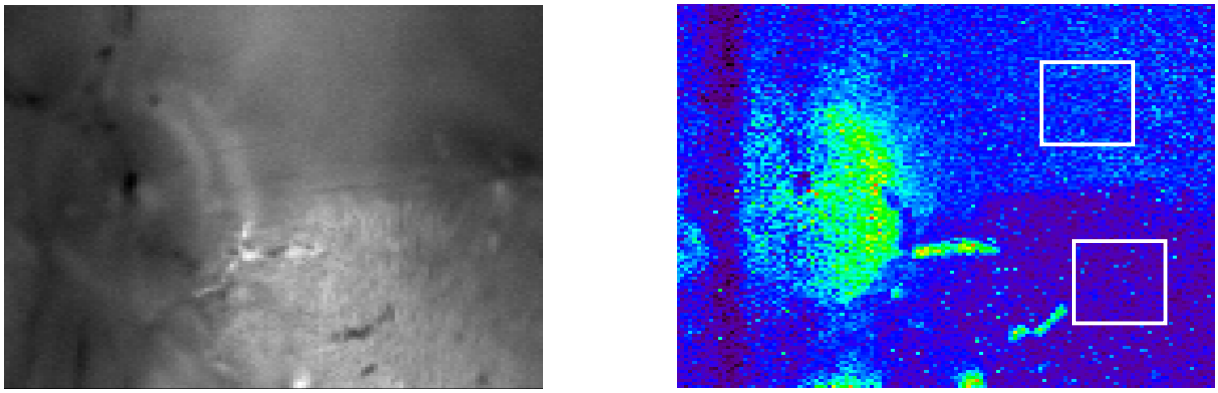


Image of fluorescence intensity Lifetime image

Fig. 8: Autofluorescence image of a human fundus specimen. In a cut through the macula, the neuronal retina was removed at the lower part. The optic disc is always located on the left. Remember that stripes in the lower part of the intensity image appear dark and bright, whereas they exhibit the same values in the lifetime image. The white fields are the investigated regions of interest.

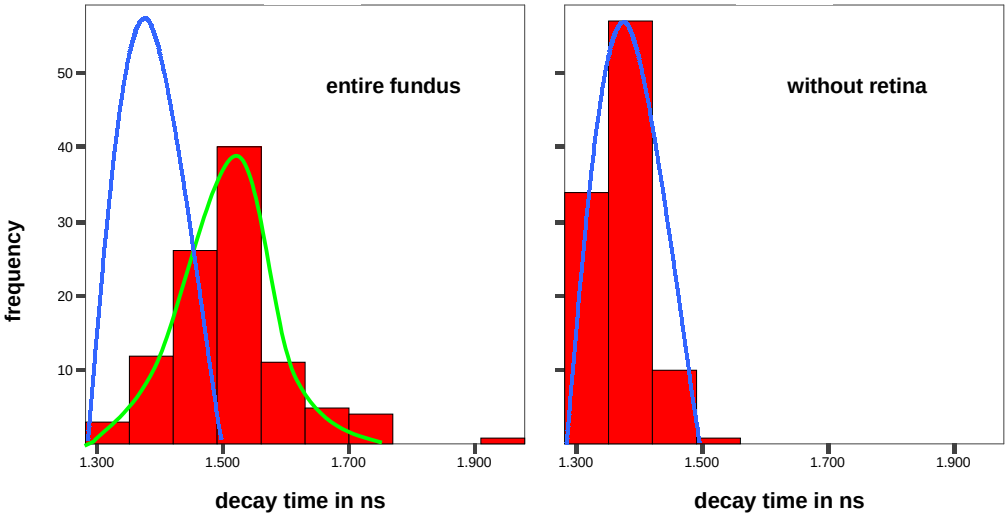


Fig. 9: Histogram of lifetimes of a human fundus specimen. Left : intact fundus, right: neuronal retina removed.

The most frequent lifetime of an intact fundus was about 1.54 ns. Investigating more retinal pigment epithelium alone, a shorter lifetime of 1.35 ns was detected most frequently. This lifetime corresponds with the lifetime most frequently detected in long-wave emission range of living fundus. As the maximum of fluorescence of lipofuscin is around 630 nm and lipofuscin is the most intensive fluorophore in retinal pigment epithelium, the mono-exponentially determined lifetime of about 1.3 ns should be assigned to lipofuscin. Measurements of the fundus specimen hint at an additional long-living fluorophore in neuronal retina.

3.2.3 Influence of oxygen breathing

In a further experiment, the influence of breathing of 100% oxygen on fluorescence lifetime was investigated. Lifetime measurements were performed before and after 6 minutes oxygen breathing, as well as 15 minutes after the stop of oxygen breathing. In Fig. 10, histograms of the detected lifetimes in the para-papillary region of interest are presented. Fluorescence light was detected between 515 nm and 700 nm. Considering the means of the detected lifetimes in the nasal side, a quenching of lifetimes is remarkable from 1.89 ns before provocation to 1.75 ns after 6 minutes oxygen breathing. The mean lifetime increased again to 1.89 ns after breathing of air for 15 minutes. Assuming normal distributions, the difference of lifetimes during and after breathing of oxygen was nearly significant ($p = 0.06$). In range temporal to the optic disc, the difference of lifetimes during and after oxygen breathing was significant ($p=0.017$).

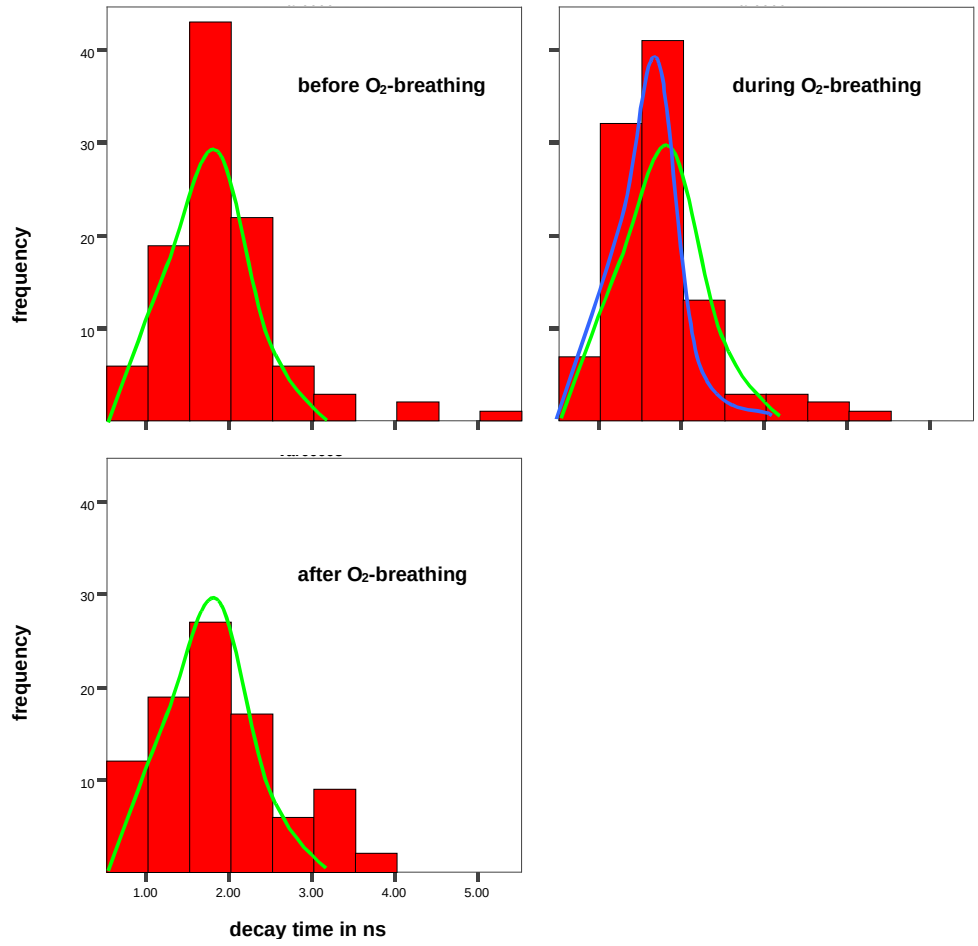


Fig. 10: Histograms of lifetimes before, during oxygen breathing for 6 minutes, as well as after 15 minutes of breathing air.

4. DISCUSSION

For an early detection of metabolic alteration, the measurements of the tissue autofluorescence can serve as a precursor of pathological processes. Under the conditions of the living eye-ground, the concentration of fluorophores is low and the excitation power is limited by the maximal permissible exposure[13]. As a consequence, the detectable fluorescence light is very weak. Due to a low transmission of ocular media in the spectral range below 400 nm[6], a discrimination of fluorophores by selection of excitation wavelengths must be excluded. Different endogenous fluorophores are excited simultaneously in the adaptable excitation range above 400 nm.

The lifetime of fluorescence is independent of the detected intensity, but the error of lifetime calculation decreases if the intensity is sufficiently high.

The time-resolved measurement of fluorescence is advantageous if weak fluororescence of one fluorophore can be discriminated from the intensive fluorescence of other simultaneously excited fluorophores. This is possible if the fluorescence lifetimes are sufficiently different.

Although extremely weak fluorescence light is expected from the fundus, the lifetimes can optimally be measured in the ns time-scale applying time-correlated single photon counting. This method is the most sensitive one. An experimental arrangement was demonstrated, allowing the measurement of fluorescence lifetime in a 2-dimensional image of the eye-ground. The well-known laser scanner technique is used as basic principle for image acquisition. Only a few hundred photons are necessary for the calculation of a mono-exponential decay. Consequently, the required mean excitation power is about one order below radiation power, usually applied in laser scanner ophthalmoscopy. First in vivo measurements from a healthy ocular fundus exhibit a lifetime of about 1,5 ns in the para-papillary region and of about 5 ns in optic disc. This large lifetime of 5 ns probably

corresponds to collagen[17]. Investigating the lifetime of autofluorescence in the para-papillary region, a mixture of fluorophores must be assumed. A partly discrimination was reached by the comparison of histograms of lifetimes for the emission range $\lambda_{em} > 515$ nm and $\lambda_{em} > 550$ nm. In long-wave emission range, $\tau \approx 1.38$ ns was detected most frequently. This lifetime was also most frequently detected in a specimen of a human ocular eye-ground, when the neuronal retina was removed. The dominant fluorophore in remaining pigment epithelium is lipofuscin[18]. Docchio et al.[19] found 4 lifetimes in human melanolipofuscin (0.22 ns/ 68.7%, 0.66 ns/22.6%, 1.97 ns/6.7% a, and 7.03 ns/2.1%). As the mono-exponential approximation of this multi-exponential behaviour results in 1.33 ns, it is quite sure that dynamic autofluorescence of lipofuscin was measured in long-wave range at living human eye-ground.

In the histograms of lifetimes detected in the whole available emission range $\lambda_{em}>515$ nm, contributions of longer lifetimes can be found. Tanaka et al.[20] found lifetimes of FAD of 47 ps for dimer, 200 ps for monomer, and 2.28 ns for free FAD. So, the accumulation of lifetimes at about 2ns hints to presence of free FAD. As the presented laser scanner ophthalmoscope permits only measurements of lifetimes longer than 500 ps, only the detection of long-living component of free FAD appears realistic. The correspondence between longer lifetimes around 2.8 ns and the concideration of fluorophores have to be solved in future. More detailed information is expected from both provocation tests and the comparison between pathologic and healthy tissue.

5. SUMMARY

It was demonstrated that lifetime of autofluorescence at fundus can be measured in nanosecond time scale using time-correlated single photon counting in connection with laser scanner technique. Surprisingly, the required power of illuminating light is one order below the normally applied intensity in laser scanning ophthalmoscopy.
The interpretation of histograms of lifetimes in regions of interest permits a first evaluation of lifetime images. Detection of fluorescene lifetime in fundus images might be developed as a tool in functional ocular diagnostics sensitive at a cellular level, already before morphological alterations are visible.

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