Quantitative Analysis of Fluorescence Lifetime Measurements of the Macula Using the Fluorescence Lifetime Imaging Ophthalmoscope in Healthy Subjects

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PURPOSE. Fundus autofluorescence (FAF) cannot only be characterized by the intensity or the emission spectrum, but also by its lifetime. As the lifetime of a fluorescent molecule is sensitive to its local microenvironment, this technique may provide more information than FAF. We report here the characteristics and repeatability of FAF lifetime measurements of the human macula using a new fluorescence lifetime imaging ophthalmoscope (FLIO).

METHODS. A total of 31 healthy phakic subjects were included in this study with an age range from 22 to 61 years. For image acquisition, a fluorescence lifetime ophthalmoscope based on a Heidelberg Engineering Spectralis system was used. Fluorescence lifetime maps of the retina were recorded in a short- (498–560 nm) and a long- (560–720 nm) spectral channel. For quantification of fluorescence lifetimes a standard ETDRS grid was used.

RESULTS. Mean fluorescence lifetimes were shortest in the fovea, with 208 picoseconds for the short-spectral channel and 239 picoseconds for the long-spectral channel, respectively. Fluorescence lifetimes increased from the central area to the outer ring of the ETDRS grid. The test-retest reliability of FLIO was very high for all ETDRS areas (Spearman’s ρ = 0.80 for the short- and 0.97 for the long-spectral channel, P < 0.0001). Fluorescence lifetimes increased with age.

CONCLUSIONS. The FLIO allows reproducible measurements of fluorescence lifetimes of the macula in healthy subjects. By using a custom-built software, we were able to quantify fluorescence lifetimes within the ETDRS grid. Establishing a clinically accessible standard against which to measure FAF lifetimes within the retina is a prerequisite for future studies in retinal disease.

Keywords: fundus autofluorescence, fluorescence lifetimes, macula, validation studies

Ophtalmic imaging has made considerable progress in the past decade. Especially the capability to diagnose structural changes with near histological resolution underlying various retinal diseases has improved enormously. However, the appearance of structural changes in retinal disease often represents irreversible functional loss with only limited treatment options. Therefore, an imaging tool to diagnose disease earlier, before occurrence of structural changes, would be desirable. Fluorescence lifetime imaging microscopy (FLIM) has been applied widely to visualize changes in metabolism and energy consumption in cells. The fluorescence lifetime represents the time a molecule spends in its excited state before returning to its ground state by emission of a longer-wavelength photon.

The fluorescence lifetime of a fluorophore is directly influenced by its microenvironment, such as pH, and interactions with other molecules. Since the first nanosecond lifetime measurement more than 50 years ago,2 numerous FLIM methodologies have been developed for clinical applications.3,4

In vivo fundus autofluorescence (FAF) lifetime imaging of the retina, which was initially described by Schweitzer et al.,5 is able to measure fluorescence lifetimes in the retina on a macroscopic level. Similar to FLIM, which can detect metabolic changes on a cellular level, this new technique may be able to detect metabolic changes in the retina due to differences in energy transfer rates caused by tissue stress. A recent report has shown that oxidative stress increases the fluorescence lifetimes in retinal pigment epithelium cells ex vivo.6 Therefore, this novel technique may not only be beneficial for clinical diagnosis, but also for investigation of novel therapies for retinal diseases.

The fluorescence lifetime imaging ophthalmoscope (FLIO) was recently developed by Heidelberg Engineering GmbH (Heidelberg, Germany). This device is able to acquire FAF...
lifetime images in approximately 2 minutes per eye and therefore may be used as a clinically viable device. To quantify FAF lifetimes from patients with retinal diseases, it is imperative to analyze lifetime data and the repeatability of FLIO measurement in a cohort of healthy subjects.

The aim of this study was to establish a measurement protocol and to assess the repeatability of FLIO measurement in healthy subjects.

**METHODS**

**Participants**

Thirty-one healthy, phakic subjects were recruited between February and March 2013 at the Department of Ophthalmology at the University Hospital of Bern. The study protocol was approved by the local ethics committee and is in accordance with the declaration of Helsinki. Informed consent was obtained from all participants before study entry. This prospective study has been registered at ClinicalTrials.gov as "Measurement of Retinal Auto Fluorescence with a Fluorescence Lifetime Imaging Ophthalmoscope (FLIO Group)" with the identifier number NCT01981148.

Subjects with any systemic medical conditions, such as arterial hypertension or diabetes mellitus, were excluded. Similarly, subjects were excluded if they had a history of eye disease or surgery. Mild refractive errors up to −3 diopters myopia or +3 diopters hypermetropia were accepted.

**Study Protocol**

All participants underwent FLIO measurement of both eyes three times with an interval of at least 1 hour in-between. Images were acquired in a dark room with no artificial light to avoid background light entering the detector. The first and the second FLIO measurements were recorded with nondilated pupils. The third FLIO examination was performed with dilated pupils. For pupil dilation, tropicamid 0.5% and phenylephrine HCl 2.5% were applied. To qualify as dilated, pupil diameters of at least 6 mm measured at standard light conditions (Goldmann perimeter, 45 lux) were required.

A spectral domain optical coherence tomogram (OCT) of the macula (Heidelberg Spectralis HRA+OCT; Heidelberg Engineering) was done of each eye to exclude structural abnormalities in the retina of the subjects and to correlate retinal thickness with fluorescence lifetimes. For statistical analysis, one randomly chosen eye was defined as the study eye (n = 31 eyes). Only for the comparison between both eyes where the study eye was compared with the fellow eye were all eyes included in the statistical analysis (n = 62 eyes).

**Fluorescence Lifetime Imaging Ophthalmoscope, Image Acquisition, and Analysis**

The setup of our FLIO system is illustrated in Figure 1. The FLIO is based on a Heidelberg Engineering Spectralis system. The basic functions of the Spectralis (i.e., infrared reflectance imaging and autofluorescence intensity imaging) are preserved. For fluorescence excitation, a picosecond pulsed diode laser at 473 nm wavelength and 80 MHz repetition rate was raster scanned at a frame rate of 9 Hz over a 30° retinal field covering the macula. The average radiation power at the corneal plane was maximally 90 µW, which is well below the limits recommended by the American National Standards Institute Z136.1-2000 Standard for protection of the human eye from laser exposure7 with a MPDh of 2.8 mW.

Emitted fluorescence light was descanned and confocally filtered using a 100-µm multimode optical detection fiber. Single fluorescence photons were spectrally separated into two spectral channels of 498 to 560 nm (short-spectral channel) and 560 to 720 nm (long-spectral channel), and detected by highly sensitive hybrid photon-counting detectors (HPM-100-40; Becker & Hickl, Berlin, Germany). The detector signals were registered using time-correlated single-photon counting modules (SPC-150; Becker & Hickl). A high-contrast confocal infrared reflection image was simultaneously recorded to the dynamic autofluorescence image so as to track eye movements in real time to ensure that every single fluores-
cence photon was registered at the correct spatial location. Each detected fluorescence photon is thus characterized by its detection time in the laser period, its coordinates in the scanning area, and its wavelength. The recording process builds up a photon distribution over these parameters from which a fluorescence lifetime map of the fundus is approximated using a multiexponential fitting procedure:

\[ I(t) = \frac{I_R}{T_0} \sum_j \left( a_j \cdot e^{-t/T_j} \right), \]  

where \( I \) is the fluorescence intensity at the time \( t \), \( I_R \) is the instrumental response function, and \( a_j \) is the amplitude with \( T_j \) as the respective lifetime.

The acquired data were analyzed with the software SPCImage 3.4 from Becker & Hickl by using a biexponential decay model (Fig. 2) and a binning factor of one. In our results, we considered the short- and long-lifetime components \( T_1 \) and \( T_2 \), their respective amplitudes \( a_1 \) and \( a_2 \) of the approximated exponentials, as well as the weighted average of the lifetime components \( T_1 \) and \( T_2 \) in each pixel according to the formula:

\[ T_m = \frac{a_1 \cdot T_1 + a_2 \cdot T_2}{a_1 + a_2}. \]  

The goodness of fit is evaluated by \( \chi^2 \):

\[ \chi^2 = \frac{1}{m - p} \sum_{j=1}^{m} \frac{(N_j(t_j) - N_e(t_j))^2}{N_j(t_j)}, \]

where \( m \) is the number of time channels, \( p \) the number of free parameters in Equation 1, \( N_j(t_j) \) the number of measured photons in time channel \( j \), and \( N_e(t_j) \) the number of calculated photons according to Equation 1 in time channel \( j \). A value of \( \chi^2 \) close to 1 indicates a good fit for an appropriate model and a random noise distribution, as defined by Poisson statistics. We analyzed our data using bi- and triexponential fitting. Because the \( \chi^2 \) values were not significantly lower and there was virtually no influence on \( T_m \) values, we used biexponential fitting.

For each eye, an average of 1000 photons per pixel over the 30° central retinal field was recorded. This corresponds to measurement periods ranging between 2 to 5 minutes and 1.5 to 2 minutes in nondilated and dilated pupils, respectively.

For the repeatability and the comparison of nondilated with dilated pupils, one eye of each subject was selected randomly and defined as the study eye, resulting in roughly equal numbers of right and left eyes (15 right and 16 left, respectively). For comparison of the right with the left eye, data of both eyes were used from the measurement with dilated pupils.

For quantitative analysis, the recorded data were imported in the purpose-built “FLIO reader” (ARTORG Center for Biomedical Engineering Research, University of Bern, Bern, Switzerland). This software allows autofluorescence and color-coded fluorescence lifetime images to be displayed in a variable intensity overlay.

To perform systematic analysis of fluorescence lifetimes in different anatomical locations of the macula, a standard Early Treatment Diabetic Retinopathy Study (ETDRS) grid was used, resulting in nine distinct areas: C (center) \( r = 0.5 \) mm, N1/2 (nasal), S1/2 (superior), T1/2 (temporal), I1/2 (inferior) (Fig. 3). Fluorescence lifetimes were averaged within these grid areas, allowing sectional analysis and comparison of the obtained fluorescence lifetime data. For convenience of analysis, N1, S1, T1, and I1 were summarized into the inner ring (IR; \( r = 1.5 \) mm) and N2, S2, T2, and I2 were summarized into the outer ring (OR; \( r = 3 \) mm).

**Statistical Analysis**

Data were processed using Excel (Microsoft, Redmond, WA). Statistical analysis was performed using the Prism GraphPad commercial software package (Prism 6; GraphPad Software, Inc., La Jolla, CA, USA).

The differences among the three analyzed ETDRS areas were assessed using Student’s \( t \)-test. The interindividual variability of fluorescence lifetime values was quantified by the coefficient of variation (CV). For the test-retest reliability, fluorescence lifetimes of corresponding areas of the ETDRS grid from the first and the second measurement were correlated using Spearman’s nonparametric analysis (\( p \)). Spearman’s correlation also was applied for the correlation of fluorescence lifetime measurements of nondilated with dilated pupils and for the correlation of values from the right and the left eyes. Bland-Altman plotting was performed for each correlation and was used to assess the clinically relevant magnitude of the variance between different measurements.
RESULTS

A total of 31 healthy subjects (21 female [68%] and 10 male [32%]) were enrolled in this study. The age ranged from 22 to 61 years with a mean of 35 years (SD: 11 years).

Fundus Autofluorescence Lifetimes of the Posterior Pole

Figure 2 shows a representative FAF image of the retina of a healthy subject and a corresponding FAF lifetime image of Tm of the short- (498–560 nm, left side) and the long- (560–720 nm, right side) spectral channel (color range: 200–1000 ps). Lifetime histograms at the foveal center, at 1.5 mm and 3 mm eccentricity temporal to the foveal center, the optic nerve, and the retinal vessels of one representative pixel are shown. The blue dots represent the distribution of arriving photons and the integral produces the brightness of the pixel. The characteristic decay times (lifetimes) of the fluorescence molecules and their corresponding amplitudes can be calculated using time-correlated single-photon counting. Assuming a biexponential decay, two lifetimes and two amplitudes can be derived. The histogram shows the upper and lower limits, where the fitting is

Figure 3. Mean fluorescence lifetime images (Tm) of the posterior pole of the retina. (A) Representative color-coded fluorescence lifetime image of the short- (498–560 nm, left side) and the long- (560–720 nm, right side) spectral channel (color range: 200–1000 ps). (B) Corresponding graphs of fluorescence lifetimes of the individual ETDRS areas: (B1) mean lifetime (Tm), (B2) short-lifetime component (T1), (B3) long-lifetime component (T2). (Box: median and 25%/75%, whiskers 10%/90%. ****P < 0.0001, **P < 0.01, ns = not significant).
conducted. The straight red line is the calculated fit curve. The $\chi^2$ value indicates the goodness of fit and is displayed within each histogram.

**Topographic Distribution of Autofluorescence Lifetimes Within the Macula**

We used a standardized ETDRS grid to quantify and compare fluorescence lifetimes of the macula. The ETDRS grid was reduced to the central area (C), the IR, and the OR. The mean FAF lifetimes ($T_m$; $n = 31$ eyes) ranged from 124 picoseconds (ps) to 390 ps in the short-spectral channel and from 189 ps to 355 ps in the long-spectral channel. Overall, the mean fluorescence lifetimes in the short-spectral channel were considerably shorter and displayed larger SDs than those in the long-spectral channel.

Mean fluorescence lifetimes ($T_m$) were statistically highly significantly different between the C, the IR and the OR for both, and the short- and the long-spectral channel ($P < 0.0001$), with the center displaying generally the shortest fluorescence lifetimes (Fig. 3). In addition to mean fluorescence lifetimes ($T_m$), box plots for the short- ($T_1$) and the long- ($T_2$) fluorescence lifetime components are summarized in Figure 3.

**Correlation of Fluorescence Lifetime Parameters**

To reduce the amount of parameters to analyze, we were interested in which parameters of the FLIO analysis correlated with each other. For this, we computed a correlation matrix (Fig. 4). In keeping with the formula to compute mean lifetimes ($T_m$, Equation 2) and because the amplitude of the long-fluorescence lifetime ($T_2$) is usually a fraction of the amplitude of the short-lifetime component ($T_1$), mean autofluorescence lifetimes were highly correlated with the short-autofluorescence lifetime component ($T_1$), whereas the long-autofluorescence lifetime component ($T_2$) correlates only weakly with the mean autofluorescence lifetime ($T_m$), especially in the long-spectral channel.

**Test-Retest Reliability of Measurement With the FLIO**

Next, we tested the test-retest reliability of the autofluorescence lifetime values in the macula of 31 study eyes using the FLIO. For this, two measurements were performed on the same eye at least 1 hour apart with nondilated pupils. The agreement of $T_m$ between two measurements in all three areas of the ETDRS grid is shown in the Bland-Altman plots in Figure 5A. In the central area, Spearman’s $\rho$ was 0.80 in the short- and 0.90 in the long-spectral channel ($P < 0.0001$), in the IR $\rho = 0.80$ and 0.87, respectively ($P < 0.0001$), and in the OR $\rho = 0.71$ and 0.88, respectively ($P < 0.0001$; Supplementary Fig. S1A). A third measurement was performed with dilated pupils. The agreement between mean fluorescence lifetimes measured with diluted and nondilated pupils for all three areas is shown in the Bland-Altman plots in Figure 5B. Here the correlation of mean fluorescence lifetime values was lower when compared with the two measurements with nondilated pupils in both spectral channels with Spearman’s $\rho$ of 0.66 and 0.69, respectively, for the C ($P < 0.0001$), the IR ($\rho = 0.62$ and 0.60, respectively; $P = 0.0002$ and 0.0003, respectively), and the OR ($\rho = 0.5$ and 0.64, respectively; $P = 0.0046$ and < 0.0001, respectively; Supplementary Fig. S1B).

**Age Dependency of Autofluorescence Lifetimes Within the Macula**

Figure 6 illustrates the age-related increase of the mean fundus autofluorescence lifetimes ($T_m$) for all study eyes in both spectral channels. Without pupil dilation, the mean fluorescence lifetime ($T_m$) was significantly correlated with age in all three analyzed areas in both spectral channels ($P < 0.00166$). With pupil dilation, $T_m$ was not significantly correlated with age in the short-spectral channel within the central area ($P = 0.102$). However, significant correlations between $T_m$ and age were measured in all other ETDRS areas of both spectral channels ($P < 0.00061$).
Next we analyzed whether the retinal thickness correlates with the mean fluorescence lifetime. Because of the variability in retinal thickness measurements in the OCT within the ETDRS inner ring, we chose the ETDRS area N1 (IR, nasal) as a representative segment. For the C and N1, there was a correlation between the retinal thickness and $T_m$ with Spearman’s $\rho$ of 0.61 for the short- and 0.63 for the long-spectral channel ($P < 0.0001$; Supplementary Fig. S2A).

The correlation of the retinal thickness with the amount of detected photons showed Spearman’s $\rho$ of 0.41 for the short- and 0.50 for the long-spectral channel ($P = 0.0011$ and $P < 0.0001$, respectively; Supplementary Fig. S2B).

**Agreement Between Eyes**

To test whether there was a difference between eyes in the same subject, we correlated right and left eyes of the FLIO session with dilated pupils of all 62 eyes. In general, there was a good correlation between the two eyes in the short- and in the long-spectral channel ($\rho = 0.52$ and 0.72, respectively; $P = 0.0024$ and $P < 0.0001$, respectively; Fig. 5C and Supplementary Fig. S1C).

**DISCUSSION**

Here, we show that a clinical prototype device of a fluorescence lifetime imaging ophthalmoscope (FLIO) allows the quantification of fluorescence lifetimes of endogenous retinal fluorophores in the picosecond range using time-correlated single-photon counting. The interindividual CVs in the fovea (central ETDRS area) were 17% for the short- and 11% for the long-spectral channel, for the inner ETDRS ring 12% and 7%, respectively, and for the OR 9% and 6%, respectively. In this report we validated this new device with 31 healthy subjects of a wide age range and showed that it provides uniform and highly reproducible measurement data.

Endogenous retinal fluorophores are considered as potential probes of metabolic function and tissue morphology and therefore have potential diagnostic importance in ophthalmology with tissue fluorescence lifetime being a potential source for contrast. The fluorescence spectra and fluorescence lifetimes of different layers of the eye have been investigated ex vivo in the porcine eye by Schweitzer et al. In the porcine eyes, the shortest lifetimes were detected in the RPE ($\tau_{max} = 260$ ps) and the neuronal retina ($\tau_{max} = 460$ ps), whereas the choroid ($\tau_{max} = 1700$ ps) and sclera ($\tau_{max} = 1780$ ps) displayed longer fluorescence lifetimes. Due to technical limitations, such as very long acquisition times and inevitable eye movements during in vivo measurements, reports so far have included only a very limited number of eyes with ophthalmic diseases, such as retinal vein occlusion, retinal artery occlusion, or drusen in macular degeneration. Although fluorescence lifetimes of individual components, such as lipofuscin, melanin, or collagen, have been characterized in vitro, investigating and assessing the accuracy of the fluorescence lifetime measurements acquired in vivo can be challenging; as it is often difficult to determine accurate values of all the nanosecond-scale lifetime components from a multiexponential decay. Typically more than one fluorophore is excited simultaneously and the measured fluorescence decay represents the superposition of all individual decay components, which have to be fitted into a multieponential decay model. However, generally these results show a significant increase in fluorescence lifetimes in eyes with ophthalmic disease. Fluorescence lifetimes
detected within shorter wavelengths (498–560 nm) may be influenced by reduced nicotinamide adenine dinucleotide (NADH), advanced glycation end products (AGE), flavin adenine dinucleotide (FAD), and connective tissue (collagen, elastin). NADH, which acts as an ubiquitous electron carrier and plays crucial roles in both glycolysis and oxidative metabolism, may allow an estimation of the availability of oxygen within the tissue and in its unbound form mainly influences the short lifetime component. On the other hand, the FAF in the long-wavelength range is dominated by lipofuscin, which accumulates in the cells of the retinal pigment epithelium. In spite of this knowledge, it remains unclear which of the lifetime components provides the best contrast for differentiating abnormal from normal retinal tissue.

It has been suggested that the short-fluorescence lifetimes or the mean lifetime, which is largely influenced by the short-lifetime component, may provide the best contrast for differentiating abnormal from normal retinal tissue. Although there is some speculation about why lifetimes increase with disease, there is no definitive pathophysiological explanation or correlation to structural findings. This is due to the lack of sufficiently large prospective studies comparing fluorescence lifetime ophthalmoscopy to conventional imaging methods.

Similarly in this context, very little is known about the influence of the different retinal layers, the blood vessels, or the optic nerve on fluorescence lifetime characteristics of fluorophores in the retina in vivo. To obtain further information on fluorescence lifetimes in the diseased retina, validation studies and characterization of fluorescence lifetimes in the healthy retina are essential. Recently, Klemm et al. investigated repeatability of fundus autofluorescence lifetimes on 10 healthy subjects with a prototype fluorescence lifetime ophthalmoscope. For repeated measurement between sessions, they achieved an average intraindividual CV of 7.1% in the fovea and 5.2% in the papillo-macular bundle.

Several salient features of the FAF lifetime measurements in these healthy subjects have implications for subsequent measurements with the FLIO. First, we have identified landmarks at the posterior pole of the retina with characteristic FAF lifetimes, such as the fovea, retinal vessels, or the optic disc, which show reproducible and consistent lifetimes in...
healthy subjects. The shortest decay curves were seen in the central fovea and the longest in the area of the optic disc and the vessels, the latter probably caused by the high collagen content in these structures. It can only be speculated whether there is a possible link between the short lifetimes and oxycarotenoids, lutein, and zeaxanthin in the fovea. The FAF lifetime contrast within these areas is most distinct when analyzing the short-lifetime components and the mean fluorescence lifetimes. Whether this also will apply to the diseased retina remains to be investigated. Second, we were able to show an age dependency on FAF lifetimes; generally, mean FAF lifetimes increased with age. Interestingly, the correlation was more pronounced in patients with nondilated pupils. This may be due to a larger influence of lens autofluorescence lifetimes when the pupil is constricted, as the time-correlated photon counts are lower. A recent article by Greenberg et al. reported a similar finding in quantitative retinal disease. Disclosure: C. Dysli, Heidelberg Engineering (F); G. Quellec, Heidelberg Engineering (F); M. Abegg, Heidelberg Engineering (F); M.N. Menke, Heidelberg Engineering (F); U. Wolf-Schnurrbusch, Heidelberg Engineering (F); J. Kowal, Heidelberg Engineering (F); J. Blatz, Heidelberg Engineering (F); O. La Schiazza, Heidelberg Engineering (F); A.B. Leichtle, Heidelberg Engineering (F); S. Wolf, Heidelberg Engineering (F, C, R), Optos (F, C); M.S. Zinkernagel, Heidelberg Engineering (F, C, R)

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