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Characterization of a new fluorescence lifetime imaging ophthalmoscope and its comparison to the gold standard

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Abstract: Fluorescence lifetime imaging ophthalmoscopy (FLIO) is a noninvasive imaging technique that allows in vivo measurement of autofluorescence intensity decays of endogenous fluorophores in the ocular fundus. So far, only devices from Heidelberg Engineering based on the Spectralis system, regarded as the gold standard, have been used in FLIO research. Here, we characterize a new FLIO device based on the RETImap system from Roland Consult and compare it with the gold standard using cuvette measurements.

We determined the maximum laser power of the new FLIO device and analyzed the instrument's behavior at three different laser power levels (150 µW, 200 µW and max.) in terms of laser spectrum and instrument response function (IRF). Then, alternating between the gold standard and the new device, we performed 80 FLIO measurements using fluorescent dyes filled in flat cuvettes (110-OS, Hellma GmbH & Co. KG, Müllheim, Germany), 20 measurements for each of the four following dyes: A (25 µM eosin Y solution mixed with 5 M potassium iodide solution), B (20 µM erythrosine B with water), C (15 µM eosin Y with 0.5 M potassium iodide), and D (3.3 µM eosin Y with water). For both devices and all measurements, the laser power was set to 101 µW. A rectangular ROI covering 1000 px was defined for each measurement. The mean fluorescence lifetimes of the ROIs were compared for the four dyes in the two spectral channels, short (498–560 nm, SSC) and long (560–720 nm, LSC). For the statistical analysis Shapiro-Wilk, Mann-Whitney U, F, and t-tests were used. Bonferroni correction was used to correct for multiple comparisons. The IRF and the full width at half maximum (FWHM) calculation and monoexponential fluorescence lifetime approximation were performed using the FLIMX software.

The maximum laser power of the new device was 280 µW. For the consecutive laser powers 150 µW, 200 µW, and 280 µW, the IRF FWHM in SSC were 298.6±1.1 ps, 341.0±2.5 ps, and 347.5±6.0 ps, respectively. In LSC, the IRF FWHM were 290.4±3.8 ps, 344.0±3.4 ps, and 358.8±1.3 ps, respectively. All results are mean fluorescence lifetime ± standard deviation. Results of the comparison measurements showed statistically significant differences in mean fluorescence lifetimes between the two FLIO devices in SSC for dye D and in LSC for all fluorescent dyes. There were no statistically significant differences in mean lifetimes in SSC for dyes A, B, and C.

A new fluorescence lifetime imaging ophthalmoscope has been characterized and is suitable for fluorescence lifetime studies. A comparison of the new device to the gold standard revealed that the devices differ significantly in the LSC and slightly in the SSC. Obtaining incompatible results in more than 60% of the cases tested implies that the results provided by the new device are not compatible with those obtained from the gold standard. It is planned to perform such a comparison on a larger number of measurements.

Keywords: Fundus Autofluorescence Imaging, FLIO, Optical Imaging, Fluorescent Dye

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1 Introduction

Fluorescence lifetime imaging ophthalmoscopy (FLIO) is a noninvasive imaging technique that allows in vivo measurement of autofluorescence intensity decays of endogenous fluorophores in the ocular fundus. These decays are called autofluorescence lifetimes or FLIO lifetimes, abbreviated as FLTs [1]. FLIO technique is based on fundus

autofluorescence (FAF) imaging while extending this well-established technique by investigating the retinal fluorophores by their unique fluorescence lifetime that is typically independent from the fluorescence intensity [1–3]. Therefore, fluorescence lifetime imaging ophthalmoscopy can provide information about metabolic and biochemical changes in the retina and not just about already existing structural changes [4]. FLIO has the potential to be valuable in detecting early systemic metabolic conditions such as diabetes, dyslipidemia, and neurodegenerative diseases [2], as well as in diagnosing functional alterations associated with eye diseases such as age-related macular degeneration, diabetic retinopathy, and glaucoma before irreversible morphological damage occurs [3]. Fluorescence lifetime imaging ophthalmoscopy is also useful for detecting Macular Telangiectasia Type 2 [5].

So far, only several prototype devices released by Heidelberg Engineering, GmbH, Heidelberg, Germany, based on the Spectralis® system, have been used in FLIO research [6]. This instrument is regarded as the gold standard in this field and has already been described elsewhere [1, 3, 7]. The results it delivers have been proven to be reproducible [4].

Here, we present and characterize a new fluorescence lifetime imaging ophthalmoscope based on the RETImap system from Roland Consult Stasche & Finger GmbH, Brandenburg a.d. Havel, Germany. Furthermore, we compare the new device to the gold standard using cuvette measurements.

2 Methods

2.1 New device characterization

The new FLIO device is based on a confocal scanning laser ophthalmoscope (cSLO, RETI-map, Roland Consult Stasche & Finger GmbH, Brandenburg a.d. Havel, Germany). Images are captured at 7.8 fps and have a resolution of 512 by 512 pixels and a field of view of 35°. For the autofluorescence excitation, a picosecond diode laser with a wavelength of 473 nm (BDL-SMN, Becker & Hickl GmbH, Berlin, Germany) is used, having a pulse width ranging from 50 ps to 70 ps (full width at half maximum, FWHM) and a repetition rate equal to 80 MHz. A continuous wave (CW) infrared (IR) laser is used to illuminate the fundus for online image registration. The fluorescence photons are collected by a multimode fiber and transmitted to a filter (BF), blocking the excitation light. A dichroic mirror (DM) with an edge wavelength of 560 nm splits the remaining photons into two spectral channels: short (SSC, 498–560 nm) and long (LSC, 560–720 nm). For each channel, there is a detector (HPM-100-40, Becker & Hickl GmbH) connected to a time-correlated single photon counting (TCSPC) device (SPC-160, Becker & Hickl GmbH). Figure 1 shows a schematic of the described ophthalmoscope.

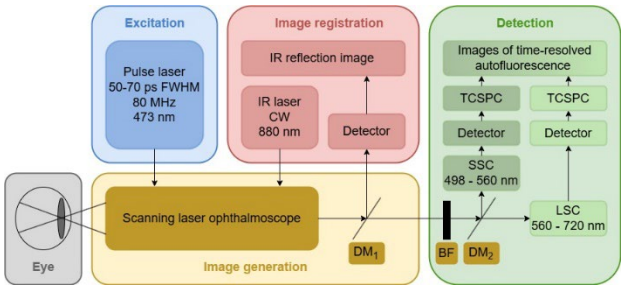

Figure 1: Schematic of the new fluorescence lifetime imaging ophthalmoscope (adapted from [3])

We determined the maximum laser power of the new device using the light meter (ILT2400, International Light Technologies, Inc. Peabody, MA, USA). Then, we analyzed the instrument's behavior at three different laser power levels: 150 µW, 200 µW, and maximum laser power, in terms of laser spectrum and instrument response function (IRF). For laser spectrum analysis, we used a spectroradiometer (CAS140CT, Instrument Systems GmbH, Munich, Germany). The instrument response function was determined using a 25 µM Eosin Y solution mixed with a 5 M solution of potassium iodide, placed in a flat cuvette (110-OS, Hellma GmbH & Co. KG, Müllheim, Germany) in front of the objective lens of the FLIO device. Fluorescence measurements of approximately 1-minute duration were performed three times for all three laser power levels. The IRF and the FWHM were calculated using FLIMX software [3].

2.2 Comparison of the devices

We determined the maximum laser power using the light meter (ILT2400, International Light Technologies, Inc., Peabody, MA, USA) in the gold standard device (101 µW) and set the same laser power in the new device. Then, we performed fluorescence lifetime measurements using four previously prepared fluorescent dyes A-D listed in Table 1. Dye A is normally used to measure the IRF in the FLIO device, and dyes B-D have fluorescence lifetimes that lie within the range of fluorescence lifetimes measured in the fundus [8]. We filled the fluorescent dyes into flat cuvettes (110-OS, Hellma GmbH & Co. KG, Müllheim, Germany) and then placed every cuvette in front of the objective lenses of the FLIO devices, setting the focus on the liquid column. Alternating between the

devices after each measurement, 20 measurements of approximately 1 to 3 minutes duration were performed for each dye, resulting in 10 measurements per device.

Table 1: Fluorescent dyes used for measurements (adapted from [8])

Cuvette	Fluorophore	FLT (ps)	Concentration and solvent (at 25 °C)
A	Eosin Y	≤10	25 µM in 5 M potassium iodide
B	Erythrosine B	89	20 µM in water
C	Eosin Y	~400	15 µM in 0.5 M potassium iodide
D	Eosin Y	1180	3.3 µM in water

We performed monoexponential fluorescence lifetime approximation using the FLIMX software and defined a rectangular region of interest (ROI) covering 1000 pixels for each measurement. We compared the mean fluorescence lifetimes calculated for ROIs for the four dyes in the two spectral channels, short and long. If the data were normally distributed, which we checked using the Shapiro-Wilk test, we tested the equality of the variances of both data samples with the F-test. Then, we checked the equality of their mean values using a t-test. When the data distribution was not normal, we compared the medians of data samples using the Mann-Whitney U test. The significance level in all tests was set to 0.05. Bonferroni correction was used to correct for multiple comparisons.

3 Results

3.1 New device characterization

The measured maximum laser power of the new device was 280 µW. The peak wavelengths of the laser spectra for the selected laser powers and the full width at half maximum of the individual spectra are shown in Table 2. The measured laser spectra are illustrated in Figure 2A.

The FWHM of the IRF in the SSC were 298.6±1.1 ps, 341.0±2.5 ps, and 347.5±6.0 ps for consecutive laser powers of 150 µW, 200 µW, and 280 µW, respectively. In the LSC, the FWHM values for these laser powers were 290.4±3.8 ps, 344.0±3.4 ps, and 358.8±1.3 ps, respectively. Results are mean ± standard deviation. Measurement results are shown in Figure 2B.

Table 2: Peak wavelengths and FWHM for different laser powers

Laser power (µW)	λ_{peak}(nm)	FWHM (ps)
150	467.6	6.86
200	467.9	6.63
280	468.0	6.34

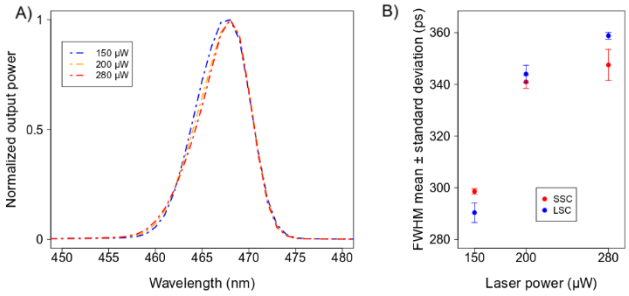

Figure 2: Instrument's behaviour analysis. A) Laser spectra for selected laser powers. B) FWHM of IRF for selected laser powers

3.2 Comparison of the devices

Table 3 shows the mean fluorescence lifetimes ± standard deviations of the ROIs for the four dyes in the two spectral channels.

Table 3: Mean fluorescence lifetime (FLT) ± standard deviation averaged over all ten measurements per device

Cuvette	Reference FLT (ps)	FLTs (ps)			
		Gold standard		New device	
		SSC	LSC	SSC	LSC
A	10	13.1±0.7	42.3±1.0	14.4±1.1	18.3±1.0
B	89	75.4±0.9	86.5±0.9	75.5±1.0	74.1±1.1
C	400	393.4±2.3	397.1±2.5	381.5±2.3	368.5±2.6
D	1180	1282.1±7.0	1258.0±7.3	1313.5±7.5	1237.8±10.2

Mann-Whitney and t-tests revealed statistically significant differences in mean lifetimes between the two FLIO devices in SSC for dye D and in LSC for all fluorescent dyes. There were no statistically significant differences in mean lifetimes in SSC for dyes A, B, and C.

Figure 3 presents coefficients of variation for each fluorescent dye in both spectral channels. The coefficients of variation for the dyes B-D were around 1%. For dye A, they had much higher values, reaching a highest value of 7.6% for the new device in the LSC.

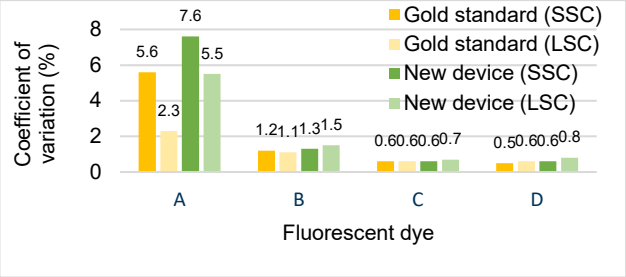

Figure 3: Coefficients of variation for both spectral channels and both FLIO devices

4 Discussion

In this work, we characterize a new fluorescent lifetime imaging ophthalmoscope and compare it with the gold standard. The obtained comparison results suggest that the devices provide different results in the majority of cases. Dye A, associated with the largest coefficient of variation, has a very short fluorescence lifetime and is used for IRF measurements. Possibly, this short lifetime is the reason for the large differences in obtained lifetimes. These different results for the new device and the gold standard for dye A are unlikely to be due to different dye samples - one sample was measured 10 times in a row, alternating between the two instruments, and then replaced by a new sample to avoid dye bleaching. The compared devices have different parameters of the individual components, and we have, therefore, tried to ensure that the initial parameters are as similar as possible. Also, the fluorescence lifetime approximation algorithm is the same (taking into account the difference in the image resolution).

The main limitation of the presented work is the small number of conducted measurements. We used four fluorescent dyes and performed 10 measurements per each device and dye, which gave us 80 measurements in total. Further steps include another comparison of the devices using fluorescent dyes with a larger number of measurements, as well as measurements with probands.

5 Conclusion

We characterized a new fluorescence lifetime imaging ophthalmoscope. The device offers a high laser power for fluorescence excitation, a large field of view, a high spatial resolution, and a sufficiently high time resolution. Thus, it is suitable for fluorescence lifetime studies.

The results of the comparison of the gold standard and the new device show that the devices differ significantly in the long spectral channel and slightly in the short spectral channel. Obtaining non-different results in less than half of the cases tested does not allow the conclusion that the results provided by the new device are compatible with those obtained from the gold standard. It is necessary to perform such a comparison on a larger number of measurements.

Author Statement

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