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IMAGING ARTIFACTS IN FLUORESCENCE LIFETIME IMAGING OPHTHALMOSCOPY

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Purpose: To investigate and quantify the influence of imaging artifacts on retinal fluorescence lifetime (FLIO) values and to provide helpful hints and tricks to avoid imaging artifacts and to improve FLIO image acquisition quality.

Methods: A systematic analysis of potential parameters influencing FLIO quality and/or fluorescence lifetime values was performed in a prospective systematic experimental imaging study in five eyes of five healthy subjects. For image acquisition, a fluorescence lifetime imaging ophthalmoscope (Heidelberg Engineering) was used. Quantitative analysis of FLIO lifetime changes due to imaging artifacts was performed.

Results: Imaging artifacts with significant influence on fluorescence lifetimes included too short image acquisition time, insufficient illumination, ocular surface problems, and image defocus. Prior use of systemic or topical fluorescein makes analysis of retinal fluorescence lifetimes impossible.

Conclusion: Awareness of possible sources of imaging artifacts is important for FLIO image acquisition and analysis. Therefore, standardized imaging and analysis procedure in FLIO is crucial for high-quality image acquisition and the possibility for systematic quantitative fluorescence lifetime analysis.

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Fluorescence lifetime imaging ophthalmoscopy (FLIO) is an imaging technique to investigate decay times of natural retinal fluorophores.[1] Retinal fluorophores are excited with a blue laser light and thereby gain a higher energy level. Within picoseconds, this gained energy is released, partially in form of light with a longer wavelength, which can be detected as autofluorescence. The mentioned time span is referred to as fluorescence decay time or fluorescence lifetime (FLT) and typically lies between 100 and 1,000 picoseconds for retinal fluorophores.[2]

One of the major fluorophores of the retina is lipofuscin within the retinal pigment epithelium[3] with a complex mixture of diretinal conjugates.[4,5] However, also other fluorophores such as melanin,[6,7] carotenoid components of the macular pigment (lutein and zeaxanthin),[1,8] connective tissue components (collagen and elastin), and possibly also redox cofactors (NAD(P)H and FAD) may contribute to the measured mean fluorescence lifetime.[7] Every fluorophore features individual excitation and emission wavelengths and an individual decay time, respectively, lifetime, which can be influenced by the local and potentially also the global environment of the fluorophore.[9,10]

Fluorescence lifetime imaging ophthalmoscopy data can be analyzed qualitatively by assessing the FLT distribution pattern. However, FLT can also be analyzed quantitatively, which allows statistical comparison of FLT within the same image, between both eyes, for follow-up examinations within the same eye, between different subjects to differentiate between healthy and diseased retina, or to investigate specific patterns of disease within a cohort of pathologies.

Previous studies investigated FLIO in healthy subjects as well as in a broad range of retinal pathologies including degenerative, hereditary, and metabolic diseases. As a noninvasive imaging procedure, FLIO provides additional

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information compared with standard imaging techniques such as optical coherence tomography, color fundus photography, and fundus autofluorescence intensity images. For example, FLIO can differentiate between newly formed flecks and older flecks in Stargardt disease.[11,12] In choroideremia, FLIO provides additional spatial resolution in areas of atrophy of the retinal pigment epithelium and allows for inference of the integrity of different retinal layers.[13] In age-related macular degeneration with geographic atrophy, areas and borders of retinal pigment epithelium atrophy can be further analyzed.[14,15] In macular telangiectasia (MacTel) Type 2, FLIO on one hand facilitates the visualization of subtle changes primarily influenced by macular pigment loss and redistribution. On the other hand, FLIO goes beyond measurement of carotenoids in patients with MacTel and provides additional information showing a unique pattern of prolonged lifetimes at the temporal side of the fovea, which appears as crescent-shaped in early stages, whereas in advanced stages, it shows a ring-like pattern.[16,17]

However, as for every imaging technique, quantitative FLIO image analysis is dependent on reproducible image acquisition. Abnormalities and changes in FLT might origin from subtle changes of retinal fluorophores; however, they might also derive from imaging artifacts. Dedicated imaging protocols have been established to standardize procedures of image acquisition and image analysis.[18] Nevertheless, FLIO features some pitfalls leading to imaging artifacts including but not limited to insufficient photon count, image illumination, camera focus, and ocular conditions. Similar as in other ophthalmic imaging techniques, imaging artifacts should be recognized, avoided, and ruled out.[19–23] Therefore, the awareness about them and the effect of individual influencing factors should be present. Well-trained photographers and clinicians/scientists for image analysis are required to allow for systematic quantitative image analysis. Otherwise, FLIO data interpretation is difficult and potentially cannot be analyzed quantitatively or compared with other images because FLT changes might also origin from imaging artifacts or differences in data analysis and interpretation.

The aim of this study was to investigate and quantitatively analyze the influence of possible sources of image acquisition artifacts in FLIO. The severity of each artifact will be discussed. An overview of suggestions to avoid or minimize FLIO imaging artifacts will be provided.

Methods

Subjects and Procedures

Systematic FLIO imaging was performed in an exemplary group of healthy eyes without lens opacifications according to a standardized protocol for clinical FLIO data acquisition (Box 1). In addition, a defined protocol was established for systematic acquisition and investigation of potential FLIO imaging artifacts within the same eye (Box 2). After FLIO imaging in nondilated and dilated pupil, the order of the subsequent measurements was variously chosen to avoid systematic imaging errors.

The FLIO study is approved by the local ethics committee and corresponds to the Declaration of Helsinki. It is registered at ClinicalTrials.gov (NCT01981148). Before FLIO imaging, all participants signed informed consent.

Box 1. FLIO Image Acquisition Protocol

1. Complete dark room environment and no light source (except dimmed computer screen).
2. Maximally dilated pupil (tropicamide 0.5% and phenylephrine hydrochloride 2.5%).
3. Correct head position and no head movement during imaging.
4. Optimal illumination of the fundus (standard 30° field of the macula).
5. Focus on small/medium vessels around the macula.
6. Use of internal fixation target if possible, alternatively external fixation light.
7. Imaging duration: minimal acquisition of 1,000 to 1,200 photons per pixel within the macular center in both spectral channels, corresponding to 2 to 3 minutes per image.

Box 2. FLIO Image Artifacts, Imaging Protocol

M1* nondilated pupil, repetition of measurement three times in a row to assess variability.
M2 dilated pupil, repetition of measurement three times in a row to assess variability.
M3 short imaging duration, 500 photons/pixel in the macular center.
M4 short imaging duration, average 200 photons/pixel for the whole image.
M5 dark edges.
M6 eye lid shadow: upper eye lid not properly opened, simulating ptosis or fatigue during measurement.
M7 bright environment light.
M8 eye movement during measurement.
M9 defocus: −2 diopters.
M10 defocus: −4 diopters.
M11 defocus: + 2 diopters.
M12 topical fluorescein.
M13 after topical fluorescein is washed out with water.
M14 shift in autofluorescence image (misalignment of detectors).
*Only M1 is imaged in miosis, M2-14 are measured in mydriasis.
The order of M3 to M11 was chosen randomly to avoid systematic errors.

Fluorescence Lifetime Imaging Ophthalmoscope

A fluorescence lifetime imaging ophthalmoscope based on a Heidelberg retina angiograph Spectralis system (Heidelberg Engineering, Germany) was used.

The excitation wavelength is set at 473 nm. The emitted fluorescence was confocally filtered and detected by time-correlated single-photon counting detectors (TCSPC; Becker & Hickl, Berlin, Germany) in a short (498–560 nm, short spectral channel [SSC]) and in a long (560–720 nm, long spectral channel [LSC]) spectral channel. This results in a cumulative fundus autofluorescence intensity image as well as a fluorescence lifetime decay trace for every pixel in the 256 × 256 pixel grid. Simultaneously, a high-contrast confocal infrared reflection image was recorded, which is used as a reference image for real time tracking of eye movements and to ensure correct spatial registration of every single detected photon.

The FLIO principles and the corresponding laser safety calculations are described in detail in previous publications.[2,11,24]

Fluorescence Lifetime Data Analysis

SPCImage Version 6.2 (Becker & Hickl, Berlin, Germany) was used for FLIO data analysis. Acquired decay times were fitted using a two exponential fitting approach resulting in a short and a long decay time (*T1* and *T2*) and their respective amplitudes ($\alpha 1$ and $\alpha 2$) corresponding to their intensities. The main outcome parameter represents an amplitude weighted mean fluorescence lifetime *Tm* and calculated as follows: $Tm = \frac{\alpha 1 \times T1 + \alpha 2 \times T2}{\alpha 1 + \alpha 2}$.

The chi-square value (χ^2) can be used as a reference value for the goodness of the applied fitting parameter where results with low chi-square generally indicate a better fitting procedure as values with high chi-square.

For dedicated ophthalmological quantitative analysis purposes, calculated FLT data were further analyzed using the "FLIO reader" software (ARTORG Center for Biomedical Engineering Research, University of Bern, Bern, Switzerland). This allows straight display of mean values ± SD for each extracted parameter (*Tm, T1, T2,* $\alpha 1$, $\alpha 2$, *photon* count, and chi-square value) for each Early Treatment Diabetic Retinopathy Study (ETDRS)[25] grid area. The main areas of interest were the macular center (1 mm diameter), the inner ring (3 mm diameter), and the outer ring (6 mm diameter).

Statistical Analysis

Fluorescence lifetime data were collected using Excel (Microsoft, Redmond, WA). Quantitative data analysis and comparison was performed using Prism GraphPad commercial software package (Prism 6; GraphPad Software, Inc, La Jolla, CA). Data were compared using a 2-tailed *t*-test with a confidence interval of 95%. *P* values below 0.05 were considered to be statistically significant.

Results

A set of 70 different FLIO images from five healthy eyes in 14 different imaging conditions according to the aforementioned imaging protocol was analyzed. The mean age of the five subjects was 33 ± 7 years with a range of 27 to 45 years. Two female and three male subjects were included, and two right and three left eyes were imaged.

Data Variability and Repeatability

In this study, the coefficient of variation within individual subjects in nondilated pupils was between 3.2% and 6.6% in the SSC and between 2.9% and 3.75% in the LSC. The highest variability was measured in the foveal center. In dilated pupils, the coefficient of variation was between 0.29% and 1.09% in the SSC and between 0.27% and 1.4% in the LSC. The coefficient of variation in this study population was between 11.13% (foveal center) and 8.01% (outer ring) in the SSC and between 8.10% (foveal center) and 7.19% (inner ring) in the LSC, respectively.

Nondilated Versus Dilated Pupil

In this case series, the difference between FLT values from nondilated to dilated pupils (M1 and M2) was between 2% and 9% (5–16 picoseconds (ps), *P* between 0.0028 for the fovea and 0.047 for the outer ring) and between 0% and 2% (1–4 ps) in the LSC (not significant) (Figure 1). The largest differences between nondilated and dilated pupils were measured in the foveal center.

Image Acquisition Time and Cumulative Photon Count Per Pixel

The mean acquisition time for a standard FLIO image with 1,000 photons per pixel in the macular center of the SSC with the slower photon count was 2 minutes 46 seconds in nondilated eyes (M1) and 1 minute 41 seconds in dilated eyes (M2). A mean photon count of 1,446 and 1,466 photons per pixel was achieved in the SSC averaged over the whole image. During this imaging period, a photon count of 1,683 and 1,772 was registered in the LSC in nondilated and dilated pupils, respectively. For a FLIO image with 500 photons per pixel in macula in the SSC, the imaging duration was 55 seconds in dilated pupils (M3) and 825 photons were acquired in

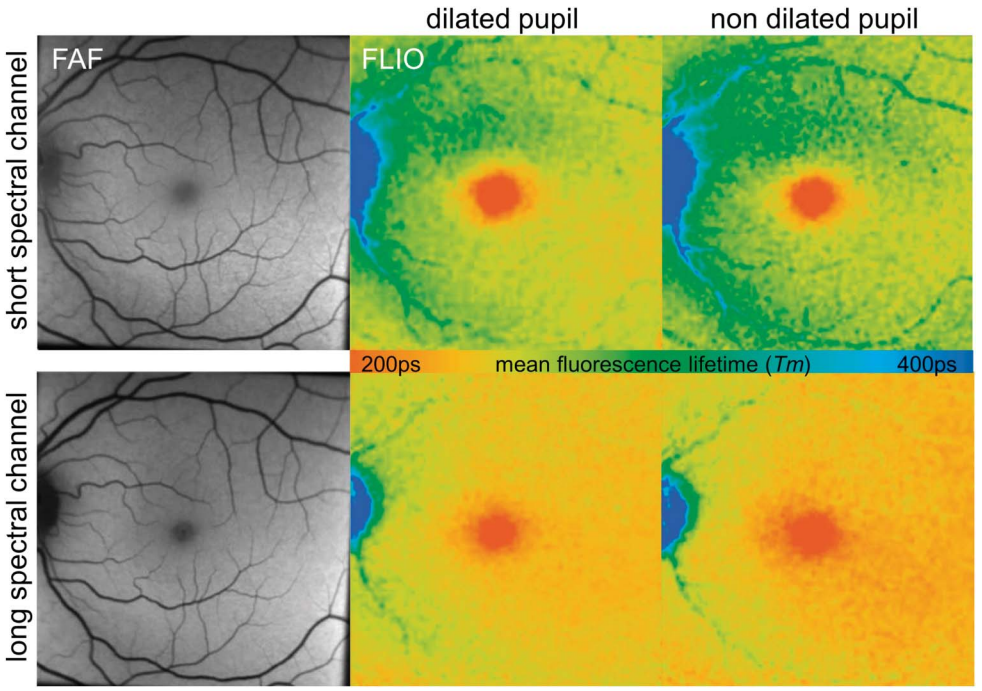

Fig. 1. Comparison of FLIO imaging in dilated vs. non-dilated pupils. Imaging in non-dilated pupils requires longer imaging time and leads to generally longer lifetimes, especially in the SSC. This difference increases with age and progressive lens opacifications that mainly influence the SSC.

average for the whole image. For an average of 200 photons per pixel (M4) for the whole FLIO image, a mean imaging duration of 14 seconds was used. In this short imaging period, an average of 130 and 193 photons were acquired in the macular center of the short and the LSC, respectively (Figure 2).

Image Illumination

Fluorescence lifetime differences of individual pixels result from variation in the fitting algorithm because of too low photon count which may result in shorter or longer lifetimes. In imaging conditions with inhomogeneous fundus illumination and dark image edges (M5, Figure 3B), especially FLT toward the periphery feature a large variation of lifetime values with mainly prolonged FLT. If no photons were detected in the insufficient illuminated edges, no mean FLT is calculated and the edges appear in black. In imaging conditions with shadow due the upper eye lid (M6, Figure 3C), large differences within the distribution map, especially the inferior part of the image was observed. FLT were prolonged by up to 18% in the SSC (+22–31 ps) and appear to be unchanged in the LSC because of a mixture of false short and false long FLT.

Environment Light

FLIO imaging in bright environmental light conditions (M7) compared with standardized condition (M2) with complete darkening of the room lead to a slight reduction of the FLT of 2% to 4% (5–11 ps). In the SSC, slightly prolonged FLTs (5.9 ps) were measured in the macular center. None of the differences was statistically significant.

Eye Movements

Eye movements during FLIO measurement (M8), including movements to the left and right, up and down, but no movement backwards with the head, lead to blurred autofluorescence intensity images as well as decreased spatial resolution in the FLIO image (M8, Figure 3D). However, quantitative FLT analysis is not significantly influenced by eye movement alone (FLT ±4%).

Camera Focus

Defocus, especially in the plus diopter range, lead to significantly prolonged FLT as well as a considerable decrease in the photon count rate, leading to a much longer image acquisition time (M9-11, Figure 4). The increase in FLT was between 7% and 30% in the SSC and 2% to 8% in the LSC, with the highest changes in the foveal center. Minus diopters lead to smaller changes with slightly shorter FLT, predominantly in the SSC (2%–7% in the inner and outer ETDRS grid ring). However, especially in the foveal center of the SSC, prolonged FLT can be measured too (+3% to 9%).

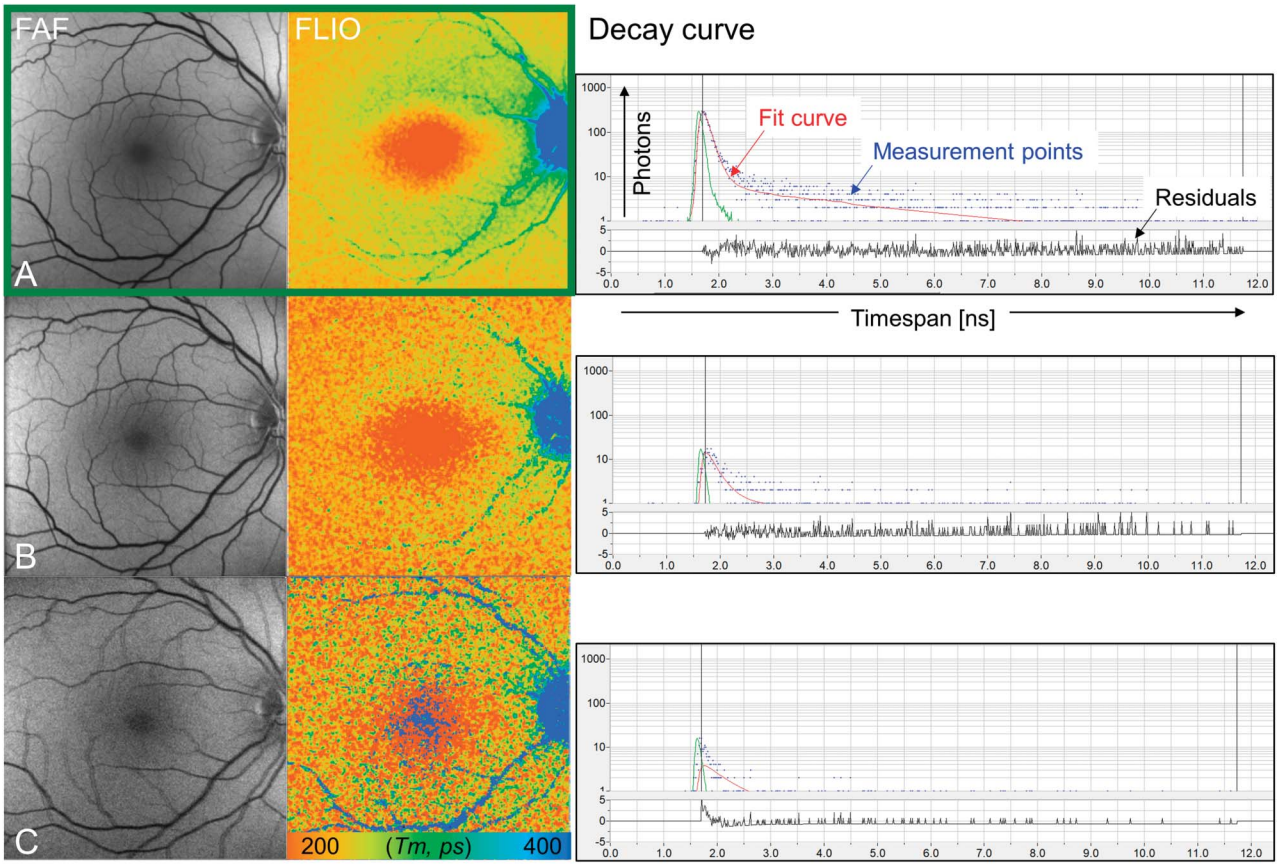

Fig. 2. Influence of a very low photon count on FLIO images. Left: autofluorescence intensity and fluorescence lifetime image, right: Corresponding decay curve of a central pixel. **A.** Reference image with a sufficient photon count (blue dots in the decay histogram) and appropriate decay curve approximation (red line). **B.** Short image acquisition with an average of 200 photons per pixel over the whole FLIO image. The decay curve on the right is calculated with significantly less photons per pixel and the decay mainly includes short decay times as the curve fitting does scarcely include long lifetimes. **C.** Acquisition time of only few seconds, leading to inappropriate curve fitting because of too few photons counted. Fitting failures appear as dots with blue (very long) lifetimes in the FLIO image.

Ocular Surface Properties

In this study, repeated measurements within the same eye revealed an increase of the measurement duration over time because of lower photon count (nondilated pupils: 3 minutes 15 seconds to 7 minutes and dilated pupils 1 minute 30 seconds to 2 minutes). However, simulating dry eyes in otherwise healthy eyes with the use of local anesthetic drops and a speculum to avoid blinking for 10 to 15 minutes did not significantly affect the measured FLT.

Fluorescein Topical and Intravenous

Topical application of fluorescein had a strong influence on retinal FLT, especially in the SSC (SSC: + 16% to 26%, LSC: + 2% to 7%). In both channels, the FLT changes were more pronounced in the foveal center (M12 + 13, Figure 5). After irrigation of the cornea and conjunctiva, FLTs are mostly normalized. However, some parameters still indicate residual influence of topical fluorescein (photon count, chi-square amplitudes) although the fluorescein cannot be detected clinically anymore.

Intravenous application of fluorescein leads to a very strong fluorescence signal and may overexert the registration capacity of the time correlated single-photon counting system (archive data). It results in a low contrast image with very long FLT between 1,500 and 3,500 ps in the SSC and between 400 and 1,500 ps in the LSC (Figure 6, example from clinical data).

Fluorescence Lifetime Imaging Ophthalmoscopy Imaging System Artifacts

Misalignment of the internal time correlated single-photon counting detectors may result in a horizontal shift in the autofluorescence intensity image (vessel doubling) and therefore also blurred FLT images (M14, Figure 7) with loss of spacial resolution; however, there was no significant change in FLT.

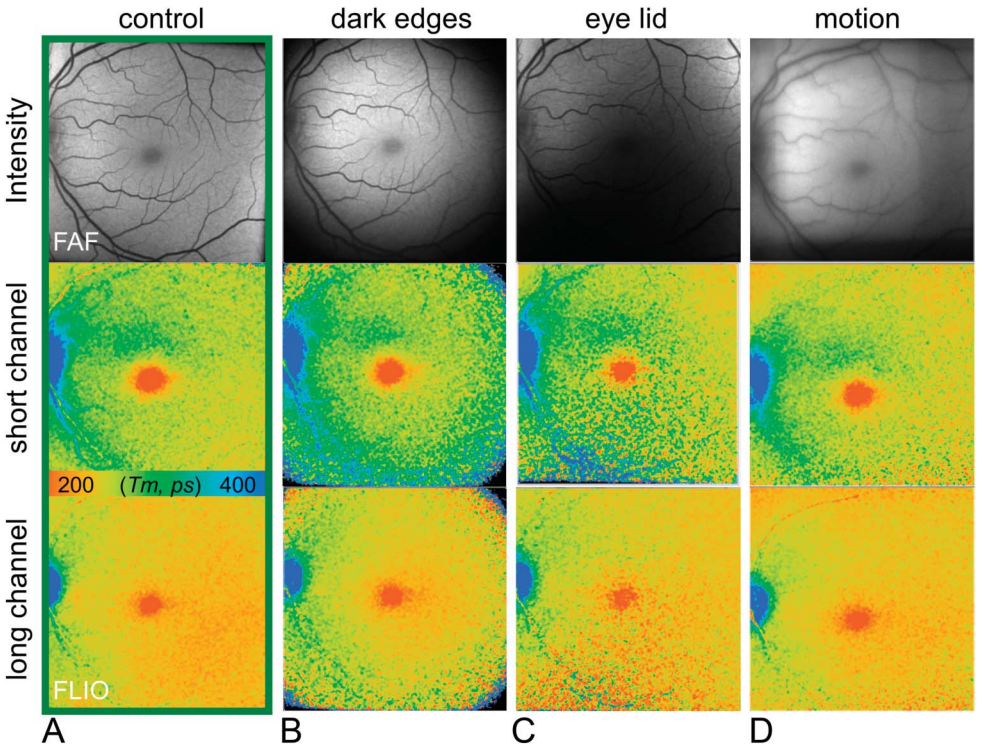

Fig. 3. Influence of image illumination and eye movement on retinal FLT. **A.** Reference image with autofluorescence intensity and fluorescence lifetime image in the SSC and LSC. **B.** Inhomogeneous fundus illumination with too dark image edges leading to prolonged lifetimes. **C.** Image artifacts due to low upper eye lid leading to a shadow on the inferior part of the image and to increased variability in FLT. **D.** Fluorescence lifetime imaging ophthalmoscopy imaging in instable fixation leading to blurred intensity images; however, the quantitative lifetime values are not significantly influenced in the macula.

Discussion

This overview of potential imaging artifacts showed a broad range from only minimal to significant changes in the measured fluorescence lifetime values. Table 1 provides an overview over the kind of artifact analyzed in this study, its cause and definition, its effect on the measured fluorescence lifetime values, and its severity and suggestions for prevention.

Data Variability and Repeatability

We already investigated the repeatability of FLIO measurements in 31 healthy subjects in 2013 and assessed the interindividual variability using the coefficient of variation in this cohort.[2] There, Spearman's ρ was 0.80 for the SSC and 0.97 for the LSC. The coefficient of variation in this previous study population was between 17% (foveal center) and 9% (outer ring) in the SSC and between 11% and 6% in the LSC, respectively. In the current study population, it was slightly lower (SSC: 11%–8%; LLC 8%–7%) probably because of a more homogeneous group of participants with a smaller age range. Similar values were described in other studies.[26,27]

Nondilated versus Dilated Pupil

In this case series, the difference between FLT values from nondilated to dilated pupils (M1 and M2) was larger for the SSC as for the LSC as described before. In our previous study from 2013, we described a mean difference of 27 ps in the SSC and 3 ps in the LSC.[2] These findings were confirmed by another study.[28] The importance of mydriasis for standardized retinal imaging was also reported for macular pigment optical density measurement.[29] To standardize imaging conditions and to avoid differences originating from the pupil size, we recommend to use dilated pupils as a standard for FLIO imaging, similar as for autofluorescence intensity imaging and for fluorescein angiography.

Image Acquisition Time and Cumulative Photon Count per Pixel

Acquisition of at least 1,000 photons per pixel throughout the whole image is recommended to ensure a sufficient photon count to allow for an appropriate fitting procedure (M2, Figure 2A). Areas with low photon count may result in an inaccurate fitting procedure as shown in Figure 2. This may lead to a general shift toward shorter mean lifetimes because of loss of long FLT values and a higher system offset (M3, Figure 2B). In addition, the risk for pixels with "fitting failures" increases, resulting in pixels with extremely prolonged FLT (M4, Figure 2C, blue pixel in the foveal center). The macular center (macular pigment), retinal vessels (connective tissue), and the optic nerve head are typical

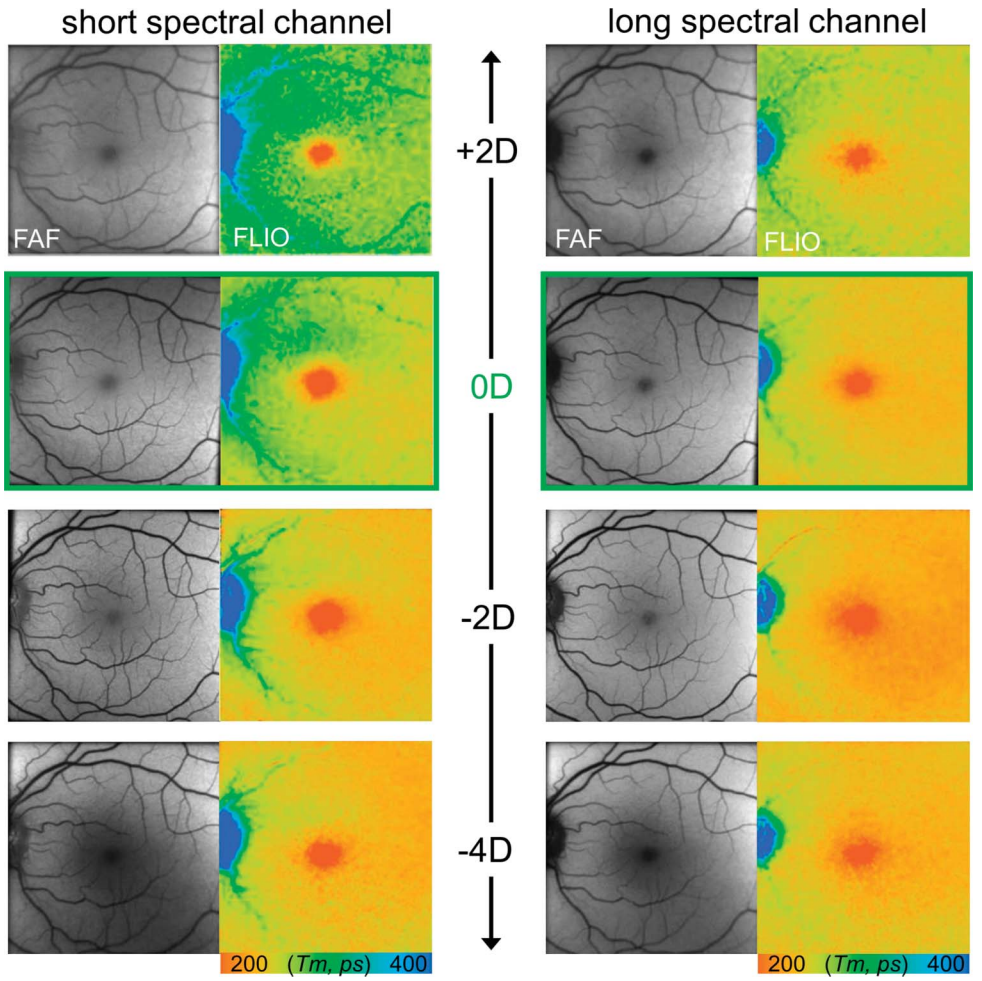

Fig. 4. Influence of image focus on retinal FLT. Plus defocus generally led to longer lifetimes, whereas minus defocus shifted the values toward shorter lifetimes.

structures with low photon count rates and appear dark in fundus autofluorescence intensity images. In addition, diseased retinal areas such as retinal pigment epithelium atrophy, scar formation, retinal deposits, and masking effects because of vitreous opacities and retinal hemorrhages may feature low photon count rates and are at risk of inadequate curve fitting when too few photons are detected in these areas.

Normally, defined regions of interest such as areas of the ETDRS grid are investigated for quantitative data analysis. Therefore, individual pixels with fitting failures normally will not significantly influence the mean value of this area. However, with increasing number of inadequately fitted pixels, this mean value per region of interest might be shifted toward long FLT, as pixels with fitting failures normally show up to ten-fold longer FLT as expected.

Therefore, collection of sufficient photons per pixel is highly recommended, especially, if the region of interest features only a low autofluorescence intensity. Individual pixels of fitting failures might be corrected manually.

Image Illumination

Similar as in other imaging modalities, proper and homogenous image illumination is crucial in FLIO. However, in FLIO in particular, FLT data—which is the main outcome parameter—might be sensitively influenced by insufficient and inhomogeneous illumination of the image section. Especially, dark edges can occur if the camera head is too close to the eye or too far away (M5, Figure 3B). In these areas, the photon count is significantly lower compared with the properly illuminated areas and the variability in FLT increases, resulting in either shortened or prolonged FLT as explained above. Similar artifacts may be found if the image is not centered, whereby one area is overexposed ("too bright") and one area is insufficiently illuminated ("too dark").

To solve this problem, camera alignment should commence far away from the eye. When the bright fundus reflex is detected, the camera can be moved slowly toward the subject, always with the brightest reflex centered on the image until the dark edges disappear.

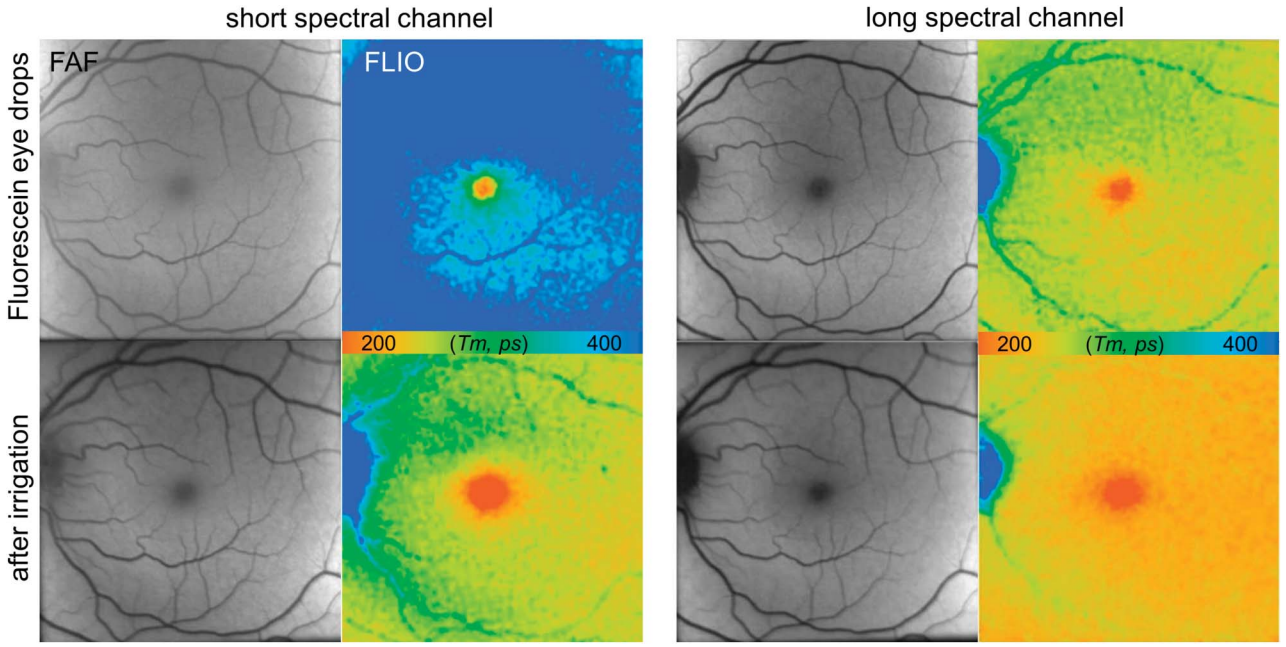

Fig. 5. Influence of topical fluorescein on retinal FLT. Topical fluorescein before FLIO imaging mainly influenced the SSC leading to a general increase in fluorescence intensity (gray shadow) and a significant prolongation of the measured lifetimes. After irrigation of the ocular surface, the lifetimes mostly normalized.

Interestingly, in our quantitative analysis using the ETDRS grid areas, changes because of inhomogeneous illumination did not substantially affect the measured mean FLT of these areas. However, if the FLT distribution of the entire image is analyzed, and/or reference areas are chosen within the periphery, illumination artifacts may have significant influence.

Another source of artifacts because of inhomogeneous illumination is insufficient eye opening, fatigue of the proband during measurement with subsequent lowering

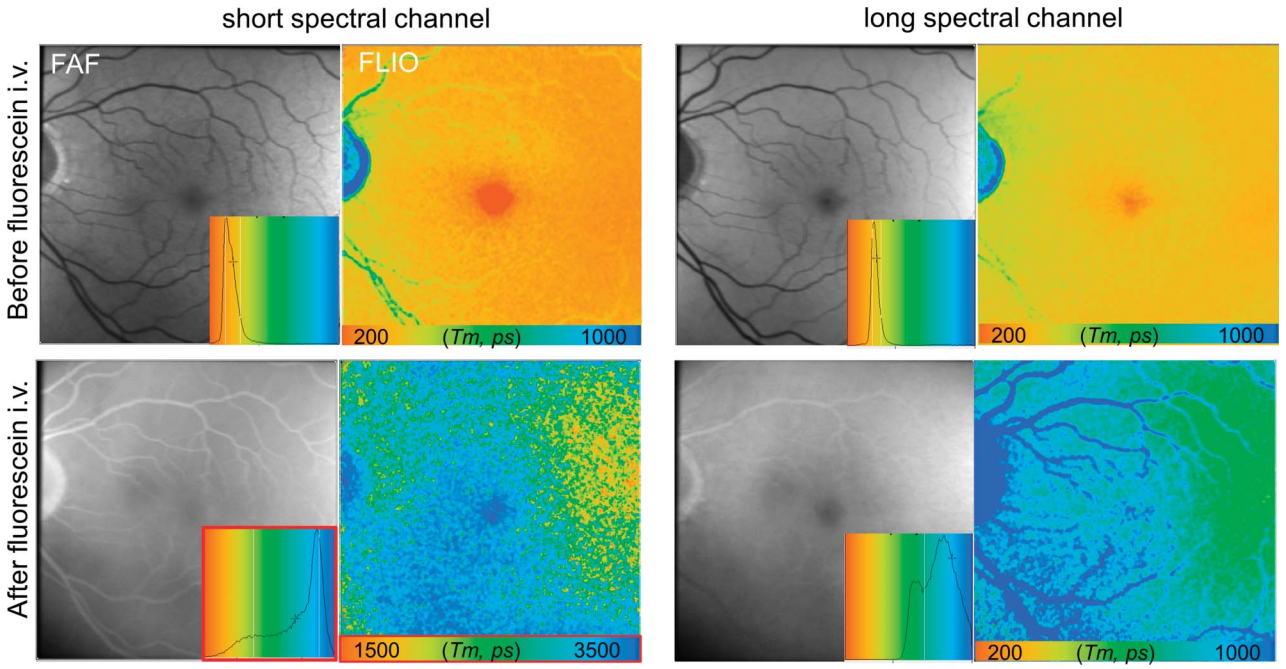

Fig. 6. Influence of systemic fluorescein on retinal FLTs. After intravenous fluorescein application, FLIO data of the retina are not evaluable any more. The distribution histogram of the calculated FLT is provided as small inlay. Note, that for the SSC, the lifetime range had to be adjusted from 200 to 1,000 ps to 1,500 to 3,500 ps.

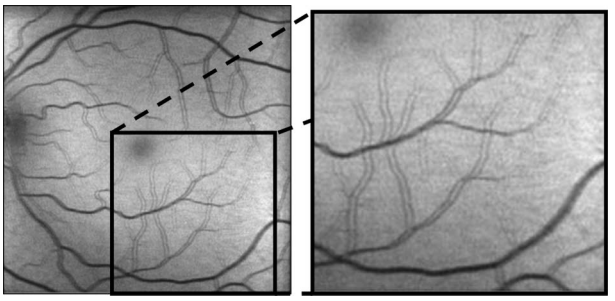

Fig. 7. Misalignment of the FLIO detectors may lead to a horizontal shift in the autofluorescence intensity image.

of the upper eyelid and ptosis (M6, Figure 3C). Upper eyelid artifacts lead to shadowing in the lower part of the image and thereby to lower photon count with a general prolongation of the FLT because of insufficient photon counts or too short FLT because of relatively too little detection of photons with long FLT.

Detailed subject instruction before the measurement is suggested as well as reminders for wide eye opening during the measurement. Blinking during the measurement—as long as the subjects open the eye afterward properly again—is explicitly encouraged to ensure a stable and homogenous tear film and therefore improve the image quality.

Environment Light

Another possible imaging artifact might origin from environment illumination and light scattering/reflection. In our study, with bright room illumination, only minor influence on the acquired FLT was measured (M7). The tendency toward shorter FLT mainly originates in a higher implicated offset value in the automatic calculation of the decay curve. Thereby, recorded long FLT components are mainly assigned to "background" scatter light and are excluded from the calculation of the mean FLT, because of the higher offset, which results in shorter FLT.

To standardize image acquisition and to minimize light scattering and in consequence increased offset values, we recommend acquiring the images in completely dark environments with a dimmed FLIO-computer screen as the only remaining light source. In addition, subjects with dilated pupils generally prefer low light conditions to avoid glare and to increase the ability for recognition of the internal fixation target.

Eye Movements

Quantitative FLT analysis was not significantly influenced by eye movement during FLIO measurement. The inbuilt infrared camera allows for very precise eye tracking. Therefore, FLIO imaging in subjects with poor fixation might be possible sometimes even in subjects with nystagmus, where other imaging modalities, such as optical coherence tomography, might fail to track the eye movement. However, eye movements lead to blurred autofluorescence intensity images as well as decreased spatial resolution in the FLIO image (M8, Figure 3D).

Camera Focus

The camera focus should be adjusted for the small-sized to medium-sized vessels around the fovea. Small changes in focus might lead to blurred autofluorescence intensity images and thereby also slightly decreased spatial resolution in FLIO images. Small defocus ($<$0.5–1 D) might not significantly influence the FLT values in our experience. However, larger amount of defocus, especially in the plus diopter range, may lead to significantly prolonged FLT as well as a considerable decrease in the photon count rate, leading to a much longer image acquisition time (M9-11, Figure 4). Minus diopters lead to only small changes with slightly shorter FLT.

By now, there can only be speculated on the reasons for the measured FLT changes and further experiments and model calculations might be advisable. In focus settings with the focal plane before or behind the retina, the excitation laser presumably does not specifically excite the expected retinal fluorophores. Still, there is excitation of fluorophores and FLT can be detected. In plus diopter defocus, the focal plane lays before the retina. Here, increased light scattering can be assumed within the vitreous body both, from the excitation laser as well as from the emitted fluorescence. According to our results, this leads to prolonged FLT. An increase in plus diopter defocus results in a decrease of the number of detected fluorophores and therefore to an increased measurement interval. Also, the FLT increase massively. On the other hand, minus diopter defocus results in a theoretical focal plane behind the retina. Thereby, probably excitation of retinal fluorophores occurs before the excitation beam reaches the calculated focal plane. Emitted photons therefore reach the detectors before they are mathematically expected and are shorter in relation to the expected instrument response rate. In this case, no significant photon scattering is expected.

Ocular Surface Problems, Cataract, and Vitreous Opacities

Ocular surface problems such as dry eye disease may also substantially affect retinal FLT. In this study, repeated measurements within the same eye led to an increase of the measurement time because of lower photon count. We recommend regular use of artificial tears between image acquisitions. Clinical experience

Table 1. Overview of artifact types

Artifact	Definition/Cause	Effect on FLT	Severity	Prevention/Solution
Pupil size	Miosis	Prolongation of FLT and SSC >> LSC	+ to +++	FLIO imaging in mydriatic pupils
Low cumulative photon count/low measurement time	Insufficient number of photons per pixel for adequate curve fitting	Fitting failures with much prolonged FLT, "dark pixels" Alternative: higher offset leading to shorter lifetimes	+ to +++	Acquisition of at least 1,000 photons/pixel also in areas with low photon count
Illumination	Camera misalignment: dark image edges	Prolonged FLT in edges, central area not severely affected	+ to +++	Uniform illumination, especially for edges of image
	Eyelid ptosis/shadow: inferior dark image	Significantly prolonged FLT (SSC > LSC)	++ to +++	Subject information before imaging, reminder for proper eye opening during imaging
	Incorrect head position: inhomogeneous illumination	Prolonged FLT	+ to +++	Subject information before imaging, control of head position during measurement
	Additional light sources	Shorter lifetimes due to higher offset	+	Complete darkening of the room and avoidance of additional light sources
Motion	Instable fixation	Blurred images, loss of contrast; FLT value barely influenced	(+) to +	Subject information before imaging, choice between internal and external fixation light, reminder for stable fixation
Defocus	Focus misalignment: blurred images	Effect SSC > LSC Small defocus (<1 D): blurred images, FLT value barely influenced Larger defocus: minus defocus: shorter FLT; plus defocus: longer FLT	(+) to ++	Focus on small to medium vessels around macula
Ocular surface	Dry eye, wet eye/tears, corneal opacifications, scar, and severe dystrophy	Prolonged FLT	+ to ++	Regular use of artificial tears between measurement
Fluorescent agent	Topical fluorescein	Prolonged FLT; SSC >> LSC	++ to ++++	No topical fluorescein before FLIO measurement (minutes 2 days)
	Fluorescence angiography	Much prolonged FLT, values not evaluable	++++	No FA/ICGA 7 days before FLIO
Detector system	Detector misalignment	Blurred images	+ to +++	Restart image
Cataract	Clinically relevant lens opacification	Prolonged FLT In severe cases retinal FLT values not evaluable	+ to ++++	Consider lens status

Overview of artifacts including their definition and cause, effect on FLT, suggestions for prevention and solution, and severity of influence to FLT. Severity grading: + light alterations, slightly blurred images, and slightly prolonged/shortened FLT possible. ++ significant alterations, fluorescence lifetime values, and lifetime distribution possibly not representative. +++ severe alterations, fluorescence lifetime values, and distribution not comparable. ++++ retinal fluorescence lifetimes and distribution not evaluable.

FA, fluorescein angiography; ICGA, indocyanine green angiography.

showed that corneal opacifications and/or dry eye conditions may have considerable effects on FLT, leading to significantly prolonged FLT. However, simulating dry eyes in otherwise healthy eyes with the use of local anesthetic drops and a speculum to avoid blinking for 10 to 15 minutes did not significantly affect the measured FLT. This result is in line with previous publications showing that oxybuprocaine drops themselves did not significantly change the tear film break up time.[30,31] However, the beforementioned studies were conducted in young healthy subjects; therefore, no direct conclusion can be drawn

for subjects with ocular surface diseases and potential instability of the tear film where shortened tear film break up time is expected. Although local anesthetic drops themselves experimentally did not directly alter fluorescence lifetime values in healthy subjects, we assume that changes in the ocular surface conditions may have a considerable effect on FLT primarily affecting subjects with preexisting surface problems.

Cataract may lead to prolonged FLT, especially in the SSC as experience and own data show. The influence of lens opacification is currently investigated in a separate study that incorporates a standardized classification system for cataract.[32] Considering the influence of the crystalline lens, we suggest to assess the lens status (e.g., clear lens, slight/advanced lens opacifications, and pseudophakic) for every FLIO measurement. There are current attempts for algorithms to correct for possible influence of the natural lens on retinal FLT, especially in subjects with significant lens opacifications.[33]

Vitreous opacities may generally lead to slightly prolonged FLT in areas of shadowing from opacities. However, as opacities may move with time, the effect on the measured FLT may vary considerably.

Fluorescein Topical and Intravenous

The topical use of fluorescein leads to prolonged FLT. Although the excitation and detection of retinal fluorescence in FLIO is designed in a confocal way, strong fluorescence from other sources than the retina may be detected as part of the mass response after fluorescence excitation. Our study shows that after irrigation of the fluorescein from the cornea and conjunctiva, FLTs are mostly normalized. However, some parameters still indicate residual influence of topical fluorescein although the fluorescein cannot be detected clinically anymore. It is important to consider that in subjects with advanced age, dry eye disease, ocular surface disease, instable tear film, or prior use of topical local anesthesia, fluorescein may remain on the surface or in the tissue for hours. Therefore, prior the use of topical fluorescein is a contraindication for FLIO imaging, and a wash out period of at least one day is recommended before FLIO imaging.

Intravenous use of fluorescein leads very long FLT mainly originating from fluorescein with no possibility to assess the component of retinal fluorescence lifetime (Figure 6, example from clinical data). Fluorescein is mostly eliminated over the kidney within 48 hours (90%). However, remaining components may be bound and hold back in different tissue, and diffusely prolonged FLT may still be detected several days after fluorescein angiography. We therefore recommend to perform FLIO images before intravenous fluorescein application or to wait with FLIO imaging at least 1 week after the angiography.

Fluorescence Lifetime Imaging Ophthalmoscopy Imaging System Artifacts

In case of detector misalignment resulting in duplication and shift in the autofluorescence intensity image, a restart of the system is needed on detection of this phenomenon.

Patients Instruction

Image quality may depend on the experience of the photographer as well as on the cooperation of the subject. Therefore, we recommend to follow a standardized imaging protocol (Box 1). To achieve the highest quality images, detailed subject instruction is an essential component before image acquisition. The subject should be informed about the importance of a stable head and gaze position (forehead and chin stable on the rest and fixation of internal [or external] fixation light) and a proper eye opening. In addition, knowledge about the imaging duration (normally 2–3 minutes) and the bright flicker lights (similar intensity as generally known autofluorescence imaging) is important as well as the recommendation for regular blinking with proper opening of the eyes afterward.

Limitations

This study is designed to summarize a broad range of possible factors influencing FLIO image quality. It includes an exemplary case series of artificially generated imaging artifacts to quantify the expected effects of individual parameters. In addition, the discussion includes a huge background of experience in FLIO imaging in healthy and diseased eye conditions. However, as expected, this study compromises several limitations. First of all, the sample size of five subjects is very small, especially for quantitative and statistical analysis. To standardize the study conditions, only healthy eyes were included. Effects of potential artifacts may be different in eye conditions such as ocular surface disease including dry eye disease, lens opacities, vitreous body opacities, retinal diseases, and possibly also systemic metabolic diseases. In addition, standardized generation of artifacts is difficult and combinations of different types of artifact are possible. In addition to the selected types of artifact, there might be other types of influences on FLT, and therefore, the mentioned selection is not final. Also, imaging techniques may differ between different photographers. In addition, hardware settings of the FLIO device may differ between different

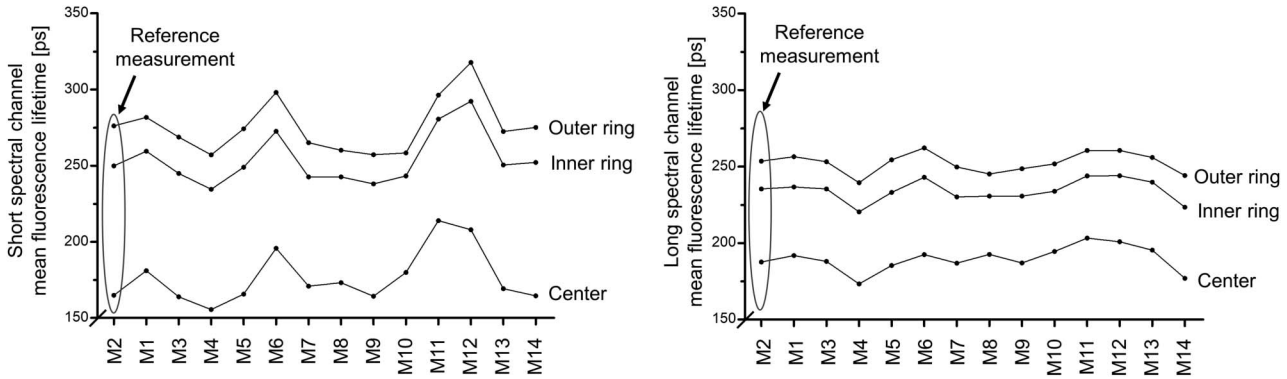

Fig. 8. Overview of the performed FLIO measurements with the measurement conditions M1-14 as described in Box 2. M2 (dilated pupil) is defined as reference value for comparison. Mean values are shown for the ETDRS grid areas center, inner and outer ring for the short (left) and long (right) spectral channel.

devices and may change over time. Finally, analysis may depend on the used software version and applied fitting parameters. Nevertheless, this article provides an overview over critical parameters which have to be taken into account independent of the numerical analysis and the software and hardware parameters.

Summary

In this study, we generated and analyzed possible artifacts and pitfalls in FLIO image acquisition. In summary, FLIO lifetime values were proven to be quite robust, especially within the area of the ETDRS grid. However, FLT toward the periphery and within the macular center generally featured a higher vulnerability for artifacts. These areas also showed the highest variability and shift toward mostly prolonged FLT (Figure 8). The largest differences in FLT were found in FLIO images after the use of topical fluorescein. Additional sources of systematic artifacts may origin from too short acquisition time with too few photon counts, uneven image illumination and considerable image defocus.

Although this study systematically illuminated and quantified potential FLIO imaging artifacts, further factors with influence on retinal FLT may exist. The experience over many years of clinical FLIO use confirms the influence of the investigated imaging artifacts and highlights the importance for structured and uniform imaging procedures.

Differences in FLT values can either origin from data acquisition or from FLT data analysis and interpretation. High-quality FLIO imaging is crucial as imaging artifacts cannot be eliminated from raw data later on.

Conclusion

The awareness about the extent of possible imaging artifacts is crucial for image acquisition, analysis, and interpretation. Standardized imaging protocols as well as image analysis procedures are essential for quantitative FLT analysis and interpretation.

Key words: fluorescence lifetimes, fundus autofluorescence, retinal imaging, FLIO, healthy eye, artifact.

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