Combination of confocal principle and aperture stop separation improves suppression of crystalline lens fluorescence in an eye model

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Abstract: Fluorescence lifetime imaging ophthalmoscopy (FLIO) is a new technique to detect changes in the human retina. The autofluorescence decay over time, generated by endogenous fluorophores, is measured in vivo. The strong autofluorescence of the crystalline lens, however, superimposes the intensity decay of the retina fluorescence, as the confocal principle is not able to suppress it sufficiently. Thus, the crystalline lens autofluorescence causes artifacts in the retinal fluorescence lifetimes determined from the intensity decays. Here, we present a new technique to suppress the autofluorescence of the crystalline lens by introducing an annular stop into the detection light path, which we call Schweitzer’s principle. The efficacy of annular stops with an outer diameter of 7 mm and inner diameters of 1 to 5 mm are analyzed in an experimental setup using a model eye based on fluorescent dyes. Compared to the confocal principle, Schweitzer’s principle with an inner diameter of 3 mm is able to reduce the simulated crystalline lens fluorescence to 4%, while 42% of the simulated retina fluorescence is preserved. Thus, we recommend the implementation of Schweitzer’s principle in scanning laser ophthalmoscopes used for fundus autofluorescence measurements, especially the FLIO device, for improved image quality.

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References and links
1. Introduction

Autofluorescence decays, generated by endogenous fluorophores in the ocular fundus, can be measured in vivo by fluorescence lifetime imaging ophthalmoscopy (FLIO). Based on the fluorescence lifetimes of the different fluorophores in the fundus, FLIO produces quantitative images that extend standard autofluorescence intensity imaging [1, 2]. The fluorescence lifetime is characteristic for each fluorophore as well its molecular environment. Thus, the fluorescence lifetime delivers additional information compared to the fluorescence intensity.

FLIO is intended to contribute to the early detection of diseases in the human eye, such as age-related macular degeneration (AMD), diabetic retinopathy, and glaucoma, before permanent morphological damage occurs. Furthermore, FLIO can be applied to basic research to understand pathological mechanisms of metabolic diseases [3]. Schweitzer et al. [4] developed FLIO, which is based on fluorescence lifetime imaging (FLIM) techniques [5] used in microscopy [6] and for in vivo tissue characterization and diagnostics [7]. In histological sections of a human donor eye, fluorescence lifetimes have been determined for retinal pigment epithelium (RPE) cells, Bruch’s membrane, as well as subretinal deposits of metabolic byproducts, e.g., drusen [8]. Furthermore, the influence of the macular pigment on the fluorescence lifetime has been investigated [9]; changes in fluorescence lifetime parameters have been found in patients with glaucoma [10], vessel occlusions [11, 12], Alzheimer’s disease [13], and Stargardt disease [14].
Figure 1 shows the fundus autofluorescence obtained with a scanning laser ophthalmoscope before and four weeks after cataract surgery and lens replacement. The preoperative image shows remarkably lower contrast than the image recorded after surgery. This can be attributed to the strong autofluorescence of the crystalline lens upon excitation at 473 nm [15]. Thus, the crystalline lens fluorescence reduces the signal-to-noise ratio (SNR) in classical fundus autofluorescence images.

Schweitzer et al. analyzed patients with diabetes without diabetic retinopathy [16] and found differences in the FLIO data between patients with phakic eyes in comparison to patients with pseudophakic eyes. The fluorescence lifetimes are shorter in the pseudophakic eyes, which is most likely caused by a contribution of the crystalline lens fluorescence in the FLIO data as the crystalline lens emits fluorescence predominantly in the short wavelength channel (498-560 nm) of the FLIO instrumentation [17]. Although the FLIO instrument is a confocal scanning laser ophthalmoscope, it is not able to suppress the autofluorescence signal of the crystalline lens entirely because of its large volume and strong autofluorescence. Especially in older patients with beginning cataract, the crystalline lens fluorescence will bias the approximated fluorescence lifetime of the retina because of its large volume and strong autofluorescence. Hence, the contribution of the crystalline lens fluorescence has to be suppressed in the FLIO data. There are three options to suppress the reflections / fluorescence of the crystalline lens that precludes fundus imaging. First, the confocal principle is already applied in FLIO but is not sufficient. Second, the aperture stop separation used in

Fig. 1. FLIO data of the short spectral channel (498 nm – 560 nm) before (left) and after (right) cataract surgery in the left eye of a 74 years old male volunteer. The improved contrast in the fluorescence intensity image after cataract surgery (B) is clearly visible. The mean fluorescence lifetime \( \tau_m \) after cataract surgery (D) is much shorter than before the surgery (C). The color scaling of the fluorescence lifetimes is identical in both images for better comparison.
fundus cameras but fundus cameras are not very well suited for autofluorescence measurements and FLIO. Both approaches are able to suppress the crystalline lens fluorescence to some extent. Third, a combination of confocal principle and aperture stop separation proposed by Schweitzer et al. [18], which we call Schweitzer’s principle. The aim of this work is to evaluate the efficacy of Schweitzer’s principle by quantifying its possible benefits in an experimental setup using a model eye based on fluorescent dyes. Furthermore, we propose parameters for an application in a scanning laser ophthalmoscope.

2. Materials and methods

2.1. Theory

The pupil of the human eye acts as aperture stop, and, thus, limits the optical flux. The corresponding entrance pupil defines the aperture angle. Widening or narrowing an aperture stop does not crop the field of view but does regulate its brightness. The aperture stop separation approach is used in fundus cameras to suppress reflections of the illumination light from the anterior segment of the eye such as the cornea and the (crystalline) lens. An annulus of light with an inner / outer diameter of 5 / 7 mm passes through the outer parts of the lens to illuminate the retina while the light reflected from retina passes through the central part of the lens, as seen in Fig. 2(A).

The confocal principle requires a point light source focused on an object. Often a laser beam in combination with an illumination pinhole is used to approximate a point light source. Scattered, reflected or fluorescence light from the illuminated spot must pass a beam splitter and a pinhole before it reaches a photodetector. This pinhole blocks light that does not originate from the focal plane, increasing contrast and reducing the depth of field. As only a small spot of the object (e.g., of the retina) is illuminated, the object must be scanned by moving the illuminated spot over the object. Thus, the image is created electronically. Confocal scanning laser ophthalmoscopes are able to suppress light reflected from the cornea and crystalline lens while imaging the retina. A schema of the confocal principle applied to the human eye is depicted in Fig. 2(B). Schweitzer et al. [18] showed that current confocal scanning laser ophthalmoscopes are not able to suppress the fluorescence of the crystalline lens sufficiently because of its large volume; the estimated suppression of the crystalline lens fluorescence may be approximately $4 \times 10^{-3}$. They proposed a combination of the confocal principle and aperture stop separation to improve the suppression of the crystalline lens fluorescence (see Fig. 2(C)). The illumination light path of the confocal scanning laser ophthalmoscope is not altered. An annular stop, optically conjugated to the pupil plane of the eye, is added to the detection light path. In comparison to the aperture stop separation used in fundus cameras (Fig. 2(A)), entrance and exit pupils are reversed for Schweitzer’s principle. The annular stop consists of a transparent annulus surrounding an opaque circular central part that blocks the directly excited fluorescence light of the crystalline lens while the fluorescence of the retina passes through the transparent outer parts of the stop.
Fig. 2. Schema of the aperture stop separation in a fundus camera (A), the confocal principle of a scanning laser ophthalmoscope (C) and Schweitzer’s principle (E). The fundus autofluorescence in a 30° field of a patient with AMD measured by a fundus camera (B; FF450, Carl Zeiss Meditec AG, Jena, Germany) using the aperture stop separation approach and by a scanning laser ophthalmoscope (D) using the confocal principle. Both approaches are able to image the fundus autofluorescence while the scanning laser ophthalmoscope delivers better contrast.
2.2. Experimental setup

An optical setup, depicted in Fig. 3, is used for the experiments. A laser (BDL-440-SMC, Becker & Hickl GmbH, Berlin, Germany) with a wavelength of 446 nm, an average power of 12 mW and a beam diameter of 0.7 mm is used as the light source. The laser beam is focused on field stop 1 (diameter 100 µm) using lens 1 (focal length 20 mm). A field stop on the order of 100 µm is often used in current scanning laser ophthalmoscopes [19]. Lens 2 (focal length 20 mm) collimates the residual light to create a point light source. The laser beam passes through aperture stop 1 (diameter 1 mm) and is reflected by a dichroic mirror (edge wavelength 495 nm) onto lens 3 (focal length 40 mm). Lens 3 is positioned such that aperture stop 1 is in its back focal plane. On the way to the model eye, the laser beam passes lens 4 (focal length 40 mm) and lens 5 (focal length 20 mm), which models the crystalline lens of the eye. The fluorescence light of the model eye with wavelengths larger than 495 nm passes through lenses 5, 4, and 3 and also through the dichroic mirror. Behind the dichroic mirror the light passes through lenses 6 and 7 (each with a focal length of 40 mm). The annular aperture stop located between lens 7 and lens 8 is required to implement Schweitzer’s principle (Fig. 2(E)). Lens 8 (focal length 20 mm), which is placed behind the aperture stop, focuses the fluorescence light on field stop 2 (diameter 100 µm) to fulfill the confocal principle. Finally, the fluorescence light is detected between 480 nm and 700 nm by a spectrometer (CAS 140CT, Instrument Systems Optische Messtechnik GmbH, München, Germany).

The fluorescence of the crystalline lens is modelled by a precision cuvette, made of special optical glass with the dimensions 52 x 12.5 x 3.5 mm³ (height x width x depth) and a light path of 1 mm (type no. 110-OS, Hellma GmbH & Co. KG, Müllheim, Germany), filled with sodium fluorescein dissolved in water (peak emission at 514 nm) and placed directly after lens 5. The retina fluorescence is modelled by a precision cuvette (type no. 110-OS, Hellma GmbH & Co. KG) filled with rhodamine B dissolved in water (peak emission at 594 nm) or by a 100 µm layer of rhodamine B dissolved in water and mixed with 2% agarose gel (peak emission at 576 nm), which is fixed between two cover slips of circa 150 µm thickness (5915100, Bresser GmbH, Rhede, Germany). The peak emission wavelengths are determined in separate measurements. The decrease of the rhodamine B peak emission wavelength of 18 nm is caused by a molecular binding of rhodamine B and agarose. The annular aperture stop is made of a non-fluorescent synthetic material by a 3D printer as shown in Fig. 4. The thickness of the material is at least 1 mm to make sure that the opaque parts do not transmit any light. Five different annular aperture stops have diameters of the inner circle \( d_i \) of 1, 2, 3, 4 and 5 mm. The outer diameter of the ring \( d_o \) is 7 mm for all annular aperture stops as this is roughly the largest achievable pupil diameter in the human eye. An aperture stop with an outer diameter of 7 mm without an inner part was also produced as a reference. A supporting structure of two small pins with a width \( d_s \) of 0.7 mm each, are required to keep the inner part in place, which was corrected mathematically according to the following equation:
\[ I_{\text{corrected}} = I_{\text{measured}} \cdot \frac{\pi \cdot d_{\text{ideal}}^2 - \pi \cdot d_{\text{ideal}}^2}{\pi \cdot d_{\text{real}}^2 - (d_{\text{d}} - d_{\text{ideal}} - d_{\text{d}}) \cdot d_{\text{real}} \cdot 4 - \pi \cdot d_{\text{real}}^2} \]  

(1)

where \( I \) is the light intensity, which is assumed to be distributed homogeneously.

Fig. 4. CAD-model of an annular aperture stop with an inner diameter of 3 mm. The length of the scale bar is 2 mm.

As the optical setup uses simple, uncorrected lenses, spherical aberrations have to be taken into account when measurements are taken with the different annular aperture stops. Thus, corrections for the focal points of each aperture stop were determined in separate measurements with a spatial resolution of 50 µm. The results, relative to a measurement without an annular aperture stop, are listed in Table 1 and used in the following experiments by placing the simulated retina at the corrected focal points.

Table 1. Properties of the annular aperture stops and the required corrections of the focal point.

<table>
<thead>
<tr>
<th>Label</th>
<th>Inner diameter (mm)</th>
<th>Outer diameter (mm)</th>
<th>Supporting structure width (mm)</th>
<th>Correction according to Eq. (1)</th>
<th>Focal point correction (µm)</th>
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</table>

2.3. Experimental design

2.3.1. Preface

To demonstrate the effect of combining the confocal principle and aperture stop separation, four experiments are performed. Because of the low suppression of the simulated crystalline lens fluorescence in case of the aperture stop separation, the signal-to-noise ratio of the simulated retina fluorescence is very low. Therefore, a higher concentration of rhodamine B is used for experiments 1 and 2 than for experiment 4. Experiment 3 is performed with both rhodamine B concentrations to allow for comparability of the measurements. Consequently, all measurements with a certain rhodamine B concentration are normalized to experiment 3, as the confocal principle is the current gold standard for scanning laser ophthalmoscopes.
2.3.2. Experiment 1: no suppression of the crystalline lens fluorescence

The motivation of the experiment is to obtain a baseline for the subsequent experiments. This measurement will serve as a reference point to quantify the suppression of the simulated crystalline lens fluorescence by the confocal principle, the aperture stop separation and the combination of both. Therefore, field stop 2 is removed from the optical setup described above and the annular aperture stop is replaced by a regular aperture stop with a diameter of 7 mm. Thus, neither confocal principle nor aperture stop separation are in effect. Fluorophore concentrations of 0.01 mM for sodium fluorescein and 2 mM for rhodamine B are used.

2.3.3. Experiment 2: aperture stop separation only

To quantify the effect of the aperture stop separation, field stop 2 is removed from the optical setup described above. Thus, only the aperture stop separation is in effect. One measurement is taken for each annular aperture stop. Fluorophore concentrations of 0.01 mM for sodium fluorescein and 2 mM for rhodamine B are used.

2.3.4. Experiment 3: confocal principle only

To quantify the effect of the confocal principle, field stop 2 is placed in the optical setup described above. The annular aperture stop is replaced by a regular aperture stop with a diameter of 7 mm. Thus, only the confocal principle is in effect. Fluorophore concentrations of 0.01 mM for sodium fluorescein and 2 mM as well as 0.1 mM for rhodamine B are used.

2.3.5. Experiment 4: combination of confocal principle and aperture stop separation

To quantify the effect of combining the confocal principle and aperture stop separation, the optical setup is used as described above with field stop 2 in place. Thus, both the confocal principle and aperture stop separation, i.e., Schweitzer’s principle are in effect. One measurement is taken for each annular aperture stop. Fluorophore concentrations of 0.01 mM for sodium fluorescein and 0.1 mM for rhodamine B are used, which is sufficient for acceptable SNR levels.

2.4. Data analysis

In each measurement, a spectrum is recorded between 480 nm and 700 nm with an integration time of 5 seconds, averaged over 5 repetitions. The spectra are filtered with a 5 nm wide pseudo-Gaussian filter to reduce noise and artifacts. From those spectra, the intensity at the peak emission wavelength of sodium fluorescein is used for further analysis as the simulated crystalline lens fluorescence. A sodium fluorescein spectrum from a separate measurement is scaled to sodium fluorescein’s peak emission and subtracted from each spectrum to extract the rhodamine B contribution at its peak emission for further analysis as the simulated retina fluorescence. The simulated retina fluorescence is corrected for the influence of the supporting structures and manufacturing variability of the annular stops according to Eq. (1). The suppressions of the simulated crystalline lens fluorescence and simulated retina fluorescence are computed on the normalized values in comparison to experiment 1. To assess the suppression efficiency, the ratio between suppression of the simulated crystalline lens fluorescence and suppression of simulated retina fluorescence is computed, which we call the ratio of suppressions. For an application of Schweitzer’s principle in a scanning laser ophthalmoscope, not only is the best ratio of suppressions important to achieve a sufficient image quality, i.e., signal-to-noise ratio but also the remaining retina fluorescence at the detector. Thus, the weighted ratio of suppressions is calculated by multiplying the ratio of suppressions with the retina fluorescence. The Michelson contrast, \( mc \) [20], is used to quantify the ratio of simulated crystalline lens and simulated retina fluorescence:
\[ mc = \frac{I_{\text{retina}} - I_{\text{lens}}}{I_{\text{retina}} + I_{\text{lens}}} \]  

Normalized values are used for the intensity of the simulated retina \( I_{\text{retina}} \) and the simulated crystalline lens \( I_{\text{lens}} \), which results in \(-1 \leq mc \leq 1\).

3. Results

The spectrally resolved measurements are shown in Fig. 5 (A: experiments 1 and 2; B: experiments 3 and 4). The reduction of sodium fluorescein, which simulates crystalline lens fluorescence, caused by the annular stops with varying inner diameters is clearly visible. The simulated retina fluorescence, modeled by rhodamine B, is reduced to a lesser degree.

Based on the extracted fluorescence intensities at the peak emission for both fluorophores from Fig. 5, the intensities for the simulated crystalline lens fluorescence and simulated retina fluorescence normalized to the confocal principle are depicted in Fig. 6. The normalization allows for a direct quantification of the effects of Schweitzer’s principle in comparison to the current state of the art. In addition, the suppressions of the simulated crystalline lens and simulated retina fluorescence in relation to the unsuppressed measurements (experiment 1) are also shown in Fig. 6.
Fig. 6. Extracted fluorescence intensities for simulated crystalline lens and simulated retina for all measurements, normalized to the confocal principle. This normalization allows for a direct quantification of the benefits and costs of Schweitzer’s principle. For example, the best compromise we propose is Schweitzer’s principle with an inner diameter of 3 mm, which reduces the simulated crystalline lens fluorescence to 4% while preserving 42% of the simulated retina fluorescence. The suppressions of simulated crystalline lens fluorescence and simulated retina fluorescence are given in dark green and red. The plots are on a logarithmic scale, values are rounded.

The confocal principle reduces the simulated crystalline lens fluorescence by a factor of 1064, while the simulated retina fluorescence is reduced only by a factor of 3.4. Schweitzer’s principle can improve the reduction of simulated crystalline lens fluorescence. For example, Schweitzer’s principle with an inner diameter of 1 mm reduces the simulated crystalline lens fluorescence to 17% while preserving 78% of the simulated retina fluorescence.

The ratio between the suppression of the simulated crystalline lens fluorescence and the suppression of the simulated retina is shown in Fig. 7. The ratio rises with increasing inner diameter of the annular stop until the best ratio of 3547 is achieved by Schweitzer’s principle with an inner diameter of 4 mm. The ratio of Schweitzer’s principle with an inner diameter of $\geq 3$ mm is at least one order of magnitude larger than the confocal principle. The aperture stop separation only achieves ratios that are about one order of magnitude lower than the confocal principle. Fig. 7 also shows the Michelson contrast between the simulated crystalline lens and the simulated retina fluorescence based on data from Fig. 6. For the aperture stop separation, the normalized simulated crystalline lens fluorescence is larger than the simulated retina fluorescence and therefore causes a negative Michelson contrast. Because the fluorescence intensities are normalized to the confocal principle, its Michelson contrast is zero. Similar to the ratio of suppressions, the Michelson contrast rises for Schweitzer’s principle as the inner diameter increases until the largest Michelson contrast is achieved at 4 mm.
Fig. 7. Ratio between suppression of simulated crystalline lens fluorescence and suppression of simulated retina fluorescence (ratio of suppressions, black) and Michelson contrast between fluorescence intensities of the simulated crystalline lens and simulated retina (blue). The highest ratio and the highest Michelson contrast are achieved for Schweitzer’s principle with an inner diameter of 4 mm.

The weighted ratio of suppression (Table 2) is a measure of the compromise between the suppression of the crystalline lens fluorescence and the remaining retina fluorescence. The largest value is attained by Schweitzer’s principle with an inner diameter of 3 mm. Inner diameters larger than 3 mm achieve considerably lower values because of their high suppression of the retina fluorescence. In contrast, although the retina fluorescence of the confocal principle is high, it achieves only a low value in the weighted ratio of suppression because of its low suppression of the crystalline lens fluorescence.

Table 2. Ratios between the suppression of the simulated crystalline lens fluorescence and the suppression of the simulated retina multiplied by the fluorescence intensity of the simulated retina for confocal principle and Schweitzer’s principle. The largest value is the best compromise between suppressing the simulated crystalline lens fluorescence and the remaining simulated retina fluorescence.

<table>
<thead>
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<th>Method to suppress the crystalline lens fluorescence</th>
<th>Weighted ratio of suppressions</th>
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<tr>
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<tr>
<td>Schweitzer's principle (d₁ = 2 mm)</td>
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<tr>
<td>Schweitzer's principle (d₁ = 3 mm)</td>
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<td>Schweitzer's principle (d₁ = 4 mm)</td>
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<tr>
<td>Schweitzer's principle (d₁ = 5 mm)</td>
<td>195</td>
</tr>
</tbody>
</table>

4. Discussion

In this work, the efficacy of suppressing the crystalline lens fluorescence while preserving as much retina fluorescence as possible was investigated in a model eye for the confocal principle, the aperture stop separation and the combination of both, called the Schweitzer’s principle. All three approaches are able to reduce the simulated crystalline lens fluorescence significantly. The highest values for the ratio between the suppression of the simulated crystalline lens fluorescence and the suppression of the simulated retina, as well as for the Michelson contrast, are observed for the Schweitzer’s principle with an inner diameter of 4 mm (Fig. 7), which reduces the simulated crystalline lens fluorescence to 1.8% and the simulated retina fluorescence to circa 20.4% compared to the confocal principle (Fig. 6).
increase in both metrics seems negligible in comparison to Schweitzer’s principle with an inner diameter of 3 mm, as this already reduces the simulated crystalline lens fluorescence to approximately 4% and the simulated retina fluorescence only to approximately 42% compared to the confocal principle. This can also be seen in Table 2. Therefore, we show that Schweitzer’s principle with an inner diameter of 3 mm is the best compromise between artifact reduction and increased acquisition time required to compensate for the loss of retina fluorescence. In case of the FLIO instrument, the required increase in acquisition time may partly be compensated for by an increased power of the excitation laser; in current FLIO measurements, the power of excitation laser often has to be attenuated to prevent an overload of the fluorescence detectors.

The optical setup is intended to be as close to a confocal scanning laser ophthalmoscope as possible. Therefore, diameters of the field stops, focal lengths of the lenses of the simulated eye and the outer diameter of the annular stops are chosen accordingly. The effect of the supporting structures of the annular aperture stops is rather large for the smaller inner diameters. This could be avoided by using annular stops made of a glass pane with a superimposed opaque layer. Disadvantages are that the glass pane introduces another optical layer to the light path, which may cause reflections and higher cost.

The fluorophores used in this work simulate the crystalline lens and the retina according to their fluorescence spectra. The artifacts caused by the crystalline lens fluorescence (Fig. 1) are known in the FLIO as well as in the quantitative fluorescence intensity imaging communities and its intensity levels in patients vary with age and pathologies. Consequently, there are no fixed fluorescence intensity levels which can be modeled. Due to the required spectra measurements, we used detectors in this work, which are not as sensitive as FLIO’s single photon detectors. Based on these arguments, the quantification of Schweitzer’s principle’s efficacy in suppressing the crystalline lens fluorescence is based on normalized data (Fig. 6) and the fluorophore concentrations are chosen for best signal-to-noise ratio instead of mimicking the autofluorescence intensity levels during *in vivo* measurements.

A software package called FLIM Explorer (FLIMX) offers an approach to correct for the influence of the crystalline lens fluorescence on the approximated fluorescence lifetime of the retina, based on a separate crystalline lens measurement [17]. Although the approach in [17] may correct for the crystalline lens fluorescence, it requires an additional measurement, which involves extra time, causes stress for the patients and introduces further uncertainty to the FLIO method. Thus, it is advisable to reduce the influence of the crystalline lens fluorescence directly during measurements by an appropriate optical arrangement.

Schweitzer’s principle can help to improve short-wavelength autofluorescence (SW-AF) imaging such as FLIO by further suppressing artifacts caused by the crystalline lens fluorescence. Another option is near-infrared autofluorescence imaging (NIR-AF) [21–23] as the crystalline lens fluorescence is not excited by near-infrared light. SW-AF originates to a large extent from lipofuscin and most of the fluorophores, which characterize the cellular metabolism, have excitation spectra in the short-wavelength range [3, 24], comparable to the excitation spectrum of the crystalline lens. NIR-AF mostly originates from melanin and related compounds in the RPE and in the choroid [25]. Thus, NIR-AF may provide additional information, e.g. about the process preceding the formation of SW-AF fluorophores in retinitis pigmentosa [26], but it is not a replacement for SW-AF.

5. Conclusion

An annular aperture stop with an inner diameter of 3 mm in combination with the confocal principle provides the best compromise between crystalline lens suppression and preservation of the retina fluorescence in our experiments. The considerable reduction of artifacts caused by the crystalline lens fluorescence outweighs the approximately doubled acquisition time, particularly in older patients, because fundus fluorescence is then correctly measured with less interference from fluorescence of the crystalline lens. We recommend the integration of
Schweitzer's principle into scanning laser ophthalmoscopes used for imaging the autofluorescence of the human retina, especially the FLIO instrument, and confirmation of the findings of this work in volunteers.

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