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Online autofluorescence measurements during selective RPE laser treatment

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Abstract *Background:* Fundus autofluorescence (AF) is derived from the lipofuscin contained by the retinal pigment epithelial cells. Using a scanning laser ophthalmoscope, two-dimensional AF measurements of the ocular fundus can be achieved. Directly after conventional photocoagulation and also after selective RPE laser treatment (SRT) with ophthalmoscopically non-visible laser lesions, irradiated areas reveal reduced AF, indicating RPE damage. Since the green treatment laser beam could also be used for AF excitation, the aim of this study was to evaluate whether absolute measurements of AF can be performed, and also possible changes in AF detected, online during SRT. *Methods:* SRT was carried out by use of a frequency-doubled Nd:YLF laser (wavelength 527 nm, pulse duration 1.7 μs, repetition rate 500 and 100 Hz, number of pulses 100 and 30, single pulse energy 50–130 μJ) in vitro (porcine RPE; retinal spot size 160 μm) and during patient treatment (retinal spot size 176 μm). During irradiation, fluorescence light from the RPE was decoupled from the laser light inside the slit lamp and detected by a photomultiplier or photodiode at wavelengths above 550 nm. Additionally, temperature-dependent fluorescence intensity measurements of A2-E, the main fluorescent component of lipofuscin, were performed in a different in-vitro setup. *Results:* The intensity of AF decreased over the number of applied pulses during laser irradiation, and this trend was more pronounced in porcine RPE samples than during human treatment. In vitro, the AF intensity decreased by about 22%; however, only a weak signal was detected. When treating patients, the AF intensity was strong and the rate of decay of fluorescence intensity with number of pulses was greater when irradiating at 500 Hz than at the 100 Hz repetition rate. However, for both repetition rates the AF decay was merely up to 6–8% over the number of pulses per laser spot. Fluorescence intensity of A2-E decreased linearly with increasing temperature at about 1% per 1°C and was completely reversible. *Conclusions:* Online measurements of AF during selective RPE laser treatment are possible and reveal a decay in AF as a function of the number of laser pulses applied to the RPE. If A2-E results can be transferred to RPE fluorescence, the AF decay could be related to the temperature increase within the tissue during treatment. Further clinical studies—in SRT as well as in conventional laser photocoagulation—might be able to show online AF changes on different areas of the retina and on different pathologies. Due to the temperature dependence of the fluorescence, on-line AF measurements during laser treatments such as photocoagulation or TTT may be able to be used as a real-time method for temperature monitoring.

Introduction

The recently developed technique of imaging autofluorescence (AF) is a non-invasive diagnostic tool and might be helpful in imaging laser lesions. AF was shown to be derived mainly from the lipofuscin within the retinal pigment epithelium (RPE); [3, 24]. Lipofuscin accumulates as a byproduct of phagocytosis of the photoreceptor outer segments in the RPE cells [3]. It contains numerous biomolecules, including proteins and lipids [20]. The main fluorophore of lipofuscin—especially in the RPE—is the Schiff base reaction product A2-E (*N*-retinylidene-*N*-retinylethanolamine), which was recently identified and synthesized [5, 6, 11]. This compound possesses toxic properties which are mediated via various pathways, e.g., inhibition of lysosomal degradative functions in RPE cells [10, 19]. However, also other fluorophores of the lipofuscin complex or other fluorophores such as FAD or flavines, which have lower fluorescence intensity, might also contribute to AF [21].

With a confocal laser scanning ophthalmoscope it is possible to illuminate the fundus (excitation at 488 nm) and to record the AF (filter above 500 nm); [2, 3, 23, 24]. It was found that non-invasive AF measurements after conventional laser photocoagulation using the Heidelberg Retina Angiograph (HRA, Heidelberg Engineering, Germany) revealed a decay of the AF intensity in the irradiated areas [7]. In selective RPE laser treatment (SRT), irradiation is applied using repetitive microsecond pulses, leading to a selective damage of the RPE cells due to the high amount of melanosomes in the RPE [9], but with sparing of the photoreceptor layer [12, 13, 14, 15, 16]. Several macular diseases are thought to be associated only with a declined function of the RPE cells [12]. Therefore selective treatment of the RPE cells without causing adverse effects to choroid and neuroretina, followed by coverage of the RPE defect due to migration and proliferation of the bystander cells [4, 22, 25], seems to be an appropriate method [12]. This RPE reaction is thought to lead to a therapeutic effect, for example, in resolution of diabetic macular edema. Using the AF measurements with the laser scanning ophthalmoscope after selective RPE laser treatment, non-invasive visualization of the ophthalmoscopically non-visible laser lesions [14, 13, 16] was possible, revealing a decay of the AF intensity in the irradiated areas comparable to the results of conventional laser photocoagulation. Thus, no invasive fluorescein angiography to confirm laser success by RPE leakage is required [8].

Since irradiation in conventional laser photocoagulation as well as in SRT is with a laser at a green wavelength (within the excitation spectrum of AF [2, 3]), it might be possible to both excite AF and to measure its intensity online during the laser treatment. Thus, possible changes in AF during irradiation could be recorded. It was the aim of this study to establish a slit lamp-adapted device for online measurement of AF during selective RPE laser irradiation, for both in vitro experiments (porcine RPE sheets) and in human treatments. We also describe the potential changes in AF related to the laser impact on the RPE.

Materials and methods

Laser

An arc-lamp excited, intracavity frequency-doubled Nd:YLF laser (Quatronix, model 527DP-H) was modified with an active-feedback electro-optical Q-switched system to generate pulses of up to several microseconds duration, at a wavelength of 527 nm [1]. Pulses of 1.7 µs duration were used throughout the study. The output pulse energy was transmitted by a 105 µm core diameter fiber (Ceram Optec, Optran UV-A 105/125/250, NA=0.1), and the length of the fiber was 50 m in order to minimize spatial intensity modulation due to speckle formation at the distal fiber tip. This fiber was directly coupled to the slit-lamp fiber (Zeiss, diameter 160 µm, NA=0.1).

AF measurements during treatment

The wavelength of the Nd:YLF laser (527 nm) is near the fluorescence excitation maximum of the ocular fundus [2, 3]. This enables the treatment laser pulse to simultaneously provide fluorescence excitation. The fluorescence from the RPE, which is radiated back into the slit lamp (30 SL/L, Zeiss, Oberkochen, Germany) is decoupled from the laser light by a dichroic beamsplitter (Schott, KV550). This beamsplitter is placed in an additional module between the fiber coupler and the slit lamp (Fig. 1). The beamsplitter was created such that the fluorescence light between 570 nm and 750 nm was coupled out at 100% and detected by a photodiode or a photomultiplier (Heidelberg Instruments / Hamamatsu, Type R1463), whereas laser light at 527 nm was transmitted at about 80%. In place of the photodiode, a photomultiplier could also be coupled to the slit lamp. This was used for in-vitro measurements on porcine RPE. Since it is known that lipofuscin accumulates with age, it is expected that porcine RPE contains just a

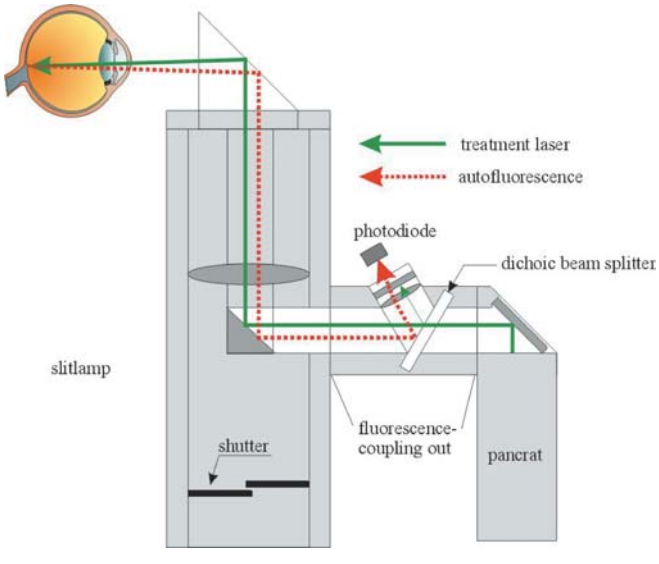

Fig. 1 Setup of the fluorescence detection device at the slit lamp

small amount of lipofuscin due to the short lifetime of feeding pigs. Thus AF intensity was expected to be much smaller in the in-vitro experiments, therefore requiring these measurements to be performed with the more sensitive photomultiplier. To reject the illumination light during the laser pulse, a shutter closes the illumination path inside the slit lamp. Data were recorded using a transient recorder (Sony / Tek, RTD710) and processed on a PC.

Treatment modalities

For the in-vitro measurements, fresh porcine RPE sheets (n=7) were used. After enucleation, globes were placed into cold, sterile NaCl solution. Under these conditions, RPE cells stay alive for about 5 h. Preparation and irradiation was performed within this time frame. Eyes were cut at the equator and adherent vitreous removed. From the posterior segment, a sample size of about 0.5 cm^2 was prepared. Neurosensory retina was removed; thus, the sample finally consisted of sclera, choroid and RPE as the superficial layer. RPE samples were then irradiated with pulse energies from 20 µJ to 40 µJ (repetition rate: 500 Hz, number of pulses: n=100, spot diameter 160 µm). AF intensity was measured over the number of the applied laser pulses and its value calibrated to each laser pulse energy, measured by the photodiode.

During patient treatment for diabetic maculopathy and central serous retinopathy (standardized focal laser treatment, n=30), irradiation took place using the following parameter sets: 100 pulses at 500 Hz repetition rate (n=10), and 30 pulses at 100 Hz repetition rate (n=20). All patients signed informed consent on the prospective nature of the study. AF was measured in the same manner as described above. Since the laser lesions are ophthalmoscopically invisible and interindividual variation of fundus pigmentation is known to exist, individual dosimetry was necessary in each patient to determine the RPE damage threshold. This was achieved by applying five to seven laser spots with increasing energy at the lower vessel arcade, and then treatment was performed with energies below the ophthalmoscopic threshold, using stable energies of about 100–120 µJ.

A2-E temperature-dependent fluorescence

A2-E shows similar broad excitation- and emission spectra as AF measurements in vivo. As described previously, lipofuscin can be excited between 450 nm and 540 nm and the emission can be detected between 500 nm and 780 nm [2]. Measurements of temperature-related fluorescence intensity changes of A2-E, which was dissolved in methanol (1.22 mM) and diluted in DMSO (1 µM), were performed (fluorescence-spectrometer SPEX2, Jobim Instruments) using the excitation wavelength of 467 nm (FWHM ±2 nm) with an excitation power of 0.87 µW. The emission was measured at a wavelength of 632 nm (FWHM ±10 nm). For the measurements a temperature-controlled cuvette holder was inserted into the sample chamber. The temperature of this holder was continuously adjusted between 20°C and 75°C. The sample temperature was measured with a thermal element (type J) in the cuvette. The temperature course as well as the corresponding fluorescence intensity of A2-E was recorded with a PC during measurement and a linear regression curve could be calculated.

Results

AF measurements in vitro on porcine RPE sheets

AF intensity was measured as a function of the number of pulses applied to the RPE. Irradiation took place with different energies ranging from 20 µJ to 40 µJ. For all pulse energies used in this trial, a decay of AF intensity was present as the number of pulses was increased to 100 (Fig. 2). On application of the higher pulse energies, AF intensity decreased by up to 22%; however, absolute AF intensity during irradiation appeared to be weak. To demonstrate RPE cell death after irradiation, specimens were stained with the vitality marker CalzeinAM, which revealed dead cells at the site of laser irradiation, indicating RPE damage. However, when also irradiating at energy levels below the RPE damage threshold, a distinct AF decay was observable, which might hint at non-dependence of AF decay and RPE damage.

AF measurements during patient treatment

Due to the high amount of lipofuscin in human eyes and the high excitation energy, fluorescence light could be detected in these measurements with a photodiode. Due to the different amount of energy applied to the tissue according to the different laser parameters (500 Hz, 100 repetitive pulses; and 100 Hz, 30 repetitive pulses), the temperature increase within the tissue is expected to be different immediately after treatment. In particular, tissue temperatures with the 500 Hz irradiation appear to be higher than with 100 Hz irradiation [17].

AF measurements at 500 Hz repetition rate

Ten patients were treated (500 Hz, 100 pulses) in whom retinal AF was measured online during laser application.

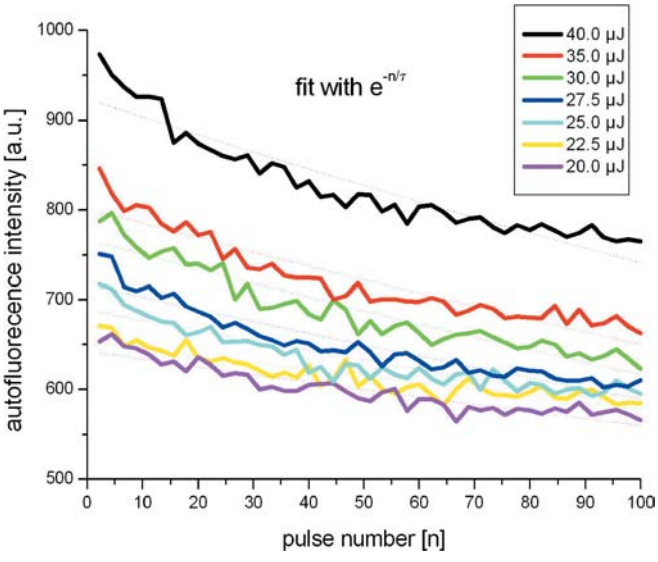

Fig. 2 Standardized AF intensity over the number of applied laser pulses for various pulse energies in vitro. Fluorescence intensity decreased over the number of pulses (down to 22%)

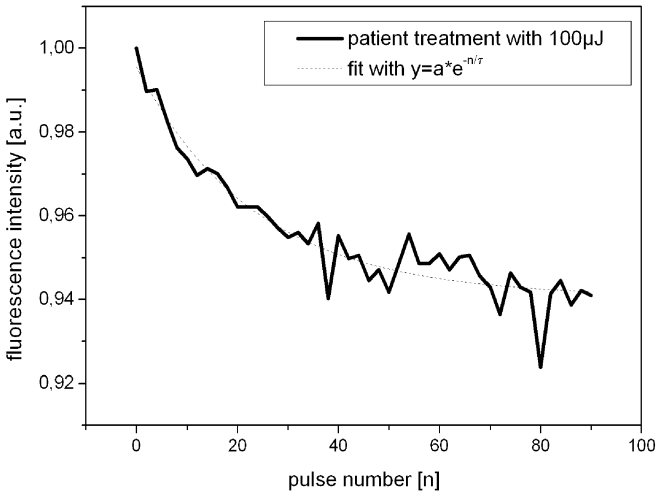

Fig. 3 In vivo AF intensity in one patient over the number of applied laser pulses at a repetition rate of 500 Hz and 100 µJ pulse energy. There was a decay of about 6% of the fluorescence intensity over the number of pulses applied. The steep decays found at 40 and 80 pulses might indicate microbubble formation and cell damage

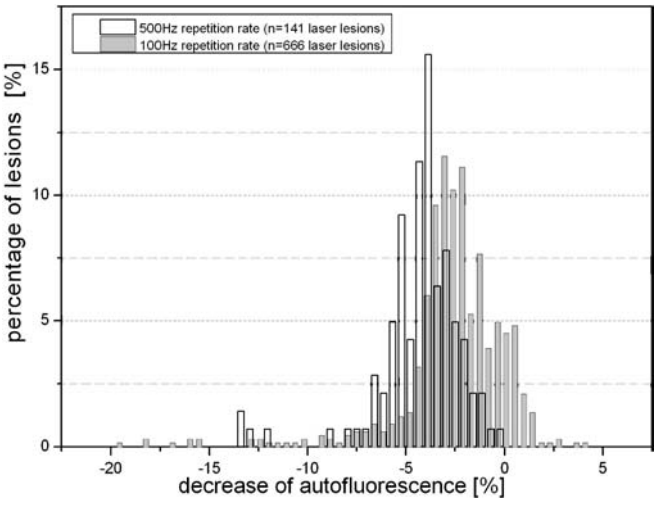

Fig. 4 Distribution of percentage reduction of AF intensity decay during laser irradiation with repetition rates of 100 Hz (50–160 µJ, 30 pulses, 20 patients, 666 laser lesions) and 500 Hz (50–130 µJ, 100 pulses, 10 patients, 141 laser lesions). Presumably due to higher tissue temperatures, the AF decay was more enhanced when using 500 Hz than with 100 Hz

Irradiation took place with pulse energies of 50–130 µJ. In all of 141 measured laser spots, the online AF showed a maximum intensity decay of 8% over the applied number of pulses, as shown in Fig. 3, using pulse energies of 100 µJ. In comparison to the in-vitro experiments, a strong AF signal was detected. Considering the distribution of the percentage AF decay for all lesions, there was a mean AF decay per lesion of about 4% during the train of laser pulses (Fig. 4). Principally, the rate of AF decay was greater with increased pulse energies.

AF measurements at 100 Hz repetition rate

In 20 patients, the retinal AF was detected for irradiation at 100 Hz and with a train of 30 pulses. Irradiation took

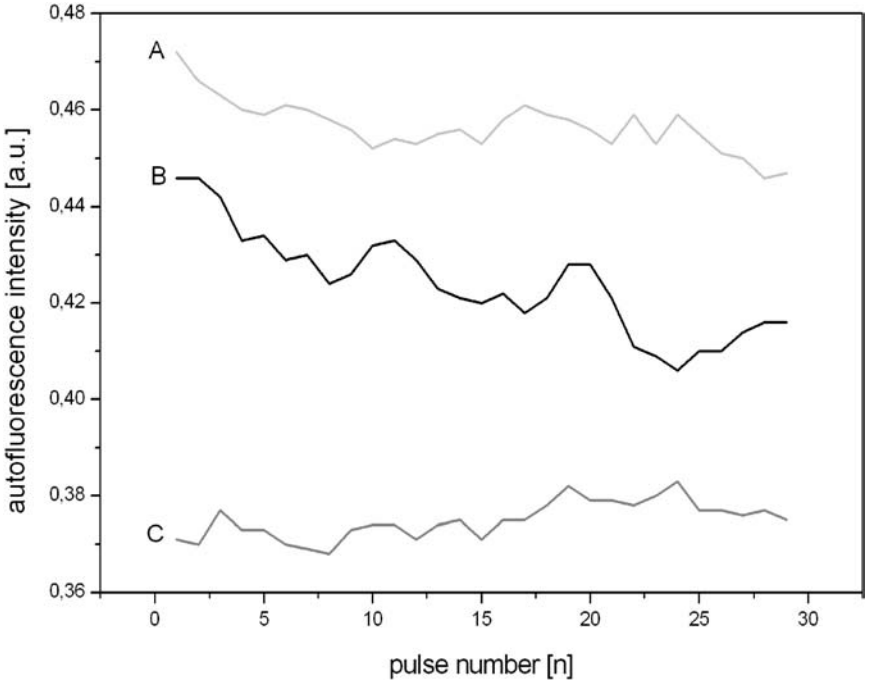

Fig. 5 In vivo AF intensity over the number of applied laser pulses at 100 Hz repetition rate and 100 µJ pulse energy in 3 patients. Particularly a decay of about 6% of the retinal autofluorescence over the number of pulses was noticed (*B*, *C*), but in some cases also no change of the fluorescence intensity was observed (*A*)

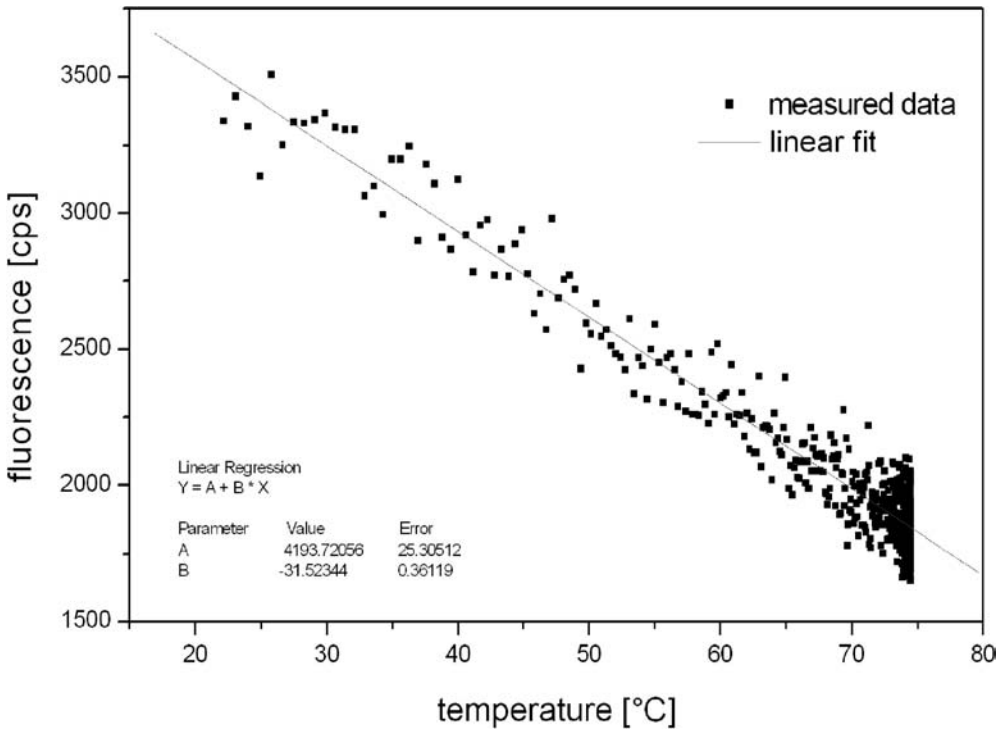

Fig. 6 Course of fluorescence intensity (cps, counts per second) of A2-E diluted in DMSO (1 μM) over temperature. A reversible linear correlation was found between decay of fluorescence intensity and temperature increase from 20°C to 75°C

place with pulse energies of 50–160 μJ, and altogether AF was measured in 666 laser lesions. A decrease of AF intensity by about 6% was noticed (Fig. 5; A, B). However, at the 100 Hz repetition rate, there were also some irradiation areas in which no change of AF was observable (Fig. 5; C). When the percentage of AF decay during irradiation for all laser lesions with 100 Hz repetition rate was considered, a mean decrease of AF intensity of about 2% was found (Fig. 4). However, in 14% of these laser lesions, no change of AF intensity was observed and in some cases even a certain increase was seen. In broad terms, the induced AF decay was smaller than when using the 500 Hz irradiation at 100 pulses (Fig. 4).

Temperature dependence of A2-E fluorescence

The temperature of diluted A2-E in a cuvette was increased from 20°C to 75°C. The fluorescence intensity decreased linearly with increasing temperature, at about 1% per 1°C (Fig. 6). This intensity change was completely reversible, even for temperatures up to 75°C. Because of this reversibility, a thermal alteration or destruction of A2-E seems to be unlikely at temperatures up to 75°C.

Discussion

It is known that postoperative AF measurements with a laser scanning ophthalmoscope (cSLO) reveal an AF intensity decay in successfully irradiated areas immediately after laser treatment [7, 8]. This study examined AF changes online during SRT. The AF decay found postoperatively was supposed to be associated with RPE damage [7, 8]. Thus, it was questionable whether online AF also decreases during laser treatment and whether this decay also contributes to the RPE damage.

A main objective in this study was the construction of an online AF detection device, adapted to the slit lamp and able to record AF during laser application. In the in-vitro experiments on porcine RPE sheets it was shown that AF intensity decreased as a function of the number of applied pulses. Due to the low amount of lipofuscin in young porcine RPE, the detected AF signal was only weak; however, a clear AF decay of about 22% was found during treatment. Initially, it was supposed to achieve this AF decay due to the damage of the RPE cells from the laser treatment. However, it was found that irradiation at energy levels below the RPE damage threshold also revealed a characteristic AF decay, which did not differ significantly from the AF decay in laser lesions with damaged RPE (Fig. 2).

Thus, if the laser-induced AF decay is not related to RPE damage, the question arose of what kind of interaction might then be responsible for it. As derived from the in-vitro experiments with A2-E, the main fluorophore

of lipofuscin, the AF decrease seems to be induced by a temperature increase. A2-E revealed completely reversible linear fluorescence decay of about 1% per 1°C temperature increase. Thus, if those results are transferable to tissue conditions in RPE sheets and in human settings, it may be speculated that the strong AF decay is derived from the temperature increase in the irradiated tissue rather than from RPE damage. In this case, the AF decay would be reversible and completely related to temperature, independently of RPE damage. Due to the irradiation with large spot sizes (160 μm) and high repetition rates (500 Hz), it is known that a strong temperature increase takes place within the tissue [17] and thus temperature-related AF changes seem likely.

Regarding AF measurements during patient treatments, the various factors contributing to this temperature-dependent AF decay could be observed more closely. However, it has to be remembered that different fluorophores might be responsible for fluorescence in porcine RPE than in human RPE, so results of the AF measurements might not be completely comparable. Treatment was performed using two different repetition rates and numbers of pulses, which we expected to lead to a different increase in tissue temperature during treatment. The temperature increase is proportional to the energy deposited per unit area and also increases with the repetition rate. Thus, significantly higher temperatures for irradiation are achieved with 500 Hz and 100 pulses than with 100 Hz and 30 pulses [17]. Using the 500 Hz parameter set, a clear AF decay could be observed in all irradiated areas, whereas using the 100 Hz repetition rate, AF changes were inconsistent, a result which is most likely derived from motion artifacts. It should be noted that the overall exposure time was 300 ms in the 100 Hz mode but only 200 ms in the 500 Hz mode. Thus, the overall AF decay using the 500 Hz irradiation was stronger than using the 100 Hz irradiation. Due to these differences it may be concluded that in humans the AF decay is also mainly due to changes in the tissue temperature during treatment.

The question remained of why AF decreases more strongly under laser impact in the in-vitro conditions than in human settings. As already pointed out, the in-vitro AF signals were weak compared to human AF signals. It might be speculated that in the young porcine RPE layer, AF of different weaker fluorophores, e.g., FAD and flavines [21], are more predominantly measured than in vivo, where AF of the older RPE–lipofuscin complex is strong and stable, and consequently AF reaction towards laser irradiation is weaker. Thus in older patients, lipofuscin AF might mask AF of the other fluorophores, which could be more dominant in porcine RPE. However, this has to remain speculative. Also, other reasons exist which may possibly explain this discrepancy, e.g., eye movements in human treatments, which could lead to uncontrolled shifting of the laser spot on the retina and thus to reduction in the amount of AF intensity change.

From the presented results, AF changes seem unable to reflect RPE damage in SRT, as initially expected from the cSLO findings [8]. The damage mechanism in selective RPE laser treatment is more thermo-mechanical than purely thermal, due to the short duration of the laser pulses in the microsecond regime [1]. Thus, microbubble formation around the melanosomes inside the RPE cell occurs during treatment, probably leading to disruption of the cell; this is in contrast to thermal denaturation in conventional laser photocoagulation [1, 18]. An abrupt AF decay due to a thermal destruction of fluorophores or to an explosive movement of fluorophores away from the laser beam would have been expected to detect RPE damage by online AF measurements. However, those characteristics were not found, which underline the proposed tissue temperature–AF interactions.

In summary, our experiments showed that it is possible to measure online AF during laser treatment and that it is also possible to detect a change of AF intensity due to the laser impact in SRT. However, online AF measurements seem unable to detect ophthalmoscopically non-visible RPE damage. Significant differences between the in-vitro and the in-vivo AF decay might suggest fluorescence originating from different fluorophores. Since the AF decay seems to be mainly temperature dependent (as derived from the A2-E fluorescence measurements) it may be possible to measure retinal temperatures non-invasively during laser impact. Especially if the various minimally invasive laser treatments such as PDT or TTT are considered where tissue temperatures during irradiation increase moderately and temperature distribution is very homogeneous, in contrast to SRT, online AF monitoring of tissue temperatures might be considered. This could be of particular advantage for TTT, where no dosimetry control is available today. For this, additional low-energy green probe laser pulses can be added during treatment to determine the tissue temperature. On the other hand, it would also be interesting to see whether online AF acts differently under laser impact using conventional laser photocoagulation or treatment of various areas in the fundus or different pathologies.

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