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Temperature dependent fluorescence of A2-E, the main fluorescent lipofuscin component in the RPE

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Abstract

Purpose. A2-E is the dominant fluorophore of lipofuscin in the retinal pigment epithelium. In an in-vitro setup, we determined the temperature-dependent changes of the A2-E fluorescence with the aim of also assessing the potential value of such measurements for determining retinal temperature by autofluorescence measurements during laser treatment.

Methods. A2-E was biosynthesized and diluted in Dimethyl Sulfoxide (DMSO) to 1 µM. Fluorescence measurements were performed with a photospectrometer under various temperatures ranging from 20°C to 75°C. Autofluorescence was excited at 467 nm, and emission was detected around 632 nm.

Results. A2-E fluorescence intensity showed a linear decrease concomitant with temperature increment. At 75°C, the fluorescence intensity decreased by 43% compared to at 20°C. Fluorescence intensity was completely reversible dependent on the temperature, which cannot be explained by thermal A2-E alteration.

Conclusions. If the A2-E temperature-dependent fluorescence *in-vitro* is transferable to human fundus autofluorescence, then it may be possible to apply an autofluorescence-based online detection device for noninvasive determination of fundus temperature during in vivo laser treatment. This is of clinical relevance, especially for the application of photodynamic therapy (PDT) and transpupillary thermotherpy (TTT).

Keywords: A2-E; fundus autofluorescence; spectral analysis; fundus laser treatment; lipofuscin; transpupillary thermotherpy; TTT

Introduction

The recently developed technique of imaging fundus autofluorescence (FAF) *in vivo* provides additional data in various retinal disorders.[1] FAF was shown to be mainly derived from lipofuscin in the retinal pigment epithelium (RPE).[1–5] Lipofuscin accumulates with age as a byproduct of constant phagocytosis of the photoreceptors' outer segments in the RPE cells.[4,5] Excessive accumulation of lipofuscin is also a common pathogenic pathway in monogenic and complex retinal diseases. With the advent of confocal scanning laser ophthalmoscopy, it is now possible to record the FAF using an excitation wavelength of 488 nm.[1,6] Normal fundus imaging revealed decreased autofluorescence at the macular area, which may account for a slower accumulation rate of lipofuscine in the fovea[7] but also to absorption of short wavelength light by the macular pigment, consisting of lutein and zeaxanthin.[6] FAF intensity also decreases towards the periphery.[6–10] Because FAF mainly originates from lipofuscin granules in the RPE and since melanin in the latter is the dominant absorber of laser energy in the green spectral region,[5,11] it can be assumed that alterations in the RPE, sec-

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ondary to laser treatment, result in changes in the FAF characteristics. This has already been demonstrated following conventional laser photocoagulation and selective RPE laser treatment.[12,13] Lipofuscin contains of numerous biomolecules including proteins and lipids.[14] The main fluorophore of the lipofuscin – especially in the RPE – is a Schiff base reaction product A2-E (N-Retinyliden-N-Retinylethanolamine), which was recently identified and synthesized.[15–17] This compound possesses toxic properties which are mediated via various pathways, for example as inhibition of lysosomal degradative functions in RPE cells.[18,19]

Here we investigated the fluorescence properties of A2-E under the impact of temperature alterations. If A2-E fluorescence intensity is temperature-dependent and if these temperature-related changes are similar to those of the lipofuscin within the RPE when laser irradiation is applied to the retina, FAF intensity would reflect RPE temperature during laser application. Knowledge of the exact tissue temperature during laser treatment would be important in terms of dosimetry for new laser treatments such as photodynamic therapy (PDT);[20] or transpupillary thermotherapy (TTT);[21] to prevent excessive heat induction with consecutive irreversible damage and degeneration of the apposing neurosensory retinal layer.

Material and methods

The lipofuscin compound A2-E was synthesized by coupling of all-trans-retinaldehyd and ethanolamin (2 : 1) as described by Parish.[17] A2-E was solved into methanol (1.22 mM) and diluted in Dimethyl Sulfoxide DMSO to 1 µM. Vaporization in DMSO occurs at 189°C, thus fluorescence measurements could be made at temperatures up to 75°C.

A fluorescence-spectrometer (SPEX2, Jobim Instruments) was used to measure the autofluorescence of A2-E. A2-E shows similar broad excitation- and emission spectra as FAF-measurements *in vivo*. As described previously,[22] lipofuscin can be excited between 450 nm and 540 nm and the emission can be detected between 500 nm and 780 nm. Measurements of temperature-related changes in fluorescence intensity were performed using the excitation wavelength of 467 nm (FWHM ± 2 nm) at an excitation power of 0.87 µW. The measured emission wavelength was 632 nm (FWHM ± 10).

A temperature-controlled cuvette holder was inserted into the sample chamber. The temperature of this holder was continuously adjustable from 20°C to 75°C. The sample temperature was measured with a thermal element (type J) in the cuvette. The temperature of the sample could be obtained with an accuracy of 0.1°C. During all measurements the sample was melted with a micro pole. Fluorescence data were collected when increasing the temperature up to 75°C within 350 seconds and later on decreasing the temperature again to baseline. The temperature course as well as the corresponding fluorescence intensity of A2-E was recorded with a PC during measurement. The integration constant was 0.2 seconds and 5 data points were averaged. The maximum duration of measurement time frames was two hours. From the fluorescence intensity data measured over the temperature of the sample a linear regression curve was calculated.

Results

The excitation spectrum of A2E, detected at 632 (FWHM ± 10) nm, had a broad range from 425 nm to 525 nm with its maximum at 467 nm. The A2E emission ranged from 575 nm to 700 nm with its peak at 632 nm, when excited at 467 nm (FWHM ± 2 nm); (Fig. 1).

In two hours of measurement at a baseline temperature of 20°C, no spectral or intensity changes of the A2E fluorescence were observed. Thus due to the low excitation intensity of 0.87 µW, any bleaching effect of A2E could obviously be prevented. By increasing the temperature from 20°C to 37°C, the fluorescence intensity of the A2E sample decreased by 17%. Subsequent decrease of temperature towards baseline (20°C) led again to the initial fluorescence intensity (Fig. 2). No delay of the fluorescence intensity response was detected within the temporal resolution of 1 second in our experiment. At high temperatures (75°C) the fluorescence intensity decreased by 43% compared to at 20°C and remained constant over a time frame of 10 minutes (Fig. 3). A linear correlation with a standard deviation of 2% was found between fluorescence intensity of the A2-E and the temperature of the sample (Fig. 4). The reduction of the fluorescence intensity was about 1% per °C of sample temperature. This intensity change was completely reversible, even from temperatures up to 75°C. Because of this reversibility, thermal alteration or destruction of A2E seems to be unlikely up to temperatures of 75°C.

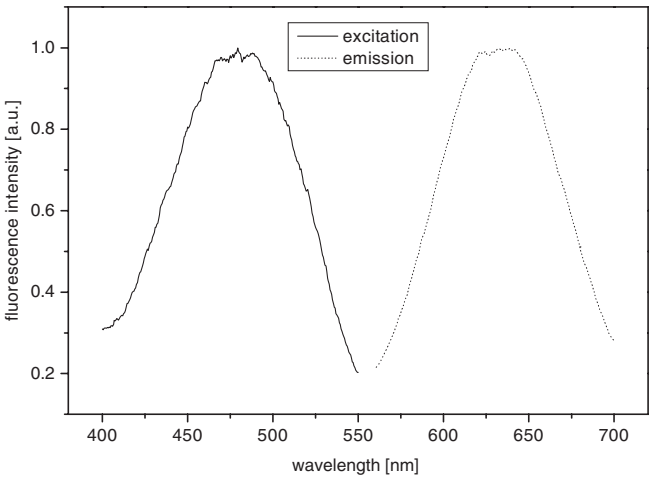

Figure 1. Measured excitation- and emission spectra of A2-E. Both spectra are broad, reaching from 425 nm to 525 nm (excitation, detection 632 ± 10 nm) and 500 nm to 700 nm (emission, excitation 463 ± 2 nm).

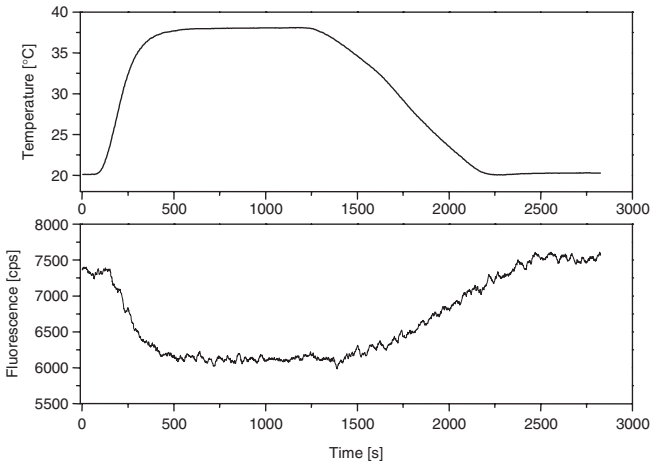

Figure 2. Course of fluorescence intensity (cps, counts per second) during increase and subsequent decrease of sample temperature containing A2E in DMSO (1 µM). During temperature increase, fluorescence intensity of A2-E reduces at about 1% per °C. If the temperature is decreased again, baseline intensity of fluorescence will be achieved.

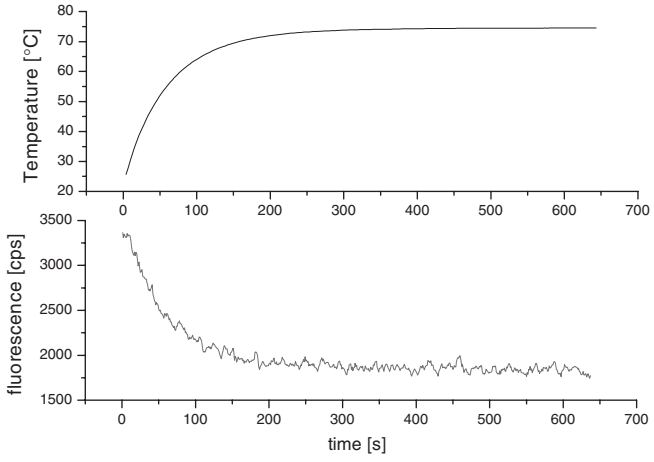

Figure 3. Course of fluorescence intensity during temperature increase of A2-E in DMSO (1 µM) over 75°C. Constant values for fluorescence intensity (cps, counts per second) over a period of 10 minutes even at these high temperatures indicate that A2E will not be destroyed.

Discussion

In this study we investigated the temperature-dependent behavior of the main fluorophore of the lipofuscin in the RPE, A2-E, *in-vitro*. We showed that A2-E is stable for temperature changes up to 75°C, that fluorescence decreases linearly by about 1% per °C increase, and that this relationship is reversible. Denaturation of A2-E was not achieved for temperatures up to 75°C.

If these results are transferred to *in-vivo* conditions, using autofluorescence for non-invasive determination of the RPE temperature during laser treatment appears to be an interesting application of this phenomenon. However, several factors have to be considered. 1) A2-E in humans is only one fluorophore of lipofuscine and the magnitude of its contribution to the *in-vivo* fluorescence is still unknown. Thus different fluorescence may arise from actual lipofuscin granules in humans, rather than from A2-E alone. 2) For the *in-vitro* experiments, A2-E was diluted in DMSO, which is different to the human medium. Thus fluorescence of A2-E may act differently *in vivo*. 3) It is very well known that fluorophores apart from the RPE are present in human tissue anterior and posterior to the RPE cell monolayer, including rhodopsin in photoreceptors, connecting tissue, melanin, collagen, eosinophiles, Flavin-Adenin-Dinucleotid (FAD) and Flavin-Mononucleotid (FMN).[23] The excitation and emission bands of some of these fluorophores overlap with lipofuscin fluorescence characteristics. Therefore, fluorescent properties of these molecules need to be considered, and also the temperature-dependent stability of those fluorophores under temperature increase is not yet known. 4) Finally the *in-vivo* fluorescent data may be largely affected depending on the individual status of the crystalline lens and also the macular pigments.

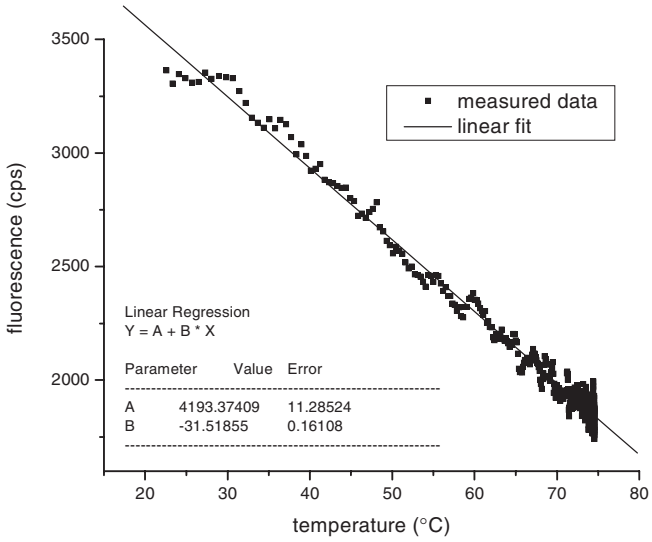

Figure 4. Course of fluorescence intensity (cps, counts per second) of A2-E in DMSO (1 µM) over temperature (from Fig. 3). A linear correlation was found between decay of fluorescence intensity and temperature increase, which was reversible.

However; according to current knowledge, A2-E is the dominant fluorophore of the lipofuscine complex.[4] Thus it may be speculated that autofluorescence of the human fundus reacts to temperature increase in a similar manner to that shown in our *in vitro* data. This would be of particular relevance for novel laser applications such as PDT or, in particular, TTT.[21] It is known that effects of TTT depend significantly on fundus pigmentation, but so far no dosimetry control is available. Clinically, inadvertent retinal burns have been described in the conventionally-applied dose range, with irreversible damage and visual loss.[24]

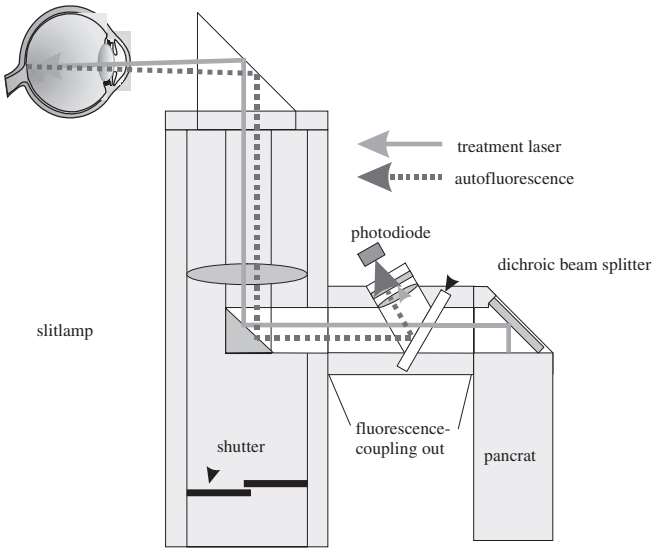

Figure 5. Setup of the slitlamp-adapted module for online measurement of autofluorescence intensity.

Since the temperature increase in retinal tissue is only moderate during PDT and TTT – in contrast to the high temperature increases during photocoagulation – RPE will not be damaged from those kinds of laser application, and therefore RPE-related online temperature determination using FAF might be feasible for these treatments. It was shown that FAF can be measured online during treatment – presenting an autofluorescence intensity decay during µs-pulsed laser heating in the green spectral range (527 nm).[25] Using the same slitlamp-adapted online setup, measurements should also be possible with IR diode lasers if a second laser emitting in the blue/green spectral region is provided to excite the fundus FAF. Within the slitlamp, the FAF can be coupled out by a dichroic beam splitter and guided to a photodiode for the FAF intensity measurements (Fig. 5). It is possible that such an "online" control can be used to adjust laser power automatically based on the FAF intensity change within the tissue, thus making e.g., TTT a more practical treatment option. However, first experiments have to address the question of general feasibility of this method *in-vivo* and in human conditions.

Secondly, it has also be remembered that laser treatment will be performed in pathologic retinal conditions as e.g., exudative age-related macular degeneration. In these diseases, FAF reveals largely variable patterns of decreased and increased autofluorescent areas, in dependence from classic or occult choroidal neovascularization, subretinal hemorrhage or RPE detachment.[1] Online temperature determination using FAF measurements during laser treatment might then reveal incorrect temperature variations. Finally, those pathologic conditions might correlate with higher levels of lipofuscine and consecutively A2-E. However, PDT and TTT therapy consist of continuous light application. One might be concerned about continuous FAF imaging during the treatment period because this additional energy deposition in the altered tissue may lead to adverse effects. However, the maximal retinal irradiation should be well below the limits established by the American National Standards Institute,[26] thus adverse effects should be ruled out. On the other hand, continuous FAF excitation seems unnecessary for proper retinal temperature monitoring. It would also be sufficient to use only small single pulses (e.g., 100 measurement points per treatment) to regulate temperature sufficiently.

In summary, using FAF based temperature measurements to guide laser application in PDT and in particular TTT techniques might be an interesting new application. *In-vivo* studies are needed to evaluate the possible transfer of the *in-vitro* results to a clinical scenario.

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