Spectrally Resolved Fundus Autofluorescence in \textit{ABCA4}-Related Retinopathy

Chantal Dysli,1,2 Philipp L. Müller,1,3 Johannes Birtel,1,3 Frank G. Holz,1,3 and Philipp Herrmann1,3

1Department of Ophthalmology, University of Bonn, Bonn, Germany
2Department of Ophthalmology and Department of Clinical Research, Inselspital, Bern University Hospital and University of Bern, Bern, Switzerland
3Center for Rare Diseases, University of Bonn, Bonn, Germany

Correspondence: Philipp Herrmann, Department of Ophthalmology, University of Bonn, Ernst-Abbe-Str. 2, Bonn D-53127, Germany; philipp.herrmann@ukbonn.de.

CD and PLM contributed equally to the work presented here and should therefore be regarded as equivalent authors.

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\textbf{Purpose.} To investigate the green and red fluorescence emission component of hyperautofluorescent flecks in patients with \textit{ABCA4}-related retinopathy.

\textbf{Methods.} A confocal light-emitting diode (LED)-based retinal imaging system (EIDON) was used for image acquisition of patients with genetically confirmed \textit{ABCA4} mutations. Using 450-nm excitation wavelengths, spectrally resolved retinal autofluorescence images were acquired in two wavelength ranges: green emission fluorescent component (GEFC, 500–560 nm), and red emission fluorescent component (REFC, 560–700 nm). Image analysis included comparison of the two emission spectra, correlation with confocal EIDON LED color fundus images, and spectral-domain optical coherence tomography (OCT).

\textbf{Results.} Eighty eyes of 40 patients with \textit{ABCA4}-related retinopathy were examined. A characteristic distribution of flecks with distinct pattern of fluorescence emission components was detected and quantified. Independent from disease manifestation, different proportions of GEFC and REFC were identified within single flecks. Centrally located flecks showed a higher proportion of GEFC and were characterized by local disruption of outer retinal bands in OCT. More peripheral flecks were more prominent in the REFC and correlated to subretinal hyperreflective deposits with only minor pathologic alterations of outer retinal layers. Individual flecks even showed spatial differences of the predominant fluorescent component.

\textbf{Conclusions.} Spectrally resolved fundus autofluorescence intensity images feature characteristic distribution of GEFC and REFC in flecks, highlighting the heterogeneity of fluorophore distribution and providing more insight into the pathogenesis of \textit{ABCA4}-related retinopathy. In view of upcoming therapeutic trials, longitudinal analysis of fluorescent components might facilitate monitoring of subtle disease progression and/or treatment effects.

Keywords: Stargardt disease, retina, OCT, EIDON, hereditary dystrophy

\textbf{A\textit{B}\textit{C}\textit{A}4-related retinopathy} is the most common inherited juvenile retinal dystrophy and is caused by mutations in the ATP-binding cassette A4 (\textit{ABCA4}) gene, encoding a transmembrane transporter in the outer photoreceptor segments.1–5 Defects in the \textit{ABCA4} protein lead to progressive accumulation of visual cycle end products such as lipofuscin, and eventually destruction of the retinal pigment epithelium (RPE) and photoreceptors with progressive visual loss.6–7

Clinically, the excessive accumulation of visual cycle end products can be visualized by short-wavelength fundus autofluorescence (FAF) imaging and is characterized by an overall increased background FAF intensity and hyperautofluorescent flecks.6,8–11 During disease progression, these flecks typically expand in a centrifugal distribution and become more hypoaurofluorescent as RPE atrophy develops.12,13 Therefore, FAF is a useful technique to monitor disease progression in \textit{ABCA4}-related retinopathy.1,4,15

The classically applied short-wavelength FAF uses blue excitation light (488 nm), and FAF emission detection between 500 and 700 nm. However, individual fluorophores feature distinct excitation as well as emission spectra.16 Emerging therapeutic approaches require monitoring options for subtle retinal changes and treatment effects, ideally over a relatively short period.17 Spectrally resolved FAF may therefore be aspired to, as it might give more differentiated insights into the composition and spreading of morphologic disease alterations. A novel confocal FAF device (EIDON; CenterVue, Padova, Italy) has recently been developed and introduced. It allows for separation of short and long FAF emission wavelengths (“color-FAF imaging”).18,19

Our study aimed to describe the distribution of FAF emission in a short- and a long-wavelength emission spectra, using the EIDON fundus camera, and to evaluate the potential clinical use of emission channel separation in \textit{ABCA4}-related retinopathy.

\textbf{Methods.} This monocentric, cross-sectional case series was performed at the Department of Ophthalmology of the University of Bonn,
Germany. The study was in adherence to the Declaration of Helsinki. Institutional Review Board approval from the University of Bonn, Germany, and patients’ written informed consent were obtained after explanation of the nature and possible consequences of the study.

**Subjects**

Patients with ABCA4-related retinopathy were recruited from the local retinal dystrophy clinic. The diagnosis of ABCA4-related retinopathy was defined by the presence of at least one disease-causing mutation in ABCA4
d and a consistent phenotype. If only one disease-causing mutation in ABCA4 was found, patients underwent next-generation sequencing to rule out other retinal diseases mimicking ABCA4-related retinopathy. Only patients with clear media and without ocular comorbidities affecting visual function or any additional retinal pathology or previous vitreoretinal surgery were included.

Severity of phenotypic presentation of the retina with flecks and atrophy was classified according to the Fishman classification for fundus flavimaculatus. Group 1 eyes showed pigmentary changes restricted to the fovea, often accompanied by a ring of flecks within one disc diameter around the fovea. Group 2 eyes revealed flecks beyond the vascular arcades, often also nasal to the optic disc. A partial resorption of the flecks is possible. Group 3 eyes presented with diffusely resorbed flecks and choriocapillaris atrophy within the macula. Finally, group 4 eyes demonstrated extensive choriocapillaris and retinal pigment epithelial cell atrophy throughout the fundus.

**Clinical Examination and Imaging**

All patients underwent a complete dilated ophthalmic examination and multimodal imaging. The imaging protocol consisted of spectral-domain optical coherence tomography (OCT; Spectralis HRA+OCT, Heidelberg Engineering, Heidelberg, Germany), as well as confocal color fundus photos (cCFPs), infrared (IR) imaging, and color fundus autofluorescence (color-FAF) using the EIDON fundus camera. The EIDON fundus camera is a fully automated retinal imaging device using autoalignment, autofocus (range, –12 to +15 diopters), autoexposure, andautocapture of the images (Sarao V, et al. IOVS 2018;59:ARVO E Abstract 4653). It enables recording of confocal light-emitting diode (LED) true color fundus photos (cCFP, 440–650 nm), IR reflectance (825–870 nm) images, and color-FAF images with blue excitation light (440–475 nm, peak at 450 nm). For signal detection, a barrier filter with cutoff at 500 nm is used. Color-FAF emission was simultaneously measured within a short-wavelength range between 500 and 560 nm (green emission fluorescent component, GEFC), and within a long-wavelength range between 600 and 700 nm (red emission fluorescent component, REFC) using a line-scanning principle; thereby, the sensor raster scans the posterior pole for illumination and imaging, using a rotating mirror. FAF images can be illustrated in gray values (as conventional FAF intensity images) or color coded (as color-FAF images).

EIDON images cover the central 60° (horizontal) × 55° (vertical) of the posterior pole in a single exposure, using an internal central fixation target. The sensor resolution equals 14 megapixels (4608 × 3288), corresponding to an image resolution of 60 pixels per degree. The retinal resolution equals 15 μm. Radiant exposure for white and blue LED lies well below the standard limits defined by ISO 15004-2 (regulatory standard for light hazard protection) and falls into the category “ophthalmic instruments for which no potential light hazard exists.” The entire imaging procedure with EIDON is illustrated in Figure 1.

**Data Analysis**

Data were collected and processed by using commercially available Microsoft Excel 2010 (Redmond, WA, USA). Values are presented as mean ± standard deviation (SD). Color-FAF images were transferred to Image J (https://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA) in order to separate the GEFC and the REFC by using the “split channels” function. No further image processing was needed. Corresponding morphologic structures were compared between the different imaging modalities. The distribution of GEFC and REFC flecks was analyzed and quantified by using an extended ETDRS grid with ring diameters of 1 mm (center), 3 mm (inner ring), 6 mm (outer ring), and 9 mm (supplementary ring). Finally, color-FAF images were imported in the custom-made analysis software “FAF Color Segmentation Tool” developed by CenterVue that allows for display of fluorescence intensity and emission wavelengths (GEFC to REFC) as a two-dimensional graph (see Fig. 6). Hence, each pixel of the image is represented in an xy-graph with the proportion of GEFC to REFC on the y-axis, and the FAF intensity on the x-axis. Thereby, characteristic distribution clouds could be identified, and specific regions of interest could be marked and highlighted within the image.
RESULTS

Cohort Characteristics

A total of 80 eyes of 40 patients (50% female) with \textit{ABCA4}-related retinopathy were included in the study. Mean age was 43 ± 6.1 years (range, 11–81 years). Biallelic \textit{ABCA4} mutations were found in 37 of 40 patients. According to the morphologic classification, 34 eyes were assigned to group 1; 32 eyes to group 2; 10 eyes to group 3; and 4 eyes to group 4. Of note, both eyes of each patient always met the same subgroup criteria. A total of six eyes (7.5%) were pseudophakic. Demographic and functional data of each subgroup are summarized in the Table.

Distribution of Flecks

In cCFP images, disease-characteristic flecks were clearly visible, accentuated at the posterior pole with or without distribution beyond the vascular arcades according to the phenotypic presentation as reflected in the subgroup classification (Figs. 2, 3). In FAF, these flecks primarily presented as hyperautofluorescent, coded bright (white) in gray-scale FAF intensity images, and reddish or greenish in color-FAF images. Resorbed flecks and areas of RPE atrophy were hypoautofluorescent, visualized by dark areas in both FAF modalities (Fig. 3). The prevalence of hyperautofluorescent flecks was 64% in eyes of subgroup 1; 100% in subgroup 2; and not assessable in subgroups 3 and 4 owing to lesions outside of the 9-mm grid and/or to very advanced disease stage without identifiable individual flecks (Table).

Green and Red Emission Fluorescent Component

Hyperautofluorescent flecks in color-FAF images revealed a characteristic distribution of predominant emission wavelength. After splitting GEFC and REFC, flecks were still visible in both spectral channels. However, most flecks revealed a primary emission in either the short (green) or the long (red) wavelength (Figs. 2, 3). The prevalence of GEFC flecks within the macular 9-mm ring was 3.33 times higher than for REFC flecks for subgroup 1, and 4.67 times higher for subgroup 2. Flecks located more centrally generally featured a higher proportion of short-wavelength autofluorescence emission coded greenish in the color-FAF images. However, more peripherally located flecks more often showed long-wavelength autofluorescence emission, and appeared reddish. In subgroup 2, the ratio of REFC to GEFC was on average 3.2

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<th>Table. Demographic and Functional Parameters of Included Patients Overall and Divided for Phenotypic Classification$^{22}$</th>
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BCVA, best corrected visual acuity.
* Values represent mean ± SD (range).
times higher in the supplementary ring (9 mm) than in the outer ETDRS ring (6 mm).

A subgroup of flecks was identified bifidly with a centrifugal sequence of predominant green to red autofluorescent components within the individual fleck, that is, the main green proportion with shorter emission wavelength was located in the central part of the fleck, whereas the main red proportion was found more peripherally (Fig. 4).

Hyperautofluorescent fundus flecks spatially correlated to subretinal deposits in OCT (Fig. 5). REFC-predominant flecks were correlated with hyperreflective deposits that disrupt external retinal layers up to the ellipsoid zone (EZ) band and displace the preserved external limiting membrane (ELM) anteriorly. In contrast, subretinal deposits associated with GEFC-predominant flecks seem to break through the ELM too. Both types of flecks showed a marked focal reduction of the outer nuclear layer thickness above the subretinal deposits (Fig. 5).

**DISCUSSION**

To our knowledge, this is the first report investigating FAF intensities of flecks secondary to ABCA4-related retinopathy on a spectrally resolved FAF imaging tool. The EIDON device allows for fully automated acquisition of three different imaging modalities (cCFP, IR, and FAF) in a large field of view within a short period of time, thus, facilitating the multimodal approach and analysis of retinal pathologies. Furthermore, the device contains two separate FAF emission detectors that

**FIGURE 3.** Spectrally resolved fundus autofluorescence in different disease stages. Representative examples for ABCA4-related retinopathies classified in four groups with Fishman stages 1 to 4. cCFP: EIDON confocal color fundus photos; color-FAF: color-coded fundus autofluorescence intensity image with enlarged region of interest. Flecks with predominant green and red emission fluorescent component are highlighted with respective arrows.

**FIGURE 4.** Green and red fundus autofluorescence emission components in flecks with pisiform growths. ABCA4-related flecks with pisiform growth featured increased GEFC located toward the macular center and increased REFC toward the retinal periphery. cCFP: EIDON confocal color fundus photography; GEFC: 500–560 nm; REFC: 560–700 nm; color-FAF: color-coded fundus autofluorescence intensity image. Green and red arrows mark flecks with increased GEFC and REFC, respectively. Arrowheads indicate pisiform flecks with both GEFC and REFC, and allow comprehensive reconstruction of centrifugal growth of the flecks.
amine (A2E). However, there are many other autofluorescent noids of RPE lipofuscin as visual cycle by-products. One of fleck. Interestingly, in a subgroup of flecks with pisiform growth, spectra could already be detected when analyzing single flecks. This might indicate areas of growth and future remodeling of the molecular composition in flecks. Thereby, areas of FAF intensity detected in the two wavelength ranges in this study. The observed distribution pattern possibly reflects the previously described centrifugal spread of flecks and disease progression over time. Of note, changes in the FAF emission spectra could already be detected when analyzing single flecks. Interestingly, in a subgroup of flecks with pisiform growth, different stages of deposits could be observed. Thereby, areas with GEFC were located more centrally, indicating a transition stage to resorption and atrophy, whereas areas with REFC might indicate areas of growth and future remodeling of the fleck.

Retinal autofluorescence mainly originates from bisretinoids of RPE lipofuscin as visual cycle by-products. One of the major fluorophores in the photoreceptor outer segments is thought to be the bis-retinoid N-retinyl-N-retinylidene ethanolamine (A2E). However, there are many other autofluorescent bisretinoids including A2E photoisomers. According to a recent FAF study on Stargardt disease investigating short-wavelength and near-infrared autofluorescence, bisretinoids in the outer segments of degenerating photoreceptors might also contribute to alterations in FAF signal, in particular during disruption of the photoreceptor cells. Cukras and coworkers have assigned variations in FAF intensity in single flecks to intracellular events such as changes of the lipofuscin and melanin content in RPE cells. This hypothesis was confirmed by evaluation of flecks in short-wavelength FAF, known to predominantly derive from bisretinoid components in lipofuscin, and in near-infrared FAF, thought to originate from the melanin compartment in the RPE and the choroid. Therefore, changes in the composition of the deposits and/or the surrounding environment might influence the proportion of FAF intensity detected in the two wavelength ranges in this study. Remodeling of the molecular composition in flecks might be one of the most subtle retinal changes in disease progression, implicating spectrally resolved FAF as a possible surrogate marker to be used in future interventional trials.

Both anatomic and functional clinical endpoints for evaluation of treatment effects in ABCA4-related retinopathy represent a challenge, as the point-of-no-return for irreversible structural and functional retinal damage is still unknown. Furthermore, longitudinal data are missing, also with regard to other imaging tools, such as quantitative autofluorescence (qAF), that visualize earliest pathognomonic retinal alterations. Future studies including prospective investigations should therefore be aspired to in order to gain further insights into the natural history and underlying mechanisms of ABCA4-related retinopathy.

Using FAF, a further assessment of the metabolic environment in ABCA4-related retinopathy besides excitation and emission spectra can be achieved by fluorescence lifetime imaging ophthalmoscopy (FLIO), which has been shown to be unique for individual fluorophores, and to reveal distinct longitudinal changes in disease progression. Thereby, peripherally located flecks initially present with short fluorescence lifetimes, whereas more central flecks exhibit longer fluorescence lifetimes, indicating differences in the composition of these deposits and their remodeling over time.

By graphic representation of the FAF intensity versus the FAF emission spectrum (Fig. 6), specific landmarks of the retina such as the optic nerve head and retinal vessels, as well as disease-specific features, could be discriminated. Thereby, specific regions of interest such as flecks and margins of atrophy can further be separated, individually depicted and analyzed, with the potential to facilitate future approaches of data quantification. Apart from ABCA4-related retinopathy, this tool might be of interest also for various other retinal diseases including age-related macular degeneration. In this context, the solely available studies using the EIDON fundus camera are described. The latter have shown that GEFC corresponds to residual debris or drusenoid material in areas of geographic atrophy, and that images correlate well to conventional FAF images acquired with 488-nm excitation wavelength. Interestingly, we observed that also structures without underlying RPE and with commonly known low or absent FAF intensity, such as atrophic areas, retinal scars, and the optic nerve head, were detected and predominantly featured GEFC.
In contrast, low FAF intensity in the area of the vessels appeared predominantly with REFC. The origin of the detected fluorescence signal has not been yet entirely clarified. Similar findings with measured autofluorescence signal in the optic nerve head, vessels, and atrophic areas have also been found when using FLIO.32,33 A possible explanation is increased autofluorescence emission after excitation with 450 nm (or 473 nm as used in FLIO), as green-emitting fluorophores are more efficiently stimulated at shorter wavelengths than at 488 nm, which is commonly used for FAF intensity imaging.16 As the emission filter has a sharp cutoff at 500 nm, filter leakage is unlikely to contribute to this phenomenon. Also, the fraction of internally reflected and scattered light is very small.

The limitations of this study included the cross-sectional design, which did not allow conclusions on the longitudinal evolution of the FAF emission pattern of flecks secondary to ABCA4-related retinopathy. We are currently conducting longitudinal observations. Furthermore, the settings of the novel device are predefined and cannot be changed to date. Modification of the excitation spectra, as well as the limits of the emission detection, might allow further information in addressing different fluorophores. Also, signal in areas of low fluorescence intensity was detected with variable intensity, as the device uses an automated intensity scaling algorithm without internal reference, in contrast to qAF imaging. Therefore, areas of low FAF can be detected with variable intensity depending on the degree of the strongest FAF.

FIGURE 6. Illustration of the autofluorescence intensity versus the green and red fundus autofluorescence emission components. Representative EIDON image (color-FAF top row left) of a patient with ABCA4-related retinopathy with corresponding fundus autofluorescence intensity (FAF, middle) image and topographic distribution of red and green emission fluorescent component (GEFC/REFC, right). Below, a correlation of the emission wavelengths (x-axis) and the emission intensity (y-axis) is shown with further analysis of specific regions of interest: flecks with increased GEFC were located predominantly centrally, whereas flecks with increased REFC were located more peripherally and surrounded the green center of the flecks, the main retina, margins of atrophy and vessels, RPE atrophy, optic nerve head, and vessels.
intensity within the same image. Furthermore, the impact of possible subclinical lens opacities on EIDON color-FAF images is not yet fully investigated. Previous data indicate that the short emission channel might especially be influenced by lens opacities. As the background- and the fleck-associated FAF intensity is increased in ABCA4-related retinopathy, good image quality can be achieved in this study cohort relatively independently of media opacities. Therefore, in the current cohort, no significant impact of subclinical lens opacities was expected.

In conclusion, this is the first study to demonstrate that flecks secondary to ABCA4-related retinopathy can be analyzed and specified by separate detection of short (green) and long (red) autofluorescence emission components. The spectrally resolved assessment of retinal pathologies may allow for refined assessments of subtle alterations over time.

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