Two-Photon–Excited Fluorescence Imaging of Human RPE Cells with a Femtosecond Ti:Sapphire Laser

Almut Bindevald-Wittich,1 Meng Han,2,5 Steffen Schmitz-Valckenberg,1 Sarah R. Snyder,5 Günter Giese,4 Josef F. Bille,5 and Frank G. Holz1

PURPOSE. To record the distribution and spectrum of human retinal pigment epithelial cell lipofuscin (LF) by two-photon-excited fluorescence (TPEF) and confocal laser scanning microscopy.

METHODS. Ex vivo TPEF imaging of the human retinal pigment epithelium (RPE) of human donor eyes was conducted with a multiphoton laser scanning microscope that employs a femtosecond Ti:Sapphire laser as an excitation laser source. The spectrum of autofluorescence of LF granules was analyzed with a confocal laser scanning microscope coupled to a UV argon laser.

RESULTS. TPEF examination allowed for imaging of RPE cell morphology and intracellular distribution of LF granules with high-contrast and submicrometer resolution. Variations in cell size and shape as well as in autofluorescence spectra of individual LF granules were recorded. The typical diameter of LF granules was found to be below 1 μm, with some RPE cells possessing larger granules. Remarkably, enhanced blue-green autofluorescence was observed from these larger LF granules.

CONCLUSIONS. TPEF imaging represents a novel tool for the investigation of morphologic and spectral characteristics of human RPE cells. Spectral variations of individual LF granules may indicate differences in the complex molecular composition. Compared to conventional single-photon excited autofluorescence, TPEF with a tunable laser source allows for reduced photo damage and deeper sensing depth. It may help to elucidate further the pathophysiological role of LF accumulation as a common downstream pathogenetic pathway in retinal diseases. With the proof of principle from this ex vivo study, further work is now planned to evaluate the safety of TPEF RPE imaging in RPE cultures and animal models. (Invest Ophthalmol Vis Sci. 2006;47:4553–4557) DOI:10.1167/iovs.05-1562

With the advent of confocal scanning laser ophthalmoscopy, it has become possible to visualize fundus autofluorescence (FAF) and its spatial distribution in vivo.1–5 This method represents a tool to evaluate the RPE during ageing and in retinal diseases.6 As shown by spectrometric investigations by Delori et al.,7 the FAF signal is mainly derived from lipofuscin (LF) granules in the RPE cell monolayer. Excessive accumulation of LF occurs as a result of lifelong phagocytosis of photoreceptor outer segments by postmitotic RPE cells and represents a common pathogenetic pathway in various monogenic and complex retinal diseases including age-related macular degeneration (AMD).8–12 Although AMD has become the most common cause in developed countries of registered blindness, its pathogenesis is still incompletely understood.13,14

Once formed, the RPE cell apparently has no means either to degrade or release LF into the extracellular space. LF has been shown to contain toxic compounds including the dominant fluorophore A2-E, a pyridinium bisretinoid, and various other modified macromolecules. These compounds can interfere with normal cell function, and various molecular mechanisms of adverse effects have been elucidated recently.15–20 Several lines of evidence suggest that oxidative damage plays an important role in lipofuscinogenesis, with antioxidant deficiency or pro-oxidant conditions being of relevance.21–24

Physiological and disease-associated RPE cell morphology has been addressed by applying conventional microscopic methods, and typical features in relation to age and fundus localization have been described. RPE cell density decreases from the fovea to the outer peripheral fundus regions, and the total number of cells decreases by approximately 0.3% per year.25–27

The advent of femtosecond laser sources led to the experimental implementation of two-photon–excited fluorescence (TPEF) microscopes.28 Compared with conventional confocal fluorescence microscopes, two-photon absorption is confined to the region of peak intensity within the focus of the illuminating laser beam. An illustration of the localization of excitation by two-photon excitation has been published by Zipfel et al.29 Diffraction-limited resolution and depth discrimination are achievable without extra pinholes, permitting high-resolution three-dimensional optical sectioning of thick tissues with reduced bleaching or phototoxic effects outside the laser focus. In contrast to the UV or blue-light excitation for one-photon-excited fluorescence (1PEF), infrared light is used for multiphoton excitation, which promises deeper sensing depth and less photodamage effects.28–30 Owing to the rich resource of fluorescent dyes available for selective and nontoxic staining and innovative recording techniques such as fluorescence resonant energy transfer (FRET) and fluorescence recovery after photobleaching (FRAP), multiphoton-excited fluorescence microscopy is widely applied in imaging living cells, probing single molecules, and investigating biochemical processes under physiological conditions. Compared with confocal microscopy, multiphoton microscopy is more suitable for imaging of living or photosensitive tissue, such as the human retina or RPE cells. Recently, the fine structure of the collagen fibrils in the

From the 1Department of Ophthalmology, University of Bonn, Germany; the 2Mannheim Biomedical Engineering Laboratories (MABEL), Faculty of Clinical Medicine Mannheim, University of Heidelberg, Germany; the 3Kirchhoff Institute for Physics, University of Heidelberg, Germany; and the 4MaxPlanck-Institute for Medical Research, Heidelberg, Germany.

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Corresponding author: Frank G. Holz, Department of Ophthalmology, University of Bonn, Ernst-Abbe-Str. 2, D-53127 Bonn, Germany; frank.holz@ukb.uni-bonn.de.
cornea and sclera has been successfully resolved with second-harmonic imaging. To the best of our knowledge, there has been no report of multiphoton microscopic imaging of human RPE cells. In this report, we introduce this novel experimental imaging method for visualizing morphologic and spectral characteristics of human RPE cells ex vivo as an initial step toward the implementation of a two-photon confocal scanning laser ophthalmoscope for in vivo investigations.

**Methods**

Four human eyecups from deceased white donors were used to prepare specimens for microscopic evaluation. The donor's ages were 19, 55, 57, and 80 years. After removal of the anterior segment for cornea transplantation, the eyes were immediately fixed in 4% paraformaldehyde solution. There were no macroscopic visible retinal alterations such as soft drusen, retinal hemorrhage, or choroidal neovascularization. The anterior segment and vitreous were carefully removed. Specimens were trephined from the macular and peripheral areas with a 5-mm trephine. After removal of the neurosensory retina and the sclera, the RPE-Bruch's membrane–choroid specimens were placed on a microscope slide and covered with a coverslip, with PBS buffer used to create a wet chamber.

Specimens were analyzed with a confocal laser scanning microscope (TCS SP2; Leica Microsystems, Bensheim, Germany) and a modified laser scanning multiphoton microscope (LSM 510 NLO; Carl Zeiss Meditec, Jena, Germany). The latter was equipped with a mode-locked femtosecond Ti:sapphire laser (Chameleon XR; Coherent Inc., Santa Clara, CA). TPEF confocal imaging can be accomplished with the same microscope, with a CW Argon ion laser (λ = 458, 488, and 514 nm) used as an excitation source. The wavelength of the Ti:sapphire laser was tuneable from 720 to 980 nm. For TPEF imaging, the laser wavelength was set to 800 nm, which is equivalent to 400 nm (UV) excitation for 1PEF. The Ti:sapphire laser produces a 150-fs pulse with a repetition rate of 90 MHz. The laser output power was attenuated to 4 mW by an acoustic optic modulator (AOM; Carl Zeiss Meditec). This value of 4 mW is the average power before the objective. Because of the attenuation by the objective, the effective excitation power is less than 4 mW, which is close to the previous reported damage thresholds for TPEF imaging of Chinese hamster ovary cells or bovine adrenal chromaffin cells. We did not observe noticeable photodamage to the fixed RPE cells at this power level. A 63×, high-numerical-aperture (NA = 1.2), water-immersion objective (Carl Zeiss Meditec) was used for TPEF imaging (lateral resolution, roughly 200 nm). The autofluorescence signals from RPE cells were collected with the same objective. Following a set of dichroic mirrors and beamsplitters, the autofluorescence signals were split into two color-coded detecting channels (non-descanned detection; green channel: 500–550 nm, red channel: 575–640 nm) and were detected by two photomultiplier tube (PMT) detectors, respectively. Imaging overlay of both channels and processing was performed by the operation software for the microscope to visualize the different autofluorescence of both channels in one image. The confocal laser scanning microscope (TCS SP2; Leica) was used to perform XYZ scanning. The complete emission spectrum of the autofluorescence from RPE cells in the range of 430 to 700 nm was measured with 8-nm accuracy through a motor-driven slit in front of the PMT detector. For both 1PEF and TPEF imaging, the acquisition time of a single 512 × 512-pixel image was generally within a few seconds, with galvanometer scanners. All the images presented in this article are single optical sections.

Human donor eyes were obtained from the eye bank of the Department of Ophthalmology, University of Bonn, Germany, and from the eye bank of the Department of Ophthalmology, University of Munich LMU, Germany. Informed consent for corneal transplantation and further use of tissue for research was obtained by a relative or documented in an organ donor pass in accordance with German law for organ donation.

**Results**

High-resolution TPEF images of LF granules in the human RPE were obtained with the modified laser scanning multiphoton microscope (LSM 510 NLO; Carl Zeiss Meditec). The LF granules were distributed in the area between cell nuclei (no autofluorescent signal) and cell membranes with a preferred location of LF granules near the cellular border, which allowed for accurate delineation of the size and shape of individual RPE cells with high contrast (Figs. 1, 2). As described previously, most of the cells displayed hexagonal cell borders. Because of large variations in cell sizes—especially in specimens from the retinal periphery—RPE cells appeared to have more or fewer than six neighboring cells, which was associated with an irregular polygonal pattern of RPE cells (Fig. 3). Besides the different cell sizes, a remarkable variability of LF granule load and distribution of LF granules in the RPE cell cytoplasm was found in the more peripheral specimens, compared with specimens from the macular area (Fig. 4). In specimens from the macular area, the arrangement of RPE cells was found to be more regular in shape and size (Figs 1, 2). Some RPE cells were found to have two nuclei, whereas most of the cells had one nucleus. Cells with more than one nucleus were generally larger than RPE cells with only one nucleus (Fig. 5).

All specimens were evaluated by using dual-channel TPEF imaging. This method enabled measurement of the autofluorescence according to different spectral ranges of LF granules. LF granules in the 19-year-old donor eye appeared overall to be darker and sparser than those in aged donor eyes. Of note, individual RPE cells in the three older donor eyes showed deviating characteristics of their LF granules. Figure 5 demonstrates two examples of these different RPE cells in a macular specimen of the 80-year-old donor eye, where few RPE cells (< 1%) presented markedly larger LF granules (with a diameter of up to, with a few more than, 2 μm) compared with the neighboring cells. Autofluorescence emission of these larger LF granules was different from that of the regular LF granules and demonstrated enhanced blue-green fluorescence. The number of these cells did not increase with longer exposure to TPEF imaging, and there was also no indication of cell membrane damage. The observed phenomenon therefore could not be attributed specifically to two-photon induced photodamage, to an artifact, or to contamination during sample preparations.

These larger LF granules were explored further with blue-shifted fluorescence by acquiring emission spectra of single LF granules with the confocal laser scanning microscope (TCS SP2; Leica). Figure 6 gives an example of a spectrum measured in individual LF granules of a peripheral RPE specimen from an 80-year-old donor eye. The typical emission line of the blue...
Argon laser lines (λ = 488, 514 nm) was not sufficient to excite the complete autofluorescence spectrum. Thus, a UV (λ = 364 nm) Argon laser was used for the spectrum measurement. The spectra shown in Figure 6 correspond to the average fluorescence derived from a group of three to four representative normal and large-type LF granules, respectively. They were acquired at 8 nm resolution and can be fitted with single Gaussian functions. In a comparison of the spectra of both types of different LF granules, the larger LF granules showed an autofluorescence emission peak at 36 nm shorter than the regular ones. The larger LF granules showed an emission peak at 520 nm, whereas the emission peak of the surrounding regular LF granules was at 556 nm.

**DISCUSSION**

In this study, TPEF imaging with a femtosecond Ti:sapphire laser allowed for investigation of morphologic and spectral characteristics of human RPE cells. The autofluorescence signal both ex vivo and in vivo mainly derived from fluorophores in LF granules. Excessive LF accumulation in postmitotic RPE represents a common downstream pathogenetic pathway in various monogenetic and complex, multifactorial retinal diseases, including Stargardt’s disease and AMD. Therefore, it is of interest to record variations in LF load in RPE cells to gain a better understanding of associations with concomitant disease processes such as the development of geographic atrophy associated with severe visual loss. We have previously shown, using FAF imaging in advanced AMD that excessive LF accumulation precedes the enlargement of preexisting geographic atrophy as well as the development of new atrophic patches.

**FIGURE 2.** TPEF imaging of RPE cells with a femtosecond Ti:sapphire laser (λ = 800 nm) with dual-channel detection: green channel, 500 < λ < 550 nm (a); red channel, 575 < λ < 640 nm (b). Specimen from a 55-year-old donor eye, center macular area. Bar, 10 μm.

**FIGURE 3.** TPEF imaging of RPE cells with a femtosecond Ti:sapphire laser (λ = 800 nm) with dual-channel detection: green channel, 500 < λ < 550 nm (a); red channel, 575 < λ < 640 nm (b). Specimen from a 55-year-old donor eye, outer macular area. Note large central RPE cell with two nuclei. Bar 10 μm.

Before atrophy occurs, areas with excessive LF accumulation (i.e., increased FAF signal) are associated with corresponding impaired retinal sensitivity. The resolution of other currently available imaging methods applied clinically including fundus photography, fluorescein angiography, optical coherence tomography, or ultrasonography neither allows for delineation of individual RPE cells nor for recording of intracellular metabolic changes, such as LF accumulation in the lysosomal compartment.

Recent biochemical analyses of isolated human LF granules revealed the presence of various compounds with toxic properties, including A2-E, which interferes with normal lysosomal function by inhibition of lysosomal degradation mediated by inhibition of the ATP-dependent lysosomal proton pump as well as by phototoxic and detergent properties. In proteome analyses we have shown that a broad spectrum of LF proteins display specific posttranslational modifications, including malondialdehyde (MDA), 4-hydroxynonenal (HNE), and advanced glycation end products (AGE), which again are thought to interfere with normal cell functions.

In the present study, the intracellular distribution of individual autofluorescent LF granules could readily be visualized with high-resolution TPEF imaging. LF granules were preferentially localized in the peripheral cytoplasm close to the cell borders when only low LF granule density was present. In RPE cells with higher LF granule density the granules also occupy central portions of the cell cytoplasm. Previously, a more basal
 localization of LF granules has been described in young eyes in contrast to diffuse spread with age. The particular distribution of intracellular LF granules allows for accurate delineation of the nonautofluorescent cell borders between neighboring RPE cells.

The ex vivo findings using TPEF are in accordance with previous reports of the use of other imaging methods. Herein, we have shown that TPEF imaging allows for visualization of human RPE cells and LF ex vivo. Besides its major advantages of large sensing depth and reduced photodamage, TPEF imaging can also be used to determine the autofluorescence spectrum of autofluorescent material in RPE cells. The broad tunable excitation source (Ti:sapphire laser, $\lambda = 720-980$ nm) for TPEF imaging is equivalent to the UV and blue excitation range ($\lambda = 360-490$ nm). Therefore, the complete spectrum of the autofluorescence can be investigated with the femtosecond infrared beam. Through TPEF imaging of RPE cells, the morphology of the individual RPE cells and the distribution of the LF granules inside the RPE cells were visualized with a lateral resolution of 0.2 $\mu m$ and an axial resolution of 1 $\mu m$. With 4 mW preobjective excitation power, TPEF did not induce any undesired photobleaching or photodamage, whereas previous studies involving TPEF microscopy have reported damage at this power level, imaging different cell lines and with other experimental setups including different pixel dwell time, scan time, numerical aperture of objective; and pulse-length.

The complexity of the damage mechanisms does not allow a general conclusion, but taking into account the presence of phototoxic photograph sensitizers and highly absorbing melanin in RPE, a further reduced average laser power or laser repetition rate may be necessary for TPEF characterization of living RPE cells.

The typical diameter of the LF granules was found to be below 1 $\mu m$, but a few were larger than 2 $\mu m$. Remarkably, enhanced blue-green fluorescence was observed from these larger LF granules. This is, to the best of our knowledge, the first description of fluorescence spectra of individual LF granules ex vivo. As a fingerprint of LF on the molecular level, blue shifted autofluorescence from LF granules may imply the presence of a different composition that may derive from other proteins or fluorophores inside the LF granules. However, the phenomenon may be related to thus far unknown byproducts in the metabolic process of individual RPE cells.

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References


