Label-free nonlinear optical imaging of mouse retina

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Abstract: A nonlinear optical (NLO) microscopy system integrating stimulated Raman scattering (SRS), two-photon excited fluorescence (TPEF) and second-harmonic generation (SHG) was developed to image fresh mouse retinas. The morphological and functional details of various retinal layers were revealed by the endogenous NLO signals. Particularly, high resolution label-free imaging of retinal neurons and nerve fibers in the ganglion cell and nerve fiber layers was achieved by capturing endogenous SRS and TPEF signals. In addition, the spectral and temporal analysis of TPEF images allowed visualization of different fluorescent components in the retinal pigment epithelium (RPE). Fluorophores with short TPEF lifetime, such as A2E, can be differentiated from other long-lifetime components in the RPE. The NLO imaging method would provide important information for investigation of retinal ganglion cell degeneration and holds the potential to study the biochemical processes of visual cycle in the RPE.

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1. Introduction

The retina is the light-sensitive tissue at the back of eve. Its morphological and functional alterations are associated with a variety of ocular diseases, leading to a special interest in the study of its fine layered structures [1]. Briefly, the innermost layers of the retina are the nerve fiber layer (NFL) and the ganglion cell layer (GCL). The retinal nerve fibers comprise the axons of retinal ganglion cells (RGCs). They relay electrical signals generated from the outer retina to the central nervous system. As the terminal sensory neurons in the visual nervous system, the RGCs in the GCL play a significant role in the visual system. Degeneration of the RGCs is observed in glaucoma and other types of non-glaucomatous optic neuropathies. The inner nuclear laver (INL) contains a variety of neuronal cells including bipolar cells, amacrine cells and horizontal cells that function as a bridge connecting the photoreceptors in the outer retina and the RGCs in the inner retina. The inner plexiform layer (IPL), located between the GCL and the INL, is formed by the neurites of the neuronal cells in the INL and the dendrites of the RGCs. Rich microvascular structures are found throughout the GCL, the IPL and the INL, supplying nutrients and exchanging byproducts of metabolic activities of the retinal neurons. Therefore, visualization of the structure and distribution of microvasculature is crucial in the study of retinal vascular diseases, such as retinal vein occlusion and diabetic retinopathy [2,3]. Similar to the IPL, the outer plexiform layer (OPL) is composed of a network of neuronal synapses connecting the bipolar cells and photoreceptors. Nuclear granules of photoreceptors are distributed in the outer nuclear layer (ONL) and the outer segments of photoreceptors extend to the outermost retina, the retinal pigment epithelium (RPE). The RPE participates intensively in the visual cycle and performs critical functions essential for homeostasis of the retina. Malfunction of the RPE may cause photoreceptor degeneration and choriocapillaris atrophy. A number of retinal diseases, for example, agerelated macular degeneration (AMD), retinitis pigmentosa, Best's disease, Stargardt's disease and Leber's congenital amaurosis are associated with abnormalities in the RPE [4-8].

In recent years, nonlinear optical (NLO) microscopy has been adopted as an efficient tool to study vertebrate ocular tissues such as the cornea, sclera and retina, with the potential of label-free imaging [9–13]. The deep penetrating power of infrared laser makes NLO microscopy an effective option to resolve the layered structures of the retina. It has been demonstrated that the two-photon excited fluorescence (TPEF) emitted from endogenous fluorophores facilitates label-free and noninvasive visualization of cellular structures [14–17]. There are significant associations between cell functions and endogenous TPEF signals [18– 22]. For example, typical endogenous fluorescent molecules including nicotinamide-adenine dinucleotide (NADH) and flavin-adenine dinucleotide (FAD) have been found to be the predominant sources of TPEF in the inner layers of the retina, while other fluorophores such as retinoids and lipofuscin contribute major fluorescence signals in the RPE [12,13]. It has been reported that several fluorophores in the RPE with different functionalities can been distinguished by their distinct fluorescence lifetimes [23-25]. Taking advantage of the inherent 3D resolution and near-IR excitation of two-photon imaging, the spectra and lifetime analysis of the RPE TPEF signal may provide more precise measurement and identification of cellular fluorescent components which can hardly be achieved by single-photon excitation methods.

Labeling of individual RGCs for imaging studies is often achieved via immunohistochemistry, retrograde labeling of neuronal tracer or utilization of transgenic technologies [26,27]. Information on the detailed morphology of RGCs can be missed because the distribution of fluorescence protein and contrast agents within a cell may not be homogenous. In this work, we develop a NLO microscope system integrating stimulated Raman scattering (SRS), two-photon excited fluorescence and second-harmonic generation (SHG) to image retinas freshly excised from mice models. We demonstrate that label-free SRS and TPEF imaging can visualize retinal neurons in an unlabeled retina. Particularly, the

SRS and NADH TPEF imaging can clearly reveal the detailed cellular morphology and nerve fibers in the GCL and the NFL. The results are validated by transgenic mice with RGCs intrinsically labeled with yellow fluorescent proteins (YFP) and retrograde-labeling with 1'-dioctodecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI). In addition, multiple native fluorophores in the RPE can be clearly differentiated via the spectral- and time-resolved analysis of TPEF signals. The results show that the fluorescence of A2E in lipofuscin has remarkably short lifetime and distinct fluorescence spectra compared with TPEF of all-trans-retinol. The visualization and differentiation of multiple fluorophores may provide important insight into the biochemical processes involved in the visual cycle.

2. Methods and materials

2.1 Instrumentation of integrated NLO microscope system

The schematic diagram of the integrated NLO microscopy is shown in Fig. 1. A femtosecond Ti:sapphire laser (Chameleon Ultra II, Coherent, Santa Clara, CA) was used as the excitation of TPEF and pump light of SRS. In SRS imaging, a portion of laser tuned at 830 nm was used as the pump beam of SRS and the rest of laser was used to pump an optical parametric oscillator (OPO) (Chameleon OPO, Coherent) to produce the infrared Stokes beam of a wavelength at 1100 nm. The differential frequency of the collinear beams matched the CH₃ stretching vibrational frequency of 2957 cm⁻¹, which were generated from proteins and lipids [28]. The dispersion of the Stokes laser pulse was compensated by two prism-based compressors, optimizing the nonlinear excitation efficiency. After compression, the Stokes beam was modulated by a 10.7 MHz acousto-optic modulator (3080-122, Crystal Technology) and then combined with the pump beam by a dichroic mirror (900DCXXR, Chroma, Bellows Falls, VT) and directed into a water immersion objective ($40 \times /1.15$ NA, Olympus). A pair of galvo mirrors (6800H, Cambridge Technology) was used for x-y scanning and the objective was driven by an actuator for axial scanning. The backscattered TPEF signals were collected by the objective and separated from the excitation light by a dichroic mirror (FF510-Di02 or FF665-Di02, Semrock). The TPEF signals were then coupled into a fiber bundle and analyzed by a spectrograph equipped with a linear array of 16 photomultiplier tubes (PMT) and a time-correlated single photon counting (TCSPC) module (PML-16-C-0 and SPC-150, Becker & Hickl). Time-resolved TPEF signals were recorded in 16 consecutive spectral bands with 13 nm resolution, to analyze the TPEF signals in both spectral and time domains. The spectral range of the detection system covered the SHG signal at 370 nm and the specific RPE autofluorescence up to 600 nm. The forward SRS signal was collected by a condenser (U-AAC, Achromat/aplanat condenser, NA 1.4, Olympus, Tokyo, Japan) and separated from the Stokes beam by a pair of filters (Fs: FF01-794/160, Semrock and 64335 shortpass 900 nm, Edmund) before being directed into a photodiode (FDS 100, Thorlabs). A high-frequency lock-in amplifier (SR844, Stanford Research Systems) was used to demodulate the SRS signal. The typical acquisition time of TPEF and SRS imaging were eight and four seconds, respectively.



Fig. 1. Schematic diagram of multimodal NLO microscope system. BS: beam splitter; M: mirror; DM: dichroic mirror; SP: short-pass filter; FB: fiber bundle; M-PMT: Multichannel PMT array; TCSPC: time-correlated single photon counting module; Fs: filter set.

2.2 Sample preparation

In this work, the ICR (CD-1) mice were used for imaging of all retinal layers. Imaging of the RGCs and optic nerve fibers was also performed in transgenic mice strain (Thy-1 YFP-G) that express YFP under the control of Thy-1 promoter. The transgenic mice were also retrograde labeled with 1'-dioctodecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) for validating the results of SRS and TPEF imaging of RGCs. In retrograde labeling, the DiI was injected into superior colliculus according to an established protocol and the mice were kept for one week following retrograde labeling before nonlinear optical imaging [26]. All the mice were sacrificed by cervical dislocation and the globes were enucleated. The retina, RPE and sclera were excised and mounted onto a glass slip. The retinal side was placed upward toward the objective. All the imaging was performed on freshly excised retinas without fixation. The study was approved by the university Animal Ethics Committee.

3. Results and discussions

3.1 TPEF and SRS imaging of the retina

TPEF and SRS imaging was first performed over a freshly dissected retina from a CD-1 mouse, from the NFL and GCL to the RPE and sclera. Based on the previous findings that most of the TPEF in the inner retina was contributed by endogenous NADH fluorescence, the excitation wavelength was set at 740 nm, which was optimal to excite NADH TPEF signal [12]. The SRS image was subsequently captured by tuning the laser wavelength to 830 nm as the pump beam of SRS and as the pump source for OPO to generate Stokes beam. The TPEF and SRS images of different retinal layers are displayed in Fig. 2(a1)-2(f1) and Fig. 2(a2)-2(f2), respectively. Each retinal layer was determined from the H&E staining histology as shown in Fig. 2(g). The sectioning depths of the images in Fig. 2(a)-2(f) are marked in the histological section (Fig. 2(g)). The TPEF spectra of all the retinal layers are shown in Fig. 2(h). The color bar on the left side of Fig. 2(a1) indicates the color coding of the TPEF spectral signal recorded at each image pixel. NADH signal coded in yellow was dominant from the GCL to the ONL, contributed by the mitochondria in optical nerve fibers and retinal neurons. On the other hand, the red-shift fluorescence in the RPE layer coded in orange was contributed by the all-trans-retinol and lipofuscin. Consistent with the finding from previous studies, the tiny bright spots observed in the RPE represent esterified storage particles of alltrans-retinol, an important product in the visual cycle [13,29]. The SHG signal generated from the collagen fibers of sclera was coded in blue as shown in Fig. 2(f1).

The SRS images in Fig. 2(a2)-2(f2) exhibit distinctive morphologies of the different retinal layers. The image recorded from the GCL clearly reveals the microstructures of individual neurons as well as the nerve fibers, as shown in Fig. 2(a2). The featureless SRS image of the IPL in Fig. 2(b2) is attributed to the small-sized staggered neurites. A variety of neuronal cells possibly including bipolar cells, amacrine cells and horizontal cells appear in the image recorded from the INL, as shown in Fig. 2(c2). Compared with RGCs in the GCL, cells in the INL show higher density and smaller size. Meanwhile, individual erythrocytes can be visualized in the microvasculature in the INL and GCL. The strong signal of erythrocytes in the SRS images may come from pump-probe or photothermal signal of hemoglobin [30,31]. The ONL shows signals originated from proteins in the nuclear granules of photoreceptors (Fig. 2(d2)). The SRS image of the RPE is shown in Fig. 2(e2). The bright spots in the RPE would represent all-trans-retinol molecules. The sclera emits strong SRS signals, which are likely related to the high protein concentrations in the collagens. Overall, we found that the integrated NLO microscope system is capable for label-free imaging of the different retinal layers. A more detailed investigation of the NFL/GCL and RPE is discussed in following sections.



Fig. 2. Representative TPEF and SRS images of different retinal layers. (a1)-(a2) TPEF and SRS images of the GCL; (b1)-(b2) IPL; (c1)-(c2) INL; (d1)-(d2) ONL; (e1)-(e2) RPE; (f1)-(f2) sclera; (g) histology image of H&E staining retinal sample; (h) TPEF spectra of each retinal layer. The field of view of (a)-(f) is 100 μ m × 100 μ m. Scale bar in (g): 40 μ m. Depths of images: a-5 μ m; b-20 μ m; c-50 μ m; d-70 μ m; e-100 μ m; f-110 μ m.

3.2 Imaging of RGCs and nerve fibers in the NFL and GCL

To further investigate the TPEF and SRS signals in the NFL and GCL, TPEF and SRS imaging were conducted in the retinas of Thy1-YFP transgenic mice in which the expression of YFP is driven by the Thy-1 promoter. In the retina, the RGCs are the predominant cell type expressing Thy-1 [27,32]. The TPEF signals and SRS images were recorded in the NFL and the GCL, as shown in Fig. 3(a1)-3(e1) and Fig. 3(a2)-3(e2), respectively. False-color coded TPEF images were composed of three fluorescence signals. The NADH signal recorded in the wavelength band from 400 to 480 nm was coded in green, the YFP signal peaked at around 530 nm was coded in orange and the hemoglobin TPEF signal of extremely short lifetime was coded in red [33-36]. Compared with the TPEF images, SRS images provide finer details of the cellular morphology. The optic nerve fibers of different widths in SRS images show excellent correspondence with the YFP-labeled nerves in the TPEF images (arrows in Fig. 3(d) and 3(e)). The relatively strong SRS signals in the nerve fibers indicate the axons of RGCs are enriched with proteins and lipids. In addition to RGCs and other neuronal cells, blood vessels in the GCL can be identified by the TPEF signals of hemoglobin in red blood cells (arrows in Fig. 3(b1) and 3(c1)). Correspondingly, individual red blood cells were also resolved by SRS imaging as shown in Fig. 3(b2) and 3(c2). The SRS and TPEF lifetime imaging of the retinal microvasculature would be useful to study the vascular abnormalities in different retinal layers [37,38].





As can be seen in Fig. 3, both NADH TPEF and SRS images reveal the individual nuclei of the neuronal cells in the GCL. The SRS imaging also allows visualization of the nucleoli. To assess SRS and TPEF imaging of RGCs, we performed retrograde labeling with DiI injected into the superior colliculus of the YFP transgenic mice, because retrograde labeling is the gold standard for the validation of RGCs in the retina with high specificity [26]. However, it is worth noting that unlike the fluorescence protein, DiI only retains on cell membranes [39]. In the GCL of the retrograde labeled retina, we recorded SRS images shown in Fig. 4(a1)-4(c1), and then recorded the DiI and YFP TPEF signals using a single excitation of 1100 nm. Although the DiI and YFP signals are highly overlapped in the wavelength domain, their TPEF lifetime decays are significantly different (Fig. 4(d)). The corresponding TPEF lifetime images from the same locations as SRS images are displayed in Fig. 4(a2)-4(c2),

where YFP signals with long fluorescence lifetime (\sim 3 ns) were coded in green and the spotted DiI signals with shorter lifetime (\sim 1.2 ns) on RGC membranes were coded in red. The merged images of SRS and TPEF signals are displayed in Fig. 4(a3)-4(c3). The DiI signals are found in over 50 percent of the cells in the SRS images, confirming that they are RGCs. On the other hand, we observed that many cells in the SRS images were not labeled with Dil. They could be displaced amacrine cells or RGCs with axonal fibers not projected to superior colliculus [26]. As shown in Fig. 4(a1)-4(c1), the SRS imaging reveals detailed cellular morphology in the GCL. In particular, it is an excellent tool to elucidate the outline of the cell nucleus and the nuclear diameters can be precisely measured [40]. In this study, 10 retina samples (each with 100 μ m \times 100 μ m) from 10 mice were used to measure the nuclear size of RGCs using SRS imaging. All the RGCs were validated with retrograde labeling. A histogram of nuclear size distribution of the DiI-labeled RGCs is shown in Fig. 4(e). The average nuclear size of DiI-labeled RGCs is 8.8 ± 1.6 µm. It has been reported that the displaced amacrine cells account for the majority of non-RGCs in the GCL of mice [1,41]. Among the displaced amacrine cells in the GCL, starburst amacrine cell is the predominant type and has the average some diameter about 8 μ m, while the some diameters of RGCs vary from 12 to 18 μ m [41–44]. Given the fact that the mean nuclear size of the measured RGCs $(8.8 \pm 1.6 \,\mu\text{m})$ is similar to the mean soma size of the amacrine cells (i.e. RGCs are larger in size than amacrine cells), it is feasible to differentiate RGCs from non-RGCs based on measurement of nuclear size. In future study, more rigorous histological validation and analysis will be conducted to evaluate the capability of SRS imaging to differentiate RGCs from other neurons in the GCL based on nuclear features.



Fig. 4. SRS and fluorescence images of transgenic mice retinas with RGCs retrograde labeled with DiI. (a1)-(c1) SRS images of the GCL; (a2)-(c2) fluorescence images of the YFP (green) and DiI (yellow) signals; (a3)-(c3) overlaid images of SRS and fluorescence; (d) TPEF lifetime of YFP and DiI signals; (e) histogram of nuclear diameter of DiI-labeled RGCs. The field of view of all images is 100 μ m × 100 μ m.

3.3 Imaging-guided TPEF spectroscopy of RPE

The RPE emits strong TPEF signal originated from lipofuscin and all-trans-retinol (or vitamin A), the two most dominant fluorophores with different optimal excitation wavelengths [13,29]. All-trans-retinol is esterified and accumulated around RPE cell membranes, forming retinyl ester storage particles (RESTs) or retinosomes [13,29]. RESTs function as storage of all-trans-retinol. Lipofuscin is mainly distributed in the cytoplasm and accumulates with age. It mainly consists of another fluorescent molecule, A2E, which is the VIII component of lipofuscin [13,23,45]. As a byproduct of the visual cycle, A2E accumulates in lysosomes of RPE cells and is associated with the pathogenesis of AMD [13,46-49]. To explore the spectral characteristics of the RPE TPEF, we examined the TPEF signals from the RPE of wild-type albino mice using a wide range of excitation wavelengths (740-805 nm). The representative TPEF images of the RPE excited by 740 nm, 780 nm and 805 nm are displayed in Fig. 5(a1)-5(c1), respectively. From the spectra-coded TPEF images, two fluorophores (alltrans-retinol and lipofuscin) with different two-photon excitation and emission characteristics in the RPE were identified [13,29]. The high-intensity RESTs granules are clearly observed in Fig. 5(a1) and 5(b1), at 740 nm and 780 nm excitation, respectively. However, the quantity of RESTs dropped significantly with excitation of 805 nm, as shown in Fig. 5(c1). On the other hand, the widely distributed lipofuscin TPEF in the cytoplasm is evident at 740 nm - 805 nm excitation, with red shift in emission spectra. Therefore, the TPEF spectrum of the RPE is a combination of two emission spectra of two different molecules. Using the imaging-guided spectroscopy technique, we extracted the TPEF signals of RESTs granules from the background lipofuscin signal in the cytoplasm in the TPEF images, and then studied the excitation and emission features of the two fluorophores separately [21,37]. Figure 5(d1) and 5(d2) show the TPEF spectra of the particles (all-trans-retinol) and background (lipofuscin) at different excitations, which agree with the results of the spectral study on pure retinoid and A2E in the previous report [13]. Comparing spectra-coded images with the TPEF spectra of the RPE, the green-colored RESTs granules contribute the short fluorescence wavelength which peaks at around 481 nm. The background lipofuscin fluorescence spectra in the cytoplasm change notably with excitation wavelength. Specifically, when the RPE was excited at 740 nm and 760 nm, the lipofuscin TPEF spectra exhibited a primary peak at 481 nm, with a small shoulder at around 533 nm. The similarity of the particle and background spectra at 740 nm excitation supports the assertion that retinoid precursors are significant compositions of lipofuscin fluorophores [50,51]. Another peak at 559 nm appears gradually from 760 nm to 780 nm excitation, which is coded in yellow in the TPEF image as shown in Fig. 5(b1). Finally, the short-wavelength peak of 481 nm totally disappears at 805 nm excitation and the A2E becomes the dominant fluorophores at this long wavelength excitation [23].

Because our NLO imaging system recorded the TPEF signals in spectral and time domains, we further studied the temporal characteristics of the RPE fluorescence. The lifetime-coded TPEF images shown in Fig. 5(a2)-5(c2) were recorded from the same locations as the spectra-coded images in Fig. 5(a1)-5(c1). In the TPEF lifetime images, the long-lifetime (photon arrival time from 2.7 ns to 10 ns) and short-lifetime (photon arrival time from 0 ns to 2.7 ns) components are coded in cyan and red, respectively [52]. The green-coded TPEF signals peaked at 481 nm exhibit a long fluorescence lifetime, coded in cyan in Fig. 5(a2) and 5(b2). On the other hand, the yellow-coded signals distributed in the cytoplasm of RPE in Fig. 5(b1) and 5(c1), with TPEF peak at 559 nm, has a short fluorescence lifetime, which is coded in red in Fig. 5(b2) and 5(c2). To differentiate the different components of the RPE more accurately, RESTs particles were extracted from background fluorescence and the fluorescence lifetime of RESTs as well as background lipofuscin fluorescence at different excitation wavelengths were calculated individually, based on our time-resolved measurement of TCSPC module.



Fig. 5. Imaging-guided spectral analysis of the RPE. (a1) TPEF spectra-coded image of RPE at 740 nm excitation; (a2) TPEF lifetime-coded image of RPE at 740 nm excitation; (b1) TPEF spectra-coded image of RPE at 780 nm excitation; (b2) TPEF lifetime-coded image of RPE at 780 nm excitation; (c1) TPEF spectra-coded image of RPE at 805 nm excitation; (c2) TPEF lifetime-coded image of RPE at 805 nm excitation; (d1) TPEF spectra of the particles in RPE at 740 nm, 760 nm and 780 nm excitation; (d2) TPEF spectra of the particle-removed background in RPE at 740 nm, 760 nm, 780 nm and 805 nm excitation. The field of view of all images is 100 μ m \times 100 μ m.

Using the imaging-guided spectroscopy technique, we analyzed the fluorescence lifetime of the TPEF signals from the RESTs granules and background cytoplasm. Table 1 shows the bi-exponential lifetime decay fitting of individual components in the RPE cells with excitation at different wavelengths [21,22]. The lifetime fitting results reveal that the fluorescence lifetime of RESTs is over 2 ns at 740 nm excitation, longer than the background cytoplasm fluorescence lifetime which is around 1.79 ns. As excitation wavelength increases to 760 nm and 780 nm, the fluorescence lifetime of the particles at 481 nm changes to 2.13 ns and 1.64 ns, respectively, while the cytoplasm TPEF lifetime decreases significantly, resulting from the appearance of A2E autofluorescence [23,53]. When the 805 nm excitation was applied, no significant RESTs fluorescence was observed and the background fluorescence was dominated by short-lifetime components with lifetime as short as 0.3 ns, which is believed to be mainly contributed from A2E [23]. There are an increasing number of indications that the formation and accumulation of RPE lipofuscin are associated with a variety of retinal disorders including Stargardt's disease, Best's disease, retinitis pigmentosa and AMD [54-58]. However, conventional autofluorescence imaging of the fundus is not able to extract the individual fluorescent components of lipofuscin [59,60]. Therefore, the imaging-guided TPEF analysis has notable advantages in removing the fluorescence emitted from RESTs granules, which would provide a more specific evaluation of lipofuscin in RPE dysfunction. Moreover,

the potential capability of differentiating multiple components in lipofuscin based on fluorescence lifetime analysis may play an important role in exploring the chemical compositions of the RPE lipofuscin.

	T_1 (ns)	A_1 (%)	T_2 (ns)	$A_{2}(\%)$	$T_{m}(ns)$
740EX _particle	0.63 ± 0.03	62.0 ± 1.4	5.25 ± 0.13	38.0 ± 1.4	2.38 ± 0.05
760EX_particle	0.75 ± 0.04	67.2 ± 9.0	5.11 ± 0.89	32.8 ± 9.0	2.13 ± 0.16
780EX_particle	0.29 ± 0.05	71.8 ± 4.0	5.07 ± 0.21	28.2 ± 4.0	1.64 ± 0.26
740EX_background	0.41 ± 0.04	72.4 ± 1.7	5.41 ± 0.11	27.6 ± 1.7	1.79 ± 0.11
760EX_background	0.26 ± 0.12	87.9 ± 4.0	4.38 ± 0.28	12.1 ± 4.0	0.76 ± 0.29
780EX_background	0.16 ± 0.003	94.7 ± 0.4	3.97 ± 0.27	5.3 ± 0.4	0.36 ± 0.03
805EX_background	0.16 ± 0.003	92.7 ± 0.2	1.99 ± 0.13	7.3 ± 0.2	0.30 ± 0.01
The values of parameters are shown in terms of the average with standard deviation over five measurements.					

Table 1. TPEF lifetime bi-exponential fitting parameters of fluorophores in RPE

 T_1 and T_2 are the two lifetime exponential components in the bi-exponential decay fitting $(T_m = A_1 \exp(-t/T_1) + A_2 \exp(-t/T_2))$. A₁ and A₂ are the amplitude coefficients of T₁ and T₂, respectively. T_m is the average fluorescence lifetime value.

4. Conclusion

In this study, we demonstrate the application of multimodal label-free NLO microscopy to extract the morphological and biochemical details in different retinal layers. High-quality label-free visualization of the nerve fibers and retinal neurons can be achieved with the SRS imaging, which captures the signals of the C-H bonds from the proteins and lipids. The combination of SRS and TPEF imaging provides unique opportunities to investigate the structure as well as the biochemical characteristics of different retinal layers. In the GCL, the label-free SRS and NADH TPEF imaging can delineate the nuclear features of the retinal neurons. We also show that the microvasculature in retinal layers can be visualized by both SRS imaging and hemoglobin TPEF signal from red blood cells. Furthermore, we studied the spectral and lifetime characteristics of RPE autofluorescence. The time-resolved spectroscopic imaging reveals that the RESTs particles distributed around the cell membranes of the RPE cells have a longer fluorescence lifetime of about 2 ns, while the intricate lipofuscin autofluorescence in the cytoplasm accounts for the short-lifetime components, which is mainly contributed by A2E with a lifetime of about 0.3 ns. The distinct difference in fluorescence lifetime between the two major RPE fluorophores would provide new insights into the functional mechanisms of the visual cycle and the mechanisms of RPE dysfunction. Finally, it should be emphasized that the label-free capability of SRS and TPEF imaging could make the wild type mouse an inexpensive and easily available model for the retina study. This may even pave the path for retina research in other animals in which transgenicor retrograde-labeling is not applicable. With the development of other advanced techniques such as adaptive optics, *in-vivo* two-photon imaging has been preliminarily achieved on mouse retinas with RGCs labeled with green fluorescent protein [61]. In-vivo two-photon excited endogenous fluorescence imaging with time-resolved detection will be developed in the near future for retinal imaging and this would afford a new approach to facilitate in vivo study of many retinal diseases.

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