Multimodal approach to reveal the effect of light irradiation on chemical composition of lipofuscin granules of human RPE tissues

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Abstract. The accumulation of lipofuscin (LF) in tissues is considered as one a hallmark of the aging process. Usually formed in yellow-brown pigment aggregates or granules primarily composed of lipid residues and proteins, the exact chemical composition of LF varies among tissues. LF of the retinal pigment epithelium (RPE) is known to contain byproducts of the visual cycle, including one of the fluorophores in LF bisretinoid N-retinyl-N-retinylidene ethanolamine (A2E). The last is characterized by high photoreactivity and implicates in age-related macular degeneration. Photoinduced production of reactive oxygen species (ROS) is shown among the main modes of A2E toxicity although particular photochemistry of A2E have yet to be completely identified. In this work we study changes in chemical composition of LF granules of human RPE under light irradiation using vibrational spectroscopy (femtosecond broadband CARS) and mass-spectrometry (TOF-SIMS) approach accompanied by measurements of fluorescence properties of the LF.

1. Introduction
Lipofuscin (LF) is a result of incomplete lysosomal degradation and represents autofluorescent material of cellular lipids and protein residues, traces of carbohydrates and metals [1]. LF is usually found as water insoluble micrometer-sized particles (lipofuscin granules, LFG) which cannot be further degraded by lysosomal enzymes or the proteasomes. Up to now, there is no any efficient way to remove LF from living organism. LF of the retinal pigment epithelium (RPE) contains byproducts of the visual cycle including bisretinoid N-retinyl-N-retinylidene ethanolamine (A2E) and its precursor phosphatidylethanolamine-bisretinoid (A2-PE) [2,3]. These constituents of LFG, especially A2E, have high photoreactivity leading to ROS and oxidized products under light illumination [4]. The A2E oxidation products are more hydrophilic and capable to leave LFG and diffuse through cytoplasm to other RPE intracellular organelles [5]. Ultraviolet radiation below 295 nm is well absorbed by cornea, however a fraction of UV light in range of 300-400 nm does reach retina [6]. The light penetration and oxygen-rich environment of the outer retina are among supporting factors contributing to enhanced oxidative processed in RPE. Moreover, in vitro experiments show various oxidation products can damage lipid membranes and cell structures, even in the absence of light [7-9]. Thus the accumulation of LFG in RPE is associated with the development of retinal diseases, particularly age-related macular degeneration (AMD), which in turn is considered as major cause of...
blindness with no satisfactory treatments yet exist [10]. Meanwhile determination of the distribution of LFG in human RPE cells reveals a progressive accumulation of LFG with increasing age, therefore LF is a hallmark of the natural aging process [11].

The amount of LF in RPE is primarily characterized by its high fluorescence originating from different all-trans-retinal conjugates [12,13]. Recent study shows significant difference in fluorescence intensity of RPE cell suspensions from the cadaver eyes with and without signs of AMD [14]. Earlier mentioned di-retinal conjugate A2E (as well as iso-A2E) is the first bisretinoid compound clearly identified in human RPE [3,4,15]. Among all other bisretinoids and products of their photooxidation and photodegradation A2E dominates in LFG fluorescence [16]. Spectral features of 21 known fluorophores fall at least into four categories: green-emitting fluorophores, a golden yellow-emitting fluorophore, yellow-green-emitting fluorophores and orange-red-emitting fluorophores [17]. High spatial resolution fluorescence microscopy shows fluorophores heterogeneous distribution across LFG meaning also LF fluorescence profile changes essentially from granule to granule [18]. Irradiation with visible light is known to alter the absorbance and fluorescence spectra of A2E and one of LFG. Earlier studies have shown A2E to photochemically initiate free-radical reactions in organized media and to photochemically produce ROS species like superoxide and peroxyl radicals in the presence of oxygen by reacting with the environment [19,20]. Among A2E oxidation products are different A2E-epoxides like 5,6-epoxy and 7,8-epoxyderivatives as well as 5,8-furanoderivatives [21]. However previous study shows light-induced changes in fluorescence characteristics of LFG and synthetic A2E system are different especially when excited in retina’s ‘blue light hazard’ region of the spectrum [22]. This implies chemical composition of known A2E photooxidation products could not fully describe LFG photochemistry which is still to be established. Recently, various methods based on mass-spectrometry or vibration spectroscopy techniques are widely used to study chemical composition of biological systems. Mass-spectrometry approach usually much more sensitive than that of absorption spectroscopy and capable to detect and quantify low level substances including A2E and oxidized A2E from RPE [23]. Thus, LF and A2E related studies recently used MALDI [24], MALDI–IMS [25], ToF-SIMS [26]. As to Raman spectroscopy approach, the one is usually represented by spontaneous Stokes modality of the spectroscopy which characterized by small cross-section of the scattering process and seems not to be practical dealing with LFG natural heterogeneity and high fluorescent properties of the LF. On the contrary, the non-linear approach of anti-Stokes Raman spectroscopy could address LFG features more effectively. In this work we study chemical profiles of large areas (containing thousands of granules) of LFG of human RPE tissues to deduce averaged effects of light irradiation using ToF-SIMS and femtosecond broadband coherent anti-Stokes microspectroscopy approach. Each studied irradiation dose is accompanied by appropriate fluorescent measurements of LFG.

2. Materials and methods

2.1 Samples

Experiments on tissue isolated from human-cadaver eyes were performed in compliance with officially accepted procedures [27]. The material without ophthalmologic diseases were obtained within 10 h of the deaths of their donors, ranging in age from 40 to 70 years. RPE cells were collected from human eyecups by gentle brushing in phosphate buffer (pH 7.2–7.4), further LF isolation was performed by the method described earlier [28,29]. The LFG were resuspended in 0.1 M potassium phosphate buffer (pH 7.3) and centrifuged. Buffer solution was replaced by mQ water to reduce potential matrix effect for ToF-SIMS. The homogenization of the LFG samples was carried out in a glass homogenizer. Obtained solution was diluted in mQ water. A drop of granules solution was placed on the cover slip and dried under gentle argon stream. All stages of sample preparation were carried out under subdued lighting. The LFG solution was illuminated by broadband LED (having 2 bell-shaped maximums at 450 and 550 nm) with long-wavelength spectrum components (λ > 500 nm) filtered out to provide ‘blue’ mode of illumination. The average irradiation power was ~10 mW/cm².
Samples were taken for ToF-SIMS and spectrofluorometric measurements at certain time intervals in range up to 160 min.

2.2 ToF-SIMS measurements
Mass spectrometry experiments were performed on the TOF.SIMS.5 instrument (ION-TOF, Germany) equipped with bismuth cluster beam [30]. An analysis area of 300×300 μm (64×64 pixels) was probed by 30 keV Bi$_3^+$ primary ions with a primary ion dose density of ~4×10$^{11}$ ions/cm$^2$ in every measurement. At least 15 measurements for each sample were recorded in both positive and negative ion modes. An electron flood gun was activated to avoid charging effect during analysis. Due to high energy of bismuth projectiles, biological species are regularly fragmented and therefore presented on mass spectrum as a set of characteristic ions. These fingerprint ions are known for lipids, amino acids, nucleotides, etc. The mass spectra were processed and analyzed by SurfaceLab 6 software. Ion yields were calculated as number of ions with specific mass divided by total ion counts.

2.3 Broadband CARS measurements
Broadband coherent anti-Stokes Raman scattering (BCARS) microspectrometer implemented as a part of femtosecond laser complex [31] representing two pulses collinear scheme for CARS generation [32]. Bell-shaped Stokes pulse (FWHM ~500 1/cm) at 800 nm negatively chirped using 1D-SLM to provide nearly bandwidth-limited pulse (~50 fs) at microscope area of the setup (M PLAN APO NIR series objectives, Mitutoyo). Narrowband (FWHM < 10 1/cm) sub-picosecond pump pulses centered at 710 and 730 nm to tune for vibrational bands of high-frequency part of the fingerprint range: 900-1300 and 1300-1800 1/cm, respectively. Measured spectra of biological samples were related to ones of UV fused silica glass to exclude non-resonant component of CARS. Finally, the related spectra transformed to Raman bands using CARS-variation of MEM described earlier [33,34]. Usual fine timing characteristics of BCARS allowed to collect large amount (~10$^5$) of spectra over different areas of control and treated samples to deal with sample heterogeneity on a micro scale.

3. Results and discussion
It is known the excitation of LFG with blue light induces fluorescence in the visible range of spectrum [35]. RPE spectral properties are sensitive to long-term light irradiation, having full recovery capabilities for illumination at $\lambda > 550$-600 nm and pronounced changes for blue part of wavelength range. The last range is supposed to produce actinic or photochemical effect at retinal irradiances while only thermal effects could be for $\lambda > 550$-600 nm. There is no sharp demarcation edge between thermal and actinic effects but actinic effects in the retina increases exponentially as the wavelength is decreased toward 400 nm [36]. Particularly, only 450 nm band of the irradiating LED leads to the change of LFG fluorescence properties (figure 1).
Figure 1. LFG fluorescence spectra for various LED exposure times with fluorescence excitation wavelength at 365 nm (a) and 450 nm (b).

As it was shown previously, irradiation with visible light results in decrease of absorption spectrum of LFG suspension at 430 nm with the simultaneous increase in absorbance intensity at 290 nm and isosbestic point close to 370 nm [22]. In other words, the species absorption in range of 370-450 nm vanish upon irradiation, while the new species absorbing in UV region are formed. This could explain general growth of LFG fluorescence intensity with increasing irradiation time for excitation at 365 nm (figure 1a). The spectra show peak close to 470 nm and some spectral peculiarities at 540-650 nm as seen before. These peculiarities are clearly seen as maximum while excitation of LFG fluorescence at $\lambda = 450$ nm (figure 1b). The A2E absorption spectrum has two characteristic maxima at 330 and 435 nm before irradiation [22]. These two maxima decrease substantially while shorter-wavelength peak at 280 nm dominates after 2 hours of irradiation. Both LFG and A2E show blue shift of fluorescence band at ~540 nm upon irradiation.

Figure 2. ToF-SIMS positive (a) and negative (b) fragments of LFG showing the most prominent change upon LED irradiation.

Our previous ToF-SIMS study of LFG profile revealed specific m/z which can be ascribed to phospholipids (phosphocholine ion, m/z 184), amino acid fragments (CH4N+, m/z 30, and C5H10N+, m/z 84) as well as A2E related peaks including singly- and doubly-oxidized A2E (A2E m/z 592; A2E-ox, m/z 608; A2E-2ox, m/z 624) [26]. Heterogeneous distribution of these m/z across LFG supports earlier conclusions of near-field fluorescent microspectroscopy [18]. The predicted fragmentation pattern of A2E includes m/z 468, 458, 442, 418, 404, 392, 376, 352 [23]. MALDI approach was able to detect many A2E precursors like phospho-A2E (m/z 672.5), A2-GPE (m/z 746.2), A2-PE (m/z 1224.8), including novel A2E-related compounds which were identified as singly oxidized and formylated A2E (m/z 634.4) and doubly oxidized and formylated A2E (m/z 650.4) [37]. Blue-light-induced modification of prepared A2E solutions also revealed a whole series of single additions of oxygen resulting in M(592)+16 ions with maximum intensity for bisoxirane (m/z 624) and ending with nonaoxirane of A2E (m/z 736) where all A2E double bounds oxidized except pyridine group [38]. Opening epoxy rings and MS cleavage along the polyene chains of A2E can give rise to group of fragments with m/z < 592: e.g. m/z 488 (M-136, cleavage of the C7/7'-C8/8' bond), m/z 458 (M-166, cleavage of the C8/8'-C9/9' bond). One hour irradiated A2E was shown to have a bunch of pronounced peaks for m/z in range of 350-550: 366, 382, 406, 422, 432, 438, 448, 454, 472, 476, 488, 492, 508, 522, 538 [39]. In this work we used ToF-SIMS to study which fragments of LFG evolve the most essentially upon LED irradiation with different exposure times. The fragments evolved for more than ~50% in terms of relative intensity (with respect to non-irradiated sample) summarized at figure 2.
and have following m/z: 41, 43, 69, 113, 60, 116, 446, 643. The last two are likely correspond to fluorophores (oxidized or non-oxidized) since similar non-monotonous behaviour is seen for the evolution of fluorescence profile over irradiation time (figure 1b). The rest fragments of figure 2 is seen to have either rise or drop of intensity over different time exposures. While m/z 41 represents common ratio (C3H5, CH2CN+H, CHN2) the others could mean the fragments with C=O bond included: m/z 43 (CH3C=O+), m/z 69 (CH3CH=CHC=O+, CH2=C(CH3)C=O+), m/z 60 (CH2-COOH+H), m/z 113 (α-cleavage of 2-octanone) [40]. Thus, the most of rising fragments of figure 2 are related to aldehydes and ketones accumulation.

LFG is a complex mixture composed of lipids, proteins and bisretinoids. There is an evidence purified granules could contain minimal amount of protein (~2%) [41]. Recently, Raman spectra from different parts of human retina sample has been reported [42]. The most of the areas contained Raman bands of lipids, proteins and DNA except one cluster where pronounced bands of carotenoids were seen. On the scale of other cell’s constituents, the amount of carotenoids usually not enough to identify the bands clearly. Therefore, resonance excitation of carotenoids can be specially used [43,44] to highlight 959 1/cm (ring methylene rocking), 1011 1/cm (methyl rocking), 1163, 1195 1/cm (C-C stretch), 1528 1/cm (C=C stretch) bands [45]. The complex chemical composition of LFG is seen on our measured averaged BCARS spectra (transformed to Raman bands) shown in figure 3.

![Figure 3](image_url)

**Figure 3.** BCARS spectra (transformed to Raman bands) for LFG before (blue) and after (red) LED irradiation for 100 min.

The lipid content of the LFG can be recognized by a typical spectral profile which is reflected in following main bands of lipids: ~1442 1/cm (CH2 scissoring mode), ~1660 1/cm (C=C stretching mode), ~1300 1/cm (in phase CH3 twisting mode), 1060–1090 and 1110–1180 1/cm (C-C stretching vibrations) including gauche C–C stretching (~1086 1/cm) and trans C–C (~1120 1/cm), ~1260 1/cm (=C–H deformations) [46]. The LFG protein content is reflected in following main bands: 1655-1670 1/cm (Amide I), ~1555 1/cm (Amide II), multiple modes (free from lipid bands) in range of 1310-1340 1/cm (including Amide III, CH3CH2 wagging and twisting vibrations), ~1050 1/cm (C-O, C-N stretching modes), ~123 1/cm (C-N, C-C stretching vibrations). Aromatic constituents of proteins like phenylalanine have typical narrow bands at: 1006 1/cm (ring vibrations), ~1030 1/cm (C-H in-plane bending mode) as well as 1586 1/cm and 1600 1/cm modes. Both lipids and proteins can contribute to bands in range of 950-990 1/cm: 968 1/cm related to lipids, 971 1/cm (C-C wagging mode), ~980 1/cm (C-C wagging vibrations in proteins) [47].

All other bands are at least less typical for lipid-protein mixture. Strong bands of pyridine ring are seen at 991 and 1030 1/cm as well as medium intensity band at ~1460 1/cm [48]. The bands 1375±10 1/cm and ~1465 1/cm are specific for alkane chain, representing C-H umbrella mode and C-H bending.
of methyl/methylene groups, respectively [49]. Moreover, five-membered aromatic heterocyclic systems like furans (possibly pyroles) have rather strong band at ~1485 1/cm (C=C stretching mode) and medium intensity band at ~1380 1/cm [50]. This is in agreement furanoderivatives could be among A2E oxidation products. Finally, the bands of aldehydes and ketones have 1390±10 1/cm (C-H bending), ~1415 and ~1465 1/cm (CH2 modes) and 1730±10 1/cm (saturated C=O stretching mode) [51]. For aromatic aldehydes and ketones, the C=O band moves in a lower wavenumber range, from 1710 to 1685 1/cm, because of conjugation. The ratio of band intensities at 1442 and 1660 1/cm represents system’s degree of saturation [52]. Using this ratio, it can be seen from figure 3 LFG system tends to be more saturated after irradiation (the ratio increment is more than 50%). On the other hand, the contribution of C=O bands (1685 and 1730 1/cm) with respect to aromatic C=C (the band of ~1600 1/cm, which seems to be unchanged) grows up to ~30% meaning LFG additional oxidation while LED irradiation.

4. Conclusion

Lipofuscin granules of human RPE tissues is a highly fluorescent mixture of lipids, proteins and bisretinoids. The last include phototoxnic substances like N-retinyl-N-retinylidene ethanolamine which is known to produce ROS species eventially changing chemical composition of LFG under the blue light illumination. These transformations can be seen indirectly by absorption and fluorescence spectroscopies while chemically sensitive methods like ToF-SIMS and B-CARS are able to trace LFG oxidation over time which is primarily seen as aldehydes and ketones accumulation.

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References

[1] Geng L, Wihlmark U, Algvere P 1999 Exp Eye Res 69 539
[2] Liu J, Itagaki Y, Ben-Shabat S, Nakanishi K, Sparrow J R 2000 J Biol Chem 275(38) 29354
[3] Crouch R K, Koutalos Y, Kono M, Schey K, Ablonczy Z 2015 Prog Mol Biol Transl Sci 134 449
[4] Lamb L E, Simon J D 2004 Photochemistry and Photobiology 79 127
[5] Dontsov A E, Sakina N L, Golubkov A M, Ostrovsky M A 2009 Dokl Biochem Biophys 425 98
[6] Boettner E A, Wolter J R 1962 Invest Ophthalmol 1 776
[7] Wu Y, Yanase E, Feng X, Siegel M, Sparrow J R 2010 Proc Natl Acad Sci USA 107 7275
[8] Sparrow J R, Vollmer-Snarr H R et al 2003 J Biol Chem 278 18207
[9] Sokolov V, Dontsov A E, Ostrovsky M A et al 2007 J Photochem Photobiol B 86 177
[10] Shaw P, Stiles T, Douglas C, Ho D, Fan W, Du H, Xiao X. 2016 AIMS Mol Sci 3 196
[11] Wing G L, Blanchard G C, Weiter J J 1978 Inv Ophthalmology and Vis Sci 17 7
[12] Sparrow J R, Wu Y, Yamamoto K, Zhou J et al 2010 Photochem Photobiol Sci, 9, 1480
[13] Yamamoto K, Yoon K D, Sparrow J R et al 2011 Invest Ophthalmol Vis Sci 52 9084 701
[14] Feldman T, Yakovleva M, Larichev A, Ostrovsky M A et al 2018 Eye (Lond) 32(9) 1440
[15] Sakai N, Decatur J, Nakanishi K, Eldred G 1996 J Am Chem Soc 118 1559
[16] Sparrow J R, Gregory-Roberts E, et al 2012 Prog Retin Eye Res 31 2 121
[17] Eldred G E, Katz M L 1988 Exp Eye Res 47 71
[18] Astafiev A, Dontsov A E, Ostrovsky M A et al 2005 Dokl Biochem Biophys 405 445
[19] Ragausskaitė L, Heckl R C, Gaillard E R 2001 Photochem Photobiol 74 483488
[20] Reszka K, Eldred G E, Wang R H et al 1995 Photochem. Photobiol 62 1005
[21] Jang Y, Matsuda H, Itagaki Y, Nakanishi K, Spar J 2005 J Biol Chem 280 39732
[22] Feldman T B, Yakovleva M A, Dontsov A E, Ostrovsky M A et al 2010 Russ Chem Bull 59 276
[23] Gutierrez D, Blakeley L et al 2010 Photochem Photobiol Sci 9(11) 1513
[24] Ablonczy Z, Higbee D et al 2013 Invest Ophthalmol Vis Sci 54 5535
[25] Adler L, Boyer N P, Anderson D M et al 2015 Photochem Photobiol Sci 141983
[26] Yakovleva M, Gulin A, Feldman T, Ostrovsky M A et al 2016 *Anal Bioanal Chem* 408 7521
[27] Feldman T B, Ostrovsky M A et al. 2015 *Anal Bioanal Chem* 407 4 1075
[28] Boulton M, Ostrovsky M A, Svistunenko D et al 1993 *Photochem Photobiol B Biol* 19 201
[29] Dontsov A E, Sakina N L, Ostrovsky M A 2012 *Rus Chem Bulletin International Ed* 61 2 442
[30] Gulin A A, Shakhov A M, Nadtochenko V A et al 2019 *Applied Surface Science* 481 144
[31] Nadtochenko V A, Aybush A V, Cherepanov D A et al 2017 *Nanomaterials* 7 371
[32] Zhang C, Zhang D, Cheng J 2015 *Annual Review of Biomedical Engineering* 17 415
[33] Vartiainen E, Rinia H, Muller M, Bonn M 2006 *Optics Express* 14 8 3622
[34] Okuno M et al 2010 *Angew Chem Int Ed* 49 6773
[35] Delori F, Dorey C, Staurenghi G et al 1995 *Invest Ophthalmol Vis Sci* 36 718
[36] Ham W T, Ruffolo J J et al 1980 *Vision Res* 20(12) 1105
[37] Ablonczy Z, Smith N, Crouch K et al 2014 *Proteomics* 14 936
[38] Ben-Shabat S, Itagaki Y et al 2002 *Angew Chem Int Ed Engl* 41(5) 814
[39] Wang Z, Keller L, Dillon J, Gaillard E 2006 *Photochem Photobiol* 82(5) 1251
[40] Brown W H et al, 2009 Organic Chemistry (Belmont: Brooks/Cole Cengage Learning)
[41] Ng K et al 2008 *Molecular & Cellular Proteomics* 7 1397
[42] Stiebing C, Popp J et al 2019 *Neurophotonics* 6(4) 041106
[43] Sharifzadeh M, Zhao D et al 2008 *J Opt Soc Am A Opt Image Sci Vis* 25(4) 947
[44] Meinhardt-Wollweber M, Suhr C, Kniggendorf A, Roth B 2018 *AIP Advances* 8 055320
[45] Tschirner N et al 2009 *Phys Chem Chem Phys* 11 11471
[46] Czamara K et al 2015 *J Raman Spectrosc* 46 4
[47] Movasaghi Z, Rehman S, Rehman I 2007 *App Spectrosc Rev* 42 5 493
[48] Corrsin L, Fax B J, Lord R C 1953 *J Chem Phys* 21 1170
[49] Smith B C 2015 *Spectroscopy* 30 9 40
[50] Kim T, Assary R et al 2011 *J Raman Spectrosc* 42 2069
[51] Smith B C 2017 *Spectroscopy* 32 11 28
[52] Sadeghi-Jorabchi H et al 1990 *JAOCS* 67 8 483