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Similar molecules spatially correlate with lipofuscin and N-retinylidene-N-retinylethanolamine in the mouse but not in the human retinal pigment epithelium

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Abstract

The accumulation of lipofuscin in the retinal pigment epithelium (RPE) has been implicated in the development of age-related macular degeneration (AMD) in humans. The exact composition of lipofuscin is not known but its best characterized component is N-retinylidene-N-retinylethanolamine (A2E), a byproduct of the retinoid visual cycle. Utilizing our recently developed matrix-assisted laser desorption/ionization imaging mass spectrometry (MALDI-IMS)-based technique to determine the spatial distribution of A2E, this study compares the relationships of lipofuscin fluorescence and A2E in the murine and human RPE on representative normal tissue. To identify molecules with similar spatial patterns, the images of A2E and lipofuscin were correlated with all the individual images in the MALDI-IMS dataset. In the murine RPE, there was a remarkable correlation between A2E and lipofuscin. In the human RPE, however, minimal correlation was detected. These results were reflected in the marked distinctions between the molecules that spatially correlated with the images of lipofuscin and A2E in the human RPE. While the distribution of murine lipofuscin showed highest similarities with some of the known A2E-adducts, the composition of human lipofuscin was significantly different. These results indicate that A2E metabolism may be altered in the human compared to the murine RPE.

Keywords

lipofuscin; A2E; retinoids; mass spectrometry; imaging; human; mouse; MALDI; profiling; RPE

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Introduction

Lipofuscin is the lipophilic non-degradable cellular waste that accumulates with age from early infancy [1] in lysosomal compartments of the RPE. The accumulation of lipofuscin is an aging marker and one of the most striking features of the aging eye [2–4]. Although in the context of the eye lipofuscin is most readily associated with the RPE, it is also present in many other post-mitotic cells including those of the central nervous system and the major organs [5] and its accumulation has been linked to diverse pathologies throughout the body [6–9]. In the RPE, where its pathophysiology has been most studied, lipofuscin has been proposed to correlate with the pathogenesis of AMD [10, 11].

Lipofuscin is usually characterized by fluorescence [2], which is detected as early as one year of age in the human RPE [3] with increasing signals observed until the age of 70–75 years [4]. The concentration of lipofuscin is highest in the center of the RPE, with the exception of the optic nerve-head [2]. Analytical studies on lipofuscin composition have been hampered by the insolubility of the material and are usually conducted on organic RPE extracts of whole-tissue preparations. The proteomics study by Ng et al. on human RPE lipofuscin granules shows a complex mixture of lipids and protein components; but with less than 2% of the material by weight containing amino acids [12]. Unfortunately, this approach prevents any spatial correlation of components to the characteristic fluorescence itself.

The molecular composition of lipofuscin is unknown, regardless of its tissue source; however, in the eye where retinoid content is high due to the visual cycle, lipofuscin is enriched in *bis*-retinoids [13]. Over 20 of these molecules have now been identified (see [14] for a review) as adducts of the retinaldehyde intermediers of the retinoid visual cycle [15–17]. The best known and most characterized of these molecules is an amphoteric quaternary amine, N-retinylidene-N-retinylethanolamine, with a chemical structure of 2-[2,6-dimethyl-8-(2,6,6-trimethyl-1-cyclohexen-1-yl)-1E,3E,5E,7E-octatetraenyl]-1-(2-hydroxyethyl)-4-[4-methyl-6-(2,6,6-trimethyl-1-cyclohexen-1-yl)-1E,3E,5E-hexatrienyl]-pyridinium, commonly abbreviated only as A2E.

In animal models, where the production of retinaldehyde is greatly suppressed, both lipofuscin and A2E are decreased in the RPE [18–20]. Additionally, in models where higher than normal amount of free retinaldehyde is present in the photoreceptors, lipofuscin and A2E can increase [21]. These observations are in agreement with the concept that the precursors of A2E (A2PE) form in the photoreceptors from retinaldehydes and phosphatidyl-ethanolamine, are internalized by the RPE via daily phagocytosis, and are partially degraded in the lysosomes where A2E eventually accumulates [18, 22]. There are numerous studies suggesting a toxic role for A2E in disease processes (reviewed in [14]). However, even though A2E can be toxic in certain conditions, studies have also suggested that they are less toxic compared to retinaldehydes, and, in this sense, their formation may be protective [23, 24]. Therefore, the role of A2E (positive or negative) *in vivo* is still unclear.

Using tissue punches of human RPE, the Bernstein laboratory found a spatial pattern of A2E surprisingly opposite to the previously reported distribution of lipofuscin (see papers from Bhosale et al. and Wing et al. [4, 25]). Since traditional immunolocalization techniques are not available to establish spatial patterns of A2E, we have recently developed a matrix-assisted laser desorption/ionization imaging mass spectrometry (MALDI-IMS)-based technique to compare the spatial distributions of A2E and lipofuscin first in mouse and then in human RPE [26, 27]. This technique is molecule-specific, has a high spatial resolution, and can collect spatial information simultaneously on many compounds. Our experiments demonstrated a high spatial correlation between the A2E signal and lipofuscin fluorescence

in the mouse but minimal correlation in the human RPE [26]. The human A2E distribution data reproduced the tissue-punch experiments [25] at higher resolution. Using representative normal (no disease) samples from the previous studies, the current manuscript is a methodical side-by-side qualitative comparison of the two species in terms of the spatial patterns of lipofuscin and A2E across the RPE tissue. The previous analyses were also extended with intensity profile information and a new image correlation strategy to identify the molecules in the complex mass spectral information with spatial patterns similar to lipofuscin and A2E. These data provided additional indirect evidence suggesting that A2E and *bis*-retinoid metabolism is altered in human compared to murine RPE.

MATERIALS and METHODS

Tissues

The human cadaver eye (age 88 year, 24 hours post mortem) was obtained from the San Diego Eye Bank (San Diego, CA). All protocols were approved by the Medical University of South Carolina Institutional Review Board. Details of tissue processing have been previously published [26]. *Sv129* mice were obtained from Harlan Laboratories (Indianapolis, IN). Procedures for the murine eye tissue preparation have been reported previously [27]. All animal work was designed and performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and protocols were approved by the Medical University of South Carolina Animal Care and Use Committee. After removal of the neural retina (and the sclera in the case of the human eyes), both human and mouse tissues were flat-mounted with their scleral-side down on indium tin oxide-coated conductive glass slides (Delta Technologies Ltd, Stillwater, MN), and desiccated under red light.

Fluorescence imaging

Fluorescence images of the human tissue were collected with a bioluminescence imaging system (IVIS 200, Caliper, Life Sciences, Hopkinton, MA) as reported previously [26]. Fluorescence images of mouse tissue of overlapping fields were collected using the SP2-RS Laser Scanning Confocal Microscope (Leica, Wetzlar, Germany) and assembled to provide a single image of the entire eyecup as detailed elsewhere [27]. For the images shown, pixel intensities were plotted on a color scale (0 – 100: red ~ 90, orange ~ 80, ~ yellow ~ 70, green ~ 50, light blue ~ 30, and dark blue ~ 15). Intensity profiles were generated along cross sectional lines in images transformed into an 8-bit absolute intensity scale without interpolation using the Image-J software (NIH, Bethesda, MD). After analysis with Image-J, the profile data were smoothed and normalized in Origin 7.5 (OriginLab, Hampton, MA).

MALDI imaging

The human tissue was hand sprayed with 20 mL of 10 mg/mL 2',5'-dihydroxyacetophenone (Sigma, St Louis, MO) in 70:30 ethanol (Thermo Fisher, Waltham, MA):water (v:v) using a 5 mL TLC sprayer (Thermo Fisher). MALDI data were collected on an AutoFlex II TOF mass spectrometer (Bruker, Billerica, MA) in the m/z 520 – 2500 range, with a raster step size of 300 μm . Additional technical details of the acquisition were described elsewhere [26]. The mouse tissue was prepared for MALDI imaging by automated matrix deposition (Portrait 630; Labcyte, Sunnyvale, CA) with 10 mg/mL 2',5'-dihydroxyacetophenone (Sigma) in 70:30 ethanol:water (v:v) with a final center-to-center spot distance of 150 μm . MALDI data were collected on an Ultraflextreme TOF mass spectrometer (Bruker) in the m/z 420 – 1400 range, with a raster step size of 150 μm . Additional technical details were published previously [27]. For both human and mouse, 8000 individual masses were collected in the observed mass ranges (see above) over the entire surface. MALDI images of

A2E were reconstructed from these data using FlexImaging 2.0 software (Bruker) with A2E (m/z 592) plotted in a mass window consisting of ± 0.5 m/z units (integrating data for 4–8 individual data points). For display purposes, data were normalized to total ion current, interpolated, and pixel intensities for all data sets plotted on the same color scale described above for “Fluorescence imaging”. Intensity profiles were generated with the same method described for “Fluorescence imaging”.

Correlation of lipofuscin and A2E with the molecules in the imaging dataset

For both human and mouse, the intensity profiles for all 8000 individually measured points in the m/z domain were exported into Analyze 7.5 format stacks of images on an 8-bit absolute intensity scale without interpolation. To calculate the correlation coefficients between the images of lipofuscin fluorescence and A2E and each individual image in the stack, the intensity distributions of lipofuscin fluorescence and A2E were transformed into an 8-bit absolute intensity scale without interpolation. Thereafter, the fluorescence images were remapped to the resolution of the MALDI images by pixel averaging. The similarity between the resultant fluorescence and A2E images and each of the individual images in the stack was quantitated by calculating pairwise Pearson correlation coefficients using Image-J. Correlation coefficients were plotted against the m/z values of the corresponding data points in the MALDI imaging dataset.

RESULTS

Intensity profiles of mouse and human lipofuscin and A2E

A comparison between the fluorescence image of lipofuscin (Fig. 1A) and the MALDI image of A2E at m/z 592 (Fig. 1B) in the RPE of the 6 month old murine RPE showed a remarkable agreement. To produce the comparison, these images were recorded consecutively (first fluorescence and then MALDI imaging) on the same flat mounted mouse eyecup (RPE/choroid/sclera), and they represent the respective intensity distributions. In the center of the tissue, the optic nerve head is clearly visible showing slightly higher fluorescence (see the asterisk) than the surrounding area (Fig. 1A) and having no detectable A2E signal (Fig. 1B). Both images indicate a left-central arrangement and exhibit visual spatial correlation. The resolution of the A2E imaging data itself is 150 μm , much less than that of the fluorescence image that was assembled from individual fields in a fluorescence microscope.

The apparent agreement between the lipofuscin and A2E distributions is further confirmed by plotting their intensity profiles along the same cross-sectional axis. The intensity profiles were extracted following the paths of the white arrows in Figs. 1A and B. Again, there is a good agreement between the lipofuscin and A2E intensity profiles (Fig. 1C).

Figure 2 shows the fluorescence image of lipofuscin (Fig. 2A) and the MALDI image of A2E (Fig. 2B) acquired from an 88 year human RPE. These images were also recorded consecutively on the same flat mounted tissue; however, due to the thickness of the human sclera, the RPE/choroid was peeled off and mounted by itself. Another difference from the mouse data was that fluorescence was not imaged with a microscope (as the human eye has a 100-fold larger surface area compared to the murine eye) but with a bioluminescence imager. Pilot experiments with fluorescent microscopy show lipofuscin distributions not significantly different from the images taken with the imaging system (data not shown). The area of the optic nerve head is seen slightly to the bottom right of center (see the asterisk) having lower fluorescence than the surrounding tissues (Fig. 2A). The signal is reduced in the optic nerve head area because for mounting the RPE/choroid the preparation needed to be completely severed from the optic nerve head, which resulted in the loss of a little tissue,

clearly visible in the flat mounted preparations. Since this is a right eye, the macular area is seen left of the optic nerve head, exhibited as a dip in the fluorescence intensity. While lipofuscin shows a bottomcentral arrangement, the distribution of A2E is rather bottom-peripheral. The observed distributions exhibit a clear lack of visual spatial correlation between the two images.

The difference between the spatial patterns of lipofuscin and A2E is further confirmed by plotting their respective intensity profiles over the same cross-sectional axis (Fig. 2C). The profiles were extracted along the lines of the white arrows from interpolation-free intensity data in Figs. 2A and B. It is evident from the two traces that lipofuscin and A2E do not overlap, rather, the profiles are the inverse of each other.

Molecules spatially correlating with lipofuscin and A2E

The MALDI-IMS datasets provide a unique opportunity to establish spatial correlation between the collected molecular data and either A2E or lipofuscin. The results of these experiments in the murine RPE are shown in Figure 3. Fig. 3A is a visual representation of the extracted mouse image stack (which consists of 8000 consecutive images in total) indicating, in this case, three randomly selected extracted images for display purposes. Fig. 3B shows the MALDI mass spectrum averaged over the whole surface (tissue profile) in the m/z 550–900 range. A2E (m/z 592) was one of the most abundant ions in this average mass spectrum and its singly oxidized version (A2E+ox, m/z 608) is also detected at significant levels. The array of molecules that spatially correlate with A2E in the mouse (Fig. 3D) are derived from charting the individual Pearson correlation coefficients between the image of A2E (Fig. 3C) and each component of the image stack against the molecular m/z value of the particular components. Figure 3C is the interpolation-free representation of the same image shown previously in Fig. 1B for A2E (m/z 592). Figure 3D indicates the correlation coefficients between A2E and each image in the stack in the m/z 550–900 range. A2E itself is identified by autocorrelation (the 0.96 correlation coefficient value at m/z 592). The autocorrelation is not perfect (1.00) as the A2E images (Figs. 1B and 3C) are generated by integration in a ± 0.5 m/z mass window, which contains several individual measurement points in the m/z domain. Figure 3F shows the array of correlations for lipofuscin fluorescence (Fig. 3E). At first sight the fluorescence image in Figure 3E is very different from Fig. 1A; however, they represent the same fluorescence images at the original resolution (Fig. 1A) compared to a resolution reduced to that of the MALDI imaging dataset (Fig. 3E). Consequently, similarly to Figs. 1A and B, there is a visually apparent correlation between Figs. 3C and E. Remarkably, the apparent correlation is confirmed by lipofuscin having essentially the same array of spatially correlating molecules as A2E. The 10 molecular masses with the highest correlation values in Figs. 3D and F respectively are shown in Table 1.

Figure 4 summarizes the molecules that spatially correlate with lipofuscin and A2E in the human dataset. Fig. 4A displays a visual representation of the extracted human image stack with three random images as examples. Fig. 4B shows the average MALDI mass spectrum calculated across of the whole tissue in the m/z 550–900 range. The MALDI profile of the human tissue is quite different from that of the mouse (Fig. 3B) and A2E is much less abundant compared to other ions in the mass spectrum of the human RPE. Singly oxidized A2E is not apparent, as its signal was barely above the baseline. The array of correlations between each image in the stack and A2E (Fig. 4C) is again derived from charting the respective Pearson correlation coefficients (Fig. 4D). It is likewise quite different from the similar mouse data (Fig. 3D). As seen previously with the mouse correlations, A2E itself is identified by autocorrelation (0.94 correlation coefficient). Figure 4F shows the correlation coefficients between lipofuscin fluorescence (Fig. 4E) and the images in the stack. Contrary to the mouse data, in the human RPE the molecules that spatially correlate with A2E and

lipofuscin are quite different, confirming the lack of correlations seen by eye (Figs. 2A and B). The 10 molecular masses with the highest correlation values in Figs. 4D and F are summarized in Table 1.

DISCUSSION

MALDI-IMS is an ideal technique to spatially resolve specific compounds (such as A2E) at the molecular level, allowing a direct comparison with lipofuscin, which is usually characterized by its fluorescence with a high spatial resolution but no molecular specificity. Although other molecularly selective techniques to determine A2E may have higher sensitivity, more robust ionization, and less dependence on the actual structure of the molecules (for additional details see [26, 27]), they involve the homogenization of the tissue and extractions with organic solvents which are very invasive compared to MALDI and preclude the gathering of spatial information. Utilizing a strategy for consecutive acquisition of fluorescence and MALDI imaging data in the same tissue, we have successfully compared the spatial distributions of lipofuscin and A2E for two murine models, *Abca4*^{-/-} mice (with a rapid accumulation of A2E [28]) and *Sv129* mice (the base strain for the *Abca4*^{-/-}) [27]. Employing a similar strategy, we have also compared lipofuscin and A2E in two dozen human cadaver eyes over ten decades of life [26]. In order to provide a more comprehensive qualitative comparison of the results in mouse and human RPE, the current study utilized a single left eye from a 6 month old *Sv129* mouse and an 88 year human cadaveric right eye. Both of these representative tissues belonged to groups considered normal as the mouse eye was from a wild-type background and the human eye had no diagnosed ocular disease. Although there is a significant difference in size (the human RPE surface is 100 fold larger compared to the mouse), which results in a huge difference in absolute A2E content (~5 pmole in mouse and ~1 nmole in human), the MALDI imaging technique is capable of detecting 200 attomole A2E in a single spot [26].

The 88-year old eye was somewhat atypical compared to previously published data due to tissue loss in the top right quadrant of the sample. Apart from the tissue loss, the fluorescence profiles of this tissue (Fig. 2A and 2C) match those published independently [4] and by our own laboratory [26, 29]. Thus, this issue did not change the overall qualitative statements about the tissue.

The basic findings of the previous studies were that in the murine RPE, there was a remarkable correlation between A2E and lipofuscin images evidenced by their mutual central distributions. The more comprehensive correlation analysis presented here clearly reinforces and extends these previous observations. On the other hand, in the human eyes, the levels of A2E were highest in the far periphery and decreased towards the central region while lipofuscin followed the central distribution described previously in detail [4]. These observations for the human RPE were somewhat unexpected but are in good agreement with the data previously obtained with lower spatial resolution from tissue punches [25].

A particular advantage of mass spectrometry is that it allows for the collection of data on multiple specific molecules at the same time (multiplexicity). In the MALDI imaging experiments, spatial distribution information is recorded on 8000 individual points in the m/z domain spanning the entire mass range. We have utilized this unique imaging information to mathematically formalize the phenomenological visual relationships between the lipofuscin and A2E images by determining the ensemble of molecules that spatially correlate with either lipofuscin or A2E respectively. The strategy for identifying the spatially correlating molecules is that for each collected data points in the m/z domain, there is a corresponding image in the extracted stack of images, exhibiting a unique intensity distribution the correlations of which can be quantitated. The utilization of the imaging

(intensity distribution) information acts as a powerful filter for identifying molecular companions in the otherwise noisy and large dataset otherwise lost to traditional techniques based on tissue homogenization.

To understand the molecular basis for the differences between the distributions of human lipofuscin and A2E, we have identified the arrays of molecules that co-localize with lipofuscin and A2E respectively in both mouse and human RPE. As expected, A2E autocorrelated with itself, and the distributions of oxidized A2E molecules followed that of A2E as was shown previously [26, 27]. Importantly, the correlation strategy presented here, identified the oxides of A2E as one of the highly co-localizing molecules with A2E; thus, validating the approach. The array of molecules that spatially correlated with lipofuscin was particularly interesting because in the human tissue, there is currently no molecular explanation for the excess fluorescence in the center of the RPE. In the mouse RPE, the molecules that correlated with lipofuscin matched with those that correlated with A2E almost completely. The actual correlation coefficient between lipofuscin and the image at m/z 592.4 (corresponding to A2E) was 0.56, somewhat lower than the value published before (0.66), likely due to the fact that the image of A2E used for the previous calculations [27] was an average over a ± 0.5 m/z mass window (which contains 4–8 individually measured data points integrated), while in this study, it represented an image corresponding to a single measurement point in the m/z domain. Interestingly, the molecules that spatially correlated with either A2E (potential A2E-adducts) or lipofuscin fluorescence were significantly different in human compared to mouse.

Although the above experiments provided additional information on the molecules that spatially correlate with lipofuscin and A2E, they left the paradox of human A2E unresolved. One possible explanation may be the interaction of A2E with light. Light is known to increase the breakdown of A2E into more harmful products [30] and it is conceivable that light exposure in the center of the human eye is stronger than in the periphery. In this regard, it is important to note that humans seek light out while mice naturally try to avoid light. Another possible explanation is that A2E accumulation is dependent on the influx of rod photopigment into the RPE, which is known to be somewhat less in the center and more in the periphery of the human eye [31, 32]. A third possibility might be the newly discovered cone visual cycle (see reviewed in [29]). It is known now that there is a substantial retinoid flux between cone photoreceptors and Muller cells [33], where 11-*cis* generation can also take place [34]. It is not inconceivable that a substantial amount of retinoids are circulating primarily within the retina in the area of the posterior pole. Supporting this idea, autofluorescent lipofuscin granules were found in human cone inner segments but not in rods [35]. However, the amount of lipofuscin was negligible in the cones compared to the RPE, perhaps because the posterior pole area of the retina contains a large amount of macular pigments [36] potentially capable of antagonizing harmful photooxidative reactions, such as the generation of A2E and its precursors. Although further research is needed to resolve these questions, the authors believe that understanding the similarities and differences in the composition of mouse and human lipofuscin is central any potential answers. The methodologies described in this paper identified some particularly interesting candidates (such as ions at m/z 678 and m/z 828). The molecular identities of these ions are yet not known, however, they do not seem to be consistent with *bis*-retinoids. Further analysis of these ions as well as of all the collected mouse [27] and human [26] data will serve in the near future to continue to answer the apparent mystery of human A2E.

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Abbreviations

A2E	N-retinylidene-N-retinylethanolamine
RPE	retinal pigment epithelium
MALDI-IMS	matrix-assisted laser desorption/ionization imaging mass spectrometry
AMD	age-related macular degeneration
HPLC	high-performance liquid chromatography

REFERENCES

- Porta EA. Ann. NY Acad. Sci. 2002; 959:57–65. [PubMed: 11976186]
- Delori FC, Dorey CK, Staurengi G, Arend O, Goger DG, Weiter JJ. Invest Ophthalmol Vis Sci. 1995; 36:718–729. [PubMed: 7890502]
- Feeney L. Invest Ophthalmol Vis Sci. 1978; 17:583–600. [PubMed: 669890]
- Wing GL, Blanchard GC, Weiter JJ. Invest Ophthalmol Vis Sci. 1978; 17:601–607. [PubMed: 669891]
- Nakano M, Oenzil F, Mizuno T, Gotoh S. Gerontology. 1995; 41:69–80. [PubMed: 8821322]
- Nakanishi H, Amano T, Sastradipura DF, Yoshimine Y, Tsukuba T, Tanabe K, Hirotsu I, Ohono T, Yamamoto K. J. Neurochem. 1997; 68:739–749. [PubMed: 9003065]
- Ulfig N. Neurosci. Res. 1989; 6:456–462. [PubMed: 2771203]
- Hashemzadeh-Bonehi L, Phillips RG, Cairns NJ, Mosaheb S, Thorpe JR. Exp. Neurol. 2006; 199:328–338. [PubMed: 16480979]
- Bajaj NP, Al-Sarraj ST, Anderson V, Kibble M, Leigh N, Miller CC. Neurosci. Lett. 1998; 245:45–48. [PubMed: 9596352]
- Sparrow JR, Boulton M. Experimental eye research. 2005; 80:595–606. [PubMed: 15862166]
- Winkler BS, Boulton ME, Gottsch JD, Sternberg P. Molecular vision. 1999; 5:32. [PubMed: 10562656]
- Ng KP, Gugliu B, Renganathan K, Davies MW, Gu X, Crabb JS, Kim SR, Rozanowska MB, Bonilha VL, Rayborn ME, Salomon RG, Sparrow JR, Boulton ME, Hollyfield JG, Crabb JW. Mol. Cell. Proteomics. 2008; 7:1397–1405. [PubMed: 18436525]
- Sparrow JR, Wu Y, Kim CY, Zhou J. J. Lipid. Res. 2009; 51:247–261. [PubMed: 19666736]
- Sparrow JR, Gregory-Roberts E, Yamamoto K, Blonska A, Ghosh SK, Ueda K, Zhou J. Progress in retinal and eye research. 2012; 31:121–135. [PubMed: 22209824]
- Eldred GE, Lasky MR. Nature. 1993; 361:724–726. [PubMed: 8441466]
- Parish CA, Hashimoto M, Nakanishi K, Dillon J, Sparrow J. Proc Natl Acad Sci U S A. 1998; 95:14609–14613. [PubMed: 9843937]
- Sakai N, Decatur J, Nakanishi K, Eldred GE. J. Am. Chem. Soc. 1996; 118:1559–1560.
- Boyer NP, Higbee D, Currin MB, Blakeley LR, Chen C, Ablonczy Z, Crouch RK, Koutalos Y. The Journal of biological chemistry. 2012; 287:22276–22286. [PubMed: 22570475]
- Katz ML, Drea CM, Robison WG Jr. Mech Ageing Dev. 1986; 35:291–305. [PubMed: 3773574]
- Katz ML, Redmond TM. Invest Ophthalmol Vis Sci. 2001; 42:3023–3030. [PubMed: 11687551]
- Maeda A, Golczak M, Maeda T, Palczewski K. Invest Ophthalmol Vis Sci. 2009; 50:5435–5443. [PubMed: 19553623]
- Liu J, Itagaki Y, Ben-Shabat S, Nakanishi K, Sparrow JR. J. Biol. Chem. 2000; 275:29354–29360. [PubMed: 10887199]

23. Maeda A, Golczak M, Chen Y, Okano K, Kohno H, Shiose S, Ishikawa K, Harte W, Palczewska G, Maeda T, Palczewski K. *Nature chemical biology*. 2011; 8:170–178.
24. Roberts JE, Kukienczak BM, Hu DN, Miller DS, Bilski P, Sik RH, Motten AG, Chignell CF. *Photochem. Photobiol.* 2002; 75:184–190. [PubMed: 11883606]
25. Bhosale P, Serban B, Bernstein PS. *Arch. Biochem. Biophys.* 2009; 483:175–181. [PubMed: 18926795]
26. Ablonczy Z, Higbee D, Anderson DM, Dahrouj M, Grey AC, Gutierrez D, Koutalos Y, Schey KL, Hanneken A, Crouch RK. *Invest Ophthalmol Vis Sci*. 2013 in press.
27. Grey AC, Crouch RK, Koutalos Y, Schey KL, Ablonczy Z. *Invest Ophthalmol Vis Sci*. 2011; 52:3926–3933. [PubMed: 21357388]
28. Mata NL, Weng J, Travis GH. *Proc. Natl. Acad. Sci. USA*. 2000; 97:7154–7159. [PubMed: 10852960]
29. Tang PH, Kono M, Koutalos Y, Ablonczy Z, Crouch RK. *Progress in retinal and eye research*. 2013; 32:48–63. [PubMed: 23063666]
30. Wu Y, Yanase E, Feng X, Siegel MM, Sparrow JR. *Proc Natl Acad Sci U S A*. 2010; 107:7275–7280. [PubMed: 20368460]
31. Tornow RP, Stilling R. *Acta anatomica*. 1998; 162:163–168. [PubMed: 9831764]
32. Young RW. *The Journal of cell biology*. 1971; 49:303–318. [PubMed: 19866760]
33. Wang JS, Estevez ME, Cornwall MC, Kefalov VJ. *Nature neuroscience*. 2009; 12:295–302.
34. Kaylor JJ, Yuan Q, Cook J, Sarfare S, Makshanoff J, Miu A, Kim A, Kim P, Habib S, Roybal CN, Xu T, Nusinowitz S, Travis GH. *Nature chemical biology*. 2013; 9:30–36.
35. Iwasaki M, Inomata H. *Invest Ophthalmol Vis Sci*. 1988; 29:671–679. [PubMed: 3366562]
36. Landrum JT, Bone RA. *Archives of biochemistry and biophysics*. 2001; 385:28–40. [PubMed: 11361022]

- Remarkable correlation between A2E and lipofuscin in the murine RPE.
- Minimal correlation between A2E and lipofuscin detected in the human RPE.
- High similarity of molecules spatially correlating with lipofuscin and A2E in the mouse RPE.
- Clear distinctions between the molecules spatially correlating with lipofuscin and A2E in humans.
- Murine but not human lipofuscin was localized similarly to known A2E-adducts.

Figure 1. Lipofuscin fluorescence and MALDI images of A2E in murine eye

Lipofuscin fluorescence image (A) was stitched together from individual fields taken with a fluorescent microscope ($\lambda_{exc} = 488$ nm) from a left eye of a 6 month old *Sv129* mouse. The optic nerve head is indicated by asterisk to the right. The MALDI image of A2E (B) was collected in the same tissue at 150 μ m resolution and was interpolated to improve optical resolution. It represents integrated signal from a ± 0.5 m/z mass window around m/z 592.4. The images are oriented: dorsal – top; ventral – bottom; nasal – right; temporal – left. The intensity is coded by the indicated false color scale. Bar = 1 mm. C, Normalized cross-sectional intensity profiles of lipofuscin fluorescence (dashed blue line, derived from A) and

A2E (red line, derived from **B**). The intensity profiles were extracted along the path of the white arrows from interpolation-free intensity data corresponding to **A** and **B** respectively and were smoothed. The optic nerve head is indicated by asterisk. There is a good agreement between lipofuscin and A2E intensity distributions.

Figure 2. Lipofuscin fluorescence and MALDI images of A2E in human eye

Lipofuscin fluorescence image (**A**) was acquired in a Xenogen IVIS 200 bioluminescence imaging system ($\lambda_{exc} = 450\text{--}490\text{ nm}$, $\lambda_{em} = 575\text{--}650\text{ nm}$) at a final resolution of $350\text{ }\mu\text{m}$ in an 88 year right human eye. The macular area is indicated by a dashed circle and the optic nerve head area is indicated by asterisk to the top left. The MALDI image of A2E (**B**) was collected in the same tissue at $300\text{ }\mu\text{m}$ resolution and was interpolated to improve optical resolution. It represents integrated signal from a $\pm 0.5\text{ m/z}$ mass window around $m/z\ 592.4$. The images are oriented: dorsal – top; ventral – bottom; nasal – left; temporal – right. The intensity is coded by the indicated false color scale. Bar = 1 cm. **C**, Normalized cross-

sectional intensity profiles of lipofuscin fluorescence (dashed blue line, derived from **A**) and A2E (red line, derived from **B**). The intensity profiles were extracted along the path of the white arrows from interpolation-free intensity data corresponding to **A** and **B** respectively and were smoothed. The macular area is indicated by # sign below and the optic nerve head by an asterisk to the right. There is little agreement between lipofuscin and A2E intensity distributions.

Figure 3. Molecules spatially correlating with lipofuscin and A2E in murine eyes

A, Schematic representation of the murine image stack, indicating three random images at m/z 555, m/z 727, and m/z 811. **B**, Average mass spectrum (MALDI profile) from the 6 month old Sv129 tissue in the m/z 550–950 range. The spectrum represents the molecules present in the entire dataset. The positions of A2E (m/z 592) and singly-oxidized A2E (m/z 608) are indicated. **C**, The interpolation-free MALDI image of A2E (comparable to Fig. 1B but at the resolution of the imaging dataset) used to determine which molecules spatially correlate with A2E. The intensity is coded by the indicated color scale. **D**, The array of Pearson correlation coefficients between the image of A2E (panel C) and every individually collected molecular component (indicated by their respective m/z value) shown in the m/z 550–950 range. **E**, The lipofuscin fluorescence image used to determine which molecules spatially correlate with lipofuscin (comparable to Fig. 1A but at the resolution of the imaging dataset). **F**, The array of Pearson correlation coefficients between the image of lipofuscin fluorescence (panel E) and each images in the stack (indicated by their respective m/z value) shown in the m/z 550–950 range.

Figure 4. Molecules spatially correlating with lipofuscin and A2E in human eyes

A, Schematic representation of the human image stack, indicating three random example molecular images at m/z 570, m/z 696, and m/z 783. **B**, Average mass spectrum (MALDI profile) from the 88 year old human tissue in the m/z 550–950 range. The spectrum represents the molecules present in the entire dataset. The position of A2E (m/z 592) is indicated. **C**, The interpolation-free MALDI image of A2E (comparable to Fig. 2B) used to determine which molecular masses spatially correlate with A2E. The intensity is coded by the indicated color scale. **D**, The array of Pearson correlation coefficients between the image of A2E (panel C) and each image in the stack (indicated by their respective m/z value) shown in the m/z 550–950 range. The location of singly-oxidized A2E (m/z 608) is indicated. **E**, The lipofuscin fluorescence image used to determine which molecules co-localize with lipofuscin (comparable to Fig. 2A). **F**, The array of Pearson correlation coefficients between the image of lipofuscin fluorescence (panel E) and each image in the stack (indicated by their respective m/z value) shown in the m/z 550–950 range.

Table 1

The ten most abundant co-localizing molecular ions.

Mouse		Human			
A2E		lipofuscin		A2E	
MW	R	MW	R	MW	R
592.38	0.96	592.49	0.56	591.99	0.94
677.59	0.87	677.59	0.56	569.63	0.82
608.42	0.86	608.53	0.51	530.65	0.80
624.46	0.76	624.57	0.48	553.52	0.79
815.81	0.75	673.6	0.47	531.69	0.77
778.15	0.73	814.9	0.47	575.87	0.76
673.72	0.7	649.71	0.46	524.67	0.73
759.72	0.7	778.15	0.46	570.67	0.71
649.71	0.69	828.78	0.45	547.28	0.70
634.58	0.68	844.82	0.45	586.01	0.68
				827.46	0.27

MW is molecular weight; R is the Pearson correlation coefficient. The correlations were calculated in the two species compared to the images of A2E and lipofuscin fluorescence respectively, as indicated.