Spatial and Spectral Characterization of Human Retinal Pigment Epithelium Fluorophore Families by Ex Vivo Hyperspectral Autofluorescence Imaging

Tal Ben Ami1, Yuehong Tong1, Alauddin Bhuiyan1, Carrie Huisingh2, Zsolt Ablonczy3, Thomas Ach4, Christine A. Curcio2, and R. Theodore Smith1

1 Department of Ophthalmology, New York University School of Medicine, New York, New York, USA
2 Department of Ophthalmology, University of Alabama at Birmingham, Birmingham, Alabama, USA
3 Department of Ophthalmology, Medical University of South Carolina, Charleston, South Carolina, USA
4 Department of Ophthalmology, University Hospital of Würzburg, Würzburg, Germany

Correspondence: Tal Ben Ami, Department of Ophthalmology, New York University School of Medicine, New York, New York, USA. e-mail: tbenami@gmail.com

Received: 6 October 2015
Accepted: 28 March 2016
Published: 17 May 2016

Keywords: hyperspectral imaging; retinal pigment epithelium; lipofuscin; autofluorescence imaging; bisretinoids

Citation: Ben Ami T, Tong Y, Bhuiyan A, et al. Spatial and spectral characterization of human retinal pigment epithelium fluorophore families by ex vivo hyperspectral autofluorescence imaging. Trans Vis Sci Tech. 2016;5(3):5, doi:10.1167/tvst.5.3.5

Introduction

The retinal pigment epithelium (RPE) is a monolayer of cells exterior to the photoreceptors of the neurosensory retina.1 The RPE rests on Bruch’s membrane (BrM), a five-layered extracellular matrix serving both as the substrate for RPE attachment and as a vessel wall at the inner aspect of the choroidal vasculature that nourishes outer retinal cells.2 Changes in the RPE are considered central to the initiation and progression of age-related macular degeneration (AMD), a major cause of vision loss among older persons worldwide. Among RPE’s various roles, generating vitamin A derivatives required for phototransduction through a series of biochemical reactions known as the visual cycle is perhaps the one best understood.

Byproducts of the visual cycle contribute to the autofluorescence (AF) signal of lipofuscin (LF) and...
melanolipofuscin (ML) granules in the lysosomal compartment of the RPE. This intense signal can be clinically visualized, and abnormalities in the clinical AF signal are strong markers for AMD and its progression. AF attributable to LF/ML presents a single broad emission spectrum, which represents a sum of multiple constituents. Knowing the individual components of this spectrum is vital to understanding the role of the RPE in health and disease. An extensive literature on bisretinoid biochemistry describes the search for candidate RPE fluorophores. Over 25 bisretinoid fluorophores have been extracted from LF/ML granules in the RPE, including N-retinylidene-N-retinylethanolamine (A2E), generally with analytic techniques that did not preserve spatial resolution.

This study used hyperspectral imaging technology, which captures images of an object or tissue at multiple wavelengths to seek unique patterns of emission, or spectral signatures, of components within it. Detection of such signatures is widely used in many scientific fields. We previously described a novel biomedical engineering algorithm based on nonnegative matrix factorization (NMF) for analysis of hyperspectral AF images and validated it on such ex vivo images of human RPE and BrM. We demonstrated that this algorithm was capable of detecting plausible spectral signatures of abundant RPE fluorophores. The goal of the present study is to apply this image analysis tool systematically on the RPE to provide data of interest specifically to vision science, namely, a comprehensive spatial and spectral catalog of the signatures of abundant RPE fluorophore families in healthy eyes. This portfolio of information can then serve as a guide for further research with techniques that are capable of precise molecular identification of the compounds themselves, such as imaging mass spectrometry (IMS); spatial localization of the enclosing organelles, such as structured illumination microscopy; and eventual extension to tissues from eyes with retinal disease. With spectra linked to specific compounds, hyperspectral imaging should achieve its full potential of spectral, molecular biopsy of the RPE, with potential for better understanding of AMD pathophysiology.

**Methods**

**Eyes and Tissues**

Twenty-μm thin flatmounts of human RPE attached to BrM (RPE/BrM) from 20 eyes lacking detectable retinal pathology, from 20 human donors, were prepared by removing the choroid and neurosensory retina, as previously described. These normal specimens were part of a research repository collected from the Alabama Eye Bank (1995–2009) and were used for the study of AF and RPE cell number with age. The ages of donors analyzed were thus dictated by the experimental design of our prior study (2 groups, >80 years and ≤51 years).

**Image Acquisition**

In each flatmount, three tissue areas were imaged and analyzed at fixed distances relative to the fovea: fovea center, perifovea (2-mm superior), and near-periphery (10- to 12-mm superior). The RPE in each of these locations relates to distinctive photoreceptor populations in the overlying retina: cones only, highest rod density, and highest rod/cone ratio, respectively.

Microscopy was performed using a Zeiss Axio Imager A2 microscope equipped with a ×40 oil objective (numerical aperture = 0.75; Carl Zeiss, Jena, Germany) and two filter cubes (436/460 and 480/510 nm band pass excitation/long pass emission; Chroma Technology Corp., Bellows Falls, VT) and connected to an external mercury arc light source (X-Cite 120Q; Lumen Dynamics Group, Inc., Mississauga, Ontario, Canada).

We refer to the two excitation band wavelengths as $\lambda_{ex}$ 436 and $\lambda_{ex}$ 480 nm. At each of the three locations for each of the 20 eyes, a hyperspectral data cube from RPE and BrM was acquired at each of these wavelengths using a hyperspectral camera (Nuance FX, Nuance 3.0.1.2 software; Caliper Life Sciences, Waltham, MA), for a total of 120 tissue datasets. Upon excitation with $\lambda_{ex}$ 436 and $\lambda_{ex}$ 480 nm, emission spectra were captured between 420 and 720 nm and between 510 and 720 nm, respectively, at 10-nm intervals. Data were recalibrated with respect to the camera’s spectral sensitivity, which is nearly linear from 450 to 700 nm.

**Extraction of Fluorophore Spectra and Tissue Localizations**

Custom MATLAB-based programs (MATLAB, release 2013a; MathWorks, Inc., Natick, MA) recovered the abundant spectra. The two hyperspectral emission data cubes, one for each excitation, were explored using NMF, an advanced mathematical tool which we extended to nonnegative tensor factorization (NTF), described below, for simultaneous decomposition of the two coregis-
terered hyperspectral data cubes. Briefly, the algorithm is initialized by the user, who samples the raw spectra of BrM and RPE from each data cube. The rest of the process is fully automated, so that data is acquired in an unbiased manner applied uniformly to each RPE/BrM tissue hyperspectral dataset. Next, a 4-Gaussian mixture model is fit to the RPE spectrum, and NMF analysis is initialized with the 4 Gaussians and the BrM spectrum. Finally, NMF analysis is performed simultaneously on the two excitation datasets by NTF, in which the simultaneous solutions for the recovered spectra are constrained to have identical spatial abundances. At this point, the solutions for the recovered spectra are constrained to excitation datasets by NTF, in which the simultaneous solutions for the recovered spectra are constrained to have identical spatial abundances. At this point, the paired datasets from the two excitation wavelengths are treated as a single dataset. The NTF process thus recovers five abundant spectra for each excitation wavelength and a tissue abundance image for each spectrum that describes its spatial localization. It is important to note, however, that the solutions are not constrained by any spectral or spatial characteristics, except that the paired abundance images for signal pairs from the two excitations are held identical. It is only after the algorithm has found a set of solutions that the number of abundant spectra, their spectral peaks, and their spatial localizations are recorded.

Smooth, well-defined spectra $S_1$ to $S_3$ and abundances consistent with LF/ML perinuclear localization were sought at all three locations (fovea, perifovea, and near-periphery) and at both excitations. Eyes were excluded, with all their hyperspectral datasets, if at least one location had a spectrum that was missing or uninterpretable (i.e., nearly flat or with multiple sharp peaks). The purpose of these exclusions was to avoid missing data when comparisons were made between locations in the final dataset. The NTF process thus recovers five abundant spectra for each excitation wavelength and a tissue abundance image for each spectrum that describes its spatial localization. It is important to note, however, that the solutions are not constrained by any spectral or spatial characteristics, except that the paired abundance images for signal pairs from the two excitations are held identical. It is only after the algorithm has found a set of solutions that the number of abundant spectra, their spectral peaks, and their spatial localizations are recorded.

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$$SAD(\hat{S}, S) = \cos^{-1} \left( \frac{S^T \hat{S}}{\sqrt{S^T S \hat{S}^T \hat{S}}} \right) $$  \hspace{1cm} (I)$$

where $S^T$ is the transpose of $S$. This angle takes the value 0 if the spectra are perfectly correlated and takes the maximum value $\pi/2$ (or $90^\circ$) if the spectra are completely uncorrelated.

For studying colocalization, overlaying abundance images in a suitable image manipulation program (Photoshop CC 2015; Adobe Systems, San Jose, CA) was more informative than side-by-side inspection. Further, by assigning standard colors (not the wavelengths of the emissions) to each of the overlaid signal abundances, with appropriate mixing, we false-colored the tissue to provide a visually intuitive interpretation of colocalizations and relative intensities. If one signal abundance is assigned the color green, and another red, then areas where they colocalize will appear yellow, or perhaps orange if the red signal is more abundant.

Tissue colocalizations of the abundances (spectral sources) were also calculated quantitatively in selected examples with the Pearson correlation coefficient using a custom MATLAB program. For tissues in which patches of bare BrM were present, a mask was first applied to eliminate BrM from the calculation of correlations between LF/ML spectra.

### Statistical Analysis

Extracted data were analyzed with descriptive and inferential statistics using SPSS Version 22 (IBM Corp., Armonk, NY). The $\chi^2$ test was used to compute categorical outcomes. Type I error was set at 0.05. The positions of the hyperspectral AF peaks assigned to each LF/ML spectral component were further compared between young and aged eyes at the different retinal locations using a Wilcoxon Mann-Whitney $U$ test. “Young” was defined as less than or equal to 51 years, and “aged” was defined as greater than or equal 80 years.

### Results

#### Subjects

The ages of the 14 donors ranged from 16 to 90 years, divided into seven young and seven aged; 29% were male, and 71% were female. Donor demographics can be found in the Table and in Supplementary Table S1.
Major Spectral Emissions

The five individual recovered spectra in the 436-nm set were labeled and color coded as follows: in the first pass, the three most abundant LF/ML signals in peak wavelength order were assigned to S1 (green), S2 (blue), and S3 (red). S0 (gray) was assigned to the spectrum localizing to BrM. However, it was apparent that in many, but not all, cases there were two signals in the 500- to 550-nm range, at which S1 typically appeared. We chose to identify these as S1A and S1B (green and azure, respectively); the next signal was then S2 (blue), and so on. S1A and S1B appeared to be the splitting of S1 into two signals, suggesting two families with similar emissions. In all, 19/42 imaging locations showed a split S1 at one wavelength or both, with 16 showing split signals at both wavelengths. When S1 was not split into S1A and S1B, then in addition to S0 (BrM) and S1, S2, and S3 (all LF/ML), a fifth, variable spectrum was recovered in some cases. It was labeled SX and assigned a new color (magenta); the abundance for SX, however, was clearly BrM and/or LF/ML in all cases. The signals at 480 nm were then tabulated in peak wavelength order to correspond with their counterparts at 436 nm, except as noted below. An example in which all signals are evident at each excitation wavelength is in Figure 1A.

Spectral Signatures by Peak Emission

The distribution of recovered spectra from all 42 tissue locations is detailed in Table. The peak emissions of the BrM (S0) and S1A, S1B, S2, and S3 signals at \( \lambda_{ex} \) 436 nm were 495 ± 14, 526 ± 15, 546 ± 19, 576 ± 20, and 635 ± 26 nm, respectively. At \( \lambda_{ex} \) 480 nm, S0, S1A, S1B, and S2 were red-shifted (relative to their \( \lambda_{ex} \) 436-nm counterparts) to 526 ± 9, 549 ± 9, 561 ± 12, and 588 ± 23 nm, respectively, and S3 was slightly blue-shifted to 628 ± 24 nm, although this last peak was more variable at both excitations due to the trimodal nature of S3 (see Discussion). In those tissues where S1 was split, S1A and S1B were added together to give an approximate version of S1, and the peak of this recombined S1 was tabulated to compare with the peaks of those S1 that were not split. A nearly uniform finding in each of the S1, S1A, S1B, and S2 signals, at both excitation wavelengths, was a secondary peak or shoulder at 601 nm (Fig. 1A, black arrows). Because of the ubiquity of this finding, we recorded it as a separate phenomenon.

Primary and secondary peaks for all spectra are presented in Supplementary Table S2.

Spectral Signatures by Shape

The shape of the spectrum is as important a descriptor as the peak, especially for complex molecular species, or families of such species. The longest wavelength spectrum S3 tended to be trimodal, with peaks and/or shoulders, P1, P2, and P3, near 580, 620, and 650 nm. The actual peak of S3 (i.e., the highest peak of P1–P3) at \( \lambda_{ex} \) 436 nm was most commonly at 650 nm, appearing in 21/38 tissue

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**Table.** Mean Emission Maxima of the Abundant Fluorophore Signals (nm)

<table>
<thead>
<tr>
<th>( \lambda_{ex}, \text{nm} )</th>
<th>S0</th>
<th>S1</th>
<th>S1A</th>
<th>S1B</th>
<th>S2</th>
<th>S3</th>
<th>S0</th>
<th>S1</th>
<th>S1A</th>
<th>S1B</th>
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<td>Prevalence*</td>
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<td>42</td>
<td>17</td>
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<td>38</td>
<td>39</td>
<td>29</td>
<td>36</td>
<td>18</td>
<td>18</td>
<td>39</td>
<td>37</td>
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<tr>
<td>Mean peak signal, nm</td>
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<td>535</td>
<td>526</td>
<td>546</td>
<td>576</td>
<td>635</td>
<td>526</td>
<td>553</td>
<td>549</td>
<td>561</td>
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<td>628</td>
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<tr>
<td>SD, nm</td>
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<td>15</td>
<td>19</td>
<td>20</td>
<td>26</td>
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<td>532</td>
<td>564</td>
<td>574</td>
<td>629</td>
<td>522</td>
<td>555</td>
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<td>566</td>
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<td>622</td>
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<td>579</td>
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<td>560</td>
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<td>Female (n = 27)</td>
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<tr>
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<td>523</td>
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<td>575</td>
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<td>562</td>
<td>594</td>
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<td>554</td>
<td>553</td>
<td>559</td>
<td>584</td>
<td>631</td>
</tr>
</tbody>
</table>

* Prevalence of recovered spectra from each family from a total of 42 locations in 14 tissues.
locations in which S3 was found. At $\lambda_{\text{ex}}$ 480 nm, S3 was again trimodal, with peak mode at 620 nm in 19/36 tissue locations in which S3 was found. Because the actual peak varied, the overall mean peak of S3 was less informative, but the shapes of the S3 spectra were recognizably the same. Thus, the SAD was particularly useful for demonstrating similarities in recovered spectra that should be assigned to S3 and distinguishing them from other spectra (Fig. 1A).

Details of the three modes of S3 are tabulated in Supplementary Table S3. For S3 at $\lambda_{\text{ex}}$ 436 nm only, an additional peak often appeared (33/42 tissues) in the blue range at approximately 475 nm, completely separate from the main S3 spectrum in the orange-red range.

### Subcellular Localization of Fluorophore Spectral Sources

Of equal importance to the peaks and shapes of the spectra recovered are the tissue sources of those spectra, shown as abundance images. In particular, appropriate perinuclear tissue localizations of the LF/ML spectra were documented in all cases.\(^{20}\) To better correlate the spectral abundances with the anatomic features (e.g., nuclei and melanosomes), a combined spectral AF image from all channels was used (Fig. 1B, RGB panel). S3 localized more specifically to melanin in all tissues examined. S1, S1A, S1B, and S2 generally showed a more homogeneous localization throughout the perinuclear region.

The tissue abundances provided by the NMF
technique unambiguously assigned a spectral signature to either BrM or LF/ML. In most cases, there was an area of bare BrM that made assignment to BrM obvious. In samples without any bare BrM, the typical short wavelength signature had abundance coincident with the RPE nuclei, consistent with presence of BrM beneath the RPE and relative lack of RPE fluorophore through the vertical light path through the nucleus. We refer to this phenomenon as "nuclear shine-through." However, in 16/42 tissue locations, the shortest wavelength spectrum S0 localized to both BrM and LF/ML compartments, and it was completely absent in 6/42. That one spectrum could localize to both RPE and BrM may signify that a certain fluorophore is indeed abundant in both, or it might simply be a limitation of the method in spectral resolution. Conversely, in some other tissues with large areas of bare BrM, two distinct BrM spectra were recovered, S0 and SX (Fig. 3B). This is not surprising, because we have already shown hyperspectral retrieval of multiple AF spectra from pure BrM.20 The remaining three spectra in these cases were abundant LF/ML spectra S1, S2, and S3.

Tissue Colocalization of Fluorophore Spectral Sources

Colocalization of S1, S1A, S1B, and S2 was moderately strong when the abundances were overlaid via color mixing. This was confirmed by the Pearson correlation coefficients, as demonstrated in Figure 3B. A high correlation between S1A and S1B (r ≥ 0.70) was found in all cases when S1 splitting occurred. Generally, correlations of either S1, S1A, or S1B with S2 were also moderately strong, with similar ranges. In Figure 3B, S3 localized to melanin on the abundance overlay, as opposed to S1 and S2, which localized to LF, with resulting low correlations between S3 and S1 or S2. This was confirmed by a low Pearson correlation between S2 and S3 (r = −0.11). In general, the correlations of either S1, S1A, S1B, or S2 with S3 varied widely with the amount of visible melanin, from essentially uncorrelated (in tissues with abundant visible melanin) to moderately correlated (r ≥ 0.70, where melanin was less abundant) (Fig. 1B).

Subgroup Analysis

Subgroup analysis of the λex 436-nm dataset for location (fovea, perifovea, and near-periphery), age
young and aged eyes), and sex showed only one possibly significant difference in spectral peaks: between age groups in the near-periphery for peak S3 (643 nm for young eyes, 635 nm for aged eyes, \( P = 0.0351, t \)-test). We further compared peak wavelength values at each excitation wavelength between young and aged eyes at each specific location using a Wilcoxon Mann-Whitney \( U \) test. Overall, the values were similar between young and aged eyes. Because results appeared similar across locations, they were combined (for more power) to compare values between young and aged eyes overall. Generalized estimating equations were used to account for the within-person correlation. Adjustment of \( P \) values for multiple comparisons was not performed. There were no significant differences between young and aged eyes overall.

**Discussion**

Hyperspectral imaging technology is well known in space science, satellite surveillance, and quality control and is still emerging in medical science. Hyperspectral reflectance imaging has previously been used to measure oxygen saturation levels in the retinal vasculature,\(^26\) as well as to demonstrate dynamic oximetry in human eyes.\(^27\) The current study extends hyperspectral technology to AF imaging of the RPE and its fluorophores.

The molecules responsible for RPE AF have received considerable attention during the last 3 decades. AF is a convenient imaging modality to follow pathological changes in a clinical setting; however, by itself, it is not molecularly specific. This is particularly true in RPE AF, where the emission spectra are broad, resulting in ambiguity in identifying individual small molecules from a family of similar molecules, particularly bisretinoid-like compounds. Here, we enhanced traditional AF imaging with hyperspectral imaging and analysis, which is a significant step toward higher specificity attainable in a clinical setting.

Herein, we build on our prior study of healthy subjects showing stability of RPE cell number and increased overall AF in older versus younger adults.\(^22\) We applied hyperspectral AF imaging and a novel...
analytic technique to these same RPE/BrM flat-mounts to identify at least three spectral signatures in the human RPE that appear to represent distinct families of RPE fluorophores. Spectrum S1 often resolved into two spectra, S1A and S1B, suggesting that two families were present in S1, making four in all. These recovered signatures provide consistent candidates for spectra of major fluorophore families across age, sex, and retinal location with appropriate perinuclear localizations. Overlaying the abundance images demonstrated further differential spectral localization to tissue compartments (Figs. 1B–3B, overlay panels) of potential biologic significance. More specifically, the LF/ML spectra S1 (or S1A and S1B) and S2 generally had perinuclear abundances that were similar, except for intensity, across broad regions of RPE, suggesting that, at our current level of resolution, these fluorophore families track together. Whether this is maintained at higher resolutions and at the level of individual granules remains to be determined. S3, however, almost always presented a coarsely granular appearance and, while maintaining largely a perinuclear localization, also appeared to colocalize more with melanosomes or ML, where present (Figs. 2B, 3B), than with LF. These observations were validated by correlation coefficients of the abundance images in selected cases. This suggests, but does not prove, that S3 is the main signal from ML.

The spectral separation of signals was incomplete in some cases, with individual spectra recovered in combinations. An example is the splitting of S1 into S1A and S1B in nearly half of the cases. Furthermore, secondary peaks and shoulders at 601 nm in S1, S1A, S1B, and S2 were very consistent, but we recovered a spectrum with a single peak at 601 nm only rarely. Whether this phenomenon represents a consistent, secondary emission in the already identified spectra of the three main fluorophore families, or just inability of the current method to separate another family spectrally, is yet to be determined. Another possibility is that these ubiquitous inflections at 601 nm were of instrumental origin. We consider this unlikely, because isolated A2E on BrM imaged using the same camera provides a smooth spectrum without secondary peaks or shoulders near 600 nm (data not shown).

Strengths of this study are the relatively large number of datasets and their broad representations of age, retinal location, and sex. The excellent quality of the RPE/BrM flatmounts analyzed and the consistency of the spectral and spatial data recovered are also of great importance. Regarding consistency, a major strength is that the extracted signals were found in an unbiased manner by a published automatic algorithm applied uniformly to each RPE/BrM tissue hyperspectral dataset.

The study had several limitations. The 20 original donor eyes excluded middle-aged donors so as to analyze AF and RPE cell numbers at extremes of age in a previous study, perhaps biasing our present findings. Studying a more complete range of ages would be a desirable future goal. Six of 20 original donor eyes were excluded from this study for technical reasons (preparation tissue loss or algorithm failure), perhaps also introducing bias, although the samples involved were randomly distributed in age, sex, and retinal location. Modifications of the algorithm will be tested in future work to see if these problems can be overcome. We limited our search with the NMF analysis technique itself to four abundant LF/ML signals and one BrM signal from each tissue, and data were obtained at only two excitation wavelengths. Further research could acquire more hyperspectral data from additional excitation wavelengths, allowing comparison with excitation spectra of known bisretinoids for molecular identification and simultaneous analysis by NTF for improved spectral recovery. Given the multiple comparisons, it is possible that some significant associations arose due to chance.

In conclusion, we discovered with ex vivo hyperspectral imaging of human RPE at least three, perhaps four, distinct LF/ML AF spectra, which have well-defined emission characteristics at two excitation wavelengths and which appear to be universally present in healthy eyes across age, sex, and retinal location. This strongly suggests that there are at least three corresponding abundant families of RPE fluorophores with these characteristic signatures that remain to be identified biochemically by techniques such as IMS and localized to specific granule populations by super-resolution microscopy techniques. The fluorophore families corresponding to these spectra should then account for essentially all significant normal human RPE fluorescence, because the consistent successful factorization by NMF of the original spectral AF data, by definition, has reconstructed the entire RPE emission from 42 representative tissues, with very small error.

Finally, our long-term goal is to translate these methods to human eyes in vivo, using a snapshot hyperspectral camera, and provide a straightforward and noninvasive method for evaluating the RPE fluorophores. Altered and/or new fluorophores may be expected in AMD, as well as redistribution of normal fluorophores, as published and preliminarily presented (Ben Ami T, et al. IOVS. 2015;56(7):ARVO...
E-Abstract 4369; Tong Y, et al. IOVS. 2015;56(7):ARVO E-Abstract 3956). Thus, although the usefulness of hyperspectral imaging in AMD is as yet unproven, the success of the method presented here in normal tissues ex vivo suggests the potential of spectral, molecular biopsy of the RPE in vivo for early detection, longitudinal follow-up, and target discovery for AMD.

Acknowledgements

Supported by grants from National Eye Institute (NEI)/National Institutes of Health (NIH) R01 EY021470 and Foundation Fighting Blindness Individual Investigator Award (RTS); NEI/NIH R01 EY06109 (CC); NEI/NIH EY19065 (ZA); German Research Foundation DFG # AC265/1-1 and # AC265/2-1 (TA); Unrestricted funds from Research to Prevent Blindness (to the University of Alabama at Birmingham, New York University School of Medicine, and Medical University of South Carolina).

Disclosure: T. Ben Ami, None; Y. Tong, None; A. Bhuiyan, None; C. Huisingh, None; Z. Ablonczy, None; T. Ach, None; C.A. Curcio, None; R.T. Smith, None

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