High-resolution imaging of autofluorescent particles within drusen using structured illumination microscopy

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ABSTRACT

Purpose Autofluorescent (AF) material within drusen has rarely been described and there is little knowledge about origin and formation of these particles. Drusen formation is still a relatively unknown process and analysis of AF inclusions might be important for the understanding of fundamental processes. Here we present a detailed analysis of drusen containing AF material using structured illumination microscopy (SIM), which provides a lateral resolution twice as high as conventional fluorescence microscopy.

Methods Eight histological retinal pigment epithelium (RPE) sections obtained from eight human donor eyes (76±4 years) were examined by SIM using laser light of different wavelengths (488 nm, 568 nm). Drusen were studied with regards to their size and shape. AF material within drusen was analysed in terms of size, shape, AF behaviour, and distribution across drusen.

Results A total of 441 drusen were found, of which 101 contained AF material (22.9%). 90.1% of these drusen were smaller than 63 μm (mean: 35.65 μm ±2.38 μm) regardless of whether classified as hard or soft drusen. AF particles (n=190) within drusen show similar spectra compared with lipofuscin granules in RPE cells. Up to 11 particles were found within a single druse. Nearly all particles were located in the outer 2/3 of the drusen (85.94%).

Conclusions SIM allows studying AF particles within drusen on a higher resolution level compared with conventional fluorescence, multiphoton or even confocal microscopy and therefore provides detailed insights in drusen. Shape and autofluorescence analysis of the material embedded in drusen suggest that these particles originate from the overlying RPE cells.

INTRODUCTION

Drusen play a key role in age related macular degeneration (ARMD) and appear as focal deposits between the inner collagenous layer of Bruch’s membrane (BM) and the basal lamina of the retinal pigment epithelium (RPE).1–4 The pathogenesis of drusen formation is still unknown. They consist of lipids and proteins and occasionally intracellular material like organelles and fragments originating from RPE cells.5–7 There are some reports (historical and in vivo) referring to autofluorescence (AF) of extracted drusen, AF of RPE cells overlying drusen or AF within drusen.6–10 In a few cases, particles/granules within drusen were observed, which consist of strongly AF agents in the visible spectrum and show similarities to lipofuscin granules (LF) of RPE cells.11–13

Although different imaging methods like conventional light microscopy,14 laser scanning microscopy15 and electron microscopy6–7 have been applied to study drusen in detail, there is no data available on frequency, size and behaviour of AF structures within drusen.

Electron microscopy is useful for examining the composition of drusen, however it cannot assess AF characteristics.6–16 Fluorescence microscopy is able to assess AF characteristics, but it is restricted by the resolution limit (wide-field microscopy: ∼230 nm, confocal microscopy: ∼180 nm, two-photon microscopy: ∼200 nm).17–19 We recently introduced structured illumination microscopy (SIM) as a new tool for high-resolution imaging of AF granules within RPE cells.20–22 This method benefits from simple sample preparation and provides a resolution that is twice as good compared with standard wide-field fluorescence microscopy. The resolving power is also superior to confocal23 or multiphoton microscopy.24 Thus SIM allows a much more differentiated analysis of the AF tissue.

In this study, SIM was used for the detection and characterisation of fluorescent structures within drusen in histological sections.

METHODS

Sample preparation

Histological sections from ora serrata to ora serrata traversing the macular region were obtained from eight human donor eyes (76±4 years) from the Department of Ophthalmology, University of Heidelberg. In all donor eyes, no macroscopic retinal abnormalities were detectable except for drusen. There were no signs of advanced dry or exudative ARMD.

Immediately after removing the anterior segment of the eyes for corneal transplantation, the remaining tissue was fixed with 4% paraformaldehyde in phosphate-buffered saline (pH 7.4) and embedded in paraffin. Sections (4 μm) were prepared and mounted on microscope slides, deparaffinised with xylene (3 ×5 min), rehydrated through graded ethanol and stored in phosphate-buffered saline until further processing. For microscopy, tissue sections were embedded with SlowFade antifade (Invitrogen, Carlsbad, California, USA), coverslipped and edges were sealed with nail polish. One section per donor was further examined.

Approval was obtained from the local ethics committee. All procedures adhered to the Tenets of the Declaration of Helsinki.
Structured illumination microscopy

SIM is a special fluorescence microscopy method, which allows lateral resolution twice as high as in commonly used wide-field microscopes, in which resolution is limited by the wavelength of light.²⁵ ²⁶

SIM is based on the moiré-effect: two overlaying gratings result in a coarser (moiré) structure, which can be resolved more easily. If the coarse grating is imaged and measured and one of the finer gratings is known, the other fine grating can be calculated (figure 1). Briefly, the samples in this study were illuminated with a fine sinusoidal pattern (350 nm period), which was shifted by 1/3×350 nm perpendicular to the direction of the grating stripes. Thus three images with different positions of the illumination grating on the sample were obtained (grating phase positions). Note that the sum of the three images corresponds to an image obtained for homogeneous illumination. In order to achieve an isotropic resolution improvement in the lateral plane the three images of changing phases were recorded for each of three orientations of the fine grating (sinusoidal pattern), which was automatically rotated (0°, 60° and 120°). Thus a total of nine images of standard resolution were acquired (three phase images, three in each direction), which were further processed using previously described custom reconstruction software.¹⁹ ²⁰ ²⁶

A custom setup was used for SIM imaging: two monochromatic excitation wavelengths were used for all experiments—a cyan 488 nm and a yellow 568 nm laser line (Coherent Sapphire 488/568 HP, 200 mW, Coherent, Dieburg, Germany). The AF signal emitted by the sample passed the high numerical objective (Leica HCX PL APO 100x/1.4 oil CS) and was separated from the excitation light by a triple-band bandpass emission filter (Z488/568/647 M V2, AHF, Tübingen, Germany). Afterwards, an image was formed by the tube lenses and detected with a charge-coupled device-camera (Sensicam QE, PCO, Kelheim, Germany). A detailed description of the microscope setup has recently been published.²⁰

The acquisition software is based on the python programming language.

Imaging

To prepare the imaging process with SIM, one histological section of each donor was analysed with regards to drusen, using a commercial epifluorescent microscope (Leica DMRB, Wetzlar, Germany; excitation: 404 nm, camera: Quantica SVGA-color, Phase, Lübeck, Germany, objective: HCX PL APO 63x/1.4 oil CS). Each druse was imaged independently of whether containing AF inclusions or not.

The same sections were then studied for drusen with the SIM setup, using low laser intensities compared with standard fluorescence microscopy (SIM excitation intensity: ∼0.17 mW/cm²±6%) in order to avoid prebleaching.

Each druse was scanned manually for AF particles from the surface to the bottom (in depth, parallel to BM; z-direction). The focal layer with the largest diameter of the fluorescent particle was chosen. Then laser power was switched to higher intensity to perform SIM-image acquisition (excitation intensity: ∼87 mW/cm²±6%; 50 ms/image). At first, the laser with the longer wavelength (568 nm) was used for image acquisition. Immediately afterwards the same druse was imaged using the shorter wavelengths (488 nm). After the image reconstruction process one image for each excitation wavelength was obtained.
RESULTS

A total of 441 drusen in eight ora serrata to ora serrata histological sections was identified using epifluorescence microscopy. Hard and soft drusen were on average equally distributed among the eight sections (table 1). Fluorescent material within drusen was found in 17.91% of all drusen (n=79) using epifluorescence microscopy (not shown in table) and in 22.68% (n=100) using SIM.

Fifty-five per cent of drusen with embedded AF material and studied with SIM were assigned to the ‘hard’ type (table 1, figure 2), whereas the remaining 45% were classified as ‘soft’ drusen (table 1, figure 3). In concordance with the histological classification of drusen by Rudolf et al,13 drusen size and shape are not necessarily related. Hard drusen (basal diameter: 29.40 μm (mean) ±19.88 μm (SD)) were on average smaller than soft drusen, however the majority of soft drusen measured less than 63 μm (43.59 μm± 26.25 μm). 90% of all drusen with embedded fluorescent material were smaller than 63 μm (mean: 35.79 μm± 23.97 μm; table 2).27

Autofluorescence intensity measurements allowed comparing the signal ratio of the fluorescent material found in drusen with the signals of LF granules deposited in RPE cells overlaying the druse, which is exemplarily demonstrated in figure 4. Here, the intensity profiles for each wavelength showed marked similarities with regards to shape and maximum intensity (figure 4) when comparing a RPE-LF granule with an AF particle embedded in the druse. Autofluorescence intensity of the RPE-LF granule was higher at 488 nm (max. intensity: 213 a.u.) than at 568 nm (max. intensity: 45 a.u.). The ratio of the maximum intensity at 568 nm excitation compared with 488 nm excitation was 0.21. A similar intensity profile and ratio was observed for the AF particle embedded in the druse (max. intensity at 488 nm: 203 a.u.; at 568 nm: 42 a.u.; intensity ratio 568/488=0.21). These similarities have been observed for other AF particles within drusen as well.

A total of 189 AF particles were found in 100 drusen. Single drusen contained 1–11 particles. Some particles appeared like LF granules in RPE cells. Others were smaller than RPE-LF granules and a few lacked the characteristic round shape, though the intensity ratio given by the two excitation wavelengths was similar to two intensity profiles of selected AF particles and RPE-LF granules were generated (one for each excitation wavelength). Calculating the characteristic ratio of the maxima given by the intensity profiles allows a comparison of RPE-LF and AF particles within drusen.
LF granules of the RPE (figure 5). AF particles within drusen had a median size of 702 nm ± 297 nm. Fifty-seven (30.16%) particles were smaller than 500 nm (figure 6). Of all AF particles, 83.07% were located in the outer two-third between BM and RPE. On average, the distance between particle and BM was 6.31 μm ± 7.12 μm. In comparison, the heights of the drusen averaged out at 11.73 μm ± 6.87 μm.

**DISCUSSION**

The pathogenesis of drusen, a major risk factor for ARMD, is still not completely understood. Drusen have been classified as hard and soft drusen and consist of several different components mainly verified in immunohistological studies. Observing soft drusen smaller than 63 μm is not in agreement with the general assumption.

**Table 2** Drusen imaged with structured illumination microscopy and found to contain fluorescent particles were distinguished into hard and soft drusen. Additionally, a size classification according to the Wisconsin grading system was conducted.

<table>
<thead>
<tr>
<th>Sample</th>
<th># Hard drusen</th>
<th># Soft drusen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt;63 μm</td>
<td>63–125 μm</td>
</tr>
<tr>
<td>80 years, female</td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td>95 years, male</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>78 years, male</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>57 years, male</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>79 years, male</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>63 years, male</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>73 years, male</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>84 years, female</td>
<td>14</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>52</td>
<td>3</td>
</tr>
</tbody>
</table>

% 52.00 3.00 0.00 38.00 6.00 1.00
Our result matches with the results presented by Rudolf et al., who also reported similar median sizes for hard and soft drusen. Earlier, Hageman and Mullins had already suggested that a ‘strict relationship between size and shape’ does not exist. However, the range covered by soft drusen is still much larger (maximum size found: 130 μm) as expected. Nevertheless, it has to be mentioned, that the data presented in this study is only referring to one section of each donor—thus we cannot provide absolute numbers of drusen in the whole eye and sections might not represent the whole donor eye.

Intracellular material like organelles and fragments presumably originating from RPE cells has been described within drusen and basal laminar deposits. While some studies report such structures as coincidental findings, Rudolf et al. found pigment granules within drusen in frequencies of 6.4% to 8.9%. However, little is known about frequency, shape and fluorescent properties of structures embedded in drusen.

SIM is a new microscopic method, which allows high-resolution fluorescent imaging. SIM provides benefits of a resolution twice as good as standard fluorescence microscopy and also provides a better resolution compared with confocal and multi-photon microscopy. The main advantage of the resolution improvement achieved with SIM emerges when analysing the fluorescent properties of structures embedded in drusen.

A detailed analysis of particle sizes yielded a mean of 702 nm±297 nm. This value is smaller than the typical size of LF granules within RPE cells, which have previously been evaluated by Boulton et al., who demonstrated a LF size of about 1–3 μm using electron transmission microscopy. Biesemeier et al. have recently reported a size of 1.5 μm×0.5 μm for granules within RPE cells using electron microscopy also but without distinguishing between LF, melanin and melanolipofuscin. We observed that almost one-third (30.16%) of AF particles in drusen were even smaller than 500 nm. This 500 nm threshold is a arbitrary selected value in this study and far below the previously reported granule sizes in literature. This emphasises the advantage of SIM. SIM is especially useful for the detection of such small particles and low autofluorescence signals. It has to be mentioned that some granules have been observed marginal to the section’s thickness. A shortcoming of measuring structures on histological sections generally is that sections are only of a certain thickness, which may result in cropped structures at the edges.

It has been postulated that inflammatory, cell-mediated and immune processes might initiate drusen formation. Injuries of the RPE caused by various events (gene mutation, light damage, oxidative stress, LF accumulation) are assumed to result in a release and accumulation of cell debris between BM and RPE. Recently, Johnson et al. have shown that even cultured RPE cells secrete several membrane-bounded and non-membrane-bounded deposits which are also found in drusen. Besides this, material originating from the RPE, choroid and plasma proteins related to
inflammatory processes have been reported within drusen,\textsuperscript{11, 33} which supports the idea that drusen biosynthesis is a response to local inflammatory events.\textsuperscript{34} Moreover electron micrograph images of ‘vacuoles’ containing LF located within the RPE monolayer were interpreted by Anderson \textit{et al}\textsuperscript{11} as degenerated RPE cells and the first stage of forming a druse.

As drusen have been identified to contain lysosomal material, observing very small granules within drusen (<500 nm) can display early stages of catabolic lysosomal processes of RPE cell material within drusen resulting in LF-like deposits.\textsuperscript{35} Alternatively, lysosomal-LF granules secreted from RPE cells might undergo further catabolic degradation processes resulting in smaller LF particles than originally excysted.

We mainly detected these LF granules within small drusen (<63 μm), which might indicate this aforementioned process of LF appearance only in early stages of drusen formation. In large drusen, LF granules are presumably completely catabolised. However, the AF characteristic must get lost during the suggested catabolic processes, as no fragments in the near vicinity of a granule were observable. It has to be mentioned that though AF particles were mainly detected within small drusen, no correlation between drusen volume and AF particle volume was observed (cf. see online supplementary material).

Furthermore, nearly all AF LF granules (83.07%) have been found in the outer two-third of the druse. This might hint at secretion of AF deposits in early stages of drusen formation. Or it indicates the time-consuming process of LF-like granules formulation within a druse while further excretion of RPE cell material is ongoing.

The data presented in this study is in concordance with the hypotheses of drusen originating partly from RPE cell material. SIM has proved to be a useful tool for analysing fluorescent particles at a resolution level, which is much better than standard fluorescence microscopy and therefore provides better insights in drusen formation.

Contributors All authors have made substantive contributions to this study.

SR: analysis and interpretation of data, drafting the article, final approval of the version to be published. TA: concept and design, critically revising the article, analysis and interpretation of data, final approval of the version to be published. GB: analysis and interpretation of data, drafting the article, final approval of the version to be published. RH: substantial contributions to conception, critically revising the article, final approval of the version to be published. CC: substantial contributions to conception, critically revising the article, final approval of the version to be published.

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We calculated the volume-fraction of drusen occupied by AF particles. To simplify the calculation we assumed spherical bodies. The diagram shows the volume of the drusen (x-axis) plotted against the volume of the AF particles embedded in the corresponding druse (y-axis). A linear and exponential trend line was applied. As clearly demonstrated no correlation between AF particle-size and drusen-size can be assumed.