Autofluorescence imaging of human RPE cell granules using structured illumination microscopy

Thomas Ach,1 Gerrit Best,1,2 Sabrina Rossberger,1,2 Rainer Heintzmann,3,4 Christoph Cremer,2,5 Stefan Dithmar1

ABSTRACT

Background/aims To characterise single autofluorescent (AF) granules in human retinal pigment epithelium (RPE) cells using structured illumination microscopy (SIM).

Methods Morphological characteristics and autofluorescence behaviour of lipofuscin (LF) and melanolipofuscin (MLF) granules of macular RPE cells (66-year-old donor) were examined with SIM using three different laser light excitation wavelengths (488, 568 and 647 nm). High-resolution images were reconstructed and exported to Matlab R2009a (The Mathworks Inc, Natick, MA, USA) to determine accurate size and emission intensities of LF and MLF granules.

Results SIM doubles lateral resolution compared with conventionally used wide-field microscopy and allows visualisation of intracellular structures down to 110 nm lateral resolution. AF patterns were examined in 133 LF and 27 MLF granules. LF granules (968 ± 220 nm) were significantly smaller in diameter than MLF granules (1097 ± 110 nm; p<0.001). LF granules showed an inhomogeneous intragranular pattern, and the average intensity negatively correlated with the size of these granules when excited at 647 nm. The autofluorescence of MLF granules was more homogeneous, but shifted towards higher excitation wavelengths in the centre of the granules.

Conclusion SIM is a useful tool for examining AF signals within single LF and MLF granules in RPE cells. This allows new insights into RPE autofluorescence patterns.

INTRODUCTION

Alterations in retinal pigment epithelium (RPE) play a key role in the pathogenesis of age-related macular degeneration (AMD). Intracellular accumulation of autofluorescent (AF) granules causes changes in RPE metabolism and, ultimately, RPE cell degeneration and apoptosis.1–3 The AF granule, lipofuscin (LF), has been shown to generate reactive oxygen species via photosensitisation with blue light.4–5 Melanolipofuscin (MLF), a complex granule exhibiting properties of both, melanosomes and LF, may reflect the onset of AMD even more closely than LF accumulation.6 Several techniques have been developed to visualise AF RPE signals. For in vivo imaging, scanning laser ophthalmoscopy systems have been established, which enable a lateral resolution in a micrometre range.7–9 For in vitro imaging, conventional fluorescence microscopes, and further developments, like two-photon-excited fluorescence imaging are available with a lateral resolution in a sub-micrometre range.10–11 We recently introduced structured illumination microscopy (SIM) for high-resolution AF imaging of RPE cells.12,13 SIM is a novel laser-based imaging technique which allows detecting AF signals at resolutions in the 100-nanometre range.14,15 In the current study, we use SIM for the characterisation of AF patterns of single RPE granules.

METHODS

Specimen preparation

The posterior segment of a 66-year-old donor was examined macroscopically and revealed no visible retinal abnormalities except for few drusen. The tissue was fixated with 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4) and embedded in paraffin. Histological sections (4 μm) of the macular region were prepared and fixed on microscope slides. Sections were deparaffinised (xylene 3×5 min), subsequently rehydrated through graded ethanol and stored in PBS. For further microscopic analysis, the slides were embedded with SlowFade antifade (Invitrogen, Carlsbad, CA, USA) and covered with cover slips. Macular regions with intact RPE were localised by light and epifluorescence microscopy (Leica DMRB, Solms, Germany; excitation wavelength: 404 nm, magnification: 40×) and marked for the structured illumination microscopy (SIM) examination process.

SIM

In SIM, a periodic illumination intensity pattern modulates the high spatial frequencies of the object as these are essential for high resolution. Hence, previously irresolvable high frequencies can be shifted to lower frequencies and, thus, be detected by the ordinary imaging process of a wide-field microscope. This effect is known as Moiré effect: if two fine irresolvable gratings are superposed, a coarser, clearly visible grid appears. The setup of the SIM microscope and sample preparation have been previously described.13 Briefly, the SIM setup is based on a modified wide-field fluorescence microscope. The structured illumination in the object plane is generated by a Twyman-Green interferometer. Two coherent beams overlap to generate the interference pattern. The beams pass the focusing lens and are then deflected by a dichromatic beam splitter (Z488/568/647, AHF, Tübingen, Germany) by 90° towards the high numerical objective (Leica HCX PL APO 100×/1.4–0.7 OIL CS, Solms, Germany). The emission light from the object is collected by the objective and then passes through the dichromatic beam splitter, an additional blocking filter and a tube lens into a CCD camera (PCO Sensicam QE, Kelheim, Germany).
After placing the specimen in the SIM setup, the previously selected and marked RPE regions were imaged. To improve the resolution in the image plane along one lateral direction, 10 frames with different illumination grating positions were acquired. To gain a homogeneous resolution in the image plane, additional acquisition series were made with the grating rotated by 60° and 120° in the plane. Thus, 30 different frames were acquired in total for each excitation wavelength. Excitation wavelengths were set to 488, 568, and 647 nm.

To minimise bleaching effects, examinations were conducted semi-automatically. This reduces excitation time to 50 ms per frame. Between measurements, specimens were kept in darkness.

**Processing SIM data**

To derive high-resolution images, the generated SIM raw data was further processed using previously described reconstruction software. For each excitation wavelength, one high-resolution image was reconstructed and exported to Matlab R2009a (The Mathworks Inc, Natick, MA, USA) to determine the accurate size and emission intensities of lipofuscin (LF) and melanolipofuscin (MLF) granules.

For size measurements, the border of every granule was drawn manually in the reconstructed image to determine the area of each single granule. The average diameters of the granules were calculated using the areas of the previously measured granules, assuming round granules.

To illustrate the emission signals within single granules simultaneously, the corresponding images were colour coded (excitation 488 nm = blue, 568 nm = green, 647 nm = red), and the channels joined to generate coloured RGB (red, green, blue) images. For further analysis, it has to be considered that the excitation laser power had to be adjusted for every wavelength to derive bright fluorescence signals. Therefore, it is not possible to compare absolute AF strength (AS) between the different excitation wavelengths. However, the relative AS changes within the examined tissue are comparable between different excitation wavelengths.

Using a self-written software tool, the ASs of LF and MLF granules were measured for every pixel within the aforementioned granule’s area, and averaged to derive the mean AS of each granule. This process was repeated for every excitation wavelength.

Finally, correlations between granule size and intensity signal at the different wavelengths were calculated.

**Statistical analysis**

All data is presented as means±SD. Wilcoxon signed rank test was performed and Pearson’s correlation coefficients were determined using SPSS V.16.0 (SPSS Inc.). The level of significance was set to $p<0.05$.

All experiments were conducted according to the tenets of the Declaration of Helsinki. The Institutional Review Board approved this study.

**RESULTS**

SIM clearly delineated AF granules within RPE cells. Figure 1 illustrates the increase in lateral resolution provided by SIM ($\sim 110$ nm) in comparison with conventional wide-field microscopy ($\sim 250$ nm).

AF intracellular granules of three adjacent RPE cells of the macular region were analysed and classified as LF or MLF granules (figure 1B). MLF granules, characterised by a central melanin core surrounded by an AF ring, could be clearly distinguished from LF granules.

A total of 133 LF and 27 MLF granules within three RPE cells were analysed (table 1). LF granules measured, on average, $968\pm220$ nm (range: 528–1510 nm) in diameter, and were significantly smaller than MLF granules ($1097\pm110$ nm, range: 680–1456 nm) ($p<0.001$).

The AF patterns within LF granules depended on the excitation wavelengths. Colour coding with the three excitation wavelengths demonstrate the inhomogeneous autofluorescence distribution within single granules (figure 2).

A correlation between size of LF granules and AS was found for 647 nm excitation in two of the three cells. Larger LF granules showed less (647 nm) autofluorescence (cell 2: $r=-0.404$, $p=0.015$; cell 3: $r=-0.325$, $p=0.05$). No significant correlation was present for granule size and AS at 488 nm and 568 nm.

SIM clearly delineates the hyperfluorescent ring and the hypofluorescent core of MLF granules. The core of MLF granules showed a significant shift in AS towards 647 nm, whereas, the hyperfluorescent ring was predominantly excitable with shorter excitation wavelengths.

When comparing emission intensity signals of MLF with LF granules, the AS of MLF granules showed a significant shift towards higher excitation wavelengths.

The size of MLF granules showed no correlation with AS at different excitation wavelengths.

**DISCUSSION**

Accumulation of AF granules within RPE cells causes changes in metabolism and is a key feature of many diseases, like age-related macular degeneration (AMD). Therefore, detailed visualisation and characterisation of these intracellular granules...
Table 1  Characteristics of autofluorescent granules in three adjacent macular retinal pigment epithelium cells

<table>
<thead>
<tr>
<th>Cell no.</th>
<th>Type of granule</th>
<th>Number of granules</th>
<th>Diameter (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>LF</td>
<td>30</td>
<td>1260±134</td>
</tr>
<tr>
<td></td>
<td>MLF</td>
<td>10</td>
<td>1206±196</td>
</tr>
<tr>
<td>2</td>
<td>LF</td>
<td>66</td>
<td>882±156</td>
</tr>
<tr>
<td></td>
<td>MLF</td>
<td>6</td>
<td>952±114</td>
</tr>
<tr>
<td>3</td>
<td>LF</td>
<td>37</td>
<td>924±164</td>
</tr>
<tr>
<td></td>
<td>MLF</td>
<td>11</td>
<td>1110±264</td>
</tr>
</tbody>
</table>

LF, lipofuscin; MLF, melanolipofuscin.

is desirable. Electron microscopy offers high resolution but is not suitable for the detection of AF signals. Conventional wide-field microscopy (lateral resolution about 200 nm) and two-photon excited fluorescence microscopy (lateral resolution in a sub-micrometre range, optimum at about 200 nm) have been used to visualise RPE autofluorescence in vitro.10–11 Recently, we introduced SIM as a novel technique for in vitro fluorescence imaging of human RPE cells.12 SIM offers a twofold higher lateral optical resolution than common techniques. Intracellular granules, like LF and MLF, could be clearly visualised with their internal structure and differentiated from each other by SIM. The distinction between LF and MLF granules might be especially important since MLF accumulation has been reported to reflect the onset of AMD more closely than LF granules.5 LF develops within lysosomes from de-metabolised rod outer segments.6 LF develops within lysosomes from de-metabolised rod outer segments. Different sizes of LF granules might reflect different stages of LF genesis with small LF granules in early stages. In our study, the AF properties of LF granules changed with growing size showing decreased average emission signals at 647 nm. This indicates an increased presence of fluorophores excitable at 647 nm. In MLF granules, there was no correlation found between size and AS.

In our study, MLF granules were significantly larger than LF granules, which might be due to fusion of LF granules and melanosomes or phagocytotic abilities of melanosomes. Warburton et al reported MLF granules to be nearly 35% larger than LF granules in flow cytometric analyses.5 SIM is able to detect autofluorescence patterns within single granules of RPE cells. This study shows that LF granules have an inhomogeneous excitation pattern suggesting the presence of multiple fluorophores differing in their excitation and emission characteristics.

This can be explained by mixtures of different fluorophores, different amounts of one fluorophore, or other mechanisms, like intra-granular energy transfer which leads to shifts in fluorescence spectra.18–20 When comparing AS throughout the examined granules, the emission signals in the hyperfluorescent rings of MLF granules are significantly shifted to shorter excitation wavelengths. This finding might be related to different properties of fluorophores in LF and MLF granules (eg, early versus later stages of LF formation).

The findings of the present study are in contrast with near-field scanning optical microscopy (NSOM) studies by Haralampus-Grynaviski and co-workers who examined extracted LF granules from RPE cells. Homogenous excitation and emission spectra were found within single LF granules, while emission spectra varied between different LF granules.18 A limited resolution of the used NSOM and different sample preparation might explain the discrepancy with our results.

The inhomogeneous fluorescence patterns within LF granules would suggest an inhomogeneous morphology of these granules. Atomic force microscopy (AFM), a specialised form of high-resolution surface microscopy, revealed components 50–100 nm in size which comprised single LF granules, and showed a homogenous LF granule surface morphology.19 Recently, Petrukhin et al who also examined extracted single LF granules with AFM and NSOM reported inhomogeneous emission patterns within LF granules and found components 50–40 and 100–120 nm in size composing a single LF granule.20 These differently sized components might reflect the inhomogeneous AF pattern within single granules, as shown in our study. Although there is better resolution in AFM, the advantages of SIM are considerable. SIM allows the examination of granules within tissues and cells, while the aforementioned methods require granules to be extracted from RPE cells. Moreover, SIM generates high-resolution images which simultaneously show the fluorescence pattern of the examined tissue.

This study has limitations, since excitation light might cause bleaching effects. Although excitation time was as short as possible (50 ms), modified AF signals are possible. Additionally, tissue needs different laser powers to derive similar excitation signals at differing excitation wavelengths, leading to varying bleaching and saturation effects. Furthermore, autofluorescence

is desired.
is influenced by preparation methods, which might explain differences in autofluorescence behaviour.\textsuperscript{21}

In conclusion, SIM is a new and useful tool in detection of AF signals within single LF or MLF granules. The high-resolution images allow accurate measurement of granule size. SIM might contribute to the understanding of LF pathogenesis and diseases like AMD.

**Contributors** All authors have made substantive contributions to this study. T Ach: conception and design, acquisition of data, analysis and interpretation of data, drafting the article, final approval of the version to be published. G Best: acquisition of data, drafting the article, final approval of the version to be published. S Rossberger: analysis and interpretation of data, critically revising the article, final approval of the version to be published. R Heintzmann: substantial contributions to conception, critically revising the article, final approval of the version to be published. C Cremer: substantial contributions to conception, critically revising the article, final approval of the version to be published. R Heintzmann: substantial contributions to conception, critically revising the article, final approval of the version to be published. G Best: acquisition of data, conception and design, acquisition of data, analysis and interpretation of data, drafting the article, final approval of the version to be published. S Dithmar: analysis and interpretation of data, critically revising the article, final approval of the version to be published. C Cremer: substantial contributions to conception, critically revising the article, final approval of the version to be published. R Heintzmann: substantial contributions to conception, critically revising the article, final approval of the version to be published.

**Funding** This study was supported by Ernst and Berta Grimmke Stiftung. The funding source had no involvement in the study design, the collection, analysis and interpretation of data, in the writing of the report or in the decision to submit the paper for publication.

**Competing interests** None.

**Ethics approval** Ethics Committee of University Heidelberg.

**Provenance and peer review** Not commissioned; externally peer reviewed.

**REFERENCES**