Article

Photodegradation of Lipofuscin in Suspension and in ARPE-19 cells and the Similarity of Fluorescence of the Photodegradation Product with Oxidized Docosahexaenoate

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Abstract: Retinal lipofuscin accumulates with age in the retinal pigment epithelium (RPE) where its fluorescence properties are used to assess the retinal health. It was observed that there is a decrease in lipofuscin fluorescence above the age of 75 years and in early stages of age-related macular degeneration (AMD). The purpose of this study was to investigate the response of lipofuscin isolated from human RPE, and lipofuscin-laden-cells to visible light, and determine whether an abundant component of lipofuscin, docosahexaenoate (DHA) can contribute to lipofuscin fluorescence upon oxidation. Exposure of lipofuscin to visible leads to a decrease of its long-wavelength fluorescence at about 610 nm with concomitant growth of the short-wavelength fluorescence. The emission spectrum of photodegraded lipofuscin exhibits similarity with that of oxidized DHA. Exposure to light of lipofuscin-laden cells leads to loss of lipofuscin granules from cells, while retaining cell viability. The spectral changes of fluorescence in lipofuscin-laden cells resemble those seen during photodegradation of isolated lipofuscin. Our results demonstrate that fluorescence emission spectra together with quantitation of intensity of long-wavelength fluorescence can serve as a marker useful for lipofuscin quantification and for monitoring its oxidation, thereby useful for screening the retina for increased oxidative damage and early AMD-related changes.

Keywords: lipofuscin, retina, retinal pigment epithelium, docosahexaenoate, docosahexaenoic acid, fluorescence, photodegradation, photobleaching, cell viability, endocytic activity

1. Introduction

Retinal pigment epithelium (RPE) is a monolayer of neuroepithelial cells separating the retina from its choroidal blood supply. RPE cells accumulate gradually with age a product of incomplete lysosomal digestion of phagocytosed photoreceptor outer segments, as well as some autophagocytosed material, known as age pigment or lipofuscin [1-3]. Its greatest accumulation occurs in the macula, which is the area of the retina responsible for acute vision [2,4-6]}. It has been determined that

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lipofuscin occupies 19% of the cytoplasmic volume of RPE cells from the macular area of 81-90-year-old cadavers [6]. Accelerated accumulation of lipofuscin is observed in some inherited retinal diseases such as Stargardt's disease, fundus flavimaculatus, Batten disease (also known as neuronal ceroid lipofuscinoses) and bestrophinopathies [7-9].

When excited with light, RPE lipofuscin emits a characteristic golden yellow fluorescence with a broad maximum at about 600 nm, and large variations between individual granules (the maxima vary from 550 to 645 m) [3,10-13]. It has been determined that, when excited with blue light, the fluorescence is emitted mostly by yellow emitting fluorophores [13]. However, a considerable part of that emission originates not by a direct excitation of these fluorophores, but by energy transfer from other blue-light absorbing chromophores, most of which still have not been identified.

Lipofuscin fluorescence can be imaged in the retina in vivo using excitation from a range of 488nm to 550 nm and is used for monitoring retinal health and progression of several retinal diseases [4,7,8,14-19].

Interestingly, it has been determined that lipofuscin fluorescence in the range of 650-750 nm, emitted when photoexcited with 550 nm light, increases in the retina in a linear fashion up to the age of 70 years, and then decreases more steeply than its prior increase despite no changes in RPE cell density [18,20]. There are several pieces of evidence suggesting that the decrease in fluorescence in the retina can be due to, at least in part, photobleaching. It has been reported in four out of five Stargardt's disease patients monitored over a period of one year or longer, that there was an overall reduction of lipofuscin fluorescence induced by excitation with 488 nm light intensity despite there was no cell loss [21]. In this study, the reduction of fluorescence intensity was smaller in fellow eyes where patients' corneas were covered during daytime by contact lenses blocking above 90% of visible light entering the eye. There are reports demonstrating that translocation of the re-attached neural retina after detachment reveals areas of increased autofluorescence intensity [22,23]. These hyperfluorescent areas visible after re-attachment match the shape of blood vessels, which, after the translocation, no longer screen the RPE behind them from incident light and these are the areas in shape of these blood vessels which exhibit autofluorescence.

Moreover, it has been shown that lipofuscin fluorescence in the macaque retina in vivo decreases by about 20% as a result of acute exposure to 210 J/cm^2 light of 568 nm wavelength while the integrity of RPE monolayer remained intact [24-26]. In case of human RPE ex vivo, a dose of 30.6 J/cm^2 of 568 nm light was sufficient to cause fluorescence photobleaching [26].

Experiments, especially on albino mice with accumulated lipofuscin also indicate that exposure to light can lead to a decrease of lipofuscin fluorescence [27,28]. Light-dependent fluorescence decrease was also observed in pigmented mice, both wild type and in mice with deleted genes coding proteins responsible for clearance of all-trans-retinal, *abca4(-/-)rdh8(-/-)*, but required longer time to be detectable [29]. Mice which were raised under light/dark cycle for 12 months exhibited 2.8-fold decreased RPE fluorescence in comparison to mice on the same genetic background raised in dark.

The decrease in lipofuscin fluorescence is likely due to photooxidation of lipofuscin. We and others have shown that exposure

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of lipofuscin isolated from human RPE to light, leads to generation of reactive oxygen species and photooxidation of lipofuscin itself [30-40].

It has been determined that the lipophilic extract from the RPE contains a number of different fluorophores [41,42] and several of them have been identified as various bisretinoids and their oxidation products [32,39,43-52]. These bisretinoids, except for the all-trans-retinal dimer, are formed as a result of condensation of two molecules of vitamin A aldehyde, all-trans-retinal with ethanolamine group of phosphatidylethanolamine, and their subsequent transformations. They are considered as the major emitter of yellow fluorescence from photoexcited RPE lipofuscin [53].

The effects of photooxidation of A2E and other bisretinoids on their fluorescence properties have been studied both in solution and on cultured cells (reviewed in [53]) but no such studies were done on lipofuscin isolated from human RPE. Therefore, the aim of this study was to monitor changes of fluorescence during photooxidation of lipofuscin isolated from human RPE in suspension and in lipofuscin-laden ARPE-19 cells, as well as to determine whether photooxidation of intracellular lipofuscin can affect cell viability and endocytic function.

Lipofuscin contains abundant polyunsaturated fatty acids, including docosahexaenoic acid (DHA) which due to six unsaturated double bonds is extremely susceptible to oxidation [48,54-56]. Oxidation products of DHA and other polyunsaturated lipids have been identified in the RPE lipofuscin [48,57-59]. Exposure of lipofuscin to light is likely to induce further oxidation of bisretinoids and DHA. We have shown that oxidation of DHA leads to the formation of products absorbing visible light with more potent photosensitizing properties than the lipophilic extract of lipofuscin suggesting that even if it contributes a small percentage to the light absorption of the lipofuscin, it can still be responsible for the majority of its photosensitizing properties [60]. While the potential contribution of lipid oxidation products to fluorescence was studied previously, it was limited to small end-products of lipid oxidation such as malondialdehyde (MDA), 4-hydroxy-nonenal (HNE) and their adducts with amines which upon absorption of ultraviolet light can emit blue fluorescence [61,62]. The absorption of visible light by oxidized DHA opens a possibility that it can be excited by visible light and contribute to lipofuscin fluorescence. Therefore, another aim of this study was to compare fluorescent properties of DHA with lipofuscin and photodegraded lipofuscin.

To achieve these aims, we isolated lipofuscin from human RPE pooled exposed it to 9.76 mW/cm^2 visible light and monitored its fluorescence after pre-selected exposure times for up to 10.5 h using photoexciation with 360 and 488 nm wavelength of light. DHA was autooxidized by exposure to the air and its fluorescence properties were compared with lipofuscin before and after photodegradation. To determine whether photodegradation of lipofuscin can occur in cultured cells without causing cytotoxicity, confluent ARPE-19 cells were supplemented with lipofuscin added to culture media changed 3 times per week over a period of 31 days. Accumulation of lipofuscin was monitored by phase contrast and fluorescence microscopy. Cells with accumulated lipofuscin and control cells were exposed daily for 14 days to 45 min of 9.76 mW/cm2 visible light to accumulate the same total radiant exposure of 369 J/cm^2 as lipofuscin in suspension. The integrity of cell monolayer, lipofuscin accumulation and its fluorescence were

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monitored by phase contrast and fluorescence microscopy, and, after solubilisation of cells in Triton X-100, by spectrofluorometry. Cell viability and endocytic activity were quantified by MTT assay of metabolic activity of cells and neutral red assay, respectively.

Our results demonstrate that photodegradation of isolated RPE lipofuscin results in a decrease of intensity of fluorescence above 550 and 500 nm, when excited with 360 and 488 nm light, respectively, and leads to an increase in lipofuscin fluorescence in the range of 380-530 nm observed with 360 nm excitation. Radiant exposure of 369 J/cm^2 leads to photodegraded lipofuscin with an emission spectrum similar to that of oxidized DHA Exposure of lipofuscin-laden ARPE-19 cells to such a dose of visible light fractionated over 14 days resulted in removal of lipofuscin from cells as well as a decrease in long-wavelength fluorescence and an increase in short-wavelength fluorescence. Importantly, photooxidation of lipofuscin did not affect cell viability nor endocytic activity. Interestingly, oxidized DHA contributed to the emission at about 600 nm thought to be characteristic for bisretinoids. Altogether, our results demonstrate that photodegradation of lipofuscin can be responsible for the observed loss of golden-yellow fluorescence observed in clinical studies. It can be suggested that the emission spectra of retinal lipofuscin can serve as a marker of oxidative damage to the RPE *in vivo;* however, a shorter excitation than 488 nm would be required.

2. Results

2.1 Exposure of RPE lipofuscin to visible light results in a decrease of long-wavelength fluorescence and an increase in the short-wavelength fluorescence

Isolated lipofuscin emitted broad-band fluorescence when excited with 360 nm or 488 nm light in agreement with previous reports [11-13] (Fig. 1A,B). The spectra corrected for changes of detection sensitivity of the spectrofluorometer showed that the emission maximum of about 610 nm is at the same wavelength as reported previously (Fig. 1C).

Exposure of lipofuscin to 9.76 mW/cm^2 visible light resulted in photobleaching of yellow/orange fluorescence and an increase in blue-green fluorescence (Fig. 1). In case of excitation with 360 nm light, the decrease in emission intensity can be seen in the range of 550 to 700 nm. In case of excitation with 488 nm light, the decrease in emission intensity can be seen over the entire range of the emission spectra starting from 500 nm.

During lipofuscin photodegradation, the intensity of the short-wavelength emission induced by 360 nm light increased in the range of 380 to 540 nm (Fig. 1A,D). After 10.5 hours of lipofuscin photodegradation providing radiant exposure of 369 J/cm^2, the intensity of emission at 450 nm increased by 3.7-fold while, at the same time, the emission at 610 nm decreased by 43% and 58%, respectively, when using excitation of 360 and 488 nm (Fig. 1D,E). Emission at 530 nm exhibited an initial increase which was followed by plateauing or a decrease depending on whether the emission was induced by excitation with 360 or 488 nm light. Similar but less pronounced changes could be seen for emission at 550 nm. We were interested in fluorescence changes at that wavelength because it has been reported that the ratio of fluorescence intensity at 550 nm to 600 nm is increased in RPE cells from eyes affected by age-related macular degeneration (AMD) in comparison with RPE cells isolated from healthy eyes of similar age [63]. The fluorescence was induced by excitation with 488 nm light. AMD retinas are exposed to

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greater levels of oxidative stress than age-matched healthy retinas as evidenced by detection of increased levels of easily cheletable iron and products of lipid oxidation [64,65]. Therefore, it can be expected that the increased oxidative stress in AMD retina can cause oxidation, including oxidation of lipofuscin, and observed changes in fluorescence. Our results demonstrate that during photodegradation of isolated lipofuscin there is an increase of the fluorescence ratio of 550 to 610 nm (Fig. 1F,G). Such an increase is even more pronounced for the ratio of emission of 530 to 610 nm. The kinetics of the ratio increase appear to be different for excitation with 360 nm than for excitation with 488 nm. The ratios of emission at 450 to 610 nm, or 470 to 610 nm, exhibit a linear increase with increasing radiant exposure (Fig. 1F).

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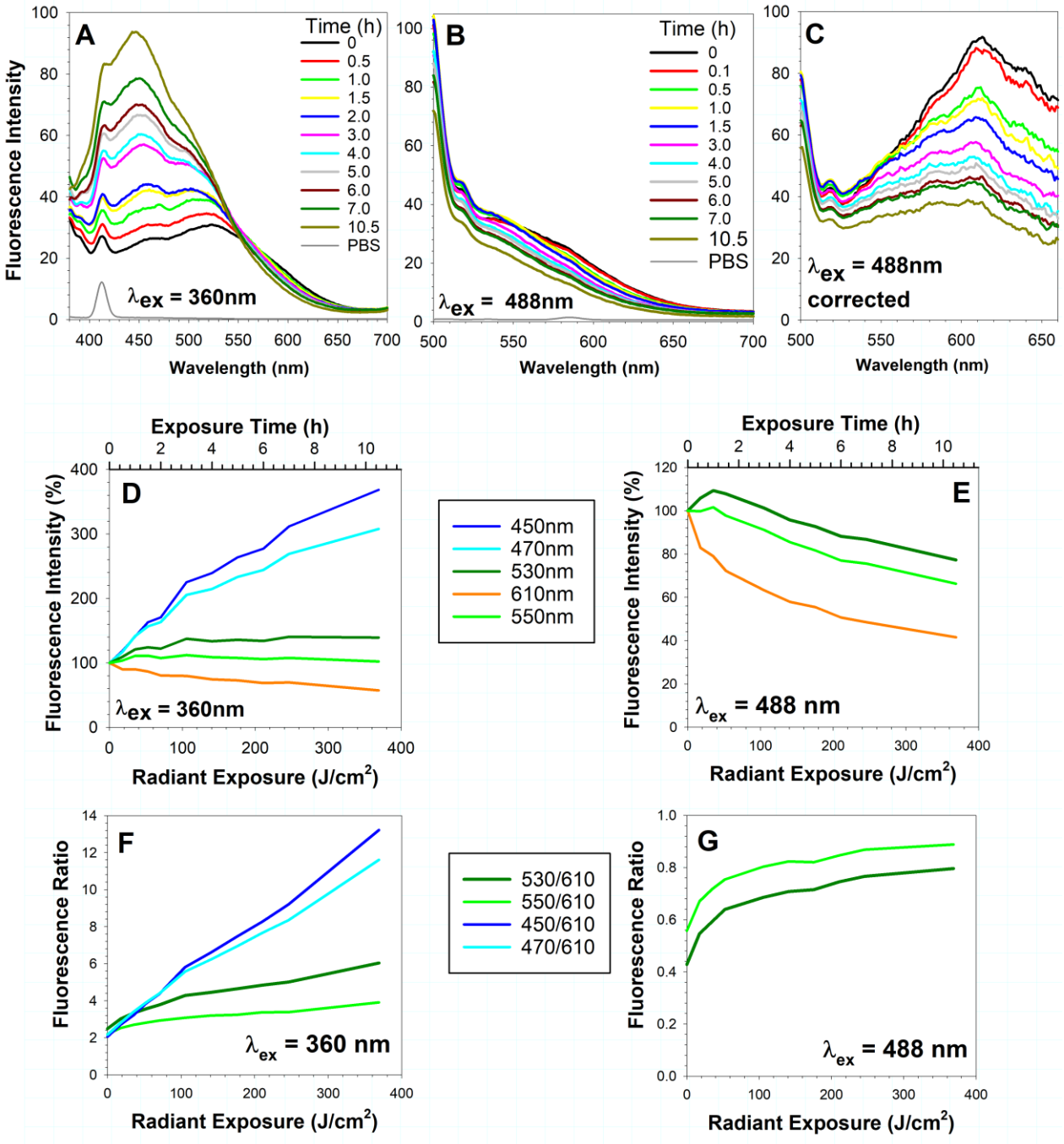

Figure 1. Irradiation of lipofuscin with visible light results in a decrease of long-wavelength fluorescence and an increase in short-wavelength fluorescence. Representative fluorescence emission spectra of lipofuscin granules suspended in PBS before and after indicated time of irradiation with visible light (9.76 mW/cm²) upon excitation with 360 nm (A) and 488 nm (B) light. C) Fluorescence emission spectra from B corrected for the spectral changes in sensitivity of the detection demonstrate that the emission maximum corresponds to the emission maximum of RPE lipofuscin at about 600 nm. Kinetics of changes of fluorescence emission intensity monitored at indicated wavelengths, normalized to the value before irradiation and expressed as percentage (D, E); and changes in fluorescence intensity ratios at indicated wavelengths (F, G) during irradiation with 9.76 mW/cm² visible light. Fluorescence was induced by excitation with 360 nm (D) or 488 nm (E) light. The ratios of fluorescence intensities were calculated based on uncorrected spectra in case of excitation of 360 nm, and based on corrected

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spectra in case of excitation with 488 nm. Fluorescence of the solvent (PBS) was recorded to show its Raman emission peaks.

2.2. The fluorescence of oxidized DHA exhibit similar spectral characteristics as fluorescence appearing during photodegradation of lipofuscin

DHA is an abundant component of lipofuscin. Due to six unsaturated double bonds, it is very susceptible to oxidation. Both, oxidized DHA and lipofuscin, can photosensitize generation of singlet oxygen and free radicals which can lead to further oxidation of DHA [60,66,67]. Oxidized DHA exhibits a broad absorption spectrum extending up to 600 nm. Considering that the quantum yields of singlet oxygen generation by oxidized DHA are 0.22 and 0.13 for photoexcitation with 355 nm and 425 nm light, respectively [60], it means that up to 78 and 87% of absorbed photons can be utilized in other deactivation pathways, such as fluorescence. Therefore, we investigated a possibility that photoexcitation of oxidized DHA can result in emission of fluorescence. Oxidized DHA emitted broad fluorescence when excited with either 360 nm or 488 nm light (Fig. 2). Interestingly, the emission spectrum obtained by photoexcitation of oxidized DHA with 360 nm light, was similar to the green portion of the emission spectrum of photodegradated lipofuscin (Fig. 2A).

Oxidized DHA also emitted fluorescence when excited with 488 nm blue light (Fig. 2B). The emission spectrum showed similar spectral characteristics as the emission spectrum of lipofuscin before or after photodegradation indicating that oxidized DHA can contribute to fluorescence emission with a maximum at 610 nm, which so far has been attributed to the fluorescence of bisretinoids [14,53,68].

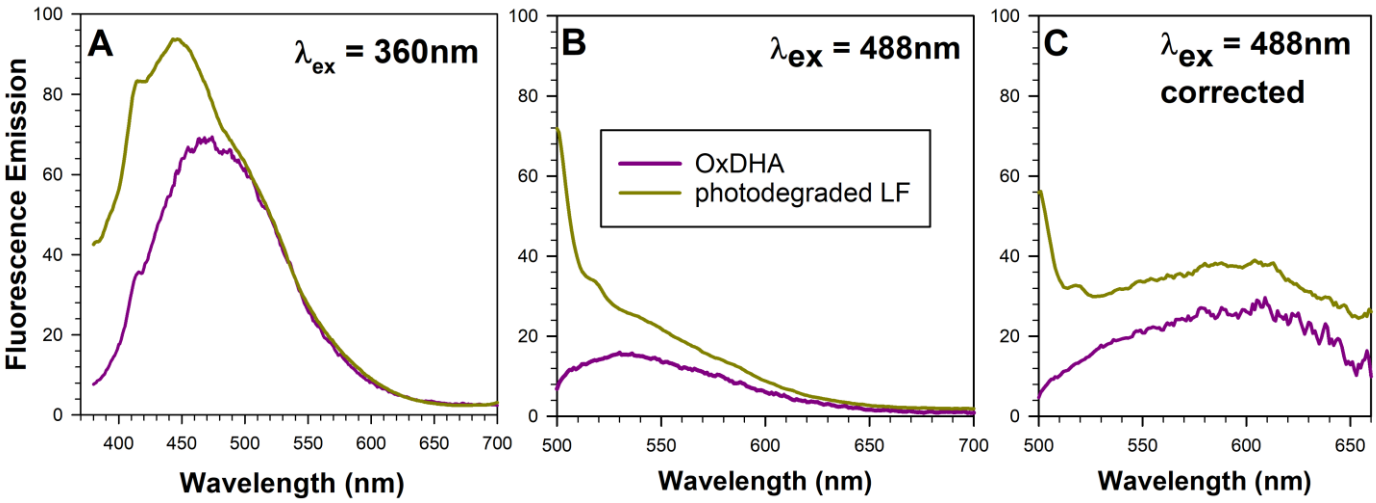

Figure 2. Fluorescence emission spectrum of oxidized docosahexaenoate (OxDHA) may contribute to the emission of photodegraded lipofuscin. Representative fluorescence emission spectra of oxDHA and photodegraded lipofuscin (LF) granules obtained by excitation with 360 nm (A) and 488 nm (B) light. C) Fluorescence emission spectra from B corrected for the spectral changes in sensitivity of the detection. Prior to fluorescence measurement lipofuscin was photodegraded by irradiation for 10.5 h with 9.76 mW/cm²visible light providing radiant exposure of 369 J/cm². OxDHA was obtained by autooxidation of phosphatidylcholine with two DHA acyl chains. The concentration used for fluorescence measurements correspond to 4.23 mg/ml of the non-oxidized phospholipid.

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2.3 Exposure of lipofuscin-laden ARPE-19 cells to light results in loss of lipofuscin from cells and spectral changes of fluorescence

To enrich ARPE-19 cells with lipofuscin, lipofuscin was added to the cell culture medium and supplemented to confluent cells during each media change over a period of 32 days. The cells were imaged using phase contrast microscopy with low light intensity for their appearance in order to monitor lipofuscin accumulation and any signs of cell loss such as rounding up or loss of confluency. Accumulation of lipofuscin granules could be clearly visible in the cell monolayer (Fig. 3).

Fluorescence microscopy with long pass filter and colour camera showed a characteristic golden-yellow fluorescence in cells fed with lipofuscin when excited with blue light, which was absent in cells without such supplementation (Fig. 3). Splitting images in Image J into green and red channels allowed to see that red fluorescence was more intense that green fluorescence in the lipofuscin-laden cells.

Based on previous reports it can be expected that the radiant exposure of 369 J/cm^2 needed for development of oxidized DHA-like fluorescence could be photoxic to cells especially if they contain lipofuscin [24-26,48,69-74]. Friedman and Kuwabara observed severe retinal damage in the photoreceptors and changes to the retinal pigment epithelium in rhesus monkeys who were exposed for 15 min to light from an indirect ophthalmoscope providing a retinal irradiance of 270 mW/cm^2 and a dose of 243 J/cm^2 [70]. Experiments of Morgan and colleagues on macaque monkey demonstrated that there is a very small difference of just 18% between a threshold dose required to bleach fluorescence, and a threshold dose causing a disruption of the RPE monolayer [24-26]. The threshold dose to cause a visible RPE photodamage in macaque monkey by 568 nm laser has been determined as 247 J/cm^2. Radiant exposures sufficient to cause photodamage to the retina were even smaller when shorter irradiation wavelengths were used [26,72]. Using photoexcitation of macaque retina *in vivo* with 488 nm light, a photobleaching of fluorescence with some disruption of the RPE monolayer was observed after radiant exposure of just 79 J/cm^2 and even smaller than that for exposure with 460 nm light [26,72]. Cultured RPE cells are also susceptible to photodamage and it has been shown that internalized lipofuscin makes them even more susceptible to phototoxicic effects of light in comparison to cells without lipofuscin [48,59,71,73-75]. Because of that, we decided to fractionate the radiant exposure of 369 J/cm^2 into 14 doses of 26.3 J/cm^2 so to make the individual dose not likely to cause a detectable damage and to give cells time to repair any sublethal damage before the next dose is applied. To do so, the cells were exposed daily for 45 minutes to 26.3 J/cm^2 of visible light giving them 23 hours each time for repair. The control plates with lipofuscin-laden and lipofuscin-free cells were incubated with PBS for 45 minutes in dark.

The accumulated lipofuscin granule density and fluorescence were stable over the period of 14 days after the supplementation ended and the cells were subjected to daily media change and 45-minute incubations with PBS in dark (Fig. 3). When the fluorescence images of lipofuscin-laden cells were split into green and red channels, the intensity of red fluorescence appeared much greater than the green fluorescence (Fig. 3). However, when lipofuscin-laden cells were exposed daily to 26.3 J/cm^2 visible light, both the granule density and fluorescence intensity decreased (Fig. 3). When inspected by microscopy a day after the first exposure, there was clearly visible lipofuscin packeted into spherical

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structures floating above the cell monolayer. The monolayer appeared confluent with no sign of cell rounding and detachment. After two exposures, the fluorescence of the packeted lipofuscin and lipofuscin in cells appeared green. When the images were split into green and red channels, the intensity of red fluorescence appeared similar to the green fluorescence (Fig. 3). After completing all 14 exposures, the fluorescence intensity in both green and red channel, as well as lipofuscin granules density, considerably decreased in comparison with cells not exposed to light (Fig. 3). Cells without lipofuscin which were exposed to light exhibited a small increase in green fluorescence in comparison with cells maintained in dark but no granules could be seen in the cell monolayer (Fig. 3).

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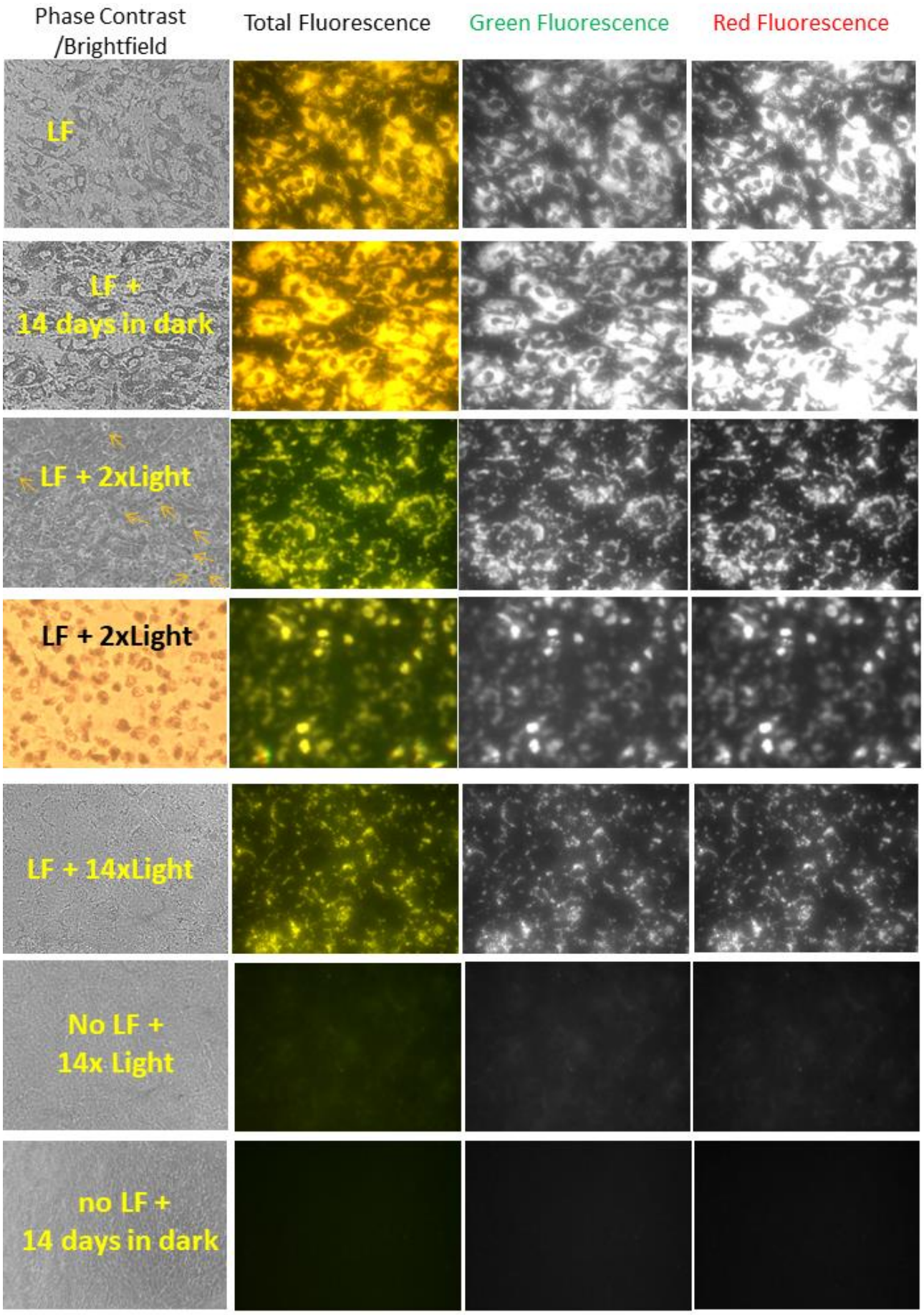

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Figure 3. Exposure of lipofuscin-laden ARPE-19 cells to 9.76 mJ/cm² visible light results in exocytosis of packeted lipofuscin granules and spectral changes in lipofuscin fluorescence. Representative images from phase contrast/bright field and fluorescence microscopy of ARPE-19 cells were supplemented with lipofuscin granules 3 times a week over a period of 32 days (13 times in total) and imaged on day 32 (LF); after additional 14 days in dark with daily media change and incubation for 45 minutes with PBS (LF+ 14 days in dark); after additional 3 days which included two 45 minute exposures to visible light (LF + 2xLight; the arrows in the phase contrast image with 100x magnification point to the packeted lipofuscin floating above the monolayer; the image below shows the packeted lipofuscin in brightfield at 400x magnification which was used for all other images; fluorescence images are focused on cell monolayer or on floating packeted lipofuscin above); after additional 15 days with 14 daily exposures to visible light (LF + 14xLight). The bottom two rows show cells not supplemented with lipofuscin but treated the same ways as the supplemented cells. Fluorescence images were acquired with colour camera capturing the colour of lipofuscin fluorescence as it appears to the human eye (Total Fluorescence). The gain used for collecting fluorescence images was two-fold smaller for lipofuscin-laden cells than for all other cells. The images were open in ImageJ as RGB stack to quantify the green (Green Fluorescence) and red (Red Fluorescence) fluorescence.

It has been reported that antioxidants can inhibit photooxidation of bisretinoids and ameliorate the phototoxic effects of lipofuscin [68,73]. To determine whether supplementation of cells with antioxidants during exposure to light can affect lipofuscin density and fluorescence, cells were exposed in the presence and absence of N-acetyl cysteine (NAC) or lipoic acid. Both of these compounds are powerful antioxidants [76]. NAC can scavenge several reactive oxygen species as well as serve as a precursor of glutathione. Lipoic acid can play an antioxidant role also by scavenging reactive oxygen species as well as by binding redox active metal ions, such as iron or copper. It can also reduce and thereby regenerate the original protective form of other powerful antioxidants, glutathione, vitamin E and vitamin C. The presence of NAC and lipoic acid during lipofuscin exposure to light did not affect lipofuscin granule density nor fluorescence in comparison with not supplemented cells (Fig. 4).

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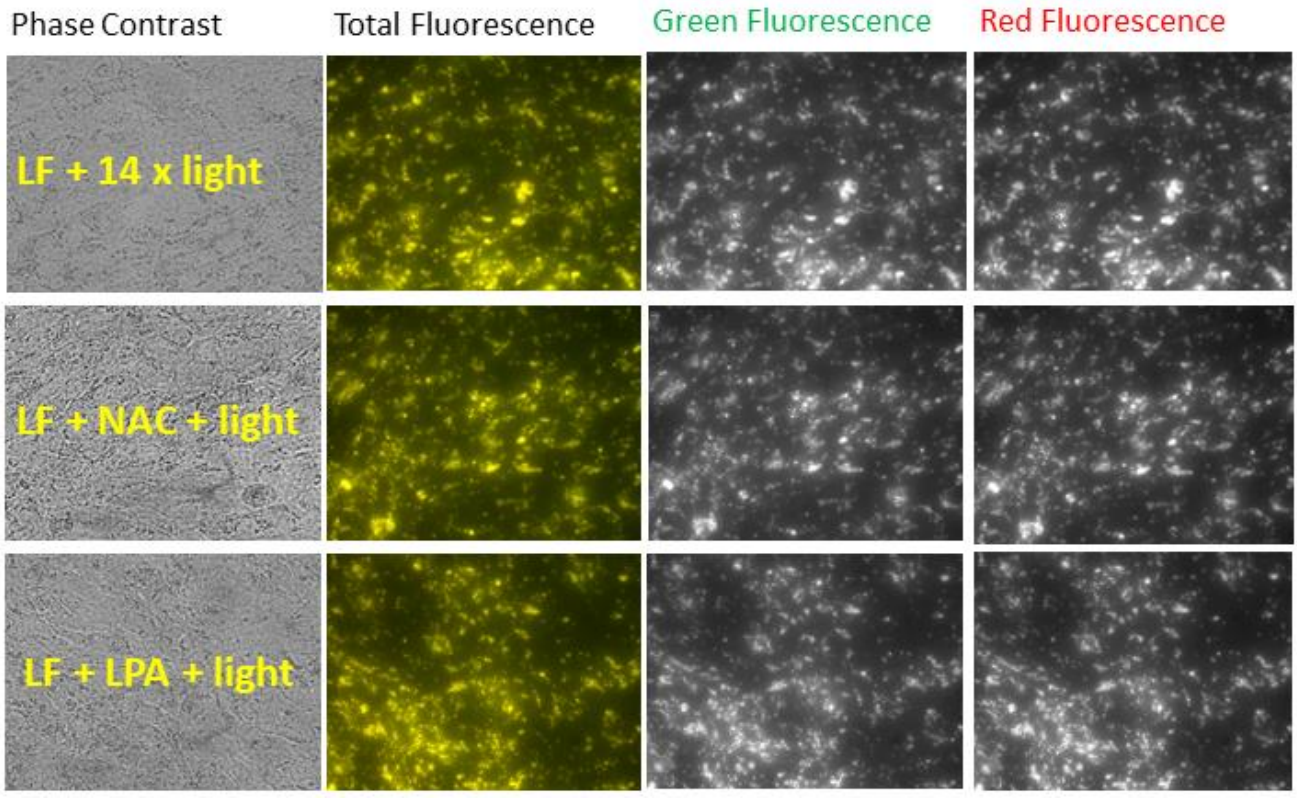

Figure 4. Supplementation with antioxidants of lipofuscin-laden ARPE-19 cells during 14 daily exposures to 9.76 mJ/cm² visible light does not affect loss of lipofuscin granules nor spectral changes in fluorescence. Representative images from phase contrast and fluorescence microscopy of lipofuscin-laden ARPE-19 cells after 14 daily exposures to visible light in the absence (LF + 14xLight) and presence of N-acetyl-cysteine (NAC) or lipoic acid (LPA). Other experimental conditions as in Fig. 3.

The quantification of lipofuscin granule density and intensity of fluorescence in the green and red channels revealed that the exposure to light resulted in a 6.6-fold decrease in granule density (Fig. 5). Both green and red fluorescence decreased by 3.6- and 5.3-fold, respectively (Fig. 5). The ratio of fluorescence in the red to green channel also decreased from 1.37 for cells incubated in dark to 0.94 for cells exposed to 369 J/cm² visible light.

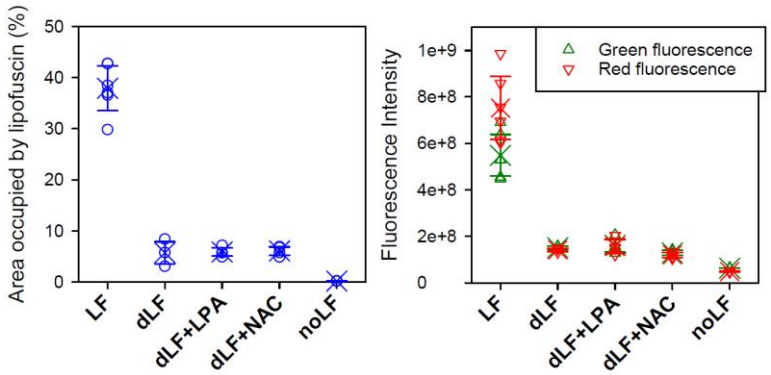

Figure 5. Quantification of area occupied by lipofuscin in phase contrast images (A) and intensity of green and red fluorescence (B) from experiments for which the representative images are shown in Fig. 3 and 4. LF: lipofuscin-laden cells maintained for additional 15 days with 14 daily media changes and

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45-minute incubations with PBS; dLF: lipofuscin-laden cells maintained for additional 15 days with 14 daily media changes and 45-minute exposures to 9.76 mJ/cm² visible light; dLF+LPA: as dLF but exposures to light were in the presence of 0.1 mM lipoic acid; dLF+NAC: as dLF but exposures to light were in the presence of 0.2 mM N-acetylcysteine; noLF: as dLF but cells were subjected to sham treatment instead of supplementation with lipofuscin.

While the colour changes in emission due to exposure of lipofuscin laden cells to light are visible to the eye equipped with a fluorescence microscopy and can be recorded with colour camera, spectrofluorymetry enables to investigate the spectral changes in fluorescence in more detail (Fig. 6). Spectrofluorometry of lipofuscin-laden cells maintained in dark and solubilized for measurements with Triton X-100 revealed that the fluorescence emission spectra resemble the spectra recorded for lipofuscin alone but after 2-3 h of photodegradation (Figs. 1, 6). The ratios of fluorescence emission from lipofuscin-laden cells at 450/610 and 470/610 nm were 5.3 and 4.9, respectively. These values are in between the corresponding values for lipofuscin exposed to 70.3 and 105.4 J/cm² light (Fig. 1E). This spectral change in fluorescence occurred even though during the supplementation period the cells were kept in dark and the media changes were done under dim light. It has been reported previously that phagocytosis by RPE cells is associated with generation of reactive oxygen species and lipid peroxidation [77]. Therefore, it appears plausible that such reactive oxygen species could lead to partial oxidation of lipofuscin resulting in changes of its composition and consequently in its fluorescence properties. It needs to be noted that cell without lipofuscin maintained in dark also exhibited fluorescence, particularly in blue-green range even though no granule accumulation could be seen in the cell monolayer. This could be due to oxidative modifications of intracellular molecules and/or formation of lipofuscin-like material due to autophagy with the size of the nondigestible remnants of autophagolysosomes not big enough to be visible with the microscope we used. It has been reported previously that lipofuscin-like material can accumulate in cells during long-term culture as a result of autophagy of intracellular organelles [1].

The initial 530 to 610 nm intensity ratios of fluorescence induced by 360 or 488 nm light in lipofuscin-laden cells were 3.7 and 0.45, respectively (Fig. 6A,C). These values are similar to the corresponding ratios for lipofuscin photodegraded for about 90 and 10 minutes, respectively (Fig. 1C,G). Such a discrepancy is not unexcpected considering that different excitation wavelengths were used. Lipofuscin is a complex mixture of various chromophores with different photosensitizing and fluorescent properties and it can be expected that their susceptibility to oxidation can vary. It can be also expected that the spectral changes of lipofuscin fluorescence upon phagocytosis-related oxidation of lipofuscin can be different than upon photooxidation because lipofuscin photooxidation involves photosensitized production of singlet oxygen whereas the phagocytosis process is accompanied by free radical generation [60,66,67,77].

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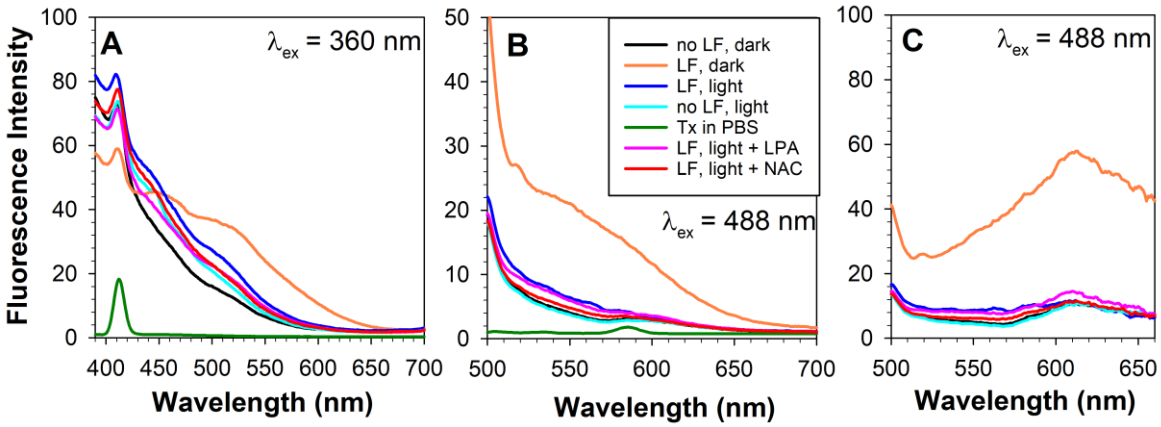

Figure 6. Representative fluorescence emission spectra from ARPE-19 cells solubilized in Triton-X-100. Cells were treated as in Figures 3 and 4. Fluorescence was induced by photoexcitation with 360 nm (A) or 488 nm (B) light. C) Fluorescence emission spectra from B corrected for the spectral changes in sensitivity of the detection. Confluent cells were supplemented with lipofuscin (LF) or sham-treated (no LF) for 32 days followed by 15 days with 14 daily media changes and 45-minute incubations with PBS in dark (dark) or 14 daily media changes and 45-minute exposures to 9.76 mJ/cm^2 visible light (light). Cells in selected wells were exposed to light in the presence of 0.1 mM lipoic acid (LPA) or 0.2 mM N-acetylcysteine (NAC). Green line shows Raman emission peak from solvent, 1% Triton X-100 in PBS (Tx in PBS).

Comparison of fluorescence emission induced by 360 nm light between lipofuscin-laden cells maintained in dark and exposed to a fractionated dose of 369 J/cm^2 visible light revealed a decrease of fluorescence for all emission wavelengths above 455 nm, and an increase in fluorescence below that wavelength (Fig. 6A). This marked increase of short-wavelength fluorescence was clearly visible despite the fact that cells exposed to light lost 85% of lipofuscin granules (Fig. 5A, 6A). It needs to be noted that cells without lipofuscin supplementation subjected to daily exposures to visible light exhibited an increase in fluorescence emission in the range of 420 and 570 nm when photoexcited with 360 nm light when compared with cells maintained in dark (Fig. 6A). These cells, however, showed no significant differences in fluorescence when photoexcited with 488 nm light (Fig. 6B,C).

The intensity of 360 nm light-induced fluorescence emission at 610 nm from lipofuscin-laden cells decreased 2.6-fold as a result of exposure to light (Fig. 6A). This value is greater than the decrease of 1.7-fold observed in lipofuscin suspension exposed to the same dose of light (Fig. 1A,C). This is consistent with 6.6-fold loss of lipofuscin granules from cells accompanied by formation of 360 nm-absorbing species which can contribute, directly or indirectly, to fluorescence emission at 610 nm.

Exposure to light led to a decrease of fluorescence induced by 488 nm light over the whole range of emission wavelengths (Fig. 6B,C). The intensity of 488 nm light-induced fluorescence emission at 610 nm from lipofuscin-laden cells decreased 5.1-fold as a result of exposure to light and became very similar to fluorescence emitted by cells not supplemented with lipofuscin maintained in dark or exposed to light (Fig. 6B,C). The expected value of 610 nm emission intensity decrease can be calculated based on i) a 6.6-fold reduction in the density of lipofuscin granules, ii) 2.4-fold reduction in lipofuscin fluorescence at that wavelength observed as a result of exposure to 3.69 J/cm^2 light, and iii) an

assumption that the lipofuscin isolated from RPE cells is the only contributor to fluorescence from ARPE-19 cells. This value of 3.6-fold is considerable smaller than the observed 5.1-fold decrease suggesting that oxidative changes in lipofuscin granules due to phagocytosis and exposure to light are greater than those occurring in isolated lipofuscin exposed to light.

Supplementation of cells with N-acetyl cysteine or lipoic acid during exposure to light exerted only a small effect on changes in fluorescence of lipofuscin-laden cells induced by these exposures (Fig. 6). The emission of fluorescence induced by excitation with 360 nm light was slightly smaller in the presence of these antioxidants than in their absence. The differences in fluorescence induced by 488 nm light between cells without antioxidants and cells supplemented with NAC or lipoic acid were even less pronounced.

2.4 Effect of lipofuscin photodegradation on cell viability and endocytic activity

Phototoxicity of lipofuscin has been reported previously and therefore our exposure conditions were designed with the aim to avoid lethal effects on cells [48,71,73-75]. Bisretinoids, such as A2E are strongly anchored within the lipofuscin granules [48], and therefore unlikely to diffuse out of the granule to exert their toxic effects; however, some their scission products resulting from degradation [49,52] are likely to be small polar molecules able to diffuse outside the granule and similar to the oxidation products of all-*trans*-retinal oxidation which were shown to be highly (photo)toxic [78].

Photooxidation of lipofuscin is likely to release polar products of oxidation of bisretinoids and polyunsaturated fatty acids which can exert toxic effects unless detoxified and removed from the cell. Therefore, the cell morphology and integrity of the monolayer was monitored by phase contrast microscopy (Fig.4). The fractionation of the dose of light enabled to deliver it with no indication of toxic effects of exposure to lipofuscin and/or light. The MTT assay of metabolic activity of cells performed 24 hours after the final 14th exposure confirmed that there were no significant differences in viability between cells with and without lipofuscin, kept in dark or exposed to light (Fig. 7A).

One of the essential roles of RPE cells is endocytosis [79]. It has been shown in previous studies that internalized lipofuscin and/or oxidative stress can inhibit phagocytosis of photoreceptor outer segments by cultured RPE cells [73,80-86]. To test whether accumulated lipofuscin and/or exposure to light can affect endocytosis, we used the neutral red assay to assess the endocytic activity of cells (Fig. 7B). The assay was performed 23 hours after the final 14th exposure to light and demonstrated that there were no significant differences in viability between cells with and without lipofuscin, kept in dark or exposed to light.

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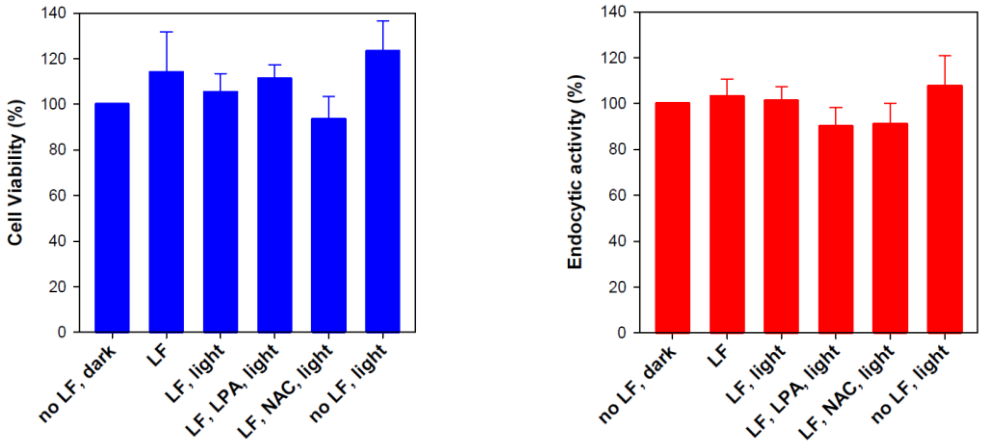

(A) (B)

Figure 7. Lipofuscin and/or fractionated exposure to 9.76 mW/cm2 visible light does not affect viability or endocytic activity of ARPE-19 cells. A) Cell viability measured nu MTT assay. B) Endocytic activity was measured by neutral red assay. Confluent cells were supplemented with lipofuscin (LF) or sham-treated (no LF) for 32 days followed by 15 days with 14 daily media changes and 45-minute incubations with PBS in dark (dark) or 14 daily media changes and 45-minute exposures to 9.76 mJ/cm2 visible light (light). Cells in selected wells were exposed to light in the presence of 0.1 mM lipoic acid (LPA) or 0.2 mM N-acetylcysteine (NAC). The assays were performed a day after the 14th exposure to light/dark. The absorbance values were normalized to the value obtained on cells not-supplemented with lipofuscin and maintained in dark. The height of the bar indicates the mean from three experiments while the error bar indicated SD. One way analysis of variance with ANOVA showed no significant differences between any groups.

3. Discussion

3.1 Changes in the long-wavelength fluorescence emission during photodegradation of lipofuscin

Our results indicate that exposure of lipofuscin to visible light both in suspension and in ARPE-19 cells results in a decrease of fluorescence at about 600 nm which is thought as being characteristic for RPE lipofuscin and is ascribed to fluorescence of bisretinoids [53].

The monotonic decrease of fluorescence at 600 nm observed during irradiation of lipofuscin granules in suspension in PBS (Fig. 1) may seem to contradict the claim that photooxidation of lipofuscin components, A2E and all-*trans*-retinal dimer is accompanied by the fluorescence increase when compared to the parent compound [47,53,68]. In the experiment descriptions reported by Kim and colleagues, only the irradiation wavelength and the irradiation times were provided but the values of the irradiance or radiant exposure were not provided [47,53,68], and therefore it is not possible to compare them with the irradiance or radiant exposures used in our experiments. There is a possibility that such an increase could have occurred before our first measurement of irradiated lipofuscin after delivering radiant exposure of 17.6 J/cm^2. Anyway, the results based on which such a conclusion about fluorescence increase was drawn [47,68] are not sufficient to support it.

In the first study [47], A2E was oxidized by exposure to endoperoxide of 1,4-dimethylnaphthalene, and then the mixture was run on HPLC with

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absorption detection at 430 nm, while the fluorescence, induced by excitation with 430 nm light, was monitored at 600 nm. The chromatograms of the mixture included A2E, isoA2E, monoperoxy-A2E, bisperoxy-A2E, as well as a number of other non-identified peaks. The ratios of fluorescence to absorption peaks in these chromatograms were greater for monoperoxy-A2E and bisperoxy-A2E than for A2E or isoA2E. This suggests that the quantum yield of emission at that particular wavelength of 600 nm with 430 nm excitation is greater for monoperoxy-A2E and bisperoxy-A2E than for A2E or isoA2E. However, this observation is not enough to conclude that fluorescence of the monoperoxy-A2E and bisperoxy-A2E is greater than that of A2E. For the meaningful comparison, it should be based on comparing the whole emission spectra obtained by excitation of equimolar concentrations of A2E and a known oxidation products, or by excitation of A2E and the whole mixture of oxidation products derived from that A2E. The same reasoning can be applied to other photooxidation products of A2E, namely monofurano-A2E and bisfurano-A2E, and of all-*trans*-retinal dimer for which it was also claimed that their fluorescence exceeds that of their parent compounds.

The second study that reported an increase in fluorescence intensity upon photooxidation of a bisretinoid [68] employed alkenyl ether lysoA2-phosphatidylethanolamine containing one plasmalogen with fully saturated 18 carbon chain (LysoA2-PE(P18:0/0:0)). This bisretinoid was solubilized in 2% DMSO in phosphate buffer, and its fluorescence spectra were acquired using excitation with 440 nm during continuous exposure to that light. The initial emission maximum of LysoA2-PE(P18:0/0:0) was 608 nm. Upon photooxidation, the emission intensity appeared to increase while its maximum shifted towards shorter wavelength of 594 nm. It needs to be considered that LysoA2-PE(P18:0/0:0) is hydrophobic and therefore likely to have low solubility in aqueous solution and precipitate when solubilized in PBS with only 2% DMSO. It can be suggested that, as a result of photooxidation, more polar molecules are generated which due to increased polarity are more soluble in the aqueous solvent. A support for such a scenario comes from experiments on A2E which, due to lack of highly hydrophobic plasmalogen, is less hydrophobic than LysoA2-PE(P18:0/0:0) but still it appears to aggregate when solubilized in PBS with 2% DMSO [87]. It was reported that A2E solubilized in 2% DMSO in PBS can be photoexcited not only with 436 and 480 but also with 545 nm wavelength and emit fluorescence with maxima of 620, 630 and 630 nm, respectively. However, A2E does not absorb 545 nm light. Therefore, it can be suggested that A2E forms aggregates in PBS with 2% DMSO which affects its absorption and fluorescence properties.

In other studies from the same group, it was reported that exposure of A2E to 480 nm light results in a decrease in intensity of fluorescence emission and a shift of the maximum from 612 nm to 586 nm, respectively [87,88]. Similar results were observed when A2E was photodegraded by 440 nm light and fluorescence was excited with 450 nm [89]. These results are consistent with the observed fluorescence decrease in our study (Fig. 1).

3.2 Decrease in long-wavelength fluorescence during photooxidation of lipofuscin and an increase in blue/cyan or green to orange fluorescence ratio as a marker of lipofuscin oxidation

Because of spectral changes occurring in lipofuscin due to oxidation, intensity of fluorescence on its own is not sufficient for lipofuscin quantification. However, in combination with information from the spectra of fluorescence emission, it can provide valuable information about the quantity of lipofuscin as well as its oxidation state.,

While the photooxidation of lipofuscin resulted in a monotonic decrease of fluorescence at 600 nm when excited with 488 nm light, the fluorescence emission at about 530 nm exhibited an initial increase up to a dose of about 50 J/cm^2, which was then followed by a monotonic decrease with increasing radiant exposure (Fig. 1E). This effect can be due to the initial oxidation of bisretinoids or other compounds resulting in the formation of oxidation products emitting fluorescence at about 530 nm. Further oxidation of these products can lead to their photodegradation accompanied by fluorescence decrease as suggested in recent reviews [53,68,90].

Our results on photooxidation of isolated RPE lipofuscin (Fig. 1F) are consistent with the results of a comparison of fluorescence emission spectra recorded in suspension of human RPE cells isolated from 74 year old eyes affected with AMD and 75 year old eyes without any signs of AMD [63,91]. The fluorescence spectra from the AMD-affected RPE cells obtained with excitation with 488 nm light show that the emission ratio at 550 nm to 600 nm is increased when compared with normal RPE of similar age. Such an increase in the ratio can be seen during photooxidation of lipofuscin in suspension and in cultured cells suggesting that it can be used as a marker of oxidative damage to the lipofuscin and/or RPE. However, due to the complex kinetics of the increase of the ration of 550/610 nm or 530/610 nm intensities, monitoring that ratio for 450/610 or 470/610 nm can be more straightforward to interpret. The disadvantage if the latter is that it would require a shorter excitation wavelength than 488 nm which is currently used in clinical instruments. The shorter excitation wavelength can also impose an increased risk of phototoxicity (reviewed in [66]). Following discovery that the phototoxicity thresholds of radiant exposures used for light safety standards were incorrect for 568 nm light [24-26], Zhang and colleagues determined the phototoxicity thresholds for several other wavelengths which include 460 nm light [72]. Further experiments are needed to determine whether using excitation of lipofuscin and retina with light of 460 nm wavelength, or shorter, can produce useful information about the lipofuscin content and its oxidation state, and whether it is feasible to use this approach in clinical setting without risking retinal photodamage.

3.2 Oxidized DHA may contribute to lipofuscin fluorescence.

Our results clearly demonstrate that oxidized DHA can emit fluorescence when excited with 360 or 488 nm light (Fig. 2). The spectral characteristics of that fluorescence indicate that it can contribute to the blue-green fluorescence of lipofuscin, particularly when lipofuscin is

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photooxidized (Figs. 1, 2). This together with several other pieces of evidence argue that oxidized DHA can contribute to lipofuscin fluorescence: i) DHA accounts for 6-9% and 7-16% of fatty acids in lipofuscin phospholipids and free fatty acids, respectively [54]; ii) upon exposure to light lipofuscin generates reactive oxygen species, such as singlet oxygen, superoxide, hydroxyl radical and hydrogen peroxide which can oxidize DHA [66,67]; an oxidation product of DHA, carboxyethylpyrrole was identified in human RPE lipofuscin [48]; oxidized DHA generates with high quantum yields similar reactive oxygen species as lipofuscin [60]. Contribution of oxidized lipids to the lipofuscin fluorescence was considered in the past but dismissed based on studies of fluorescent properties of end-products of lipid oxidation, such as MDA and HNE, and their adducts with amines [10]. These particular products of lipid oxidation are very small molecules, which do not absorb visible light, so UV light is required for their photoexcitation, upon which they elicit the blue fluorescence. It can be suggested that products of oxidation of other lipids with fewer unsaturated double bonds, such as docosahexapenoic acid, arachidonic acid and linolenic acid, as well as small scission products of bisretinoids degradation can contribute to the short-wavelength emission spectrum of lipofuscin but this suggestion requires experimental verification.

3.3 Physiological relevance

It is important to consider the physiological relevance of our study. The irradiance levels employed in our study of 9.76 mW/cm^2 are about 100 times greater than the highest estimates of those encountered in daylight, which is 0.1 mW/cm^2 for sunlight reflected from snow [92]. However, the cultured cells were exposed to it for only 45 minutes per day for 14 days providing single radiant exposures of 26.4 J/cm^2 and total radiant exposure of 369 J/cm^2. Such a dose can be expected to accumulate in the human retina over a period of 1025 h of exposure to 0.1 mW/cm^2, or ten-fold longer for 0.01 mW/cm^2, which is considered as irradiance for the retina under typical lighting conditions [92]. Assuming further that the exposure to daylight us for 16 hours per day, it gives 640 days over which such a dose could accumulate.

An interesting observation from our experiments is loss of lipofuscin granules from cultured cells as a result of exposure to light. While ARPE-19 cells accumulated large amounts of lipofuscin which remained there for the following 15 days when cells were maintained in dark and appeared healthy, the exposure of lipofuscin-laden cells to light resulted in a substantial loss of lipofuscin granules which appeared as lipofuscin packeted into membranes-enclosed structures floating in the culture medium above the cell monolayer. This packeted lipofuscin was removed when the culture medium was replaced with PBS before the next exposure to light. However, in the retina such packeted lipofuscin may contribute to the age-related deposits accumulating between the RPE and collagenous layer of Bruch's membrane which separates the retina from the choroidal blood supply. This is consistent with observations of histologic sections of human retina of Burns and Feeney-Burns, where they saw membrane-enclosed parts of RPE cells, containing lipofuscin, and budding of the cells or being a part of deposits between the RPE and Bruch's membrane, known as drusen

[93]. Similar observations were made by Gouras and colleagues who studied the retina of aged monkeys [94]. More recently, it has been reported that during early stages of AMD there is a formation of lipofuscin-dense structures within RPE cells and loss of lipofuscin granules from RPE [95]. It appears from our results that oxidative damage to the lipofuscin can trigger such effects. This can also explain why there is loss of lipofuscin granules and therefore fluorescence in the aged retina and even more in AMD, where oxidative stress and oxidative damage in increased as evidenced by the increased levels of easily chelatable iron and products of lipid oxidation [48,59,96,97]. Further investigations are clearly needed to understand the mechanism trigerring the externalization of lipofuscin.

Our results showing loss of lipofuscin granules from ARPE-19 cells and spectral changes of fluorescence as a result of exposure to light can also help explaining why there an age-related decrease in lipofuscin fluorescence and can be no spatial correlation between retinal fluorescence and bisretinoids as observed in the human and monkey retina [98-103].

4. Materials and Methods

4.1. Reagents

Chemicals, at least analytical grade, including lipoic acid, N-acetyl-cysteine (NAC) and dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), neutral red, were purchased from Sigma (Sigma, St. Louis, MO, USA) or Fisher Scientific (Loughborough, UK) unless stated otherwise. Phospholipid containing docosahexaenoate (DHA) acyl chains, 1,2-di-(4Z,7Z,10Z,13Z,16Z,19Z-docosahexaenoyl)-sn-glycero-3-phosphocholine (Di22:6PC) was from Avanti Polar Lipids (Alabaster, AL, USA). Chromatography grade organic solvents were from Fisher, Merck or VWR International, and used as supplied.

4.2. Isolation and purification of RPE lipofuscin

Research on human tissue was approved by the School Research Ethics Audit Committee, School of Optometry and Vision Sciences, Cardiff University. Human eyes were obtained from the Bristol Eye Bank, Bristol, UK. The research adhered to the tenets of the Declaration of Helsinki. Lipofuscin was isolated and purified from RPE cells from 74 pairs of human cadaver eyes (range of age: 43-95 years, mean of 74 years) as described previously [38,48,60].

4.3. Preparation of liposomes and oxidation of DHA

Multilamellar lipid vesicles (liposomes) from Di22:6PC were prepared as described previously [38,60]. The lipid film was exposed to the air to allow for its oxidation and then hydrated with phosphate buffered saline (PBS) to form liposomes.

4.4. Absorption and fluorescence spectrometery

Optical density spectra of liposomal or lipofuscin suspensions were measured in a quartz cuvette with a 1 cm path length using U-1800 UV-

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VIS spectrophotometer (Hitachi) equipped with Hitachi UV Solutions software. Fluorescence spectra were measured using a F4500 fluorescence spectrophotometer (Hitachi) equipped with Hitachi FL Solutions software. Fluorescein and rhodamine B were used to determine the correction of the spectral sensitivity of the spectrofluorometer detection.

4.5. ARPE-19 cell culture

ARPE-19 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured as described previously [48,78,104] using 24-well plates and DMEM/F12 with addition of, L-glutamine, and penicillin-streptomycin and 10% or 2% heat-inactivated foetal calf serum (FCS) (Sigma-Aldrich Chemical Co., St Louis, MO, USA). All experiments were performed on confluent cell monolayers seeded in 24-well plates, passage numbers 24 and 25, with media changes done under dim room light (with the fluorescent light under the class II cabinet switched off).

4.6. Enriching ARPE-19 cells with lipofuscin

To enrich cells with lipofuscin, cells were fed with culture medium supplemented with 2.9 x 108 lipofuscin granules per ml as described previously [48]. Cells were fed with lipofuscin three times per week up to 13 times. Control cells were fed with culture medium supplemented with PBS as vehicle. Incorporation of lipofuscin was monitored by bright field/phase contrast microscopy using dim light to avoid unnecessary photooxidation due to imaging [105].

4.7. Irradiation of lipofuscin and ARPE-19 cells with visible light

Suspensions of lipofuscin in PBS or cultured ARPE-19 cells were irradiated with visible light in spectrofluorometric cuvettes and in 24-well cell culture plates, respectively. A solar simulator Sol lamp (Honle UV Ltd, Birmingham) was used as a light source as described previously [48,78]. Filters absorbing heat and the residual UV light (Lee Heat Shield and #226 Lee UV filter, respectively; Lee Filters, UK; and a UV-cut off filter, AMO Inc., Santa Ana, CA, USA; 2g/100 ml solution of copper sulphate) were placed between the lamp and the glass plate where the cuvette or cell culture plate were positioned. Fluence rates and irradiance spectra were measured using a spectroradiometer (Specbos 1201 with JETI LiMeS software; Glen Spectra, UK) (Fig. 8). The irradiance used in experiments was 9.76 mW/cm², illuminance 35.3 klx.

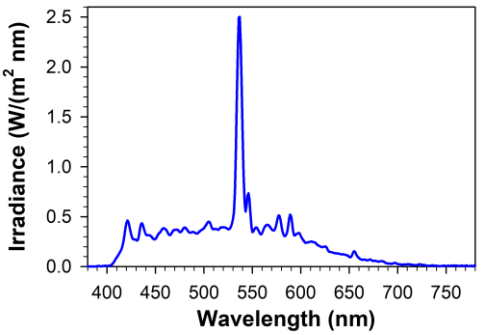

Figure 8. Irradiance spectrum of light used for photodegradation of lipofuscin and exposure to light of ARPE-19 cells.

ARPE-19 cells were irradiated daily for 14 days, for 45 min each time, at a temperature of about 26^{0}C. Before the exposure, the medium was removed, cells were washed twice with Dulbecco's phosphate buffered saline with calcium and magnesium (PBS), and PBS with or without antioxidants (0.2 mM N-acetyl cysteine, NAC or 0.1 mM lipoic acid, LPA with 0.2% DMSO) were added to wells for the exposure time. After exposure, PBS was replaced with culture medium containing 2% FCS and cells were returned to the incubator for 23 h when the next exposure, or assessments of fluorescence, cell viability or endocytic activity assays were performed. Dark-maintained control cells were treated the same way except the exposure to light was replaced by incubation of plates wrapped in blackened aluminium foil in the same room as the light source.

4.8. Quantification of pigment granules in cell monolayers and their fluorescence

Cells were visualized using an Olympus IX70 inverted microscope with phase contrast and fluorescence [78]. Fluorescence was induced by excitation with blue light; emission was collected after passing through a long-pass emission filter. Images were obtained using a Spot RT colour CCD camera and Spot Advanced software (Diagnostic Instruments Inc, UK).

ImageJ was used for quantification of lipofuscin granule density and their fluorescence. In brightlight and phase contrast images, lipofuscin granules were visible as dark dots. They were quantified as an area above the threshold, which was determined on images of cells not supplemented with lipofuscin, and expressed as percentage of total image area. Images of fluorescence were opened in ImageJ as RGB stack which splits them into three colour channels. Fluorescence in green and red channels was quantified as total fluorescence intensity per image in each channel separately.

4.9. Monitoring of ARPE-19 cells viability and endocytic activity

Cell viability was assessed by monitoring the morphology of ARPE-19 cell monolayer by phase contrast microscopy using an Olympus IX70 inverted microscope and by MTT assay of reductive activity where cells were exposed for 1 h to cell culture medium containing 0.5 mg/ml of MTT as described previously [78,104]. Then cells were washed with PBS and MTT-derived formazan was solubilised by acidified isopropanol and absorbance at 570 nm was red using a Multiscan Ascent plate reader. Endocytic activity was determined by neutral red assay where cells were exposed for 1 h to cell culture medium containing 0.1 mg/ml of neutral red followed by extraction and absorbance reading at 545 nm [106].

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