Photochemistry and Photobiology, 2009, 85: 234–238

Monitoring the Accumulation of Lipofuscin in Aging Murine Eyes by Fluorescence Spectroscopy

Prasun Mukherjee[1], Sayantan Bose[1], Alyse A. Hurd[1], Ramkrishna Adhikary[1], Holger Schönenbrücher[2], Amir N. Hamir[2], Jürgen A. Richt[2], Thomas A. Casey[3], Mark A. Rasmussen[3,4] and Jacob W. Petrich*[1]

[1]Department of Chemistry, Iowa State University, Ames, IA
[2]Virus and Prion Diseases of Livestock Research Unit, National Animal Disease Center, Agricultural Research Service, USDA, Ames, IA
[3]Pre-Harvest Food Safety and Enteric Disease Research Unit, National Animal Disease Center, Agricultural Research Service, USDA, Ames, IA
[4]SarTec Corp., Anoka, MN

Received 3 April 2008, accepted 3 June 2008, DOI: 10.1111/j.1751-1097.2008.00425.x

ABSTRACT

The integrated fluorescence of murine eyes is collected as a function of age. This fluorescence is attributed to pigments generally referred to as lipofuscin and is observed to increase with age. No difference in fluorescence intensity is observed between the eyes of males or females. This work provides a benchmark for further studies that are planned in order to use such signatures as markers of central nervous system (CNS) tissue or even of diseased CNS tissue and provides a basis for determining the age of a healthy animal.

INTRODUCTION

It is believed that the aging process in humans and animals is accompanied by the progressive accumulation of yellow-brown intracellular pigments (1). These age-related fluorescent pigments are classically known as ''lipofuscin.'' The pigment lipofuscin is a heterogeneous, high-molecular weight granular material that has been shown to be accumulated in high concentrations mainly in postmitotic cells, such as neurons, cardiac muscle and retinal epithelium. It has also been observed to accumulate in cases of human Creutzfeldt–Jakob's disease (CJD) and in other cases of experimental transmissible spongiform encephalopathies. For example, Boellaard *et al.* have demonstrated a relationship between lipofuscin production, the presence of autophagocytosis and the experimental induction of CJD in mice (2–11). Lipofuscin is believed to be the product of ''oxidative stress.'' It accumulates in central nervous system (CNS) tissues, which are nonregenerative. Lipofuscin also accumulates in the eye, especially in the case of disease, and its accumulation may be related to age-related macular degeneration and other pathologies (12–29).

Using specific excitation wavelengths, brain and spinal cord samples show characteristic fluorescence. Earlier, we reported solid state fluorescence measurements with front-faced geometry from solid bovine brain and spinal cord samples (30). The fluorescence from these tissues was attributed to the accumulation of lipofuscin. There are numerous studies that deal with the study of lipofuscin pigments in the eye (15–20,31,32). Analytical chemists have tended to view the autofluorescence arising from lipofuscin as a nuisance (33), whereas we propose to exploit it.

The goal of this study was to monitor the accumulation of lipofuscin in murine eyes, to compare these spectra with those from eyes from other species, and to compare these data with other tissues. Previous attempts to characterize age-related changes in eyes were based on absorption study of primate lens (34,35) and transmission of light to the human retina (36). Roberts *et al.* have investigated age-related changes in the human lens by monitoring porphyrin triplet state kinetics (37). Wing *et al.* have demonstrated an increase in the concentration of lipofuscin in human retinal pigment epithelium as a function of age (24). In this report, we monitor the increase in the fluorescence of the murine eye as a function of age. To our knowledge, this is the first time that this has been systematically analyzed over the life span of mice. Ettershank *et al.* (38) performed a similar but less exhaustive study with the fleshfly, *Sarcophaga bullata*. They inspected lipofuscin accumulation in the larva over a period of up to 24 days. Our work provides an important benchmark for further studies investigating the potential of lipofuscin fluorescence to be used as a marker of CNS tissue and perhaps an indicator of diseased CNS tissue.

MATERIALS AND METHODS

Collection of murine eye samples. The animal work was approved by the National Animal Disease Center animal care and use committee and the mice were housed and fed according to Federal Animal Welfare Act Guidelines. Litters of CD-1 mice were raised from birth and weaned at 3 weeks of age. Mice were housed in cages with littermates of the same sex with no more than six female or four male per cage. All mice were fed a standard ration of mouse chow (Harlan Teklad, Madison, WI). Artificial fluorescent lights were cycled on and off every 12 h during the entire course of the rearing period. The animals were reared behind double door entryways and never exposed to sunlight. Beginning at 3 weeks of age, and continuing each week until 26 weeks of age and then every 2 weeks afterwards until 86 weeks of age, typically groups of 10 mice (five male and five female) were selected and euthanized by exposure to 100% carbon dioxide (CO_2)

*Corresponding author email: jwp@iastate.edu (Jacob W. Petrich)

until cessation of breathing. CO_2 exposure was continued for a minimum of 15 additional minutes until necropsy. Mice were not decapitated so as to avoid intracranial pressure which could cause hemorrhages in the eyes which might interfere with the subsequent analysis. The eyes from a total of 515 mice were examined.

Preparation of sample. Following transport to the laboratory, the eyes were removed at necropsy and the tissues prepared for fluorescence spectroscopy. To obtain total fluorescence from the eye, each eyeball was crushed and spread in a thin layer on a Fisherbrand plain microscope slide ($25 \times 75 \times 1$ mm).

Collection of murine CNS tissue samples. CNS tissue samples (brain and spinal cord) were collected from healthy male ($n = 5$) and female ($n = 5$) C57 BL/DK mice between 6 and 8 months of age. For this purpose the animals were killed with CO_2 followed by decapitation. These samples were kept frozen at −20°C until processing. In addition, brain and spinal cord of C57BL, Tg20 PrP overexpressing and BALB/c PrP knockout (Ko) mice were collected from male ($n = 2$) and female ($n = 2$) groups at 6 and 12 months of age.

Collection and dissection of murine retina samples. Healthy ($n = 59$) C57BL/DK mice and BALB/c PrP Ko mice ($n = 11$) were raised and killed at the age of 6 months according to the Animal Care and Use guidelines. Both eyeballs were collected, stored in an Eppendorf tube and placed on ice before dissection of the retina. The retina from each eyeball was dissected using a dissecting stereo microscope (Stereo Star Zoom; Reichert Scientific Instruments, NY) for magnification. Lens and vitreous body were removed after circular cutting in the ora serrata. The retina was removed from the eye cup and placed plain on a microscope slide, which was prepared with a drop of distilled water. Each sample was covered with a cover slip for additional enlargement of the surface area of the retina. To avoid dehydration of the samples they were stored in a moist chamber and placed at ≤ +7°C until fluorescence spectra were collected within the next 12 h.

Collection of cow and sheep retina samples. Cow and sheep eyeballs were removed from normal animals at necropsy, and the samples were kept frozen at −20°C until processing. Retina samples were prepared by dissecting the eyeball and removing the retina to a glass microscope slide.

Steady-state measurements. For age-related accumulation of fluorescent pigment in murine eyes steady-state fluorescence spectra were obtained on a SPEX Fluoromax for age group of 3–60 weeks and on a SPEX Fluoromax-2 for the remaining age group samples up to 86 weeks using a 4 nm bandpass and corrected for lamp spectral intensity and detector response. The fluorescence intensities obtained from the two different instruments were normalized to the corresponding intensities of the water Raman signal. Spectra were obtained with solid samples using a front-faced geometry. All measurements were made at room temperature with freshly prepared tissues that were never frozen. Typically all experiments were carried out within ∼10 h after euthanasia. For all other measurements the steady-state fluorescence spectra were obtained on a SPEX Fluoromax-2.

RESULTS AND DISCUSSION

Choice of excitation wavelength

Initially we focused on excitation of the samples at wavelengths of 350, 410, 470 and 520 nm. However, during spectral measurements using the early age group, samples indicated negligible fluorescence from all excitation wavelengths except 410 nm. Samples, up to week 40, were excited at each of the four excitation wavelengths but after week 40, fluorescence was only checked occasionally at all four wavelengths. Significant (*i.e.* greater than baseline noise) fluorescence was never observed (except for older group of samples, see later part of the discussion) with any excitation wavelength except 410 nm and we chose to use 410 nm exclusively for all samples from the entire experimental period (86 weeks). Use of these excitation wavelengths completely eliminated the background fluorescence from fluorescent amino acids, namely tryptophan, tyrosine and phenylalanine.

Age-related accumulation of lipofuscin

During the first few weeks of the study we saw a significant change in fluorescence intensity (weeks 3–4 and onward). This indicated that within approximately 1–1.5 months of age a significant amount of fluorescent material (lipofuscin) began to accumulate in the eye. Figure 1 presents total fluorescence from the eyes of male and female mice as a function of age from 3 to 86 weeks. We did not maintain the animals past 86 weeks as they were approaching the full extent of their life span, approximately 2 years. There was an increase in fluorescence emission with age, although the effect was less pronounced on closely adjacent age groups but the effect became more prominent for widely separated age groups. Over the time span investigated, we observed an increase in fluorescence intensity of ∼20 times. The increase in fluorescence emission suggests lipofuscin accumulation in eye with respect to age. We did not see a distinct gender-based correlation in fluorescence intensity for the time span investigated. There was also no difference in spectra from different mouse lines investigated at the same age.

Representative, baseline-corrected, fluorescence spectra from mice eyes from different age groups are presented in Fig. 2. In general, when excited at 410 nm, we observed broad fluorescence spectra with a single peak centered at ∼580 nm. The shape of the spectra was conserved with age, and, as indicated above, the spectra were obtained when $\lambda_{ex} = 410$ nm (but not 350, 470 or 520). This is a curious phenomenon. In our earlier work, considerable structure was observed for bovine brain and spinal cord tissues using an excitation wavelength of 480 nm (30). Consequently, we

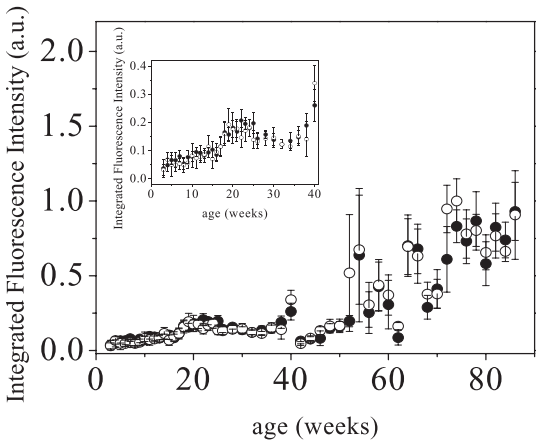

Figure 1. Total fluorescence from male (solid circles) and female (open circles) mice eyes as a function of age. All samples were excited at 410 nm with a front-faced geometry. An interference filter at 410 nm was placed in the excitation beam and a 425-nm long-pass filter was placed in the emission beam. This eliminated interference from scattered light from the solid samples. All data were normalized to a surface area of 2 cm^2. For each spectrum the integrated emission intensity was calculated using a wavenumber scale. For clarity, data up to 40 weeks are shown in the inset separately. The point corresponding to a particular age is typically an average of 10 data points with error bars representing the standard deviation in the measurement. The plot clearly indicates the age-related accumulation of fluorescent pigments (lipofuscin) in the eye. For a given age we did not see any clear distinction of fluorescence intensity between male and female mice eyes.

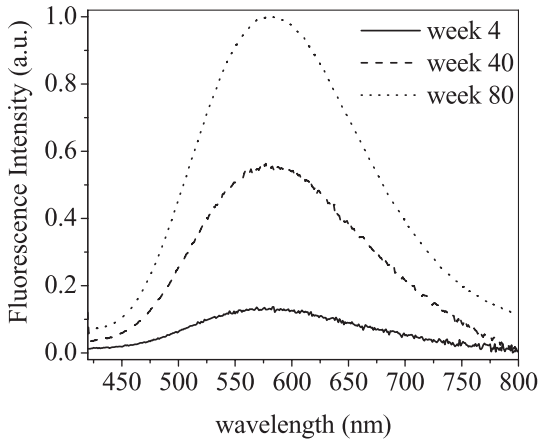

Figure 2. Representative, baseline-corrected, solid state fluorescence spectra from mice eyes for different age groups. Experimental conditions are the same as those given for Fig. 1. The spectra showed a broad peak centered at ~580 nm and the nature of fluorescence spectra was conserved throughout the course of our study. The three spectra shown from a specified age group were selected randomly for purposes of comparison.

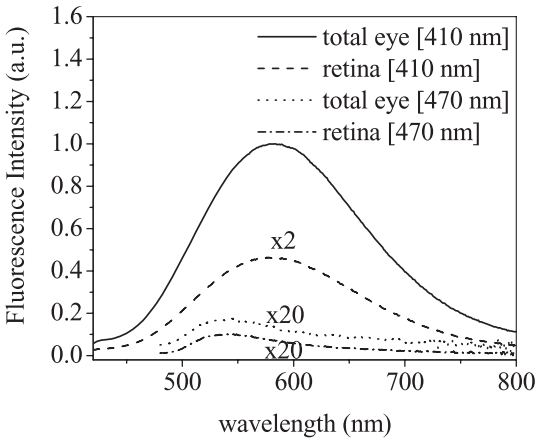

Figure 4. Representative, baseline-corrected, solid state fluorescence spectra from mice retina with excitation wavelengths of 410 and 470 nm. For comparison, spectra from total eye samples (~1.5 years) were also included. The numbers in the parentheses are the excitation wavelengths. Experimental conditions are the same as those given for Fig. 1 with the replacement of appropriate interference and long pass filters required for particular excitation wavelength. We did not observe any structure in the spectra of mice retina. Excitation at 470 nm gave a blueshifted spectrum compared to that obtained with 410 nm excitation. Some of the spectra were multiplied by a scaling factor for clarity.

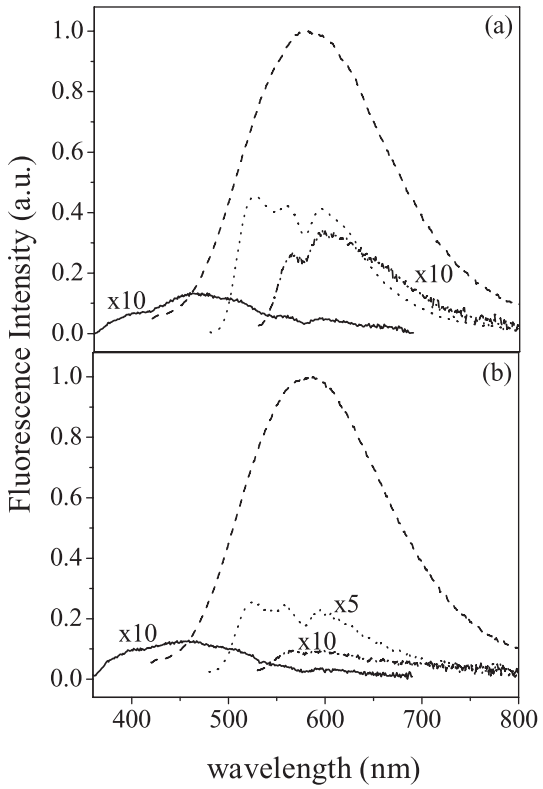

Figure 3. Representative, baseline-corrected, solid state fluorescence spectra from mice: (a) brain; (b) spinal cord. Excitation wavelengths are 350 nm (solid line), 410 nm (dashed line), 470 nm (dotted line) and 520 nm (dash dotted line). Experimental conditions are the same as those given for Fig. 1 with the replacement of appropriate interference and long-pass filters required for a particular excitation wavelength. Excitation at 410 nm yields a structureless spectrum for both samples, whereas other excitation wavelengths give structured spectra. Some of the spectra were multiplied by a scaling factor indicated in the figure for clarity.

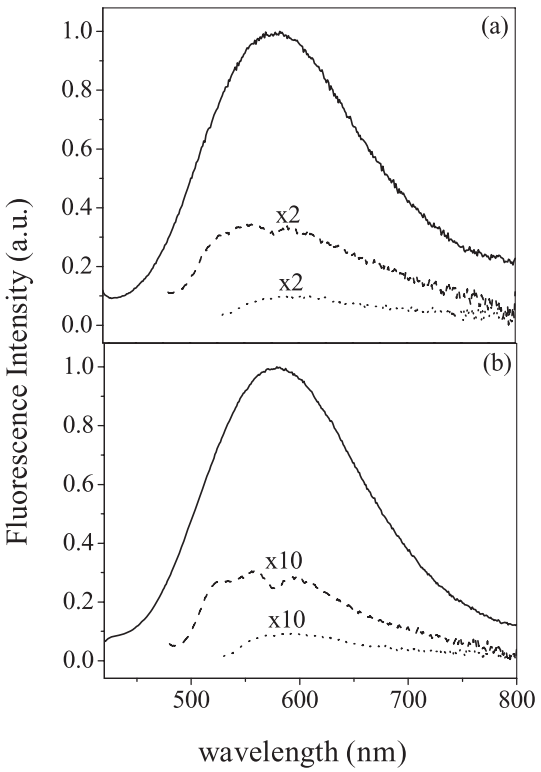

Figure 5. Representative, baseline-corrected, solid state fluorescence spectra of retina from (a) cow and (b) sheep samples with excitation wavelengths of 410 (solid line), 470 (dashed line) and 520 (dotted line) nm. Experimental conditions are the same as those given for Fig. 1, with the placement of appropriate interference and long-pass filters for the specific excitation wavelengths. Excitation at 410 nm yields a structureless spectrum in both cases, whereas other excitation wavelengths give structured spectra. Some of the spectra were multiplied by a scaling factor for clarity. The spectra are significantly different from those of mice retina, where no structure was observed.

investigated other murine CNS tissue. In fact, mouse brain and spinal cord exhibited structured fluorescence when excited at 350, 470 and 520 nm; but this structure disappeared if λ_{ex} = 410 nm (Fig. 3). In contrast, at all ages, the mouse eye yielded structureless fluorescence regardless of the excitation wavelength. There are two factors most likely involved with these discordant observations. First, as lipofuscin is a highly heterogeneous substance, its fluorescence is a composite of several fluorophores, so it is unreasonable to assume that we excited one particular pigment. As an excitation wavelength, 410 nm fortuitously produced a smooth, nearly symmetric, fluorescence spectrum which arose from this heterogeneous substance. In contrast, the spectra of the entire mouse eye were structureless regardless of the excitation wavelength. It would seem, then, that the absence of structure is a result of the spectrum of the entire eye but this is not the case. When individual portions of the eye are examined, they too yield structureless spectra (Fig. 4, mice retina and other tissue). This phenomenon is not observed in other mammals, such as cows or sheep (Fig. 5, cow and sheep retinas). A more complete understanding of these observations awaits further investigation.

This study indicates that fluorescence may be used as an indicator of age. We note that our present study with mice can be considered as a representative marker for age which appears related to the accumulation of fluorescent pigment (lipofuscin) in eyes, which could be easily extended to similar studies in other animals like sheep, cow and perhaps even in humans.

CONCLUSIONS

We present fluorescence spectra from the eyes of mice over a wide range of age (3–86 weeks) that almost covers the complete life span of the mouse. The study indicates age-related accumulation of fluorescent pigments including lipofuscin, in mice eyes and suggests that fluorescence spectroscopy might be used as an age marker. We suggest that the present study could be successfully extended to age-related pigment accumulation in other species.

Acknowledgements—We thank Deb Lebo and Nathan Horman for technical assistance and Dr. G. Krishnamoorthy for his work on the early stages of this project.

REFERENCES

1. Yin, D. (1996) Biochemical basis of lipofuscin, ceroid, and age pigment-like fluorophores. *Free Radic. Biol. Med.* **21**, 871–888.
2. Boellaard, J. W., W. Schlote and J. Tateishi (1989) Neuronal autophagy in experimental Creutzfeldt–Jakob's disease. *Acta Neuropathol.* **78**, 410–418.
3. Ironside, J. W. (1996) Review: Creutzfeldt–Jakob disease. *Brain Pathol.* **6**, 379–388.
4. Ironside, J. W. (2006) Pathological diagnosis of variant Creutzfeldt–Jakob disease. *Acta Pathol. Microbiol. Immunol. Scand.* **110**, 79–87.
5. Ironside, J. W. (2006) Variant Creutzfeldt–Jakob disease: Risk of transmission by blood transfusion and blood therapies. *Haemophilia* **12**, 8–15.
6. Hilton, D. A. (2006) Pathogenesis and prevalence of variant Creutzfeldt–Jakob disease. *J. Pathol.* **208**, 134–141.
7. Pauli, G. (2005) Tissue safety in view of CJD and variant CJD. *Cell Tissue Banking* **6**, 191–200.
8. Ironside, J. W. and M. W. Head (2004) Variant Creutzfeldt–Jakob disease: Risk of transmission by blood and blood products. *Haemophilia* **10**, 64–69.
9. Dabaghian, R. H., P. P. Mortimer and J. P. Clewley (2004) Prospects for the development of pre-mortem laboratory diagnostic tests for Creutzfeldt–Jakob disease. *Rev. Med. Virol.* **14**, 345–361.
10. Burthem, J. and D. J. Roberts (2003) The pathophysiology of variant Creutzfeldt–Jakob disease: The hypotheses behind concerns for blood components and products. *Br. J. Haematol.* **122**, 3–9.
11. Ironside, J. W. and M. W. Head (2003) Variant Creutzfeldt–Jakob disease and its transmission by blood. *J. Thromb. Haemost.* **1**, 1479–1486.
12. Terman, A. (2001) Garbage catastrophe theory of aging: Imperfect removal of oxidative damage? *Redox Rep.* **6**, 15–26.
13. Terman, A. and U. T. Brunk (1998) Lipofuscin: Mechanisms of formation and increase with age. *Acta Pathol. Microbiol. Immunol. Scand.* **106**, 265–276.
14. Tsuchida, M., T. Miura and K. Aibara (1987) Lipofuscin and lipofuscin-like substances. *Chem. Phys. Lipids* **44**, 297–325.
15. Mata, N. L., J. Weng and G. H. Travis (2000) Biosynthesis of a major lipofuscin fluorophore in mice and humans with ABCR-mediated retinal and macular degeneration. *Proc. Natl. Acad. Sci. USA* **97**, 7154–7159.
16. Rózanowska, M., J. Jarvis-Evans, W. Korytowski, M. E. Boulton, J. M. Burke and T. Sarna (1995) Blue light-induced reactivity of retinal age pigment. *J. Biol. Chem.* **270**, 18825–18830.
17. Boulton, M., F. Docchio, P. Dayhaw-Barker, R. Ramponi and R. Cubeddu (1990) Age-related changes in the morphology, absorption and fluorescence of melanosomes and lipofuscin granules of the retinal pigment epithelium. *Vision Res.* **30**, 1291–1303.
18. Sundelin, S. P. and S. E. G. Nilsson (2001) Lipofuscin-formation in retinal pigment epithelial cells is reduced by antioxidants. *Free Radic. Biol. Med.* **31**, 217–225.
19. Katz, M. L. and M. J. Shanker (1989) Development of lipofuscin-like fluorescence in the retinal pigment epithelium in response to protease inhibitor treatment. *Mech. Ageing Dev.* **49**, 23–40.
20. Gaillard, E. R., S. J. Atherton, G. Eldred and J. Dillon (1995) Photophysical studies on human retinal lipofuscin. *Photochem. Photobiol.* **61**, 448–453.
21. Delori, F. C. (2004) Autofluorescence method to measure macular pigment optical densities fluorometry and autofluorescence imaging. *Arch. Biochem. Biophys.* **430**, 156–162.
22. Han, M., G. Giese, S. Schmitz-Valckenberg, A. Bindewald-Wittich, F. G. Holz, J. Yu, J. F. Bille and M. H. Niemz (2007) Age-related structural abnormalities in the human retina-choroid complex revealed by two-photon excited autofluorescence imaging. *J. Biomed. Opt.* **12**, 024012-1-7.
23. Docchio, F., M. Boulton, R. Cubeddu, R. Ramponi and P. D. Barker (1991) Age-related changes in the fluorescence of melanin and lipofuscin granules of the retinal pigment epithelium: A time-resolved fluorescence spectroscopy study. *Photochem. Photobiol.* **54**, 247–253.
24. Wing, G. L., G. C. Blanchard and J. J. Weiter (1978) The topography and age relationship of lipofuscin concentration in the retinal pigment epithelium. *Invest. Ophthalmol. Vis. Sci.* **17**, 601–607.
25. Delori, F. C., D. G. Goger and C. K. Dorey (2001) Age-related accumulation and spatial distribution of lipofuscin in RPE of normal subjects. *Invest. Ophthalmol. Vis. Sci.* **42**, 1855–1866.
26. Delori, F. C., C. K. Dorey, G. Staurenghi, O. Arend, D. G. Goger and J. J. Weiter (1995) In vivo fluorescence of the ocular fundus exhibits retinal pigment epithelium lipofuscin characteristics. *Invest. Ophthalmol. Vis. Sci.* **36**, 718–729.
27. Weiter, J. J., F. C. Delori, G. L. Wing and K. A. Fitch (1986) Retinal pigment epithelial lipofuscin and melanin and choroidal melanin in human eyes. *Invest. Ophthalmol. Vis. Sci.* **27**, 145–152.
28. Dorey, C. K., G. Wu, D. Ebenstein, A. Garsd and J. J. Weiter (1989) Cell loss in the aging retina. Relationship to lipofuscin accumulation and macular degeneration. *Invest. Ophthalmol. Vis. Sci.* **30**, 1691–1699.
29. Delori, F. C., G. Staurenghi, O. Arend, C. K. Dorey, D. G. Goger and J. J. Weiter (1995) In vivo measurement of lipofuscin in Stargardt's disease—Fundus flavimaculatus. *Invest. Ophthalmol. Vis. Sci.* **36**, 2327–2331.

30. Chowdhury, P. K., M. Halder, P. K. Choudhury, G. A. Kraus, M. J. Desai, D. W. Armstrong, T. A. Casey, M. A. Rasmussen and J. W. Petrich (2004) Generation of fluorescent adducts of malondialdehyde and amino acids: Toward an understanding of lipofuscin. *Photochem. Photobiol.* **79**, 21–25.
31. Glickman, R. D. (2001) The origin of photo-oxidative stress in the aging eye. In *Progress in Brain Research*, Vol. 131 (Edited by K. H. H. Ripps and S. Wu), pp. 699–712. Elsevier, Amsterdam.
32. Haralampus-Grynaviski, N. M., L. E. Lamb, J. D. Simon, J. R. Krogmeier, R. C. Dunn, A. Pawlak, M. Rózanowska, T. Sarna and J. M. Burke (2001) Probing the spatial dependence of the emission spectrum of single human retinal lipofuscin granules using near-field scanning optical microscopy. *Photochem. Photobiol.* **74**, 364–368.
33. Billinton, N. and A. W. Knight (2001) Seeing the wood through the trees: A review of techniques for distinguishing green fluorescent protein from endogenous auto fluorescence. *Anal. Biochem.* **291**, 175–197.
34. Gaillard, E. R., L. Zheng, J. C. Merriam and J. Dillon (2000) Age-related changes in the absorption characteristics of the primate lens. *Invest. Ophthalmol. Vis. Sci.* **41**, 1454–1459.
35. Ervin, L. A., J. Dillon and E. R. Gaillard (2001) Photochemically modified α-crystallin: A model system for aging in the primate lens. *Photochem. Photobiol.* **73**, 685–691.
36. Dillon, J., L. Zheng, J. C. Merriam and E. R. Gaillard (2004) Transmission of light to the aging human retina: Possible implications for age related macular degeneration. *Exp. Eye Res.* **79**, 753–759.
37. Roberts, J. E., S. J. Atherton, E. R. Gaillard and J. Dillon (1995) Age-related changes in the human lens as monitored by detection of porphyrin excited states. *Photochem. Photobiol.* **62**, 339–341.
38. Ettershank, G., I. Macdonnell and R. Croft (1983) The accumulation of age pigment by the fleshfly, *Sarcophaga bullata* Parker (Diptera: Sarcophagidae). *Aust. J. Zool.* **31**, 131–138.