**Purpose.** To image and quantify the spatial distribution of fundus autofluorescence in normal subjects, to determine its age dependence, and to document the deviation from normal in patients with age-related macular disease.

**Methods.** Using a confocal laser scanning ophthalmoscope (cLSO), the intensity and spatial distribution of fundus autofluorescence was studied in 33 normal subjects, 97 eyes with drusen only, and 111 eyes with visual loss caused by age-related macular disease.

**Results.** Fundus autofluorescence intensity in normal subjects was highest at the posterior pole and dipped at the fovea. Autofluorescence increased with age at the posterior pole. Fundus in eyes with age-related maculopathy showed localized high autofluorescence that did not correspond with drusen. Linear pigmentation at the level of the retinal pigment epithelium (RPE), whether detached or flat, fluoresced brightly, whereas plaques of melanin did not. Areas of low and high levels of autofluorescence were seen in lesions containing choroidal new vessels. In areas of geographic atrophy, autofluorescence was low.

**Conclusions.** The spatial distribution of background fundus autofluorescence and the correlation of autofluorescence with age in normal subjects imply that autofluorescence is derived from lipofuscin at the level of the RPE. Focal accumulation of autofluorescent material occurs at the level of the RPE in patients with drusen, but the drusen do not show marked increases in autofluorescence. It is likely that melanolipofuscin accounts for the high levels of autofluorescence, corresponding to linear pigmentation at the level of the RPE. Low-intensity autofluorescence occurs in the presence of retinal photoreceptor loss, and variable levels over disciform lesions probably relate to variations in metabolic activity of the RPE.

Visual loss in age-related macular disease results from either subretinal neovascularization, detachment of the retinal pigment epithelium (RPE) from Bruch’s membrane, or geographic atrophy. It is thought that these changes occur in response to the accumulation of debris in Bruch’s membrane, and there is good evidence that this material is derived from the RPE. Age-related change in the RPE is indicated by the presence of increasing quantities of residual bodies rich in lipofuscin. Thus, it is likely that accumulation of lipofuscin in the RPE plays a role in the pathogenesis of age-related macular degeneration. However, the relationship between RPE lipofuscin content, as indicated by autofluorescence, and the dynamics of the changes in Bruch’s membrane with age has not been elucidated. Until recently, information concerning lipofuscin content of the RPE has been based largely on in vitro observations, which do not allow the recording of change with time or the correlation of autofluorescence with other funduscopic features. In vivo recordings of fundus autofluorescence show good evidence that the autofluorescence is derived from lipofuscin in the RPE. This conclusion is based on the distribution and the spectral characteristics of the autofluorescence, and its accumulation with age, which corresponds with the information derived from in vitro studies. In addition, the variation of intensity in disease is in accordance with that found by microscopy. It is hoped that knowledge of lipofuscin accumulation may give important clues to the
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FIGURE 1. A 10-year-old boy (A), a 35-year-old woman (B), and 78-year-old man (C) with normal fundi and visual acuity of 20/20 showing increasing fundus autofluorescence intensity with age. Autofluorescence imaging showed diffuse fundus autofluorescence with decreased intensity at the fovea and with blood vessels and optic disc shown as dark structures.

SUBJECTS AND METHODS

This study was conducted on 208 eyes of 104 patients with age-related macular disease. In 97 eyes, there were drusen only (23 eyes with hard drusen and 74 eyes with soft drusen), 36 eyes had active subretinal neovascularization, 21 eyes had retinal pigment epithelial detachments, 18 eyes had geographic atrophy, and 36 eyes had a disciform scar. The age range of the patients with age-related macular disease was 61 to 86 years (mean age, 72 years; 58 females, 46 males). All patients had clear lenses or minimal nuclear sclerosis as determined by slit lamp examination. The normal population was comprised of 33 white subjects between 6 and 78 years of age (17 females, 16 males) with no detectable fundus disease. All those in the comparison group had clear lenses. To localize the origin of autofluorescence, we imaged and measured the fundus autofluorescence in five patients with stage 4 macular hole and central neurosensory retinal defect.

Autofluorescence was recorded using published techniques. The pupil was dilated with cyclopentolate 10% (Mydriate; Boehringer, Berks, UK) and phenylephrine 2.5% (Moorfields Eye Hospital NHS Trust, London, UK) before imaging, and all pupils were larger than 6 mm (at which pupil size had a negligible effect). The manufacturer's standard confocal aperture sizes were used. Reflectance images of the distribution of autofluorescence at the posterior pole were produced using a 40° field-of-view mode and the confocal aperture 3, which provided a depth of resolution of less than 200 μm. The ametropic corrector was used to correct for refractive error. After focusing on the structure of interest, a series of images was recorded. The barrier filter was moved into place to capture the fundus autofluorescence.

FIGURE 2. Fundus autofluorescence of normal subjects measured at the fovea (A) and at the site of maximum intensity, at 7° to 15° of eccentricity (B).
FIGURE 3. Fundus photograph (A) and fluorescein angiogram (B) of a 53-year-old man with stage 4 macular hole (A). The central neurosensory defect corresponds with an area of increased autofluorescence, demonstrating that the autofluorescence detected by the confocal scanning laser ophthalmoscope lies posteriorly to the neuroretina and luteal pigment.

in front of the detector in the cLSO, and a series of images was recorded again. Focusing on the retinal surface and on knowledge of the axial resolution of the cLSO ensured that the autofluorescence recorded after moving the barrier filter in front of the detector was derived from the ocular fundus.

The cLSO was a prototype SM 30-4024 donated by Zeiss (Oberkochen, Germany). Argon laser light (488 nm, 250 μW) was used for illumination; to record autofluorescence, a wide band-pass filter was inserted in front of the detector with a short wavelength cut off at 521 nm extending beyond 650 nm for the longer wavelengths. The cLSO images were recorded at standard video scanning rates on SVHS video tape and digitized at 256 × 256 resolution using a Wild Vision V-10 frame grabber (Wild Vision, Tyne and Wear, UK) with an Acorn Archimedes computer (Acorn Computers, Cambridge, UK). Each pixel was rectangular to preserve the aspect ratio of the display of the image, and the size of each pixel was calculated to be 35 μm wide × 26 μm high on the retina. An averaging procedure was used for all eyes as described previously. A reference frame was chosen from the sequence, and each of the other images of the sequence was flickered between this reference frame and the frame to be aligned. If there was displacement of the image to the eye movement, this was evident by apparent motion between the images. The computer mouse was used to move each image relative to the reference frame to eliminate or minimize apparent motion and to register the entire sequence.

The system was calibrated using cuvettes with standard sodium fluorescein solutions of varying concentrations, and it was found to be approximately linear. Autofluorescence intensity was measured in gray scale units from 0 to 255. The dark signal was found to be approximately 60 gray scale units, and it ranged between 60.9 and 61.86 on the normal optic nerve head. In all measurements, the noise (the intensity measured over the optic nerve head) was subtracted from the average intensity of the pixel box over the area of interest, thereby denoting a dynamic range of 0 to 194.

In normals, background measurements were made in the temporal retina at the site of maximum intensity.
FIGURE 5. Reflectance image (A), fluorescein angiogram (B), and autofluorescence image (C) of a 68-year-old man with soft, scattered, subconfluent and confluent drusen, pigment clumping retinal pigment epithelial hypopigmentation, and a small, central area of geographic atrophy. Visual acuity was 20/30. Focal changes show that the highest autofluorescence intensity corresponded to dark areas on fluorescein angiogram and were present in the areas with pigment clumping, adjacent to old-looking, resolving, and mineralized drusen areas; that in the areas of confluent drusen and retinal pigment epithelial pallor, there was focal, mild increased autofluorescence that did not correspond to the drusen pattern; that there were no focal areas of increased autofluorescence in the scattered drusen areas; and that the area of geographic atrophy showed decreased autofluorescence (C).

FIGURE 6. Fundus photograph (A) of a 65-year-old man with large, longstanding, confluent drusen, retinal pigment epithelial hypopigmentation, and pigment clumping. Visual acuity in the right eye was 20/30. Autofluorescence imaging (B) showed focal areas of increased autofluorescence corresponding to the pattern of pigment clumping and retinal pigment epithelial hypopigmentation and no marked change in autofluorescence intensity over drusen.

intensity between 7° and 15° from the fovea and at the fovea. The background autofluorescence in diseased eyes was measured in an area in which there was no abnormal appearance on ophthalmoscopy and no focal changes of fundus autofluorescence. We chose to use a small box of 8 × 8 pixels, corresponding to a retinal area of 208 × 280 μm, so that small areas of variation of fluorescence could be detected. Because in the normal comparison group fundus autofluorescence shows no focal variation greater than 5 U, focal decreased and increased autofluorescence was graded as mild (10 to 20 U different from background), medium (20 to 30 U different from background), and intense (>30 U different from background).

Autofluorescence images of patients with age-related macular degeneration were compared with reflectance images, fundus photographs, fluorescein angiograms, and autofluorescence images of the normal age-matched comparison group. The research followed the tenets of Declaration of Helsinki, and it was approved by the Hospital Ethics Committee. Informed consent was obtained from subjects involved in the study.

RESULTS

Normal Population

Normal fundi showed a consistent pattern in subjects older than 6 years of age; and diffuse autofluorescence was most intense between 5° and 15° from the fovea without focal variations > 5 U in an 8 × 8 pixel box. The optic disc and retinal blood vessels were shown
FIGURE 7. Reflectance image (A), fluorescein angiogram (B), and autofluorescence images (C,D) of a 71-year-old woman with retinal pigment epithelial detachment reducing visual acuity in her left eye to 20/120 (A). Focusing on the surface of the pigment epithelial detachment on autofluorescence imaging (C), a high autofluorescent area that corresponded to the pattern of blocked fluorescence in the angiogram (B), became evident. Focusing closer to the area of attached retina (D), an area of low autofluorescence that contained focal highly autofluorescent features became evident inferiorly; the two spots of maximal fluorescence intensity corresponded to the dark spots on the angiogram, and the low autofluorescent area corresponded to an atrophic retinal pigment epithelium.

as dark structures (Fig. 1). This pattern of distribution of fundus autofluorescence was consistent regardless of age.

Autofluorescence intensity as a function of age was plotted to scale at two fundus locations: at the fovea, and temporal to the fovea at the site of maximal autofluorescence intensity (Fig. 2). The recorded autofluorescence intensity increased significantly with age at both sites \( R^2 = 0.811, P < 0.001, \) and \( R^2 = 0.798, P < 0.001 \). The ratio of foveal autofluorescence to maximal autofluorescence intensity was 0.378, which did not vary with age \( R^2 = 0.0287, P = 0.846 \).

The high autofluorescence recorded in the patients with stage 4 macular hole associated with neurosensory retinal defect demonstrates increased fluorescence at the site of the hole and decreased fluorescence at the site of detachment, demonstrating that the signal was derived from posterior to the neurosensory retina (Fig. 5).

Age-Related Macular Disease. The intensity of autofluorescence over hard and soft drusen was within or below the range of the background autofluorescence in that eye and when compared with agematched normal controls (Fig. 4). Background autofluorescence was no greater in those eyes with visible drusen than in fundi without drusen. A striking finding was the consistent presence of localized high autofluorescence that did not correspond with drusen but that often appeared to be derived from areas of retinal pigment epithelial pallor (Fig. 5). Pigment figures at the level of the retinal pigment epithelium, whether detached or flat, that were hypofluorescent on fluorescein angiography showed intense autofluorescence (Figs. 5 to 7).

Retinal pigment epithelial detachments older than 6 months showed a mild, diffuse, increased autofluorescence corresponding exactly with the detached area (Fig. 7). The mild, increased autofluorescence persisted for more than 2 months after the detachment flattened (for example, after successful laser treatment).

In patients with choroidal neovascularization,
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FIGURE 8. Reflectance image (A) and autofluorescence image (B) of a 67-year-old man with a retinal pigment epithelial detachment and occult neovascularisation in the right eye, which reduced visual acuity to 20/100. Autofluorescence imaging shows diffuse, mild, increased autofluorescence at the detached area but not at the site of the drusen. Autofluorescence was irregular; there were regions with more and less than background levels of fluorescence (Figs. 8 to 10). High autofluorescence often was seen extending beyond the edge of the lesion. Autofluorescence intensity was less than background over disciform scars than over the whole lesion.

In subjects with areas of geographic atrophy, the absence of autofluorescence corresponded well with atrophy, but autofluorescence was present in the adjacent regions in which geographic atrophy was not evident (Figs. 5, 11). Reduced autofluorescence was seen in areas of crystalline drusen. There was often a band of increased autofluorescence around the edges of geographic atrophy.

DISCUSSION

Normal Population

Several lines of evidence indicate that the image is derived from lipofuscin at the level of the retinal pigment epithelium. The confocal nature of the optics of the cLSO ensures that the autofluorescence recorded is derived from the ocular fundus provided that the focus is on the retinal surface. That the source of autofluorescence is located posterior to the neurosensory retina is supported by the distribution of autofluorescence at the site of macular holes, which could be accounted for by the lack of neurosensory retina bearing luteal pigment, an observation also made by others. The distribution of fundus autofluorescence imaged with the cLSO is consistent with our knowledge of lipofuscin distribution derived from histologic studies; the autofluorescence intensity is highest at the macula, dips at the fovea, and decreases toward the periphery. Decreased autofluorescence intensity at the fovea is probably caused by lower lipofuscin content and absorption of short wavelength light by melanin and luteal pigment. The blue-green excitation light at 488 nm and a short wavelength cut-off filter at 521 nm are appropriate for detecting autofluorescence from lipofuscin. Delori showed that the spectral characteristics of in vivo fundus autofluorescence are consistent with those of lipofuscin.

FIGURE 9. Fundus photograph (A) and fluorescein angiogram (B) of a 69-year-old man with a disciform scar, which reduced visual acuity to 3/200. Autofluorescence imaging (C) shows low autofluorescence in the central lesion and focal high autofluorescence toward the periphery of the lesion. The focal autofluorescent area of maximal intensity corresponded to the blocked fluorescence on angiography (arrows).
and he made reference to the identification of individual lipofuscin fluorophores by Eldred.24 He also demonstrated that the excitation spectrum of the orange-red fluorophores extended into the visible range making them accessible for in vivo excitation. The finding that autofluorescence intensity increases with age corresponds with the knowledge derived from histologic studies,3,5,6 as well as with in vivo spectrophotometric measurements at the fovea7 and at 7° temporal to the fovea.12 Using the current technique of in vivo imaging of the fundus autofluorescence with a cLSO, it is evident that the highest level of background autofluorescence did not have a fixed location with respect to anatomic landmarks. Because we measured at a different location eccentrically, we cannot directly compare the fundus autofluorescence measured by us and that measured by others using a fundus spectrophotometer, although the results of the two are consistent. The slightly lower rate of the age-related rise in autofluorescence in our study compared to that found by others12 may have been caused by the different excitation wavelength used.

The dark appearance of the optic disc indicates that the image was not caused by reflected light because this is the most reflectant feature of the fundus. The quantitative relationship to lipofuscin levels would be affected by such factors as loss of melanin in the apical domain of the retinal pigment epithelial cells; this also occurs with age and may contribute to the increased signal in the elderly.

Age-Related Macular Degeneration

As expected, the autofluorescence over drusen did not differ greatly from that of the background unless they had a crystalline appearance. Spectral analysis also demonstrated that the autofluorescence over drusen could not be differentiated from that of the background.25 Delori measured fundus autofluorescence in vivo and showed that the aging Bruch’s membrane exhibits autofluorescence that may account for 10% of the total fundus autofluorescence at 620 nm using an excitation wavelength of 510 nm.26 We cannot rule out that some contribution may be derived from Bruch’s membrane, but it is likely that the bulk of the signal is derived from the RPE. A striking and consistent finding was localized high autofluorescence

FIGURE 10. Fundus photograph (A) and fluorescein angiogram (B) of a 74-year-old woman with a pigmented disciform scar, which reduced visual acuity to 1/60. In the central region with pigmentation, the autofluorescence was low (C). The periphery of the lesion showed focal areas of increased autofluorescence.

FIGURE 11. A 76-year-old woman with scattered drusen with crystals and geographic atrophy, which reduced visual acuity in the right eye to 20/120 (A). The low autofluorescent areas on autofluorescence imaging (B) corresponded to the area of geographic atrophy and calcified drusen.
not derived from visible drusen. In some cases, this corresponded exactly to the pattern of pigmentation at the level of the RPE, and this would be best explained by the pigment being melanolipofuscin. Those areas of increased autofluorescence not visible by ophthalmoscopy may correspond to a group of RPE cells containing higher quantities of lipofuscin than their neighbors.

It is widely thought that debris that accumulates with age in Bruch's membrane is derived from the RPE, which discharges cytoplasm contents into the inner portion of Bruch's membrane to achieve cytoplasmic renewal.\(^{27-29}\) This material is thought to be cleared by the choroid, and that incomplete clearance causes thickening of Bruch's membrane. Thus, a relation between accumulation of debris in the RPE, as shown by autofluorescence, and in Bruch's membrane might be expected, and variation in background and focal autofluorescence may bear some relationship to the outcome of disease. The significance of focal pigment epithelial accumulation of fluorescent material to disease is uncertain; it may be elucidated by longitudinal studies. The observation that the number of photoreceptor cells is reduced in the presence of increased lipofuscin content in the RPE led to the proposal that the increased accumulation of autofluorescent material may occur before cell loss.\(^{3}\) The observation that focal pigmentation indicates a high risk of visual loss in age-related macular disease would support this concept. Our findings imply that this does not occur evenly over the fundus but that it is focal. If high levels of autofluorescence presage geographic atrophy, its focal nature would be in keeping with clinical experience.\(^{17}\) It has been suggested that the high volume of residual bodies may interfere with RPE function, though it could equally well be a consequence rather than a cause of dysfunction.

In more advanced stages of macular degeneration, areas of retinal atrophy showed decreased autofluorescence. This was seen in areas of overt geographic atrophy and in those containing crystalline drusen, which indicate the onset of geographic atrophy. This observation might be expected if the accumulation of fluorescent material in the RPE reflects the level of metabolic activity largely determined by the quantity of photoreceptor outer segment renewal and if the residual bodies had finite half-lives. The first premise is widely thought to be true, and there is evidence to support the second. There appears to be constant degradation of residual bodies in the RPE,\(^{30,31}\) and progressive loss of lipofuscin in the retinal pigment epithelium would be expected if metabolic demand were to be reduced by the demise of photoreceptor cells. Alternatively, it is possible that the RPE discharges material into Bruch's membrane before cell death or metabolic failure, which itself leads to photoreceptor loss. Areas of increased autofluorescence around disciform lesions corresponded to areas of irregular pigmentation and may have been caused by a multilayered RPE, a phenomenon well illustrated by histopathology.\(^{17}\) Phagocytosis of debris derived from exudation from the new vessel complex may contribute to the accumulation of intracellular material. The high levels of autofluorescence in detached RPE are not explained readily.

It is evident that interpretable images of RPE autofluorescence can be obtained in age-related macular disease by which it may be possible to establish the relationship between changes in the RPE and in Bruch's membrane caused by aging. It will take time to determine the potential value of this new information for our understanding of the disease process and its management. Incorporation of this technique in cross-sectional surveys and in longitudinal studies may add greatly to the value of these research efforts.

**Key Words**
age-related macular degeneration, fundus autofluorescence, laser scanning ophthalmoscope, lipofuscin, retinal pigment epithelium

**Acknowledgments**
The authors thank Mr. John Dart for supplying a large part of the data on the normal study population and Dr. Sarah Owens for the data on the population of patients with soft drusen.

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