In Vivo Fluorescence of the Ocular Fundus Exhibits Retinal Pigment Epithelium Lipofuscin Characteristics

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**Purpose.** To characterize the intrinsic fluorescence (autofluorescence) of the human ocular fundus with regard to its excitation and emission spectra, age relationship, retinal location, and topography, and to identify the dominant fluorophore among the fundus layers.

**Methods.** Using a novel fundus spectrophotometer, fluorescence measurements were made at 7° temporal to the fovea and at the fovea in 30 normal subjects and in 3 selected patients. Topographic measurements were made in 3 subjects. Ex vivo measurements of fluorescence of human retinal pigment epithelium (RPE) were obtained and compared to in vivo data.

**Results.** Fundus fluorescence reveals a broad band of emission between 500 and 750 nm, a maximum of approximately 630 nm, and optimal excitation of approximately 510 nm. Exhibiting a significant increase with age, this fluorescence is highest at 7° to 15° from the fovea, shows a well-defined foveal minimum, and decreases toward the periphery. In vivo fluorescence spectra are consistent with those obtained ex vivo on human RPE. Measurements with short wavelength excitation are strongly influenced by ocular media absorption and reveal an additional minor fluorophore in the fovea.

**Conclusions.** Spectral characteristics, correlation with age, topographic distribution, and retinal location between the choriocapillaris and the photoreceptors suggest that the dominant fundus fluorophore is RPE lipofuscin. The minor fluorophore is probably in the neurosensory retina but has not been identified. Invest Ophthalmol Vis Sci. 1995;36:718–729.

Lipofuscin is an autofluorescent pigment that accumulates in the lysosomes of most aging eukaryotic cells. Intensively studied as a biomarker for cellular aging and as a cumulative index of oxidative damage, mounting evidence across the biologic spectrum indicates that lipofuscin may play an important role in cellular aging and disease.

In the human retinal pigment epithelium (RPE), lipofuscin accumulates as a byproduct of phagocytosis of photoreceptor outer segments. In advanced age, lipofuscin is a major constituent of the RPE cell because it may occupy 20% of the free cytoplasmic space of the cell.1 It has been postulated that excessive levels of lipofuscin in the RPE could compromise its essential functions and that lipofuscin contributes to the pathogenesis of age-related macular degeneration (ARMD).1,2–8 The concept that lipofuscin may impair essential RPE functions is supported by the observations that elevated levels of lipofuscin are associated with decreased numbers of photoreceptors in humans.9 The spatial topography, age relationship, and racial distributions of lipofuscin exhibit remarkable similarity to patterns seen in ARMD (summarized by Dorey et al9). Loss of visual function in inherited diseases such as Stargardt’s disease10 and Batten’s disease (ceroid lipofuscinosis)11 also has been attributed to excessive accumulation of lipofuscin-like materials in the RPE. The biologic significance of lipofuscin remains controversial because most of the evidence is circumstantial, having been obtained from dead tissue in which the temporal information is lost. A means for noninvasive measurement of lipofuscin in vivo is essential to resolve these questions.
Although it is routinely excited by ultraviolet light, lipofuscin has a broad range of excitation, from 300 to 500 nm. The ability to excite lipofuscin with visible light made it feasible to elicit this fluorescence in vivo. We have developed a novel fundus spectrophotometer for noninvasive measurement of intrinsic fluorescence of the ocular fundus. Our technique allows for the determination of fluorescence excitation and emission spectra from discrete sites of the fundus. We have shown that although the absolute fundus fluorescence measurement is affected by lens absorption, it is minimally influenced by the strong fluorescence of the crystalline lens. We have also demonstrated that detailed spectral information is attainable with our method, which is essential in identifying the nature of the fluorophores and understanding the influence of other ocular pigments. In this article, we demonstrate that the dominant fluorophore detected noninvasively exhibits the spectra, topographic distribution, age relationship, and retinal location of RPE lipofuscin.

METHODS

Subjects

The study population included 30 subjects between 21 and 67 years of age (6 per decade, 14 women and 16 men). All subjects were white, had no fundus pathology, and had no or minimal nuclear sclerosis. In addition, we measured the fluorescence in three patients with selected pathology to help localize the fundus fluorophores. One patient was a 60-year-old woman with full-thickness, longstanding, macular hole (stage 3, no neurosensory retina) in one eye and a normal fellow eye. The macular hole had a diameter of 400 μm and was surrounded by a distinct whitish cuff of about 600 μm in diameter. The second patient was a 70-year-old woman with ARM and extended atrophy (few remaining RPE cells). The third patient was a 50-year-old woman with Stargardt's disease (in which histologic sections display dramatic increases in lipofuscin-like material in RPE lysosomes). Fluorescein angiography demonstrated the dark choroid sign but no evidence of obvious atrophy. Visual acuity was 20/20 in both eyes.

The tenets of the Declaration of Helsinki were followed, informed consent was obtained for all subjects, and Institutional Human Experimentation Committee approval was granted. The pupil of one eye was dilated to a diameter of at least 6.5 mm with 1% tropicamide.

Fundus Spectrophotometer

Intrinsic fundus fluorescence was characterized using our fundus spectrophotometer. The technique, described in detail elsewhere, allows measurement of fluorescence spectra from discrete sites on the fundus within 35° of the fovea. A simplified diagram of the instrument is presented in Figure 1. Fluorescence excitation was derived from a xenon arc lamp, and interference excitation filters were centered at 430, 450, 470, 490, 510, 530, and 550 nm (full width at half maximum [FWHM] = 20 nm). The excitation field was defined by aperture EA and corresponded to a 3° diameter retinal field. The exposure duration for each excitation was 180 msec, and retinal radiant exposures were between 10 and 17 mJ/cm². The fluorescent and reflected light were collected by aperture DA that defined a 2° (585 μm) diameter sampling field on the retina (centered in the excitation field). Reflected light was rejected by high-pass emission filters, and the fluorescence—after dispersion by a monochromator and amplification by a cooled image intensifier—was spectrally analyzed by an optical multichannel analyzer (OMA, EGG-PAR Instruments, Bedford, MA). Fluorescence was integrated simultaneously in 512 wavelength channels in the spectral range 360 to 900 nm with an effective spectral resolution of 6 nm.

The subject's pupil was aligned under infrared illumination, and the site of interest was selected while the operator viewed the fundus (540 to 620 nm light); the subject's gaze was directed with an internal fixation target (668 nm). Accurate focus was achieved by concentrically aligning images of the excitation and the sampling fields on the subject's fundus. The fluorescence spectrum measurement at each excitation wavelength was followed by a baseline spectrum mea-
for the two measurements at $\lambda = 510$ nm were averaged. For all subjects, the coefficient of variation associated with these two measurements was 2.5% on average at $7^o$ temporal (range, 0.08% to 15%) and 3.2% on average at the fovea (range, 0.05% to 23%).

Excitation spectra were obtained by plotting the fluorescence intensity at $\lambda = 620$ nm as a function of the excitation wavelength $\Lambda$, that is, $\Phi(\lambda, 620)$. Emission maxima were determined by a 50 nm wide parabolic fit around the maximum. The widths of the emission spectra were expressed by the FWHM.

**Ex Vivo Measurements**

In vivo spectra were compared to spectra obtained from flat preparations of human RPE. Four donor eyes (donor ages, 60, 63, 64, and 72 years) were fixed in 4% paraformaldehyde in 0.1 M cacodylate buffer (pH, 7.2 to 7.4) for several hours and stored in phosphate buffer. After removing the anterior segment and vitreous, specimens were punched (7 mm diameter) from a site centered at 8 to 10 mm temporal to the fovea (26° to 33°). The macular tissue was used in other studies. Fluorescence measurements were made from the center of the punched area using all excitation wavelengths. Ex vivo fluorescence was also evaluated for a flat mount of unfixed RPE and choroid from a 65-year-old donor. Fluorescence measurements were made from two sites at approximately 5 mm from the fovea (about 16°). All ex vivo measurements were performed with the same exposure time and filters used in vivo. The preparations were located at the focal plane of a 36.5-mm lens placed in front of the fundus spectrometer. Equation (1) is used with $f_e = 36.5$ mm and $n = 1$.

## RESULTS

### Intrinsic Fluorescence in Individual Subjects

Representative fluorescence spectra measured in two individuals (ages, 23 and 62 years) at $7^o$ temporal and at the fovea are shown in Figure 2. Fundus fluorescence is emitted across a broad band (500 to 800 nm) with a maximum at 620 to 640 nm, and optimal excitation occurs for $\lambda = 510$ nm excitation. The emission spectra at $7^o$ temporal (Fig. 2, top) have approximately the same shape for different excitation $\Lambda$, indicating the predominance of a fluorophore with peak emission at approximately 630 nm. Fluorescence at the fovea is considerably lower than at $7^o$ temporal in both subjects, especially for short excitation wavelengths as demonstrated by the differences in the shape of the excitation spectra at the fovea and at $7^o$ temporal (Fig. 2). The foveal emission spectra obtained with long excitation wavelengths ($\lambda = 510$ and 550 nm) have shapes similar to those seen at $7^o$ temporal. With short wavelength excitations ($\lambda = 430$ and 470 nm) the
In Vivo RPE Lipofuscin Fluorescence

Ags: 23 Age: 62

FIGURE 2. Fluorescence spectra measured at 7° temporal to the fovea (top) and at the fovea (bottom) in two normal subjects, ages 23 (left) and 62 (right) years. Sampling field, 2°. In each panel, the continuous curves are emission spectra for the excitation wavelengths 430, 470, 510, and 550 nm, and solid squares connected by a line is the excitation spectrum for fluorescence emission at 620 nm. The interrupted line (upper left, for $(430,620)$) illustrates how the excitation spectrum is related to the emission spectra. Emission spectra for 450, 490, and 530 nm excitation were omitted for clarity. Spectral distortions caused by the minor fluorophore. In each panel, the continuous curves are emission spectra for the excitation wavelengths 430, 470, 510, and 550 nm, and solid squares connected by a line is the excitation spectrum for fluorescence emission at 620 nm. The interrupted line (upper left, for $(430,620)$) illustrates how the excitation spectrum is related to the emission spectra. Emission spectra for 450, 490, and 530 nm excitation were omitted for clarity. Spectral distortions caused by the minor fluorophore.

shapes are different, particularly in the young subject (Fig. 2, bottom left) in whom the spectra exhibit a broad maximum in the 520 to 580 nm spectral range (double arrows), suggesting that another fluorophore is being excited in that spectral range. Subtle distortions seen on other spectra (single arrows) are also consistent with added fluorescence from a minor fluorophore.

Age Dependence

Figure 3 illustrates the age-related increase of $\Phi(510,620)$ at 7° temporal and at the fovea for all normal subjects in the study. Table 1 gives the average $\Phi(\Lambda,620)$ for different $\Lambda$ and sites, as well as the correlations with age. The increase in fundus fluorescence with age is highly significant at both sites when $\Lambda > 470$ nm. Fluorescence excited at $\Lambda = 470$ nm correlates less strongly with age, and no correlation is observed for $\Lambda = 430$ nm. This results from the effect of ocular media absorption: The age-related increase of fundus fluorescence, clearly seen with $\Lambda > 470$ nm, is canceled out by the age-related decrease in ocular media transmission at $\Lambda = 450$ nm. The short wavelength end of the excitation spectrum steepens substantially as age increases (Fig. 2). This is demonstrated by the very strong negative correlation of the ratio $\Phi(430,620)/\Phi(510,620)$ with age ($r = -0.93, P < 0.0001$).

Emission Maxima and Spectral Widths

Table 2 gives average values for the wavelengths of maximum emission ($\lambda_{\text{max}}$) and the spectral width (FWHM) for different excitation wavelengths $\Lambda$. The emission maximum shifts toward longer wavelengths, and the spectrum narrows as the excitation wavelength increases. The foveal spectra are broader than those at 7° temporal, and their $\lambda_{\text{max}}$ occurs at shorter wavelengths for $\Lambda = 430$ and 470 nm and at slightly longer wavelengths for $\Lambda = 510$ and 550 nm.

Intersubject variability in $\lambda_{\text{max}}$ and in spectral width, and hence in the shape of the spectra, is smallest when the fluorescence is intense ($\Lambda = 510$ nm) and highest when it is low (fovea, $\Lambda = 430$ and 470 nm). The broad emission maxima in the 520- to 580-nm spectral range, seen in Figure 2 (double arrows), are found for approximately one third of the foveal spectra ($\Lambda = 430$ and 470 nm), particularly when the fluorescence is low. Indeed, $\lambda_{\text{max}}$ shifts significantly toward longer wavelengths when $\Phi(\Lambda,620)$ increases (for $\Lambda = 430$ nm: $r = +0.56$, $P = 0.0008$; for $\Lambda = 470$ nm: $r = +0.79$, $P < 0.0001$).

FIGURE 3. Fluorescence at 620 nm for excitation at $\Lambda = 510$ nm, $\Phi(510,620)$, for normal subjects ($n = 30$). (top) At 7° temporal to the fovea. (bottom) At the fovea. Sampling field, 2°.
TABLE 1. Fluorescence at 620 nm Obtained at 7° Temporal to the Fovea and at the Fovea Using Different Excitation Wavelengths (λ)

<table>
<thead>
<tr>
<th>Excitation Wavelength λ (nm)</th>
<th>A = 430</th>
<th>A = 470</th>
<th>A = 510</th>
<th>A = 550</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluorescence at 7° temporal*</td>
<td>85 (21)</td>
<td>241 (24)</td>
<td>315 (33)</td>
<td>223 (41)</td>
</tr>
<tr>
<td>Correlation with age (r)</td>
<td>-0.14</td>
<td>+0.45</td>
<td>+0.79</td>
<td>+0.77</td>
</tr>
<tr>
<td>P = 0.45</td>
<td>P = 0.011</td>
<td>P &lt; 0.0001</td>
<td>P &lt; 0.0001</td>
<td></td>
</tr>
<tr>
<td>Fluorescence at the fovea*</td>
<td>27 (34)</td>
<td>61 (37)</td>
<td>137 (41)</td>
<td>132 (51)</td>
</tr>
<tr>
<td>Correlation with age (r)</td>
<td>-0.08</td>
<td>+0.32</td>
<td>+0.78</td>
<td>+0.80</td>
</tr>
<tr>
<td>P = 0.65</td>
<td>P = 0.08</td>
<td>P &lt; 0.0001</td>
<td>P &lt; 0.0001</td>
<td></td>
</tr>
<tr>
<td>Ratio of foveal to temporal fluorescence</td>
<td>0.32 (28)</td>
<td>0.25 (29)</td>
<td>0.43 (16)</td>
<td>0.59 (18)</td>
</tr>
<tr>
<td>Correlation with age (r)</td>
<td>-0.02</td>
<td>+0.07</td>
<td>+0.34</td>
<td>+0.42</td>
</tr>
<tr>
<td>P = 0.90</td>
<td>P = 0.72</td>
<td>P = 0.066</td>
<td>P = 0.019</td>
<td></td>
</tr>
</tbody>
</table>

* Average fluorescence in (nJ nm⁻¹ sr⁻¹)/J (n = 30 for all averages). All numbers in parentheses are coefficient of variation (in %).

Topographic Distribution

The distribution of fundus fluorescence recorded along a horizontal line through the fovea (Fig. 5) shows a well-delineated minimum in fluorescence at the fovea, maximal fluorescence at 7° to 15° from the fovea, and a decrease in fluorescence toward the periphery. The optic disk fluoresces less intensely than does the surrounding retina, and it is characterized by different excitation and emission spectra, indicating that a different fluorophore is involved.

The depth of the foveal minimum is more pronounced when shorter excitation wavelengths are used: The ratio of foveal fluorescence to temporal fluorescence is on average 0.59 at λ = 550 nm, but it decreases to 0.25 at λ = 470 nm (Table 1). If the excitation is attenuated by absorption of a pigment in the fovea, the difference spectrum Δ(λ) = log Φ(λ,620)₇°temporal - log Φ(λ,620) should reflect the absorption spectrum of that pigment. The difference spectra (Fig. 6) are consistent with macular pigment absorption, indicating that excitation of the foveal fluorescence is attenuated by macular pigment (emission wavelengths are not affected because they occur outside the absorption range of macular pigment). In other words, the fluorophore must be located posterior to the macular pigment.

Bleaching Experiment

If fundus fluorescence originates from layers posterior to the photoreceptors, it should be affected by bleaching of photopigments. We tested this prediction by measuring the fluorescence (λ = 450 nm) at 10° to 15° temporal to the fovea after a period of dark adaptation (15 minutes). Results for one 52-year-old subject (Fig. 7) show that bleaching occurs and that the difference spectrum between the light- and dark-adapted state, Δ(λ) = log Φ(450,λ)₇°temporal - log Φ(450,λ)D, can

TABLE 2. Wavelengths of Maximum Emission λₘₐₓ and Full Width at Half Maximum

<table>
<thead>
<tr>
<th>Excitation Wavelength (nm)</th>
<th>A = 430</th>
<th>A = 470</th>
<th>A = 510</th>
<th>A = 550</th>
</tr>
</thead>
<tbody>
<tr>
<td>λₘₐₓ (7° temporal)</td>
<td>620 ± 8</td>
<td>621 ± 6*</td>
<td>631 ± 4†</td>
<td>641 ± 6†</td>
</tr>
<tr>
<td>λₘₐₓ (fovea)</td>
<td>596 ± 29†</td>
<td>603 ± 25*†</td>
<td>634 ± 6§</td>
<td>649 ± 8§</td>
</tr>
<tr>
<td>FWHM (7° temporal)</td>
<td>209 ± 12</td>
<td>183 ± 6†</td>
<td>167 ± 4†</td>
<td>—</td>
</tr>
<tr>
<td>FWHM (fovea)</td>
<td>217 ± 15§</td>
<td>210 ± 15§</td>
<td>182 ± 11§</td>
<td>—</td>
</tr>
</tbody>
</table>

Average ± SD in nm (n = 30, for all averages).
Comparisons by one-tailed paired t-tests: *greater than value on the left (P < 0.05); †greater than value on the left (P < 0.0001); §smaller than value above (P < 0.061); ¶higher than value above (P < 0.001).
FWHM = full width at half maximum.
In Vivo RPE Lipoicuacin Fluorescence

**FIGURE 4.** Estimated emission spectra of the minor fluorophore for excitation at \( \lambda = 430 \) and 470 nm. (A) Younger normal subject in Figure 2. Difference spectra obtained by subtracting the scaled temporal spectra (\( \lambda = 430 \) and 470 nm) from the corresponding foveal spectra (left scale). (B) Patient with unilocular macular hole (Fig. 9). Difference spectra obtained by subtracting the scaled spectra from the macular hole (\( \lambda = 450 \) and 470 nm) from the corresponding foveal spectra of the fellow eye (right scale). In both patients, we assumed that the minor fluorophore did not substantially affect the long wavelengths (675 to 750 nm). The scaling was obtained by ensuring that the average of each difference spectrum between 675 and 750 nm was zero.

Be fitted to absorption spectrum of rhodopsin. Because the difference spectrum is the result of a double pass through rhodopsin (once at \( \lambda \) and once at \( \lambda' \)), one would expect the long wavelength plateau of \( A(700) \) to correspond with the effect of bleaching at the excitation wavelength (\( \lambda = 450 \) nm). However, as shown in Figure 7, only part of \( A(700) \) can be accounted for by bleaching at 450 nm, indicating that another minor bleaching process is occurring. This experiment was repeated in two other subjects with similar results. For the three subjects (ages, 52, 42, and 32 years), the single-pass rhodopsin densities (at 500 nm) were 0.047 ± 0.002 (SD), 0.070 ± 0.003, and 0.047 ± 0.002 DU, respectively. The magnitude of the unexplained bleaching component \( x \) (Fig. 7) was 0.024 ± 0.002, 0.010 ± 0.003, and 0.023 ± 0.002 DU, respectively (\( r^2 \) if the linear fit were 0.83, 0.79, and 0.84, respectively).

**Ex Vivo Measurements**

Figure 8 presents the comparison of emission spectra (\( \lambda = 430, 470, \) and 510 nm) and excitation spectra obtained ex vivo from flat-mounted RPE of donor eyes to those from our in vivo measurements in the 10 oldest subjects of this study. The data for the four fixed retinas, for the unfixed retina, and for the 10 subjects were normalized and averaged. The spectral shape obtained for the unfixed sample (not shown in Fig. 8) did not differ substantially from that of the
fixed tissue. The average fluorescence $\Phi(510,620)$ for the four fixed retinas, the unfixed tissue (two sites), and the 10 oldest subjects was $286 \pm 43$ (SD), $292 \pm 92$, and $408 \pm 73$ (nJ·nm$^{-1}$·sr$^{-1}$)/J, respectively. The wavelength of maximal emission of the ex vivo spectra increases with $\Delta$ from $588 \pm 7$ nm at $\Delta = 430$ nm to $610 \pm 4$ nm at $\Delta = 510$ nm, similar to that observed in vivo, though at somewhat longer wavelengths (Table 2). The normalized ex vivo emission spectra are shifted toward shorter wavelengths (by approximately 10 to 25 nm) compared to the in vivo measured spectra. The normalized ex vivo excitation spectra are also shifted by approximately 50 to 50 nm toward shorter wavelengths compared to the in vivo spectra.

Selected Clinical Cases

Table 3 gives the fluorescence $\Phi(510,620)$ at 7° temporal and at the fovea for the selected clinical cases (subject ages, 50 to 70 years). For comparison, the average fluorescence and wavelengths are given for the 10 oldest normal subjects (mean age, 61 years). For the patient with macular degeneration, the fluorescence in atrophic areas was significantly lower than normal ($P < 0.0001$). The spectra had some signature of the dominant fluorophore but were very noisy and somewhat distorted. In the patient with Stargardt's disease, the fluorescence was significantly higher ($P < 0.0001$) than normal, and the spectra had essentially the same shape as those obtained in normal subjects.

A comparison of the normalized fluorescence spectra in the fovea in the two eyes of a patient with a unilocular macular hole is presented in Figure 9. Four distinct observations can be made: (1) For each...

![Figure 7](image-url)

**Figure 7.** Effect of bleaching on the fluorescence at 10° to 15° temporal to the fovea (2° sampling field, $\Lambda = 450$ nm). (top) Dark-adapted (DA, first measurement) and light-adapted (LA) emission spectra in a 52-year-old subject. Curves a and b show the variation of fluorescence at 620 and 520 nm, respectively, for the successive measurements performed after dark adaptation (vertical markers). (bottom) Difference spectrum $A(\lambda) = \log \Phi(450, \lambda)_{LA} - \log \Phi(450, \lambda)_{DA}$. The smooth line is the linear fit (between 495 and 700 nm) of the rhodopsin spectrum to the experimental data: Single-pass rhodopsin density at 500 nm was 0.047 DU ($r^2 = 0.83$). The long wavelength plateau $A(700)$ can be decomposed into a component $r$ due to rhodopsin bleaching at 450 nm, and a component $x$ due to an additional minor bleaching process.

![Figure 8](image-url)

**Figure 8.** Comparison of ex vivo and in vivo fundus fluorescence. Normalized emission spectra obtained from donor eyes (solid continuous lines) and from in vivo measurements (interrupted lines) at excitation wavelengths $\Lambda = 430, 470,$ and $510$ nm (top to bottom). The ex vivo data are the average from four fixed donor eyes (donor ages, 60 to 72 years, thick line), and the in vivo data are the average from the 10 oldest subjects (mean age, 61 years, open circles). The open squares are the estimated spectra after correction for ocular media absorption (see Discussion). Error bars correspond to standard deviations of the normalized spectra. The bottom panel also contains the normalized excitation spectra $\Phi(\Lambda, 620)$ corresponding to the same ex vivo and in vivo data (with identical symbols).
TABLE 3. Fluorescence $(510,620)$ at 7° Temporal to the Fovea and at the Fovea of Special Clinical Cases and Normal Control

<table>
<thead>
<tr>
<th></th>
<th>Fluorescence* at 7° Temporal</th>
<th>Fluorescence* at the Fovea</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal eyes (10 oldest subjects)†</td>
<td>408 ± 73</td>
<td>187 ± 49</td>
</tr>
<tr>
<td>Eye with atrophy</td>
<td>72</td>
<td>48</td>
</tr>
<tr>
<td>Eye with Stargardt's disease (OS)</td>
<td>746</td>
<td>295</td>
</tr>
<tr>
<td>Eye with Stargardt’s disease (OD)</td>
<td>629</td>
<td>442</td>
</tr>
<tr>
<td>Eye with macular hole (OS)</td>
<td>337</td>
<td>232</td>
</tr>
<tr>
<td>Normal fellow eye (OD)</td>
<td>339</td>
<td>128</td>
</tr>
</tbody>
</table>

* Fluorescence in $(\text{fJ nm}^{-1} \text{sr}^{-1})/\text{J}.$ †Average ± SD.

A, the shape of the emission spectrum measured in the macular hole closely resembled that measured at 7° temporal, indicating that the dominant fluorophore detected at 7° temporal was the same as that detected when the neurosensory retina was absent. (2) The fluorescence $\Phi(510,620)$ was higher in the hole than at the fovea of the fellow eye (Table 3) because its excitation was not reduced by macular pigment absorption. This is confirmed by the fact that the excitation spectrum (Fig 9, bottom panel) at the macular hole was not distorted by the macular pigment absorption signature. (3) When excited at short wavelengths, the foveal emission spectrum of the normal fellow eye was broader and shifted toward shorter wavelengths. This is consistent with the presence of a minor short-wavelength fluorophore in the fovea of the fellow eye. Its emission spectra (for $\lambda = 430$ and 470 nm) can be estimated by spectral subtraction of the foveal spectra of both eyes. The derived emission spectra of the minor fluorophore (Fig. 4, spectra B) had shapes similar to those obtained in the normal eye (spectra A), and they revealed a maximum emission at 520 to 540 nm. (4) The fact that the minor fluorophore was not seen in the macular hole indicated that it may have been located in the neurosensory retina.

DISCUSSION

Our results demonstrate that intrinsic fundus fluorescence results from at least two fluorophores—a dominant fluorophore with peak emission at 620 to 630 nm (optimal excitation at 510 nm) and a minor fluorophore with peak emission at 520 to 540 nm (optimal excitation at 430 to 470 nm). The minor fluorophore was best seen when the fluorescence of the dominant one was low, suggesting that these are indeed two fluorophores.
dominant fundus fluorophore are consistent with those of lipofuscin as measured in the RPE of human donor eyes. Ex vivo lipofuscin studies used measurements of fluorescence emitted from the RPE in section and counts of the number of lipofuscin granules observed by electron microscopy.

The topographic distribution of fundus fluorescence (Fig. 5) is consistent with that of lipofuscin obtained in donor eyes. It is highest at the posterior pole, there is a localized minimum at the fovea (of approximately 83%), and it decreases toward the periphery. Because the foveal measurements are affected by macular pigment and by the high local concentration of RPE melanin, it is important to consider whether the decrease in foveal fluorescence is real or is a consequence of the measurement geometry. Macular pigment clearly contributes to the local minimum when excitation wavelengths within the absorption range of the macular pigment are used (Fig. 6, Table 1). However, the foveal minimum cannot be attributed entirely to absorption by macular pigment because foveal fluorescence excited at A = 550 nm, outside the macular pigment absorption range, still averages 59% of that at 7° temporal (Table 1). Similarly, RPE melanin absorption could lower the foveal fluorescence because it is denser at the fovea. Using our previous data on RPE melanin densities, and assuming that all melanin is located in the apical RPE cell, we estimate that melanin would lower the foveal fluorescence to 83% of the parafovea on average, not enough to account for the observed average decrease of 59%. Thus, even when the effects of macular pigment and RPE melanin are estimated, the foveal fluorescence remains low, which is consistent with the ex vivo observations.

Localization Within the Fundus Layers. Reflectance spectra of the fundus always reveal various degrees of the signature of absorption by choroidal blood because light penetrates into the choroidal layers. The fundus fluorescence emission spectra (Fig. 2) are continuous curves without a detectable signature of absorption by blood, that is, no sudden increase in fluorescence emission is observed above 580 nm, at which absorption by blood decreases rapidly. In contrast, the fluorescence spectra from the optic disk reveal a distinct blood absorption signature because the fluorescence emanates from within the capillary-filled disk tissue. Thus, the absence of the spectral signature of blood indicates that the fluorophore is located anterior to the choriocapillaris.

In contrast, the foveal fluorescence excitation spectra are affected by the spectral signature of the macular pigment (Fig. 6). Our macular pigment density estimates (0.42 and 0.51 DU) are consistent with densities obtained from psychophysical methods. Because the macular pigment is located in the superficial layers of the fovea, this locates the fluorophore between the inner retina and the choriocapillaris.

Similarly, the fluorescence is affected by bleaching of rhodopsin. The difference spectra at approximately 500 nm are very noisy because the fundus fluorescence is low at these wavelengths. The rhodopsin densities (at 500 nm) at 10° to 15° temporal from the fovea calculated from our spectral fits (Fig. 7) in three subjects were between 0.045 and 0.075 DU (single pass). This is consistent with double-pass densities obtained by rod densitometry at 15° to 20° (maximum density of rods), that is, 0.15 to 0.20 DU (double pass). Thus, the fluorescence of the dominant fluorophore is fully attenuated by the rods, and the fluorophore is therefore located posterior to the photoreceptor layer. The nature of the additional bleaching process detected in our experiment is not known and will require additional investigation to identify.

Finally, the evidence of the macular hole measurement parallels the other findings (Fig. 9, Table 3). Histopathologic studies have shown lack of neurosensory retinal tissues in patients with full-thickness macular holes, whereas the RPE complex remains intact. Partial atrophy of the photoreceptors at the margin of the hole is present in most cases. Our sampling field (385 μm diameter) included an area of exposed RPE (400 μm diameter, ~50% of sampling area) and a somewhat atrophic cuff of retina (600 μm diameter). If the dominant fluorophore were located primarily in the neurosensory retina, removal of at least half of the retina (fluoresphore and macular pigment) would cause a strong decrease in fluorescence. Instead, we see an increase compared to the fellow eye (Table 3) because removal of macular pigment in the hole has exposed a fluorophore located posterior to the neurosensory retina.

Fluorescence Spectra. Comparison of the fluorescence spectra obtained in vivo with those from flat preparations of human RPE demonstrate good correspondence in the shape of the spectra but show a shift of the in vivo spectra toward longer wavelengths. This shift, in part, can be accounted for by increases in the ocular media transmission with wavelength throughout the visible spectrum. We corrected the in vivo spectra to account for media absorption using the algorithm of Pokorny. The corrected spectra (square in Fig. 8) show a better correspondence with those obtained ex vivo. However, ocular media absorption only partially accounts for the difference in the excitation spectra, probably because of the difficulties in accurately correcting for media absorption at the shortest excitation wavelengths.

Most published emission spectra have focused on chloroform methanol extracts of lipofuscin excited with ultraviolet light. These spectra demonstrate peak emission at approximately 570 to 590 nm, which is consistent with spectral measurements of emission...
from individual lipofuscin granules in paraffin sections of RPE. As seen in Figure 10, the emission spectra obtained with 430 nm excitation in vivo exhibit good agreement with previous measurements of lipofuscin. However, when excited at 510 nm, the in vivo emission spectrum displays a marked shift toward longer wavelengths with maximum emission at ~630 nm. It is now known that lipofuscin is a mixture of at least 10 fluorophores, all of which are primarily excited in the ultraviolet range. However, three of those lipofuscin fluorophores ("orange-red" emitters) have excitation spectra that extend substantially above 400 nm and dominant emission at 620 to 630 nm. The emission spectrum we obtained in vivo with 510 nm excitation indicates that this fluorescence may be predominantly derived from the group of orange-red fluorophores described by Eldred.

**Minor Fluorophores**

The minor fluorophore (peak emission at 520 to 540 nm, Fig. 4) only substantially affects the fundus fluorescence if the fluorescence of lipofuscin is low, that is, at the fovea, at \( \lambda < 510 \) nm, and for younger subjects. Its fluorescence then affects, to various degrees, the short wavelength side of the emission spectra and may cause—for very low lipofuscin—a broad maximum between 520 and 580 nm. Although it appears to be highest at the fovea, its presence may not be restricted to the fovea because traces of its signature may be seen in young subjects at 7° temporal with \( \lambda = 430 \) nm (Fig. 2, top left). The fluorescence of the minor fluorophore does not appear to increase with age, as does the lipofuscin fluorescence, and its contribution appears negligible for \( \lambda > 470 \) nm.

If the minor fluorophore is in the neurosensory retina, as suggested by the macular hole evidence, it could be FAD (flavin adenine dinucleotide, a component of the respiratory enzyme chain), the highest concentration of which is found in the mitochondria of the photoreceptor's inner segment. The fluorescence characteristics of FAD (excitation peak, \( \lambda \approx 460 \) nm; emission peak, \( \lambda \approx 520 \) nm) are consistent with our observations. In attempting to demonstrate FAD fluorescence, Teich used an excitation at 457 nm and reported changes in fundus fluorescence (500 to 700 nm) in one monkey with varying breathing conditions. However, no spectral identification of the FAD spectral signature was provided, and the work was not pursued. At this point, however, we cannot entirely exclude the possibility that the minor fluorophore is in the RPE in normal eyes but is degraded when the cells no longer phagocytose outer segments (i.e., in a macular hole). Definite identification and more precise localization of the minor fluorophore awaits further studies and detailed analyses by spectral deconvolution.

Our fluorescence measurement would not be significantly affected by either vitamin \( \text{A} \) or by other components of the respiratory enzyme chain (e.g., NADH and NAD) because these fluorophores are optimally excited in the 300 to 360 nm range with blue light emission. Melanin has been reported to fluoresce, but its emission must be faint because melanin appears as dark granules in fluorescence microscopy observation of RPE. Although fluorescence of photopigment has been reported from intermediates of bleaching, demonstration of these transient states requires low temperatures or time-resolved measurements. It is unlikely that they contribute to the fundus fluorescence reported here. Finally, Bruch's membrane has a blue-green fluorescence in fluorescence microscopy observation of RPE under ultraviolet excitations, consistent with emission spectra of collagen and elastin. Further studies are needed to assess the influence of Bruch's membrane fluorescence on our measurements, especially in older subjects.

**CONCLUSIONS**

Spectral characteristics, correlation with age, topographic distribution, retinal location between the
choriocapillaris and the photoreceptors, and results from selected clinical cases provide strong evidence that the fundus fluorescence is primarily from lipofuscin and that it can be reliably measured noninvasively. Ongoing studies will further evaluate the relationship between lipofuscin and aging and will determine whether onset or progression of ARMD is significantly related to lipofuscin.

Key Words

lipofuscin, intrinsic fluorescence, autofluorescence, excitation spectrum, emission spectrum, retinal pigment epithelium, age-related macular degeneration, macular hole, Stargardt’s disease

Acknowledgments

The authors thank Dr. Stephen Burns for his encouragement and advice and Dr. Frank Hole (RKU, Heidelberg, Germany) for sharing donor eyes obtained through the eye-banks of Moorfields Eye Hospital (London, England).

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