Macular pigment density measured by autofluorescence spectrometry: comparison with reflectometry and heterochromatic flicker photometry

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We present a technique for estimating the density of the human macular pigment noninvasively that takes advantage of the autofluorescence of lipofuscin, which is normally present in the human retinal pigment epithelium. By measuring the intensity of fluorescence at 710 nm, where macular pigment has essentially zero absorption, and stimulating the fluorescence with two wavelengths, one well absorbed by macular pigment and the other minimally absorbed by macular pigment, we can make accurate single-pass measurements of the macular pigment density. We used the technique to measure macular pigment density in a group of 159 subjects with normal retinal status ranging in age between 15 and 80 years. Average macular pigment density was 0.48 ± 0.16 density unit (D.U.) for a 2°-diameter test field. We show that these estimates are highly correlated with reflectometric (mean: 0.23 ± 0.07 D.U.) and psychophysical (mean: 0.37 ± 0.26 D.U.; obtained by heterochromatic flicker photometry) estimates of macular pigment in the same subjects, despite the fact that systematic differences in the estimated density exist between techniques. Repeat measurements over both short- and long-time intervals indicate that the autofluorescence technique is reproducible: The mean absolute difference between estimates was less than 0.05 D.U., superior to the reproducibility obtained by reflectometry and flicker photometry. To understand the systematic differences between density estimates obtained from the different methods, we analyzed the underlying assumptions of each technique. Specifically, we looked at the effect of self-screening by visual pigment, the effect of changes in optical property of the deeper retinal layers, including the role of retinal pigmented epithelium melanin, and the role of secondary fluorophores and reflectors in the anterior layers of the retina.

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1. INTRODUCTION

Macular pigment (MP) is a blue-absorbing pigment that is especially dense in the axons of the cone photoreceptors at the center of the macula (Fig. 1).[1–4] It is composed of a mixture of two carotenoids, lutein and zeaxanthin, that have similar absorption spectra in the 400–540-nm spectral range, and this mixture has a maximum absorption at approximately 460 nm.[3–7] The role of the MP (Ref. 1) has been thought of as a spectrally selective filter that reduces chromatic aberrations by attenuating the short wavelengths and as a blue-light absorber and/or antioxidant to protect the retina from photo-oxidative damage by short-wavelength radiation. In recent years, there has been growing evidence that carotenoids in the diet (such as in spinach and corn) and/or in the blood plasma are associated with a lower risk for age-related macular degeneration,[1,8,9] an ocular disease that affects vision in older people. Recent studies have also shown that MP can be increased by a carotenoid-rich diet[10] or by lutein and/or zeaxanthin supplementation.[11] This opens the possibility of supplementing individuals with low MP in order to reduce the purported risk of degeneration of the retina. Monitoring the supplementation requires robust methods to estimate the MP density. These methods must be reliable, reproducible, and readily applied to aging populations that are often afflicted by poor fixation,

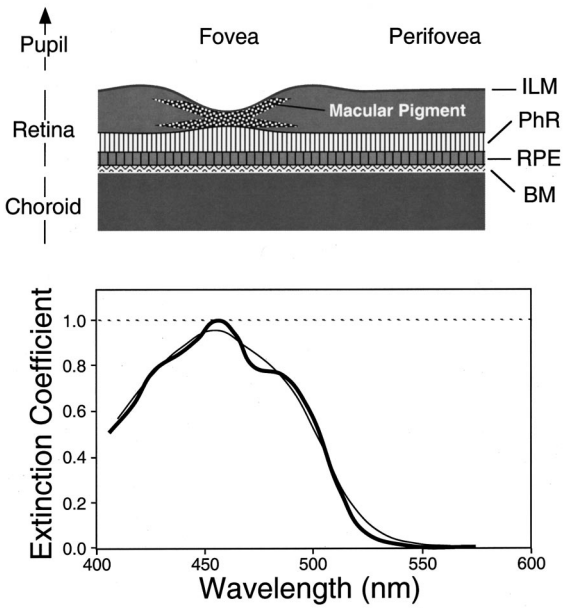

Fig. 1. (top) Schematic diagram (top) representing a section through the retina at the fovea, where the outward displacement of retinal cells causes a thinning of the retina. The symbols are PhR, photoreceptors; ILM, inner limiting membrane of the retina; RPE, retinal pigmented epithelium (containing both the fluorophore lipofuscin and the absorber melanin), and BM, Bruch's membrane. The choroid is rich in blood and provides nutrients for the photoreceptors. The blue-absorbing macular pigment (MP) is located in the cone axons at the center of the fovea. (bottom) Absorption spectrum of MP (thick curve) measured by Brown[6] and normalized to its peak absorption at 455 nm. The thin curve is the same spectrum averaged over the excitation spectral band by using Eq. (10).

diminished physical skills, absorbing and scattering ocular media, and possibly by early degeneration of the macula.

The optical density of MP can be measured *in vivo* by both psychophysical and optical methods. The most commonly used psychophysical method is heterochromatic flicker photometry (HFP),[12–19] in which the subject performs flicker matches at both the fovea and at a peripheral site using two wavelengths of light. Optical methods are dominated by reflectometry techniques in which the reflectance spectrum of the retina at the fovea is compared with the reflectance spectrum at the perifovea.[20–24] Imaging reflectometry has also produced estimates of the spatial distribution of the MP density.[24–27] Another optical method, based on Raman spectroscopy of the MP, has been shown to provide estimates of MP in excised eyes[28] and has recently been adapted to *in vivo* measurements.[29] Unlike other techniques to measure MP, the Raman method requires a correction to account for ocular media absorption.

In this paper, we present a noninvasive, optical method for measuring the optical density of the MP. This method was initially introduced by us in 1993.[30] The method utilizes the autofluorescence (AF) of lipofuscin, which is located in the retinal pigmented epithelium (RPE) cells of the retina (Fig. 1). This fluorescence is emitted in the 520–800-nm spectral range, and it can be excited *in vivo* between 400 and 570 nm.[31,32] The method is based on the observation that the excitation light of foveal RPE fluorescence is attenuated by MP and that the absorbance spectrum, *in vivo*, is closely related to the absorption spectrum of MP.[32] Thus, by using two excitation wavelengths that are differentially absorbed by the MP and detecting the fluorescence outside the absorption range of the MP, one is able to determine the single-pass optical density of the MP. MP densities obtained by this AF method in subjects with normal retinal status and a large age range were compared with MP densities determined in the same subjects by reflectometry and, in a subset of subjects, with MP densities estimated psychophysically by using HFP.

2. PRINCIPLES

A. Autofluorescence Method

Typical fluorescence spectra acquired at the fovea and at 7° temporal to the fovea in a 56-year-old subject are shown in Fig. 2. The excitation spectrum at the fovea has a shape different from that measured at the perifoveal site: MP absorption causes an increase in the attenuation of light reaching the RPE in the 450–510-nm range as compared with that at 550 nm, where MP does not absorb. The logarithm of the ratio of perifoveal to foveal excitation spectra is closely related to the absorption spectrum of MP (Fig. 2). Similar difference spectra

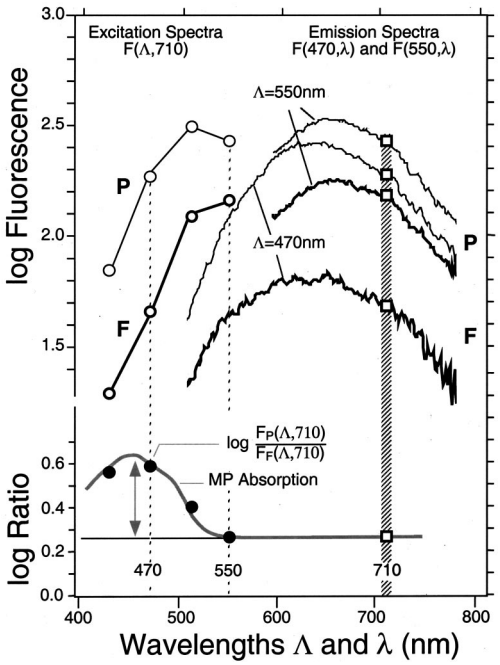

Fig. 2. (top) Fluorescence excitation (measured at 710 nm) and emission spectra (for 470- and 550-nm excitation) measured at the fovea (F) and at 7° temporal to the fovea (P) in a 56-year-old male subject. Fluorescence was measured with a circular test area of 585 μm (2°) in diameter. Fluorescence is expressed in nJ nm^{-1} sr^{-1}/J. Excitation spectra represent the fluorescence intensity at 710 nm as a function of the excitation wavelength (430, 470, 510, and 550 nm); to avoid confusion, only the emission spectra for 470- and 550-nm excitation are shown. (bottom) Log-ratio spectrum of perifoveal to foveal fluorescence (solid circles) and fitted MP spectrum (gray curve). The vertical arrow represents the term in the square brackets in Eq. (5), and this quantity is proportional to the MP density.

from two subjects, with the use of seven excitation wavelengths, were presented in a previous paper.[32]

Let $F_F(\Lambda, \lambda)$ and $F_P(\Lambda, \lambda)$ be the AF (Λ is the excitation wavelength, and λ is the emission wavelength), measured at the fovea and at the perifovea (7° temporal to the fovea), respectively, let D_F and D_P be the optical density of the MP at both sites, and let $F_F^*(\Lambda, \lambda)$ and $F_P^*(\Lambda, \lambda)$ be the fluorescence of all layers located posteriorly to the MP. Assuming that *all* the detected fluorescence has been affected by MP absorption (no fluorophores anterior to the MP), we can express the foveal and the perifoveal fluorescence as

$$F_F(\Lambda, \lambda) = F_F^*(\Lambda, \lambda)10^{-D_F(\Lambda)-D_F(\lambda)}, \quad (1)$$

$$F_P(\Lambda, \lambda) = F_P^*(\Lambda, \lambda)10^{-D_P(\Lambda)-D_P(\lambda)}. \quad (2)$$

Combining Eqs. (1) and (2), we obtain the "log-ratio" equation:

$$\log\frac{F_P(\Lambda, \lambda)}{F_F(\Lambda, \lambda)} = \log\frac{F_P^*(\Lambda, \lambda)}{F_F^*(\Lambda, \lambda)} + [D_F(\Lambda) - D_P(\Lambda)] + [D_F(\lambda) - D_P(\lambda)]. \quad (3)$$

We replace the density differences ($D_F - D_P$) by $\mathbf{D}_{AF}$, the unknown MP density difference (at Λ or λ) between the fovea and the perifovea. Applying Beer's law, we can express $\mathbf{D}_{AF}(\Lambda)$ as a function of the density difference at 460 nm and of the corresponding extinction coefficients; we used $\mathbf{D}_{AF}(\Lambda) = \mathbf{D}_{AF}(460)K_{mp}(\Lambda)$, where $K_{mp}(\Lambda)$ is the extinction coefficient of MP relative to that at 460 nm. Substituting in Eq. (3), we obtain

$$\log\frac{F_P(\Lambda, \lambda)}{F_F(\Lambda, \lambda)} = \log\frac{F_P^*(\Lambda, \lambda)}{F_F^*(\Lambda, \lambda)} + \mathbf{D}_{AF}(460) \times [K_{mp}(\Lambda) + K_{mp}(\lambda)]. \quad (4)$$

Equation (4) is visualized as a spectrum at the bottom of Fig. 2. We further assume that the ratio $F_P^*(\Lambda, \lambda)/F_F^*(\Lambda, \lambda)$ is constant over the range of excitation wavelengths employed. This implies that the fluorophore at the fovea is the same as that at the perifovea (although the concentration of that fluorophore might be different)[33] and that foveal–perifoveal differences in absorption by other pigments located between the MP and the fluorophore (retinal blood, visual pigments, RPE melanin) are negligible. We use Eq. (4) for two excitation wavelengths Λ_1 and Λ_2 in the high- and low-absorption ranges of MP, respectively, and measure the fluorescence at the same emission wavelength λ. Subtraction of the two equations eliminates $F_P^*(\Lambda, \lambda)/F_F^*(\Lambda, \lambda)$ and all terms containing the emission wavelength λ. After rearrangement we obtain

$$\mathbf{D}_{AF}(460) = \frac{1}{K_{mp}(\Lambda_1) - K_{mp}(\Lambda_2)} \times \left[\log\frac{F_P(\Lambda_1, \lambda)}{F_F(\Lambda_1, \lambda)} - \log\frac{F_P(\Lambda_2, \lambda)}{F_F(\Lambda_2, \lambda)}\right]. \quad (5)$$

The density $\mathbf{D}_{AF}(460)$ is the peak optical density difference between the fovea and the perifoveal reference site. The expression in the square brackets in Eq. (5) is visualized at the bottom of Fig. 2 (arrow). It should be stressed that selecting the common emission wavelength λ outside the MP absorption range is not a necessary condition for this technique: In contrast to differential absorption at Λ, differential absorption at λ by MP or by any other absorber does not influence the measurement.

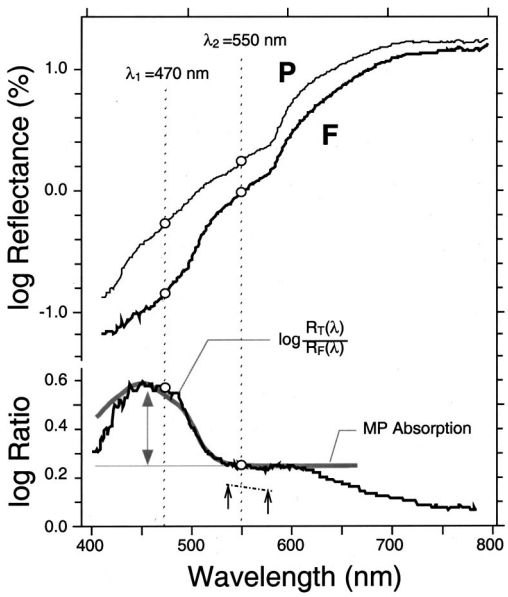

Fig. 3. (top) Reflectance spectra measured at the fovea (F) and at 7° temporal to the fovea (P) in the same subject as that in Fig. 1. Reflectance was measured in a circular test area of 585 μm (2°) in diameter. (bottom) Log-ratio spectrum of the perifoveal to foveal reflectance (black curve) and fitted MP spectrum (gray curve); the gray arrows correspond to the term in the square brackets in Eq. (9), which is a quantity proportional to the MP density. The two black arrows near 550 nm highlight the decrease of the log ratio with wavelength, which is associated with differential melanin absorption between the fovea and the peripheral site (Subsection 5.D).

B. Reflectance Method

Typical reflectance spectra acquired at the fovea and at 7° temporal to the fovea of a normal subject are shown in Fig. 3. The reflectance spectrum at the fovea is attenuated at all wavelengths compared with that at the perifovea, particularly in the absorption range of the MP (400–550 nm).

The derivation of MP density for the reflectance method, originally proposed by Brindley and Willmer,[20] is similar to that of the fluorescence method described in Subsection 2.A. Let $R_F(\lambda)$ and $R_P(\lambda)$ be the reflectances (with wavelength λ) at the fovea and at the perifovea, respectively, let $D_F(\lambda)$ and $D_P(\lambda)$ be the optical densities of the MP, and let $R_F^*(\lambda)$ and $R_P^*(\lambda)$ be the reflectance of all layers located posteriorly to the MP. Assuming that *all* the reflected light that is detected has been attenuated by MP (no reflectors/scatterers anterior to the MP), we can express the foveal and perifoveal equivalent reflectances[34] as

$$R_F(\lambda) = R_F^*(\lambda)10^{-2D_F(\lambda)}, \quad (6)$$

$$R_P(\lambda) = R_P^*(\lambda)10^{-2D_P(\lambda)}. \quad (7)$$

The factor 2 conforms to an assumed double pass of the probing light through the MP [corresponds with $D(\Lambda) + D(\lambda)$ in Eqs. (1) and (2)]. Performing substitutions similar to those in the derivation of Eq. (4), one obtains

$$\log\frac{R_P(\lambda)}{R_F(\lambda)} = \log\frac{R_P^*(\lambda)}{R_F^*(\lambda)} + 2\mathbf{D}_{\mathrm{RE}}(460)K_{\mathrm{mp}}(\lambda), \qquad (8)$$

where $\mathbf{D}_{\mathrm{RE}}$ is the MP density difference between the fovea and the perifovea. Equation (8) is visualized as a spectrum at the bottom of Fig. 3. We assume that the ratio $R_P^*(\lambda)/R_F^*(\lambda)$ is constant over the range of wavelengths employed, or that the reflectance spectrum of the deeper layer at the fovea is proportional to that at the perifovea. This implies that foveal–perifoveal differences in absorption by other pigments (retinal and choroidal blood, visual pigments, RPE, and choroidal melanin) do not cause large differences in the shape of the log-reflectance spectra at both sites. We then apply Eq. (8) to two wavelengths λ_1 and λ_2 in the high- and low-absorption ranges of MP, respectively. The density difference $\mathbf{D}_{\mathrm{RE}}(\Lambda)$ between the fovea and the perifovea is then

$$\mathbf{D}_{\mathrm{RE}}(460) = \frac{0.5}{K_{\mathrm{mp}}(\lambda_1) - K_{\mathrm{mp}}(\lambda_2)} \times \left[\log\frac{R_P(\lambda_1)}{R_F(\lambda_1)} - \log\frac{R_P(\lambda_2)}{R_F(\lambda_2)}\right]. \qquad (9)$$

Equation (9) is similar to Equation (5). The expression in the square brackets in Eq. (5) is visualized at the bottom of Fig. 2 (arrow).

3. METHODS

A. Subjects

The study population consisted of 159 subjects, 16 to 80 years of age (mean age: 52 ± 17 years), with normal retinal status and no systemic disease other than hypertension (Table 1). All subjects were Americans of European origin, except for four African-Americans and two Asians. All subjects had best-corrected visual acuity of 0.6 or better, refraction between −6 and +4 diopters, and no ocular pathology (28 older subjects had hard drusen, discrete thickenings in Bruch's membrane, which are nonpathologic aging changes). Table 1 details the number, the age, and the gender of subjects that participated in studies on interocular comparisons, reproducibility, long-term repeat testing, and comparison with the psychophysical method.

Table 1. Population

Substudies	*n*	Mean Age ±SD.	Age Range	Females/ Males
Normal subjects	159	52 ± 17	15–79	86/73
Interocular comparison	20	59 ± 17	21–78	13/7
Reproducibility (<1 month apart)	9	46 ± 17	21–72	1/8
Retesting (12–24 months apart)	22	57 ± 16	22–78	13/9
Comparison with psychophysics	30[a]	59 ± 15	29–78	16/14

[a] Includes six subjects not included in the main group because of missing information ($n = 3$) or with minor ocular pathology ($n = 3$).

The tenets of the Declaration of Helsinki were followed, Institutional Review Board approval was granted, and informed consent was obtained for all subjects. The pupil of the test eye was dilated with 1% Tropicamide to at least 7 mm in diameter. All eyes had a complete retinal examination by an ophthalmologist. Subjects with a clinical nuclear sclerosis score > 2+ (based on slit lamp examination of the lens) or with ocular media density at 510 nm higher than 0.375 density unit (D.U.) (Ref. 35) were not included in the study.

B. Fluorometry and Reflectometry

Data were acquired by fundus spectrophotometry[31] according to protocols and analyses routinely utilized in our studies of lipofuscin. Excitation light, derived from a xenon arc lamp with excitation filters centered at 430, 470, 510, and 550 nm [full width at half-maximum (FWHM): 20 nm], irradiated a 3°-diameter retinal field during 180 ms (radiant exposures: 10–17 mJ/cm^2). The fluorescent light and the reflected light were collected from a 2°- (585-μm-) diameter sampling field (centered in the excitation field). After filtering, the fluorescence was spectrally analyzed by an optical multichannel analyzer (EGG-PAR Instruments, Bedford, Mass.) with a spectral resolution of 6 nm. For reflectance spectra measurements, the excitation and blocking filters were removed, and a 2-D.U. neutral-density filter was inserted in the excitation channel. All light levels used for excitation, illumination, and focusing were within the safety limits recommended by the American National Standards Institute standards.[31]

The subject's pupil was aligned under infrared illumination, and the fundus was observed with 540–620-nm light (0.12 mW/cm^2 at the retina for approximately 2–3 min). An internal fixation target (668 nm) was used to direct the subject's fixation either at the center of the sampling area or at a site 7° temporal to the fovea (the perifovea). Accurate focus was achieved by concentrically aligning the retinal images of the excitation and sampling fields (550 nm; 2 mW/cm^2 for 3–5 s).[31] For all subjects, we measured the emission spectra at each site for excitation wavelengths of 430, 470, 510, and 550 nm, as well as a reflectance spectrum. Each fluorescence and reflectance measurement was followed by acquisition of a baseline spectrum, which was subtracted from the original spectrum; this baseline was obtained by displacing the excitation spot on the retina (by ≈3.5°) without altering the incident beam in the pupil. Since the detection area was unchanged, this baseline spectrum measures contributions of lens fluorescence and scatter, stray fluorescence in the instrument, and dark/leakage current from the detector.[31] Acquisition of the four spectra needed for the measurement of MP is accomplished in approximately 5 min, including alignment and focusing.

The illumination light [5.6 log photopic trolands (td) for 2–3 min] and the focusing light (6.8 log photopic td for 3–5 s) ensure that >99% of the cone pigments are bleached before the first excitation is used. For rods, respective illuminances of 4.7 and 6.1 log scotopic td for the same durations cause 59%–68% of the rhodopsin to be

bleached before data collection commences. This estimate was confirmed experimentally.[36] Additional exposures by the excitation lights will further bleach the pigments. We shall see that the influence of this unbleached rhodopsin causes only a minor error in the MP estimates (Subsection 5.F).

All spectra were corrected for the excitation energies, for the spectral sensitivity of the detecting system, and for the spectral distribution of the incident light (reflectance).[31] Furthermore, the data were individually corrected to account for absorption by the ocular media.[35] This correction is part of our standard analysis protocol and is not strictly needed for the MP density determination [it does not affect the ratios in Eqs. (5) and (9)]. We also measured the sampling efficiency of our system over the sampling aperture: It was uniform in the central 1° but dropped off slightly toward the edge of the field.[37]

C. Computation of Macular Pigment Densities D_{AF} and D_{RE}

For the MP density measured by the AF method (D_{AF}), we primarily used data obtained at the fovea and at the perifovea with the excitation wavelengths $\Lambda_1 = 470$ nm (high MP absorption) and $\Lambda_2 = 550$ nm (low MP absorption). The fluorescence was measured at $\lambda = 710$ nm (spectral band: 20 nm) to minimize errors associated with the presence of secondary fluorophores (see Subsection 5.G). Results of the four fluorescence measurements $F(\Lambda, 710)$ were substituted in Eq. (5) together with the effective extinction coefficients $K_{mp}(\Lambda)$ of MP. For the latter, we used the MP absorption spectrum $K(\Lambda)$ measured by Brown[6] on unfixed retinal tissue from a rhesus monkey (Fig. 1). We averaged these data over the spectral distribution $E(\Lambda)$ of the excitation light to obtain the effective extinction coefficient $K_{mp}(\Lambda)$:

$$K_{mp}(\Lambda) = \frac{1}{D(460)} \log \frac{\sum E(\Lambda) S(\Lambda) 10^{-D(460)K(\Lambda)} \Delta\Lambda}{\sum E(\Lambda) S(\Lambda) \Delta\Lambda}, \tag{10}$$

where $S(\Lambda)$ is the sensitivity of the light detection system, which is the excitation spectrum in the case of the AF method. We calculated the effective extinction coefficients for a MP density $D(460) = 0.4$ D.U. (mean single-pass MP density) and found that $K_{mp}(470) = 0.852$ and $K_{mp}(550) = 0.014$. The conversion factor $1/[K_{mp}(470) - K_{mp}(550)]$ in Eq. (5) was then 1.193.[38] This scales the MP density as if it were measured by a single wavelength at the peak absorption.

For MP density measured by the RE method (D_{RE}), we used the reflectance at 470 and 550 nm for the same sites. The extinction coefficients $K_{mp}(\lambda)$ were calculated by using Eq. (10) for $D(460) = 0.8$ D.U. (double pass) and with the 16-nm-wide spectral band $E(\lambda)$ of our detection system. We found that $K_{mp}(470) = 0.850$ and $K_{mp}(550) = 0.006$, and the conversion factor $0.5/[K_{mp}(470) - K_{mp}(550)]$ in Eq. (9) was then 0.593.

D. Psychophysical Measurement D_{HFP}

MP density was determined by comparing the sensitivity at the fovea with that at the perifovea with use of the technique of heterochromatic flicker photometry (HFP).[14,18,39] The test field (0.8° diameter) was presented near the center of a 460-nm, 10°-diameter, 2.2-log-photopic-td, steady background. The test wavelengths were 460 and 550 nm (FWHM: 10 nm). The 460- and 550-nm fields were superposed and presented in square-wave alternation at a temporal frequency of 12–15 Hz. The subject's task was to adjust the intensity of the 460-nm test light to minimize the flicker, thus defining the point of equal luminance for the two wavelengths. This task was performed at the fovea while subjects fixated the center of the test field and at a perifoveal site at 5.5° in the temporal retina. Assuming that all the detected light has been attenuated by the MP, we can then equate, for each site, the luminance match as

$$E_F(460) 10^{-D_F(460)} S_F(460) = E_F(550) 10^{-D_F(550)} S_F(550), \tag{11}$$

$$E_P(460) 10^{-D_P(460)} S_P(460) = E_P(550) 10^{-D_P(550)} S_P(550), \tag{12}$$

where E_F and E_P are the radiant powers needed to obtain minimal flicker at each site and S_F and S_P are the photoreceptor sensitivities at the fovea and at the perifovea, respectively. Since $E_F(550) = E_P(550)$, one obtains the following from Eqs. (11) and (12):

$$\mathbf{D}_{HFP}(460) - \mathbf{D}_{HFP}(550) = -\log \frac{E_P(460)}{E_F(460)} + \left[\log \frac{S_P(550)}{S_P(460)} - \log \frac{S_F(550)}{S_F(460)} \right], \tag{13}$$

where $\mathbf{D}_{HFP}$ represents (as above) the density difference between the fovea and the perifovea. If the sensitivity spectrum $S(\lambda)$ at the fovea is proportional to that at the perifovea, then the term in the square brackets equals 0. This assumption implies that self-screening is negligible (see Subsection 5.C), that selective chromatic adaptation and flicker are sufficient to suppress the influence of the short-wavelength cones,[12] and that the proportion of middle- and long-wavelength cones is the same in the fovea and in the perifovea. Furthermore, interference of the rod system is minimal because rods are largely saturated (2.8 log scotopic td)[5] and because flickering biases the response away from the rod system. The density measured by HFP is then, after introduction of the extinction coefficients, given by

$$\mathbf{D}_{HFP}(460) = \frac{-1}{K_{mp}(460) - K_{mp}(550)} \log \frac{E_P(460)}{E_F(460)}. \tag{14}$$

The density is scaled to represent the density that would be measured by a single wavelength at peak absorption. The extinction coefficients, averaged over a 10-nm-wide spectral band by using Eq. (10), were $K(460) = 0.973$ and $K(550) = 0.011$, and the conversion factor $1/[K_{mp}(460)\text{-}K_{mp}(550)]$ was then 1.039.

4. RESULTS

A. Macular Pigment Density Determined by the Autofluorescence Method

MP densities determined by the AF method for 159 normal subjects show a large individual variability (Fig. 4), as has been found in other studies.[10,12–26] The average density D_{AF} was 0.48 ± 0.16 D.U. [±standard deviation (SD)] and the coefficient of variation was 34% (Table 2). Older subjects (ages: 65–80 years) in this population had MP densities ≈15% higher than those of young subjects (ages: 15–30 years).

B. Macular Pigment Density Measured by the Reflectance Method

Macular pigment densities D_{RE} determined by the RE method in the same subjects also show a large individual variability (top of Fig. 5), but the average density D_{RE} was 0.23 ± 0.07 D.U., significantly smaller than D_{AF} (Table 2). The coefficient of variation was 29%, or slightly smaller than that of the AF method. The MP densities for older subjects (ages: 65–80 years) were ≈17% higher than that for young subjects (ages: 15–30 years). The mean D_{RE}/D_{AF} ratio was 0.50 ± 0.13, not significantly affected by age ($r = 0.0$, $p > 0.9$). MP density estimates by the AF and RE methods were highly correlated with each other ($r = 0.73$, $p < 0.0001$), confirming earlier results.[30] The linear regression line (bottom of Fig. 5) exhibited a slope of ≈0.3 and a significant positive intercept. It is noted that we measured a higher D_{RE} than D_{AF} for only one of the 159 subjects.

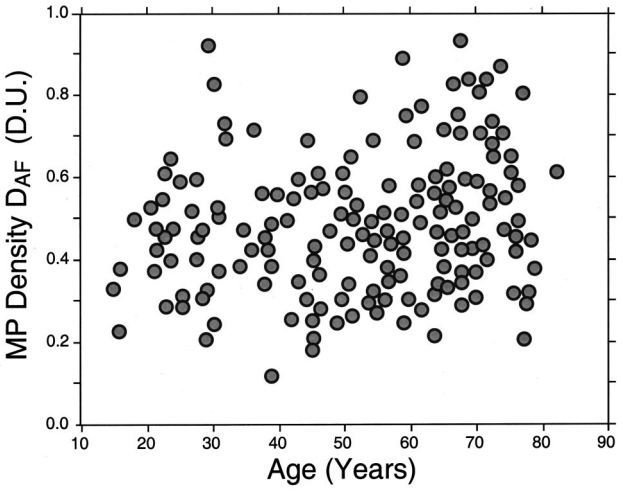

Fig. 4. MP densities determined by the AF method (D_{AF}) for normal subjects as a function of age ($n = 159$). The test field was 2° in diameter.

C. Validation of the Autofluorescence and Reflectance Methods

Validation of the AF and RE methods requires that Eqs. (4) and (8) be valid, that is, the measured log ratios $\log[F_P(\Lambda)/F_F(\Lambda)]$ and $\log[R_P(\lambda)/R_F(\lambda)]$ must be linearly related to the extinction coefficient $K_{mp}(\Lambda)$ of the MP. We fitted the log-ratio data for different wavelengths (Λ or λ) to the known extinction coefficients at these wavelengths. Results for such fits in seven subjects with both the AF and RE methods are presented in Fig. 6, where we plotted the log ratios and the scaled extinction coefficients as a function of wavelength. These fits were all statistically significant ($p < 0.03$), indicating that the log-ratio *in vivo* measurements were well predicted by the MP spectrum (measured *ex vivo*).

Fits of the MP spectrum to the AF and RE log-ratio data of 147 subjects were performed (12 subjects had incomplete data at $\Lambda = 430$ nm). Data at 430 nm were assigned a half-weight because the residuals at 430 nm were large and the data most variable; this can be attributed in part to the low signal available for the 430-nm data (Figs. 2 and 3). The median r^2 values of the fits were 0.97 ($n = 4$, $p = 0.01$) and 0.98 ($n = 6$, $p < 0.0001$) for the AF and RE methods, respectively. Analysis of the residuals revealed small systematic deviations from the MP spectrum characterized by low observed values at 430 nm (≈−0.05 log unit) and high observed values at 470–510 nm (≈0.03 log unit). This could mean that the true MP spectrum is shifted slightly toward longer wavelengths (compared with the spectrum that we used) or

Table 2. Macular Pigment Density by the Autofluorescence and Reflectance Methods

	Autofluorescence Method	Reflectance Method	Comparison
Macular Pigment Density ($n = 159$)			
MP density (460 nm) (D.U.)	0.48 ± 0.16[a]	0.23 ± 0.07	$p > 0.0001$
Range (D.U.)	(0.11–0.92)[b]	(0.07–0.44)	
Coefficient of variation (%)	34.1	29.1	$p = 0.05$[c]
Testing on Same Day or within 30 days ($n = 9$)			
MP density (D.U.)	0.45 ± 0.18	0.18 ± 0.07	
Mean of $\lvert D_{MP,1} - D_{MP,2}\rvert$ (D.U.)	0.042 ± 0.019	0.039 ± 0.029	
SD of $(D_{MP,1} - D_{MP,2})$/mean (D.U.)	0.11	0.27	$p = 0.01$[c]
Retesting 10–24 Months Apart ($n = 22$)			
MP (D.U.)	0.46 ± 0.22	0.22 ± 0.07	
Mean of $\lvert D_{MP,1} - D_{MP,2}\rvert$ (D.U.)	0.053 ± 0.048	0.042 ± 0.042	
SD of $(D_{MP,1} - D_{MP,2})$/mean (D.U.)	0.15	0.27	$p = 0.02$

[a] Mean±SD.
[b] Range in parentheses.
[c] *F*-test of equality of variance (two-tailed).

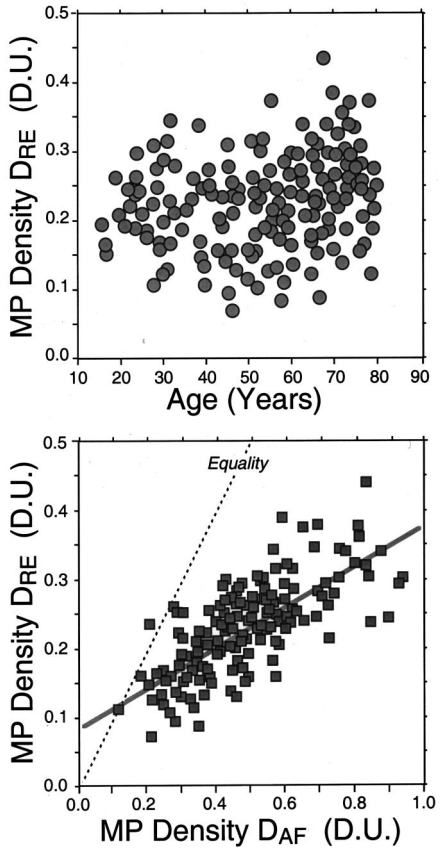

Fig. 5. (top) MP densities determined by the reflectance method (D_{RE}) as a function of age. The subjects are the same as those in Fig. 4. The test field was 2° in diameter. (bottom) D_{RE} as a function of the MP densities determined by the fluorescence method (D_{AF}). The dashed line represents equality of the two estimates, and the solid line is the linear regression $D_{RE} = 0.086 + 0.30D_{AF}$ ($r^2 = 0.54$, $p < 0.0001$). The 95% confidence intervals were 0.06 and 0.11 D.U. for the intercept and 0.25 and 0.34 for the slope.

that a spurious effect causes the observed values to increasingly underestimate the MP spectrum as wavelength decreased. However, we obtained different distributions of residuals when we fitted the data to other published MP spectra.[3–5] We concluded that the spectrum of MP *in situ* is not known with enough certainty to put a lot of weight on the observed deviations from the "MP spectrum." Nevertheless, the overall correspondence of the measured log-ratio spectra and the MP spectrum suggests that MP, within the uncertainties associated with the MP spectrum *in situ*, is in large part responsible for the attenuation of the foveal excitation spectrum and the foveal reflectance spectrum. Both methods accurately sample the MP when wavelengths longer than 450 nm are used.

D. Biologically Significant Characteristics of Macular Pigment Measured by Optical Methods

To test the biological utility of the AF method, we measured two characteristics of MP that have previously been established by HFP—interocular correlation and the effect of cigarette smoking. These two characteristics are appropriate because one comparison (interocular) shows no difference between two groups,[11,14] whereas the other comparison, smoking,[40] shows a large effect. For the interocular comparison, we measured MP densities D_{AF} and D_{RE} in both eyes of 20 normal subjects. Strong interocular correlations in MP were found ($r = 0.91$ and 0.88, $p < 0.0001$). For each method, we found that the slope of the regression line of right eye density versus left eye density was not significantly different from 1 and that the intercept was not significantly different from 0. The mean absolute differences between eyes were 0.055 ± 0.045 and 0.027 ± 0.025 D.U. for D_{AF} and D_{RE}, respectively.

Similarly, we compared age-matched groups of smokers ($n = 27$, mean age: 46 ± 16 years) and nonsmokers ($n = 102$, mean age: 49 ± 15 years); there were no significant differences between groups in regard to gender and ocular pigmentation. Smokers had significantly ($p = 0.01$) lower MP density than nonsmokers: Mean densities D_{AF} were 0.40 ± 0.15 and 0.49 ± 0.16 D.U., respectively. In our population, we did not find group differences between MP density and gender ($p = 0.5$)[41] or between MP density and ocular pigmentation ($p = 0.4$).[42] Density estimates D_{RE} obtained by reflectometry were not significantly affected by any of the above factors (including smoking, $p = 0.3$).

E. Influence of Hard Drusen and Lens Scatter

We investigated the possible spurious effect of changes in fluorescence/reflectance caused by hard drusen on the MP density by comparing MP estimates in a group of 18 nonsmoking subjects with hard drusen (mean age: 69 ± 6 years) with an age-matched group of 39 nonsmoking subjects with no visible drusen (mean age: 68 ± 5 years). The MP densities were 10% higher for the group with hard drusen, but the difference was not significant ($p = 0.2$). A slight shift of the foveal emission spectrum toward shorter wavelengths was detected in subjects with hard drusen: The ratio $F(470, 560)/F(470, 660)$ was higher in these subjects than in those with no visible drusen ($p = 0.02$). No significant difference was found for D_{RE} in the two groups.

Furthermore, to assess the efficacy of our baseline correction procedure (Subsection 3.B), we examined whether the MP estimates were affected by the crystalline lens densities, on the premise that this would reflect the spurious influence of lens scattering. In a group of 57 older subjects (ages: 58–79 years), we found, after accounting for the strong age dependence of lens density, no significant dependence of D_{AF} upon lens density ($p = 0.38$) and of D_{RE} upon lens density ($p = 0.31$). In addition, we computed MP density estimates both accounting for and without accounting for our baseline in a group of young (ages: 15–29 years, $n = 20$) and old (ages: 65–78 years, $n = 25$) subjects. For the AF method, baseline-corrected MP densities were on average $1\% \pm 3\%$ (paired test, $p = 0.2$) and $13\% \pm 16\%$ ($p = 0.01$) higher than the uncorrected values for the young and the old group, respectively. For the RE method, corrected MP densities were on average $5\% \pm 4\%$ ($p < 0.0001$) and $34\% \pm 34\%$ ($p < 0.0001$) higher than the uncorrected values for the young and the old group, respectively. These results sug-

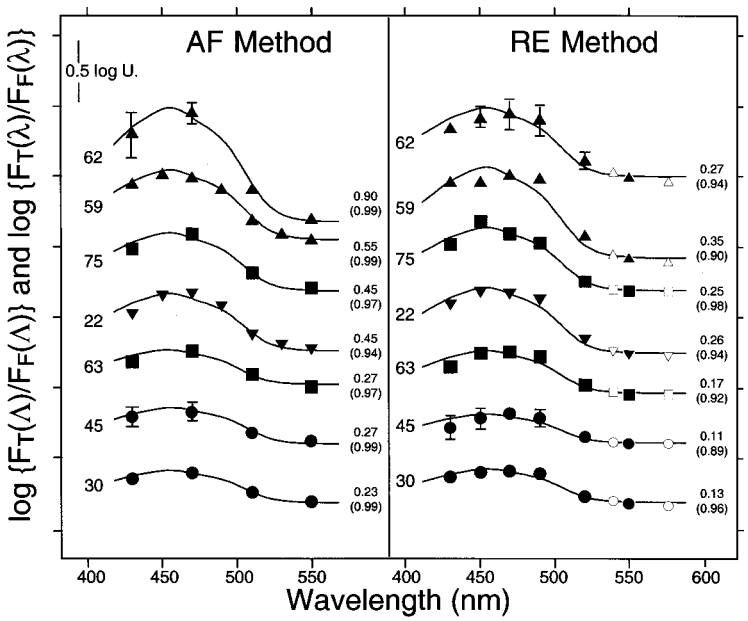

Fig. 6. Variation of the measured log ratio (symbols) and the fitted MP spectra (curves) for both the AF method (left) and the RE method (right). The spectra are for the same seven subjects (ages given to the left of each spectrum). The derived MP densities (D_{AF} and D_{RE} for the AF and RE methods, respectively) are given on the right of each spectrum together with the regression's r^2 (in parentheses). The data for two subjects (ages: 62 and 45 years) were measured twice, and the fits made on the averaged data (error bars: SD). The log ratios for the AF method were measured at 710 nm with Λ = 430, 470, 510, and 550 nm (as for all subjects in this study). Additionally, we used Λ = 450, 490, and 530 nm in two subjects. The log-ratio fits for the RE method were made by using λ = 430, 450, 470, 490, 520, and 550 nm (solid symbols). Two additional wavelengths, λ = 540 and 575 nm, were used in the determination of RPE melanin (open symbols; Subsection 5.D). The three lowest spectra in each panel (ages: 63, 45, and 30 years) correspond to the three subjects for which the MP density estimates by the HFP method were less than 0.05 D.U. (see Fig. 7); the MP spectral signature is seen on the AF and RE log-ratio spectra.

gest that our correction method minimizes the influence of scattering in the lens and ocular media.

F. Comparison of Optical and Psychophysical Methods

MP densities were determined by all three methods in a subset of 30 subjects (Table 1). The diameter of the test field was 0.8° and 2.0° for the psychophysical and optical methods, respectively. The results (Fig. 7) show significant intermethod correlations between both D_{AF} and D_{HFP} ($r = 0.77$, $p < 0.0001$) and between D_{RE} and D_{HFP} ($r = 0.61$, $p = 0.0003$). Mean densities for these 30 subjects were $D_{AF} = 0.48 \pm 0.20$ D.U., $D_{RE} = 0.21 \pm 0.06$ D.U., and $D_{HFP} = 0.37 \pm 0.26$ D.U. The densities D_{AF} were generally larger than those obtained by HFP, particularly at low densities. The densities obtained by reflectometry were generally much lower than HFP estimates, except at low MP densities, where they are higher. Regression analysis showed that zero estimates by flicker photometry ($D_{HFP} = 0$) corresponded with densities of $D_{AF} = 0.26 \pm 0.04$ D.U. ($\pm$standard error) and $D_{RE} = 0.16 \pm 0.02$ D.U., both significantly larger than zero ($p < 0.0001$).

To account for differences in test field diameter, we converted the measured HFP densities to their equivalent values for a 2° test field by using a conversion based on an exponential MP distribution.[43] After conversion the mean MP density was $D_{HFP} = 0.30 \pm 0.22$ D.U. We found no significant change in the intercept (Fig. 7) of the regression line between D_{AF} and the converted D_{HFP} (~0.27 D.U., $p < 0.0001$), and the slope of the regression line increased from ≈0.58 to ≈0.67 (Fig. 7, dashed lines) but was still significantly different from 1.

G. Reproducibility of the Three Techniques

Reproducibility of the optical methods was evaluated by comparing successive MP density estimates measured in nine subjects [five on the same day and four within 30 days (Table 1)]. The absolute difference between the two measurements was on average 0.042 and 0.039 D.U. for the AF and the RE method, respectively (Table 2). To compare reproducibility of methods that produce different average estimates of MP density, we have expressed the absolute test–retest difference as a percentage of the mean density. The mean test–retest differences were then 9% and 22% of the mean density estimates for the AF and the RE method, respectively. Analysis of the differences in the variances confirms that the AF method was significantly more reproducible than the RE method ($p = 0.01$, Table 2). Additionally, we have compared successive measurements in a group of 22 subjects (Table 1) at an interval of 8–24 months. The absolute difference between the two measurements was on average 0.053 and 0.042 D.U. for the AF and the RE method, respectively (Table 2). This corresponds to 11% and 19% of the mean densities, respectively. Again, analysis of variances showed that the AF method was more stable than the RE method ($p = 0.02$). The high concordance between MP densities over long-time intervals attests to the high stability of MP in the retina.[17]

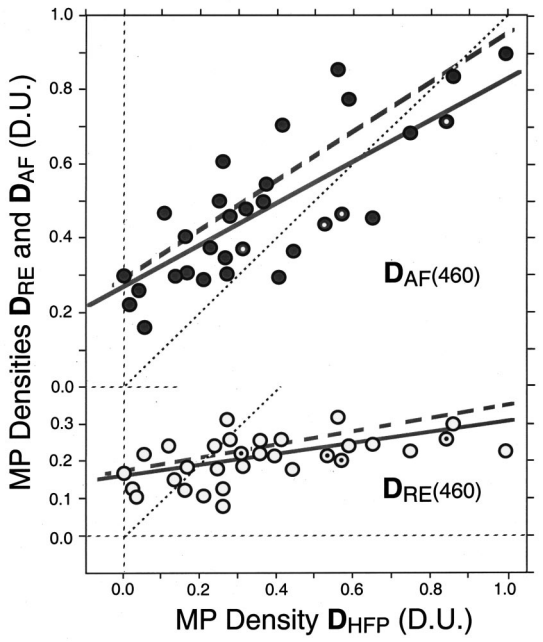

Fig. 7. Comparison of MP densities measured in 30 subjects by the fluorescence method (D_{AF}, top) and the reflectance method (D_{RE}, bottom) with MP densities measured by HFP (D_{HFP}). Test fields were 2.0° and 0.8° in diameter for the optical and psychophysical methods, respectively. Plots were displaced to avoid overlap. Symbols with a central dot correspond to subjects in which contralateral eyes were tested. The black dashed lines at 45° are lines of equal density for both methods, and solid lines are regression lines. The regression line for the AF method was $D_{AF} = 0.26 + 0.58D_{HFP}$ ($r^2 = 0.60$, $p < 0.0001$). The 95% confidence intervals were 0.18 and 0.35 D.U. for the intercept and 0.40 and 0.77 for the slope. The regression line for the RE method was $D_{RE} = 0.16 + 0.15D_{HFP}$ ($r^2 = 0.38$, $p = 0.0003$). The 95% confidence intervals were 0.12 and 0.19 D.U. for the intercept and 0.08 and 0.23 for the slope. The two thick, gray dashed lines are the regression lines after adjusting for differences in test field diameter.

For the comparison with HFP, we referred to published values from several studies: Average absolute differences between paired tests were found to be 0.05 ± 0.03 D.U. (16% mean MP density, ages: 19–42 years),[14] 0.08 ± 0.07 D.U. (23% of mean, 30–65 years),[10] 0.10 ± 0.10 D.U. (33% of mean, 19–22 years),[15] and 0.04 ± 0.04 D.U. (18% of mean, 26 ± 5 years).[19] Some of these studies were performed on trained and experienced subjects; as a result, it is difficult to assess the effect of subject experience on reproducibility. Two very experienced observers obtained reproducibilities of 0.02–0.03 D.U.,[11] but reproducibility data on untrained naive subjects with a fully optimized HFP protocol are lacking, particularly for older subjects.

In summary, the reproducibility of the RE method (19–22%) appeared to be comparable with that of HFP (15–35%), and the reproducibility of the AF method (9–11%) was better than that of the two other methods. However, these comparisons were made on different populations using protocols that may not have been optimal. For example, AF and RE data in this study were acquired for lipofuscin evaluation and not specifically for MP measurements; in most cases, we measured only one spectrum for each of the four conditions needed for MP determination (two wavelengths, two sites). Thus comparison of the reproducibility of the optical and psychophysical methods awaits further test–retest evaluations with optimized protocols for each technique.

5. DISCUSSION

Our method to measure MP noninvasively utilizes the AF of lipofuscin that is normally present in the human retinal pigment epithelium (RPE). By exciting the lipofuscin with two wavelengths, 470 nm (well absorbed by MP) and 550 nm (minimally absorbed by MP), and by detecting the fluorescence outside the MP absorption range (at 710 nm), we can measure the differential absorbance of the excitation lights and thus obtain a single-pass measure of the optical density of the MP. As with all methods to measure MP, we estimate the MP density from a comparison of foveal and perifoveal measurements, minimizing thereby the influence of the spectral characteristics of the underlying tissues, of the ocular media, and of the instrument.

MP densities determined by the AF method are comparable with those measured by psychophysics[12–19] and exhibit the large biological variability associated with MP. MP densities measured by the AF method are highly correlated with those measured by reflectometry (RE method) and by HFP in the same subjects. The reproducibility of the AF method compares quite favorably with those of HFP and the RE method. We have confirmed that there is a high interocular correlation in MP densities[14] and that MP densities are reduced in people who smoke.[40] We did not find in our population significant correlations between MP density and gender[41] or between MP density and ocular pigmentation.[42] In regard to the higher MP densities found in the older subjects of our population, we believe that this is in part due to selection biases within our population. Other studies have reported no change of MP density with age[12] or a decrease with age.[19,25] In one study, however, Hammond and colleagues[16] reported that MP densities in older individuals were higher than in young individuals. Interestingly, most of the older subjects in that study were from the same subject pool from which we recruited in this study.[44] It is probable that the subjects in that pool had better general and ocular health than the overall population.

The AF method is rapid, safe, and easily applied to measurements in individuals of all ages. While pupil dilation and a reasonable fixation (each measurement is done in 0.2 s under visual control by the operator) are needed, the method otherwise requires only minimal participation from the test subject. The current AF method requires four independent measurements (four separate light exposures) compared with two measurements for other techniques. Other methods combine these four measurements in two pairs of measurements either by rapidly changing wavelengths at each site (HFP), by recording the two wavelengths simultaneously (RE method), or by recording both sites together in one image at each wavelength (reflectometry or fluorescence imaging). Our technique could be improved by adopting rapidly alternating excitations in a dedicated instrument.

A. Comparison of Optical and Psychophysical Methods

MP densities obtained by either the AF method (D_{AF}) or the RE method (D_{RE}) correlate highly with MP densities determined by HFP (D_{HFP}). After we accounted for differences in test field diameter, densities D_{AF} were larger by ≈0.23 D.U. than D_{HFP} (Fig. 7), particularly at low densities. Densities D_{RE} were generally much lower than D_{HFP}, except again at low densities. For individual subjects with low MP densities, both optical methods gave a larger MP density estimate than the HFP estimate. For the three subjects in Fig. 7 with the lowest D_{HFP} (0.02 ± 0.02 D.U.), we found mean D_{AF} and D_{RE} to be 0.28 ± 0.04 and 0.13 ± 0.04 D.U., respectively. The three lowest log-ratio spectra of Fig. 6 correspond to these three subjects: Both the AF and RE spectra exhibited the characteristic MP signature. The discrepancy is not caused by RPE melanin, because the log-ratio spectrum would then increase monotonically toward shorter wavelengths (see also Subsection 5.D). Thus flicker photometry appears to underestimate the amount of MP present in the retina for individuals with low MP densities.

In comparing the optical and psychophysical methods, one must also consider that these methods sample the MP spatial distribution differently. As illustrated in Fig. 8, the AF and RE methods measure an average light transmission through the MP over the sampling field, whereas the psychophysical estimates correspond to the density at the edge of the test stimulus.[12,17] We found that for the same test field diameter, AF densities are always higher than HFP densities (Fig. 8). The discrepancy at low densities between the AF and HFP estimates cannot be explained by predictions based on an exponential MP distribution; all techniques tend to measure essentially zero density for very low peak densities. However, if a square spatial distribution is assumed at the center of the fovea, then AF densities could be higher than HFP densities, even with unequal field diameters. A theoretical limit can be envisioned by an infinitely high MP density over a central area just under 1° in diameter. While HFP would measure zero density, the AF method (2°-diameter field) would still measure a density of $-\log(3/4) = 0.125$ D.U., since 1/4 of the sampling area is not contributing to the optical measurement. These considerations explain only a small part of the observed difference between the AF and HFP estimates at low density. We conclude that HFP underestimates, for an unknown reason, the MP density in subjects who clearly have MP as measured by both the AF and RE methods.

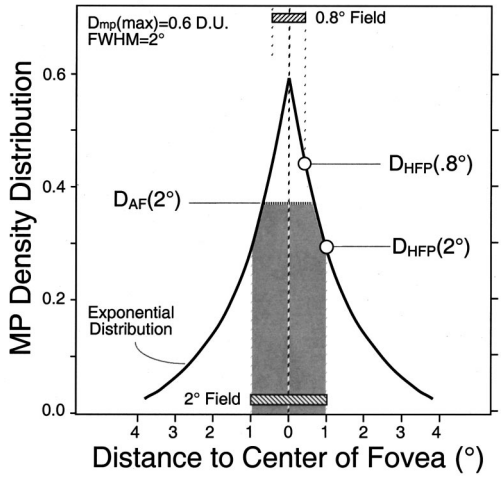

Fig. 8. Relationship between the density distribution of the MP and MP density estimated by the AF method (2°) and by HFP (0.8° and 2°). Different densities simulating different techniques were computed for an exponential distribution of MP.[43] The FWHM is 2°, similar to the mean width observed *in vivo*.[17] Flicker photometry (D_{HFP}) estimates the density at the edge of the test stimulus, whereas the AF method (D_{AF}) provides an average density over the test field. For equal test field size, AF estimates are thus higher than HFP measures.

B. Tissue Properties Affecting Macular Pigment Density Determination

Although the MP density estimates obtained by different methods correlate very well with each other, systematic differences exist that cannot be explained by differences in test parameters (wavelength, test field sizes, location of reference site). AF estimates are higher than HFP estimates, and both are substantially higher than estimates obtained by reflectometry. We therefore investigated possible violations of the main assumptions on which the techniques are based. The first assumption states that all the light that is sampled by the photodetector (photoreceptor) must have been attenuated by MP absorption. This implies that, for the optical methods, there are no sources of fluorescence or reflection anterior to the MP.

Since the lens and ocular media, as well as superficial retinal tissues, all have scattering and/or fluorescence properties, we investigate whether substantial errors arise secondary to such spurious contributions (Subsections 5.G and 5.H). The second assumption states that the spectral characteristics of the underlying tissues must be similar at the fovea and at the perifovea. Specifically, the spectral sensitivity of the photoreceptors (HFP), the excitation spectrum (AF method), and the reflectance spectrum (RE method) at the perifoveal reference site must be proportional to the respective spectrum at the fovea. In this regard, we investigate the effect of self-screening on HFP (Subsections 5.C) and the effect of RPE melanin on both optical methods (Subsections 5.D and 5.E).

C. Photoreceptors' Self-Screening and the Heterochromatic Flicker Photometry Method

To verify a key assumption of the HFP method, we assessed the effect of the difference in self-screening between the fovea and the perifovea. In Eq. (13), the term in the square brackets was set to 0 by assuming, among other conditions, that self-screening by the visual pigment was small. To investigate the importance of this (generally made) assumption, we calculated the change in HFP sensitivity $S(\lambda)$ by using

$$S(\lambda) = k[1 - 10^{-\omega(\lambda)D_{vp}(550)}], \qquad (15)$$

where $\omega(\lambda)$ is the extinction spectrum of the visual pigment (normalized at 550 nm) and $D_{vp}(550)$ is the optical density of that pigment at 550 nm. For small absorption, this expression is approximated by $k\omega(\lambda)D_{vp}(550)$, and

the term in square brackets is then eliminated from Eq. (13). We computed the expression in the square brackets of Eq. (13) by using extinction spectra $\omega(\lambda)$ for the L and M cones[45] that were assumed to be the same for the fovea and for the perifovea.

We used a ratio of L/M cone optical densities of 2 (Refs. 46 and 47); this ratio remains relatively constant for the eccentricities used in HFP.[48] The contribution of self-screening can then be expressed as

$$\left[\log\frac{S_P(550)}{S_P(460)} - \log\frac{S_F(550)}{S_F(460)}\right] \approx -0.347[D_{\mathrm{vp},F}(550) - D_{\mathrm{vp},P}(550)]. \quad (16)$$

This approximation does not deviate by more than 0.01 D.U. from the exact values if the density difference on the right-hand side of relation (16) is $\leqslant$0.4 D.U. ($D_{\mathrm{vp},P} = 0.1$ D.U.). With photopigment density difference between the fovea and the perifovea being 0.1–0.2 D.U. (as estimated psychophysically with test fields of 0.8°–1.0° diameter),[46,49,50] we find an underestimation of the MP densities determined by HFP of 0.03–0.07 D.U. for all MP densities. This effect should decrease with age because the density difference decreases with age.[50]

D. Retinal Pigmented Epithelium Melanin and the Reflectometry Method

Fundus reflections, for wavelengths shorter than 580 nm, occur predominantly at the level of the RPE (interfaces, melanin scattering) and/or at Bruch's membrane.[21,22,51] Reflections at the cone's photoreceptor disks, whose existence was invoked by van de Kraats and colleagues,[23] in an optical model of foveal reflectance (which incorporates directionality of photoreceptors),[52,53] is not believed to play a large role in our technique, because the light is incident on the retina and is detected from the retina at large angles (≈5° from the normal on the retina).[31] Thus fundus reflectance will in large part be affected by RPE melanin, and MP densities may be overestimated because melanin is denser at the fovea than at the perifovea[54,55] and because it absorbs more at short wavelengths.[56]

Our estimation of the amount of RPE melanin is based on the observation that the log-ratio spectrum associated with reflectance generally exhibits a slight decrease with increasing wavelengths between 540 and 575 nm (Fig. 3, arrows; Fig. 6). Because blood absorption is the same at these wavelengths,[57] we believe that this is caused in large part by the difference in the concentration of RPE melanin between the fovea and the perifovea. Therefore we equate the log ratio, similarly to Eq. (8), as

$$\log\frac{R_p(\lambda)}{R_F(\lambda)} = \log\frac{R^*_P(\lambda)}{R^*_F(\lambda)} + n\mathbf{d}_{\mathrm{me}}(460)K_{\mathrm{me}}(\lambda) + 2\mathbf{D}_{\mathrm{RE}}(460)K_{\mathrm{mp}}(\lambda), \quad (17)$$

where $\mathbf{d}_{\mathrm{me}}$ is the single-pass melanin density difference (at 460 nm) between the fovea and the perifovea, $K_{\mathrm{me}}(\lambda)$ is the extinction coefficient of melanin,[56] and $R^*(\lambda)$ is the fundus reflectance in the absence of MP and RPE melanin. The factor n equals 2 if all the light is reflected by Bruch's membrane or is less than 2 if light is reflected within the RPE by scattering of melanin granules. The difference in melanin density sampled between both sites is $n\mathbf{d}_{\mathrm{me}}$.

Estimates of RPE melanin were obtained by fitting Eq. (17) to the reflectance log-ratio spectra (450–575 nm) for 147 subjects. The median r^2 for all fits was 0.992. Mean $n\mathbf{d}_{\mathrm{me}}$ was found to be 0.13 ± 0.11 D.U., with a large intersubject variability (Fig. 9). A fovea–perifovea melanin density difference of ≈0.25 D.U. was found *ex vivo* for single-pass measurements through human RPE (Refs. 55 and 58); our results then suggest that $n \approx 0.5$ or that only 1/4 of the melanin layer is being sampled. Our melanin density differences were not correlated with age. While the number of melanin granules in the RPE decreases with age,[54] their size and optical density increase[59]; these opposing effects may explain why we found no age dependence and why *ex vivo* RPE melanin concentration in the macula[60] and RPE optical density[55] were not found to change with age. There was also no significant correlation in the melanin differences with ocular pigmentation as defined by iris color ($p = 0.6$), which may be expected since, unlike choroidal melanin, RPE melanin is not significantly affected by race[55] and iris color.[60]

MP densities D_{RE} derived from the same fits of Eq. (17) were not correlated with $n\mathbf{d}_{\mathrm{me}}$ ($p = 0.5$) and had a mean value of 0.20 ± 0.05 D.U., significantly lower than the mean density of 0.23 ± 0.06 D.U. found with the RE method (paired, $p < 0.0001$). The mean difference, 0.03 ± 0.03 D.U., represents the overestimation of the MP density in the RE method resulting from the presence of melanin in the RPE. The reason for an ≈0.13-D.U. melanin density to have only an ≈0.03-D.U. effect on the MP density resides in the difference in the absorption spectra of both pigments. Indeed, a relationship between the MP density $\mathbf{D}_{\mathrm{RE},c}$, accounting for melanin, and the observed MP density $\mathbf{D}_{\mathrm{RE}}$ can be derived by applying Eq. (17) for $\lambda = 470$ and 550 nm, as we did in deriving Eq. (9):

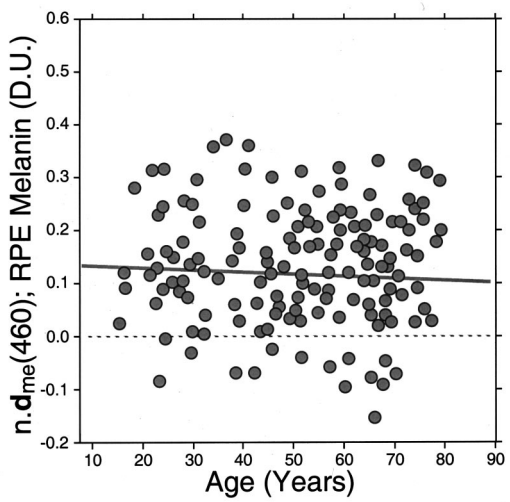

Fig. 9. Difference in the amount of RPE melanin at the fovea and at the perifovea as a function of age. The melanin density difference $n\mathbf{d}_{\mathrm{me}}(460)$ was derived by fitting Eq. (17) to the log-ratio reflectance data. Regression line: $n\mathbf{d}_{\mathrm{me}}(460) = 0.12 - 0.0003 \times (\mathrm{age})$ with $r^2 = 0.001$ ($p = 0.7$). More melanin was sampled at the perifovea than at the fovea in 14 out of 147 subjects (negative values).

$$\mathbf{D}_{\mathrm{RE},c}(460) = \mathbf{D}_{\mathrm{RE}}(460) - \frac{n}{2}\mathbf{d}_{\mathrm{me}}(460) \times \left[\frac{K_{\mathrm{me}}(470) - K_{\mathrm{me}}(550)}{K_{\mathrm{mp}}(470) - K_{\mathrm{mp}}(550)}\right]. \quad (18)$$

The multiplier in the square brackets was computed by using known extinction coefficients of MP (Ref. 6) and melanin[56] and was found to be 0.472. Thus an $n\mathbf{d}_{\mathrm{me}}$ of 0.13 D.U. corresponds to an MP density of 0.03 D.U. We conclude that melanin difference across the retina does not strongly affect our reflectometric estimates of MP.

E. Retinal Pigmented Epithelium Melanin and the Autofluorescence Method

RPE lipofuscin is intermixed with melanin in the RPE cell, causing partial attenuation of the excitation and the emission from lipofuscin. Furthermore, RPE melanin and lipofuscin are not uniformly distributed; melanin has a higher concentration at the apical side (corneal side) of the cell, and lipofuscin has a higher concentration on the basal side.[55] Evidence that RPE melanin causes MP densities by the AF method to be overestimated can be found in the fact that D_{AF} exhibited a significant and positive correlation ($r = 0.3$, $p < 0.0001$) with the melanin density difference $n\mathbf{d}_{\mathrm{me}}$ (determined independently by reflectometry). To quantify this error, we added $m\mathbf{d}_{\mathrm{me}}[K_{\mathrm{me}}(\Lambda) + K_{\mathrm{me}}(\lambda)]$ to the right-hand side of Eq. (4) and derived the relationship between the MP density $\mathbf{D}_{\mathrm{AF},c}$, accounting for melanin, and the observed MP density $\mathbf{D}_{\mathrm{AF}}$:

$$\mathbf{D}_{\mathrm{AF},c}(460) = \mathbf{D}_{\mathrm{AF}}(460) - m\mathbf{d}_{\mathrm{me}}(460) \times \left[\frac{K_{\mathrm{me}}(470) - K_{\mathrm{me}}(550)}{K_{\mathrm{mp}}(470) - K_{\mathrm{mp}}(550)}\right], \quad (19)$$

where $\mathbf{d}_{\mathrm{me}}$ is again the single-pass melanin density difference between the fovea and the perifovea. The factor m equals 1 if all the lipofuscin is located on the basal side of the melanin or is less than 1 if lipofuscin and melanin are intermixed within the RPE. Since the density difference associated with emitted fluorescence was eliminated in the derivation of Eq. (19) (see Subsection 2.A), $m\mathbf{d}_{\mathrm{me}}$ corresponds only to differential absorption of the excitation light. The multiplier in the square brackets is 0.476.

We calculated the values of $m\mathbf{d}_{\mathrm{me}}$, assumed to be proportional to $n\mathbf{d}_{\mathrm{me}}$, that would eliminate the above-mentioned correlation between D_{AF} and melanin. We found that a melanin density difference of $m\mathbf{d}_{\mathrm{me}} \approx 1.06n\mathbf{d}_{\mathrm{me}}$ would entirely eliminate a dependence of D_{AF} upon melanin ($r = 0$, $p > 0.99$). This approximation corresponds to a mean melanin density difference between the fovea and the perifovea of $m\mathbf{d}_{\mathrm{me}} = 0.14 \pm 0.11$ D.U. This estimate was confirmed by a different method based on AF.[61] Although $n\mathbf{d}_{\mathrm{me}}$ and $m\mathbf{d}_{\mathrm{me}}$ are practically equal, interpretation must include the fact that $n\mathbf{d}_{\mathrm{me}}$ represents the amount of melanin sampled by reflected light (equivalent to a double pass through an average layer with density $0.5n\mathbf{d}_{\mathrm{me}}$) whereas $m\mathbf{d}_{\mathrm{me}}$ represents the amount of melanin affecting only the excitation light. Thus the excitation light probes a layer that is approximately twice that sampled by reflected light.

The melanin density difference $m\mathbf{d}_{\mathrm{me}}$ was substituted in Eq. (19) to yield MP densities corrected for the effect of RPE melanin: A mean MP density of 0.41 ± 0.15 D.U. was found, compared with 0.47 ± 0.16 D.U. for the measured $\mathbf{D}_{\mathrm{AF}}$ values ($n = 147$). The average overestimation in the MP densities was thus 0.07 ± 0.05 D.U. (range: -0.08 to 0.19 D.U.). Accounting for melanin does not substantially alter our conclusions in regard to subjects with low D_{HFP}: The mean melanin density difference for the three subjects with low MP (Fig. 7) was 0.04 ± 0.05 D.U., resulting in a decrease of the mean MP density by only 0.01 D.U.

F. Other Optical Factors Affecting the Assumptions about Posterior Tissues

Lipofuscin in the RPE cells is composed of at least ten fluorophores that are universally observed in the RPE of aging donors.[62] Only three of these fluorophores have excitation spectra that extend into the visible spectrum (all others have only UV excitations) and thus could be expected to be excited *in vivo*. Their excitation and emission spectra are similar (Fig. 4 in Ref. 62). Little is known about changes in the composition of RPE lipofuscin with age and with retinal location. Since lipofuscin is derived from phagocytosis of both rods and cone outer segment membranes, one might expect differences in the composition and the spectra at the fovea and at the perifovea. Such differences would result in systematic errors,[33] but it is currently not possible to evaluate their magnitude, except that they are not sufficient to distort the shape of the detected MP spectrum (Subsection 4.C).

Fluorescence and/or reflection at Bruch's membrane (BM, Fig. 1) change with age (drusen), and this may alter the reflectance and fluorescence spectra of the posterior layers and affect our MP estimates.[33,63] However, these changes occur throughout the posterior pole[64] and may not have marked effects on MP estimates. This may be confirmed by the fact that MP densities were not significantly affected by the presence/absence of hard drusen, despite changes in the foveal emission spectrum.

Unbleached visual pigments could also affect the comparison between the two sites. Since rods, unlike cones, were not completely bleached in our protocol (Subsection 3.B), we evaluated the effect of their differential absorption on the MP measurement. We use Eq. (19) with $m = 1$ (single pass) and after replacing $\mathbf{d}_{\mathrm{me}}$ by $\mathbf{d}_{\mathrm{rods}}$ (the fovea–perifovea single-pass density difference for rods) and K_{me} by K_{rods} (the extinction coefficients for rhodopsin). Using $K_{\mathrm{rods}}(470) = 1.19$ and $K_{\mathrm{rods}}(550) = 0.85$ for the extinction coefficients normalized at 460 nm, we find that the multiplier of $\mathbf{d}_{\mathrm{rods}}$ is 0.41. For a single-pass, dark-adapted, density difference of $\mathbf{d}_{\mathrm{rods}} = -0.06$ D.U. at 460 nm (-0.1 D.U. at 500 nm), one would underestimate the MP estimate by 0.02 D.U. [Eq. (19)]. With rod bleaching of least 60% in the worst case, we can expect an error of at most 0.01 D.U. for the AF method. The reflectance spectra are measured at each site after multiple excitation exposures; the rods are then bleached, and no error can be expected for the RE method.

Other differences between the fovea and the perifovea, such as difference in retinal thickness and directionality in bleached photoreceptors, are not expected to strongly affect the optical methods because their contributions are likely to be largely wavelength independent. Moreover, the large angles of incidence of the excitation and detected lights (≈5°) used in our technique precludes strong photoreceptor directionality influences.[52,53] The effect of retinal capillary blood at the perifovea (capillary-free zone at the fovea) can also be evaluated. For an equivalent peripheral blood layer of ≈2 μm in thickness[65] or a single-pass density difference of $\mathbf{d}_{\text{blood}} \approx 0.02$ D.U. at 460 nm,[57] we found, using Eqs. (18) and (19), an overestimation of ≈0.01 D.U. for both methods [the multiplier in the square brackets of Eqs. (18) and (19) is −0.58].[57]

G. Anterior Fluorophores and the Autofluorescence Method

The effect of a secondary fluorophore $S_a(\Lambda, \lambda)$ located in front of the foveal MP can be computed by replacing F_F in Eq. (5) by $F'_F = F_F + S_a$, where F'_F denotes the fluorescence measured at the fovea and corrected for lens absorption.[66,67] After rearrangement one obtains a relationship between the density D'_{AF}, which one would estimate if S_a were ignored, and the true MP density D_{mp}:

$$\mathbf{D}'_{\text{AF}}(460) \approx \mathbf{D}_{\text{mp}}(460) - \frac{1}{K_{\text{mp}}(470) - K_{\text{mp}}(550)} \times \left\{ \log\left[1 - \frac{S_a(550,\lambda)}{F'_F(550,\lambda)}\right] - \log\left[1 - \frac{S_a(470,\lambda)}{F'_F(470,\lambda)}\right] \right\}. \quad (20)$$

The ratios S_a/F'_F are always smaller than 1. MP absorption causes $F'_F(470,\lambda)$ to be smaller than $F'_F(550,\lambda)$, but $S_a(470,\lambda)$ is usually larger than $S_a(550,\lambda)$, since the excitation spectra of other ocular fluorophores generally decrease with wavelength.[68–71] Thus the term in braces in relation (20) will be positive, and MP density will be underestimated, particularly if the foveal fluorescence F'_F is low.

Evidence of a secondary fluorophore was derived from the fact that MP density increased with the wavelength (λ_{det}) at which the fluorescence was measured (top of Fig. 10). This effect was most pronounced in young subjects. In deriving Eq. (5), we have noted that MP density determination by the AF method cannot be affected by differential absorption at the emission wavelength. Thus, if absorption is not involved, then the changes must be explained by a secondary fluorophore. We hypothesize that a secondary fluorophore is located in front of the MP and that it has a high emission at 500–540 nm, which causes a shift of the foveal emission spectrum toward shorter wavelengths (Fig. 2). The emission tail of this fluorophore must extend to long wavelengths (inset; top of Fig. 10), since the MP densities converge to a constant value beyond this. Thus our MP densities are underestimated, but the error should be minimized because we chose to measure the fluorescence at 710 nm.

If the secondary fluorophore is located in front of the MP, then one would expect its effect to be inversely related to the measured foveal fluorescence [relation (20)]. We use the density difference ΔD (top of Fig. 10), as a measure of that effect.[72] The bottom of Fig. 10 shows how ΔD exhibited a significant negative correlation ($r = -0.42$, $p < 0.0001$) with the foveal fluorescence $F'_F(470, 710)$. The factor ΔD was also negatively correlated both with the amount of RPE lipofuscin [estimated by $F'_F(550, 710)$ outside the absorption range of MP; $r = -0.30$, $p < 0.0001$] and with the transmission of MP ($r = -0.37$, $p = 0.0001$). This indicates that both higher MP and lower lipofuscin serve to increase the influence of the secondary fluorophore. The effect of age seen in the top of Fig. 10 is a direct result of the marked increase in RPE fluorescence with increasing age.[32,73] After accounting for the dependence $F'_F(470, 710)$ upon age, we found no correlation between ΔD and age ($p > 0.9$). Thus the underestimation caused by the secondary fluorophore becomes less pronounced with increasing age, and this could contribute to the slow increase of the density estimates with age (Fig. 4).

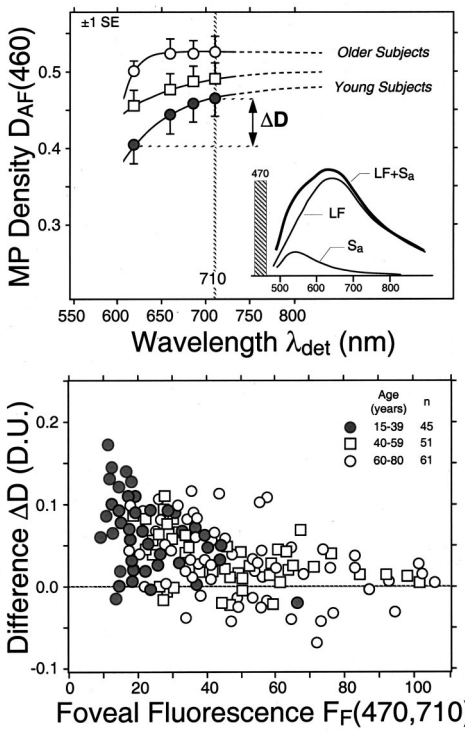

Fig. 10. Effect of an anterior fluorophore on the MP density estimates D_{AF} by the AF method. (top) Average MP densities determined with the AF method ($\Lambda = 470$ and 550 nm) as a function of the wavelengths λ_{det} at which AF is measured. Mean densities are given for three age groups. Dashed lines are extrapolations that tend toward the average correct MP densities in each group. The AF method uses 710 nm as the detecting wavelength, resulting in a slight underestimation in MP density. The density difference ΔD between the densities measured with $\lambda_{\text{det}} = 710$ nm and $\lambda_{\text{det}} = 620$ nm is a measure of the magnitude of the secondary fluorescence.[72] This measure decreases significantly with age ($p < 0.0001$). (top inset) Schematic representation of the emission spectrum of lipofuscin (LF), of a secondary fluorophore (S_a), and of the combined fluorophores (LF + S_a). (bottom) Density difference ΔD as a function of the measured foveal fluorescence ($\Lambda = 470$ nm).

We have not, to date, identified the exact nature of the secondary fluorophore(s), principally because its magnitude and detailed emission spectrum are not known. We know that its excitation spectrum decreases with increasing wavelength,[33] but this is the case for most ocular fluorophores. Several candidates can be considered. Stray lens fluorescence,[68] which increases with age,[69] was minimized by our baseline spectrum correction (Subsection 3.B). This correction seems to be effective, since we found no significant correlation between ΔD and age or between MP estimates and crystalline lens density (Subsection 4.E). However, lens fluorescence reflected by the limiting membrane is still a possibility because lens fluorescence and inner limiting membrane (ILM) reflectance respectively increase and decrease with age.[69,74] Fluorescence of collagen fibers in the vitreous in close proximity to the retina[70] would not be corrected by the baseline procedure and could thus also play a role. Finally, we cannot exclude fluorescence of Henle fibers,[75] of metabolically active components in the superficial retina,[71] and of MP itself[76] or its binding protein.

In summary, the influence of an unidentified secondary fluorophore located in front of the MP can be minimized by proper selection of the wavelength at which the AF is measured. With 620 nm as the detection wavelength as was used in an earlier protocol,[30] average underestimation in MP densities ranged from 0.03 D.U. for the older group to 0.08 D.U. for the younger group (Fig. 10). However, this error was reduced to a mean of 0.015 $\pm$ 0.013 D.U. (range: 0–0.06 D.U.)[72] by using a detection wavelength at 710 nm.

H. Anterior Reflectors/Scatterers and the Reflectometry Method

Although our reflectometry estimates of MP density $D_{\mathrm{RE}}(460)$ are in the same range as those obtained in other reflectometry studies,[20–27] they are all substantially lower than those found by either psychophysics or the AF method. It is often suggested that these low values are the result of anterior reflections/scattering by the ILM and/or the ocular media,[13,18,23] and we will examine this issue in the following paragraphs.

The effect of an anterior reflector $R_a(\lambda)$ on MP density can be calculated, in the same manner as that for relation (20), and is given by

$$\mathbf{D}'_{\mathrm{RE}}(460) \approx \mathbf{D}_{\mathrm{mp}}(460) - \frac{0.5}{K_{\mathrm{mp}}(470) - K_{\mathrm{mp}}(550)} \times \left\{ \log\left[1 - \frac{R_a(550)}{R'_F(550)}\right] - \log\left[1 - \frac{R_a(470)}{R'_F(470)}\right] \right\}, \quad (21)$$

where the notation is that of relation (20) and R'_F denotes the reflectance measured at the fovea and corrected for lens absorption.[66] Again, the term in the braces is generally positive, and the MP density can be expected to be underestimated as a result of anterior reflectors, particularly if fundus reflectance is low.

Figure 11 shows the extent of the underestimation of the MP densities $D_{\mathrm{RE},c}$ compared with the densities $D_{\mathrm{AF},c}$, where both densities have been corrected to account for RPE melanin (Subsections 5.E and 5.D). If we assume that the MP densities estimated by reflectometry are underestimated as a direct result of anterior reflectors, then we can evaluate the magnitude of this reflectance. To do this, we use the AF estimates $D_{\mathrm{AF},c}$ and ask how much reflectance is required to reduce $D_{\mathrm{AF},c}$ to the level of measured $D_{\mathrm{RE},c}$. Transforming relation (21) to render R_a explicit, we obtain

$$R_a = \frac{\Psi - 1}{\Psi/[R'_F(550)] - 1/[R'_F(470)]}$$

$$\text{with } \Psi = 10^{-2[\mathbf{D}_{\mathrm{AF},c}(460) - \mathbf{D}_{\mathrm{RE},c}(460)][K_{\mathrm{mp}}(470) - K_{\mathrm{mp}}(550)]}, \quad (22)$$

where the R'_F's are the measured foveal reflectance corrected for ocular media absorption[66,67] and R_a is assumed to be a spectrally neutral reflector. Applying Eq. (22) to the individual data of all subjects, we obtained a mean equivalent reflectance R_a of 0.39% $\pm$0.21% (range: 0.0%–1.3% reflectance). This anterior reflectance represents 63% $\pm$ 18% and 23% $\pm$ 9% of the total foveal reflectance at 470 and 550 nm, respectively. The ratio $R_a(470)/R'_F(470)$ was not correlated with age ($p = 0.4$). The reflectance R_a increases with age, but this could result from an overestimation in our media correction.[77] If the anterior reflectance decreases with wavelength (as it would be for scattering in the media or the superficial retina), then the estimate for R_a will be lower; we found that $R_a(470) \approx 0.31\%$ in the extreme case of $R_a(550) \approx 0$. The average effect of an anterior reflector is illustrated in Fig. 11 by single-parameter curve fits[78] to the

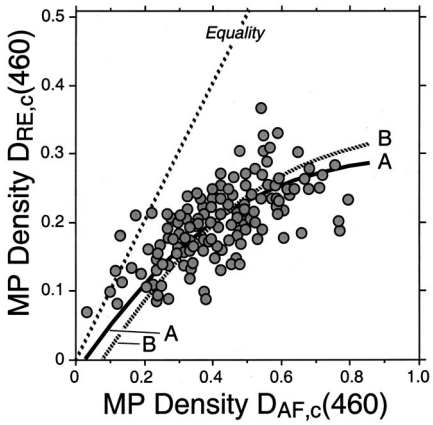

Fig. 11. Effect of an anterior reflector/scatterer on the MP densities estimated by the RE method. The MP density $D_{\mathrm{RE},c}$ derived from the RE method is given as a function of the MP densities $D_{\mathrm{AF},c}$ derived from the AF method. This plot is similar to that at the bottom of Fig. 5, except that both densities were corrected to account for the effect of RPE melanin. The curves are fits of relation (21) to the data,[78] assuming that the anterior reflector is spectrally neutral (curve A) or that the reflectance R_a decreases sharply [$R_a(550) = 0$] with increasing wavelength (curve B). The fitted parameters $R_a(470)/R^*_F(470)$ were 0.38 and 0.25 for curves A and B, respectively. The corresponding reflectances $R_a(470)$ were, respectively, 0.39% and 0.33%, similar to the mean reflectances found with Eq. (22).

data: The densities $D_{RE,c}$ increasingly deviate from the densities $D_{AF,c}$ as the MP density increases.

Our results are in good agreement with those of van de Kraats and colleagues,[23] who used a model-based approach, incorporating some of the same assumptions to analyze reflectance spectra from the fovea in ten normal subjects. They found an anterior foveal reflectance of 0.26% ± 0.09%, representing ≈60% of the total reflectance at 470 nm. After accounting for the anterior reflection, they found a mean MP density (460 nm) of ≈0.54 D.U., not inconsistent with values found by the AF method.

Is the presence of this anterior scatterer compatible with quantitative measurement of other foveal pigments? In retinal densitometry, the amount of photopigment is determined by measuring the change in reflectance of the retina between dark-adapted and bleached conditions. Modern densitometers regularly measure double-pass density differences as high as 0.4–0.6 D.U. at ≈550 nm.[79–82] If we invoke the above-estimated amount of an anterior reflectance [$R_a(550) = 0.39\%$], which does not change with dark adaptation, and ask what density difference would be measured if the cone photopigments were infinitely dense, we find that we should measure double-pass density differences not larger than $-\log(0.38/1.66) = 0.64$ D.U. at 550 nm, where 1.66% is the mean foveal reflectance $R'_F(550)$ in our population ($n = 147$).[66] Thus this limiting double-pass density is not necessarily incompatible with current densitometry results. Our estimate $n\mathbf{d}_{me}$ for RPE melanin difference between the fovea and the perifovea (Subsection 5.D) will also be affected by an anterior reflector. The resulting underestimation may be small because the melanin density difference was small and the reflectance at 540–575 nm higher. In any case, this will not affect our estimation of the effect of RPE melanin on the AF method, since the latter was entirely based on the absence of a correlation between D_{AF} and $n\mathbf{d}_{me}$ (Subsection 5.E).

Among the possible sources of reflection/scattering located in front of MP, the foveal reflex may be the most obvious because it is visible as a small bright image of the entrance pupil. This reflex is a specular reflection occurring at the concave interface of the ILM at the fovea (Fig. 1).[22] ILM reflection is most intense and specular in young subjects but could also be present in a more diffuse manner in older eyes. The reflectance characteristics of the ILM were measured directly by Gorrand and Delori.[74] They found that, for a test field of 2° in diameter, the equivalent reflectance from the foveal ILM was 0.019% ± 0.013% in young subjects.[83] This is at least 1 order of magnitude lower than the above-mentioned estimates of the anterior reflectance, and thus it is unlikely that the foveal reflex is the source of the hypothesized anterior reflectance.

Crystalline lens scatter would in large part be accounted for by our baseline correction (Subsection 3.B), as was demonstrated by a 34% increase in MP density when the correction was implemented in the RE method. Furthermore, the MP estimates were not affected by the individually measured media densities (Subsection 4.E). However, reflectometry methods that do not account for lens scattering would likely be significantly affected by scattering and stray light. Vitreous scattering close to the retina would not be corrected by our baseline procedure and could contribute to anterior scattering.[23] However, MP densities estimated by scanning laser opthalmoscope (SLO) imaging also have low-density values, despite the fact that vitreous scattering would be decreased by the confocal arrangement of the SLO optics.

Several of our findings suggest that the cause of low MP estimates in the RE method may be related not necessarily to a reflector located in front of the MP but rather to a difference in the interaction of light with the retina. For instance, the facts that D_{RE} and D_{AF} correlated highly with each other, that both show similar changes with age, that the coefficient of variation of MP estimates is slightly smaller for the RE method (Table 2), that the RE method was found to accurately predict the MP spectrum (Subsection 4.C), and that both densities exhibit nonzero values when D_{HFP} is zero (Fig. 7) all tend to suggest that the low MP densities are caused by a mechanism that does not introduce much variability into the MP measurement and/or that the MP may be sampled by less than a double-pass process [factor larger than 1/2 in Eq. (9)]. This is further supported by the fact that studies using a wide variety of reflectometry techniques ranging from small-field spectrophotometry[20–24] to retinal imaging (conventional cameras and confocal SLO)[24–27] all found low MP densities.

We hypothesize that scattering of light by Henle's fibers[75] contributes to the anterior reflections as well as reduces the path through the MP to less than a double pass. MP molecules are believed to be oriented and located on the membranes of Henle's fibers.[2,84] The retinal nerve fiber layer reflects light, as is observed by the bright striations of fiber bundles visible in red-free illumination and by the lower fundus reflectance in areas of nerve fiber defects. Knighton and colleagues[85,86] demonstrated that the reflectometric properties of the nerve fiber layer are consistent with light scattering by thin cylinders. They determined that the equivalent reflectance of a 10-μm-thick layer of fibers is ≈0.4%.[86] Since Henle's fiber layer is at least this thick,[75] it is logical to expect that scattering by nerve fibers could contribute to the anterior reflection at the fovea. Since the MP is intimately linked to these fibers, one can further predict that the path through the MP will be reduced, thereby requiring less anterior reflectance to explain the discrepancy between the RE and AF methods.

6. CONCLUSIONS

We have presented a technique for measuring macular pigment (MP) densities based on autofluorescence (AF) of the human retina. The AF method is reliable, sensitive, and reproducible. It is noninvasive, rapid, and easily applied to measurements in individuals across the age range of interest and produces estimates of MP density that correlate highly with both psychophysical and reflectometric estimates. The MP densities determined by the AF method are higher [by ≈0.25 density unit (D.U.)], particularly at low densities, than the MP densities estimated psychophysically by heterochromatic flicker photometry (HFP). MP density estimates by reflectometry

(RE method) are substantially lower, by approximately 50%, than those determined by the AF and HFP methods, are somewhat less reproducible, and may suffer from the effect of crystalline lens scattering in reflectometry techniques that do not account for such scattering.

By incorporating detailed considerations on light–tissue interactions, we have analyzed and tested the underlying assumptions of the methods. Modeling the sources of error in all three techniques does not fully account for the quantitative differences between the various estimates. The analysis suggests that the discrepancy between MP densities determined by the AF and HFP methods can be explained only in part by the differences in test field size, self-screening of visual pigment, RPE melanin, blood, or secondary fluorophores. By numerically estimating the effects of these factors (Fig. 12), we conclude that the MP densities estimated by the AF method are generally higher than the HFP estimates, particularly at low densities. The range of corrected densities overlaps the range that would be expected, for equal test fields, on the basis of difference in the sampling criteria (Fig. 8).[43] However, the fact that HFP appears to measure little or no MP in subjects who clearly have MP as measured by both the AF and RE methods remains unexplained.

The lower-density estimates obtained by the RE method can be modeled by an anterior reflector or scatterer, although the low apparent variability of such a spurious contribution suggests that a different mechanism for the interaction of light with the MP may exist.

For situations where the desire is to measure MP density differences between similar groups or changes in MP's for single individuals that are due to nutritional intervention, the difference between MP densities estimated by the AF method and by HFP are probably less critical. Selection of a technique will undoubtedly be based on other considerations such as reproducibility, robustness, cost and complexity of the instrumentation, the need to dilate the subject's pupil in optical methods, and the ability to reliably test older subjects, who are often afflicted by poor fixation and diminished psychophysical skills. The AF method may be applicable in situations where detailed comparisons are desired because it provides higher-density estimates and should be able to measure early changes following nutritional manipulation, particularly in individuals with low amounts of MP. Finally, our method is applicable to AF imaging[87] and may be used in studies of MP distributions.

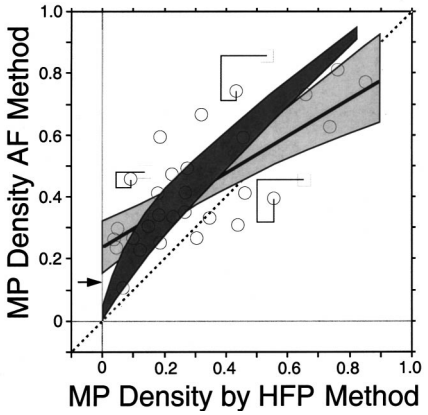

Fig. 12. Comparison of MP densities D_{AF} estimated by the AF method and densities D_{HFP} estimated by the HFP method (both with a 2°-diameter test field). In these data, we accounted for field size differences (Subsection 4.F) and for those effects that were identified in Section 5 (self-screening of visual pigment, RPE melanin, retinal capillaries, and secondary fluorophore). The open circles are the corrected data. The lightly shaded area represents the 95% confidence interval of the linear regression of D_{AF} on D_{HFP} ($n = 30$, intercept ≈ 0.23, and slope ≈ 0.62). The three jagged lines illustrate the different density corrections; starting from the open square (original data of Fig. 7), we implement the corrections for field size scaling (left), for RPE melanin and blood (down), for self-screening of visual pigment (right, 0.05 D.U.), and for the secondary fluorophore (up). The darkly shaded area represents the range of predictions for D_{AF} and D_{HFP} based on the difference in sampling criteria used in both methods (Subsection 5.A).[43]

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33. An error in $\mathbf{D}_{AF}(460)$ occurs if the excitation spectrum of tissues posterior to the MP at the fovea is not proportional to that at the perifovea. We model this; we equate foveal fluorescence as $F_F^*(\Lambda, \lambda) = kF_P^*(\Lambda, \lambda) + \Delta(\Lambda, \lambda)$, where F_F^* and F_P^* are the fluorescences at the fovea and at the perifovea, respectively, k is a constant, and Δ accounts for the spectral difference between the fovea and the perifovea (can be negative). Substitution of F_F^* in Eq. (4) and derivation of Eq. (5), assuming that $\Delta/F_P^* \ll 1$, gives the following approximation for the measured density:

$$\mathbf{D}'_{AF}(460) \approx \mathbf{D}_{AF}(460) + \frac{k \log(e)}{K_{mp}(\Lambda_1) - K_{mp}(\Lambda_2)} \times \left[\frac{\Delta(\Lambda_1, \lambda)}{F_P^*(\Lambda_1, \lambda)} - \frac{\Delta(\Lambda^2, \lambda)}{F_P^*(\Lambda_2, \lambda)}\right].$$

If the foveal excitation spectrum is shifted toward shorter wavelengths, then Δ decreases with increasing Λ (positive to negative), and the term in square brackets >0, resulting in an overestimation of the MP density. The opposite will occur if the shift is toward longer wavelengths.
34. Equivalent reflectances of the fundus use a perfect Lambertian reflector located at the retina of an artificial eye as reference.[21,22]
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36. For a subset of the population, we performed the fluorescence measurements at the peripheral site twice in the following order: excitations at 550, 510, 470, 430, 550, 510, and 470 nm. Rods were bleached only by the illumination and focusing lights before the first 550-nm exposure, whereas they were fully bleached (≈98%) by four excitation exposures before the second 550-nm exposure. For 30 subjects, the fluorescence at the second 550-nm exposure was 1.023 ± 0.035 times higher than that at the first exposure ($p = 0.001$). This corresponds to a single-pass rod density of 0.02 ± 0.03 D.U. at 500 nm. Thus approximately 60%–80% of the rods were bleached by the illumination and focusing lights (assuming a single-pass rod density of 0.05–0.1 D.U. at 500 nm).
37. The distribution of retinal irradiance and sensitivity of the fluorometer in the 2° test field was measured optically by displacing a small reflecting surface (equivalent to ≈0.2° diameter) in a focal plane located in front of the instrument. The product of both distributions at different radii was 1.00, 1.00, 0.90, 0.80, and 0.20 at 0°, 0.25°, 0.50°, 0.75°, and 1.00°, respectively.
38. The conversion factor used in this study was slightly larger than that calculated by using Eq. (10) from other MP spectra cited in the literature. A difference was found of 1% for the spectrum of a mixture of liposome-bound lutein and zeaxanthin in Bone *et al.*,[4] of 5% for the psychophysically determined spectrum in Wyszecki and Stiles,[5] and of 6% for the spectrum in fixed primate retinas obtained by microspectrometry in Snodderly *et al.*[3]
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43. We assume that the MP distribution $D(r)$ is exponential[12,17] and that it is given by $D(r) = D(0)10^{-2\ \log(2)(r/\mathrm{FWHM})}$, where r is the distance to the center. To convert D_{HFP} estimated with a 0.8°-diameter field to equivalent values for a 2° test field, we further assumed that the peak density $D(0)$ and the full width at half-maximum (FWHM) were linked by the experimentally derived relation $D(0) = 0.13 + 0.22\mathrm{FWHM}$.[17] Using various values of FWHM, we obtained a nonlinear relationship between the densities for the 2°- and the 0.8°-diameter field: $D_2 = -0.03 + 0.31D_{0.8} + 1.14D_{0.8}^2$ (for $D_{0.8} < 0.8$ D.U.). Tp predict the densities D_{AF} measured by the AF method (test field of radius R), we calculate

$$D_{\mathrm{AF}} = \log\left[\sum_0^R A(r)P(r)r\Delta r\right] - \log\left[\sum_0^R A(r)P(r)10^{-D(r)}r\Delta r\right],$$

where $P(r)$ is the distribution of fundus AF in the absence of MP and $A(r)$ is the product of the distribution of retinal irradiance and sensitivity of the fluorometer. Densities D_{AF} were computed with a constant $P(r)$, the measured distribution $A(r)$,[37] and an increment of $r = 0.005°$.
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58. *Ex vivo* data from the paper of Weiter *et al.*[55] for RPE melanin was multiplied by 2.2 to account for the fact that melanin transmission in that study was measured in the 500–600-nm spectral range and is expressed here at 460 nm. Mean melanin densities at 460 nm for single pass through the RPE were computed as 0.56 ± 0.19 and 0.31 ± 0.10 D.U. for the fovea and for sites at 6°–7° on either side of the fovea, respectively. Mean density difference in melanin between both sites was 0.25 ± 0.15 D.U.
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61. *Ex vivo* measurements in sections of RPE showed that lipofuscin fluorescence at the fovea was 81% ± 14% of that at the perifovea.[55] This ratio was calculated from the fluorescence measurements in sections of the RPE (bleached melanin) and from RPE cell heights at both sites. *In vivo* fluorescence at the fovea, measured with $\Lambda = 550$ nm (no MP absorption), was on average 64% ± 10% of that at the perifovea. If we assume that the difference between these ratios results from a higher apical melanin absorption at the fovea, then we can calculate that the melanin density difference is $\log(64/81)/[K_{\mathrm{me}}(550) + K_{\mathrm{me}}(710)]$ or 0.14 ± 0.14 D.U. at 460 nm.
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66. Fluorescence F' and reflectance R' incorporate absolute corrections to account for ocular media absorption. This combines our individually determined lens densities, which are relative to the density of a 44-year-old average observer,[35] and mean lens densities at age 44 years from Pokorny *et al.*[67] Average media-corrected fluorescences F' and reflectances R' at the fovea (F) and at the perifovea (P) were ($n = 159$)

$$\begin{aligned}
\text{At } F: &\quad F'_F(470, 710) = 81 \pm 44\ \mathrm{AFU}\\
&\quad F'_F(550, 710) = 136 \pm 62\ \mathrm{AFU}\\
\text{At } P: &\quad F'_P(470, 710) = 310 \pm 129\ \mathrm{AFU}\\
&\quad F'_P(550, 710) = 214 \pm 91\ \mathrm{AFU}\\
\text{At } F: &\quad R'_F(470) = 0.61\% \pm 0.26\%\\
&\quad R'_F(550) = 1.66\% \pm 0.61\%\\
\text{At } P: &\quad R'_P(470) = 2.74\% \pm 0.89\%\\
&\quad R'_P(550) = 3.10\% \pm 0.91\%
\end{aligned}$$

The AF unit AFU is $\mathrm{nJ\ nm^{-1}\ sr^{-1}/J}$.
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68. Crystalline lens AF was measured in three study subjects (ages: 37, 46, and 57 years) by focusing the sampling vol-

ume within the lens (using an additional ophthalmic lens). Excitation spectra decreased by 76% ± 3% between 470 and 550 nm. Emission spectra were maximal at 520 ± 5 nm for $\Lambda = 470$ nm and decreased to 10% of that maximum fluorescence at 655 ± 8 nm. Fluorescence intensity increased with age of the three subjects.

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72. By rewriting relation (20) for two-emission wavelengths, $\lambda = 620$ nm and $\lambda = \lambda_{det}$, and assuming that S_a is small compared with the foveal fluorescence and that its emission decreases exponentially, we can show that

$$\Delta D \propto S_a(470,\ 620)\{1 - [F'_F(470, 620)/F'_F(470, \lambda_{det})] \times \exp[-\alpha(\lambda_{det} - 620)]\}.$$

The measure ΔD is then proportional to S_a and increases in a quasi-exponential manner with increasing λ_{det} toward the true value (as sketched in the top of Fig. 10). By performing detailed fits for a number of subjects, we estimated that the amount of underestimation is approximately 1/3 of ΔD.
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77. Reflectances R_a are positively correlated with age ($r = 0.4$, $p < 0.0001$) as a direct result of the increase with age of media-corrected reflectances $R'_F(470)$. This increase in reflectance cannot be accounted for by known age-related retinal changes. As we mentioned before,[35] we believe that this increase may result from a slight overestimation of our media correction method.
78. The foveal fluorescence F'_F in relation (21) was expressed as a function of the reflectance R^*_F of layers posterior to the MP [as in Eq. (6)] by

$$R'_F(\lambda) = R_a(\lambda) + R^*_F(\lambda)10^{-2K_{mp}(\lambda)D_{AF,c}(460)}.$$

The parameter R_a/R^*_F is then the single free parameter in the fits.
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83. Direct measurements[74] of the ILM reflectance ρ used a perfect mirror as reference (with curvature equal to that found in the eye). To convert this reflectance into an equivalent reflectance (reference: perfect diffuser),[34] we used Eqs. (27) and (28) of Gorrand and Delori's paper.[74] The equivalent reflectance of the ILM is then $R_{ILM} = (\pi\rho r^2)/(4A)$, where the reflectance is $\rho = 0041\% \pm 0.0019\%$ (seven young subjects), r is the radius of curvature of the foveal depression ($r = 1.22 \pm 0.22$ mm), and A is the area of the retinal test field (0.26 mm^2 for a 2°-diameter field). Using the original data ($n = 7$, ages: 16–26 years), we found that $R_{ILM} = 0.019\% \pm 0.013\%$ (range: 0.007%–0.04%).
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