Comparison between Macular Pigment Optical Density Measurements Using Two-Wavelength Autofluorescence and Heterochromatic Flicker Photometry Techniques

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Purpose. The association of macular pigment (MP) with age-related macular degeneration has been extensively studied in recent years, and interest in a rapid, objective, and accurate technique to measure MP optical density (MPOD) has increased. The purpose of this study was to compare the MPOD values at specific loci around the fovea using the heterochromatic flicker photometry (HFP) and the two-wavelength autofluorescence (AF) methods in a young, healthy population.

Methods. Ten patients (20 eyes) were enrolled. Subjects with any ocular or systemic disease were excluded. All patients underwent HFP and AF examination. The AF examination was performed using a modified scanning laser ophthalmoscope. Values for both devices were measured at four eccentricities around the fovea (0.25°, 0.5°, 1.0°, and 1.75°). Each eye was tested three times with each method. Statistical analysis was based on paired *t*-test and linear regression analysis.

Results. At all retinal eccentricities, the HFP values were consistently lower than the HRA values ($P < 0.001$). There was, however, a significant correlation at almost all locations. The strongest correlation between two methods was found at 1.75° from the center of the fovea ($r = 0.73$).

Conclusions. The modified-HRA AF method for MPOD generated results that were highly correlated with the standard HPF method but consistently higher at all eccentricities. These findings suggest that HRA can be reliably used in patients unable to perform HPF, which is important for wider clinical application of MP testing. (*Invest Ophthalmol Vis Sci.* 2010;51:3152–3156) DOI:10.1167/iovs.09-3608

A yellowish pigment, composed of xanthophyll carotenoids (lutein, zeaxanthin, and meso-zeaxanthin), accumulates around the fovea and is referred to as macular pigment (MP).[1,2] Actually, the name macula derives from the Latin expression, macula lutea (yellow spot), which refers to the appearance of the accumulation. Lutein and zeaxanthin are of dietary origin, whereas meso-zeaxanthin is formed in the retina by conversion from lutein.[3] MP is deposited preferentially in the photoreceptor axons and inner plexiform layers of the retina. It acts as a short-wavelength light filter for the foveal photoreceptors and protects the retina from photo-oxidative damage by short wavelength radiation.[1–3] Lower levels of MP have been associated with known risk factors such as smoking and elevated lipid profiles for developing age-related macular degeneration.[4–7] Moreover, although still controversial, there is growing evidence supporting that higher levels of carotenoids in the diet and in the blood plasma are associated with a lower risk for age-related macular degeneration.[8,9] Other studies have demonstrated that MP levels can be manipulated by increasing carotenoids in the diet or by providing lutein and/or zeaxanthin supplementation.[10–13] These findings support the need for an objective and accurate clinical technique for measuring MP density, which must be reliable, reproducible, and sensitive. Such a method could be useful to study the effects of dietary manipulation in diverse populations, which may be at risk for progression of a variety of degenerative and age-related retinal diseases.

Several objective techniques have been used to measure MP density indirectly and noninvasively in vivo. They are divided into either psychophysical or optical methods. They have each been well described, and each has certain merits and limitations. The most commonly used psychophysical methods are heterochromatic flicker photometry[14] and motion photometry.[15] Optical or physical methods include reflectometry,[16] imaging reflectometry,[17] Raman spectrometry,[18] and autofluorescence spectrometry.[19]

Psychophysical methods are the most common. Among them, heterochromatic flicker photometry (HFP) is the most used and is recognized for its accuracy and repeatability. It can measure visual sensitivity with a test wavelength that is maximally absorbed by MP and a reference wavelength that is not absorbed by MP.[14] Several different locations or eccentricities from the center of the fovea are tested sequentially, and the results are used to map the MP optical density (MPOD) profile.[20] This noninvasive method is simple and relatively low cost. It does not require pupillary dilation, and it has a high test-reliability in cooperative and alert subjects.[20,21] Its major limitation is that it requires very good cooperation from patients who can understand and follow instructions.

The autofluorescence (AF) method has recently been introduced and allows objective measurements of the MP distribution.[22] It uses the fluorescence of lipofuscin normally present in the retinal pigment epithelium (RPE) to determine MP density. Carotenoids partially absorb the excitation light, reducing the fluorescence in areas of the fundus underlying the MP. The device captures sets of images at two excitation wavelengths. These images are averaged and aligned and were used to produce a map of MPOD extending in a 10° radius around the center of the fovea.[23–25] This is noninvasive, rapidly performed, and automated but more expensive and less available. Although it requires less patient cooperation and fewer physical skills, stable fixation and clear optical media are necessary.

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Although HFP is widely used, as a psychophysical test it is substantially subject dependent and may be difficult for older patients with degenerative macular diseases to perform accurately. Given that these patients are most likely to benefit from MP assessment because of possible therapeutic intervention, the value of using an easier optical technique, such as AF, to obtain measurements could be substantial. Understanding the correlation between HFP and AF measurements in normal eyes could provide the means to compare the values obtained with each study in patients.

The present study was designed to determine whether the MPOD values obtained from an AF technique are comparable to those obtained with HFP at similar locations around the center of the fovea in a young, healthy population.

Patients and Methods

This prospective study was conducted at the Retina Service, Department of Ophthalmology, of The New York Eye and Ear Infirmary and adhered to the tenets of the Declaration of Helsinki. Informed consent was obtained from all subjects.

Ten healthy subjects were enrolled in this study. Demographic data collected included age, sex, and race. All subjects underwent comprehensive ophthalmic evaluation that included best-corrected visual acuity (BCVA), biomicroscopy, and funduscopy. Eyes with any disease were excluded. If eligible, both eyes were included. After that, each eye was tested at least three times with both HFP and AF methods. First, all subjects underwent HFP testing, and subsequently their pupils were dilated with tropicamide 1% and phenylephrine 2.5% drops for AF testing on the same day.

The HFP technique has been well described elsewhere in the literature.[14] In brief, visual sensitivity was measured using a test wavelength that is maximally absorbed by MP (460 nm) and a reference wavelength that is not absorbed by MP (550 nm). These measurements were made at a retinal location with highest MPOD, the center of the fovea, and in an area where MPOD is minimal, 7° in the temporal retina. Sensitivity was measured using flicker photometry, which presented the two test stimuli in temporal square wave alternation of 12 to 15 Hz at the fovea and of 6 to 7 Hz in the parafoveal region. Temporal resolution of small stimuli is higher in the fovea than in the parafoveal area. The flicker rate must be reduced to match parafoveal flicker sensitivity. The subjects were instructed to adjust the dial until the flickering of the two lights appeared to stop, the null point. At the point where the intensity of the 460-nm blue light matches the 550-nm green light, the subject is unable to distinguish when the two wavelengths alternate. Blue and green diodes are configured to create targets of varying sizes presented against a blue background. A computer interface analyzes the value of the subject's response to obtain the (MP) filtration density value, which correlates to the MPOD at a specific locus. This measurement is repeated four times for each target, and the values are averaged to obtain the mean blue light flicker frequency at which the flicker rate of the target is most suppressed for the subject. This blue light frequency is proportional to the amount of MP present.

The AF method for measuring MPOD has also been previously described.[19,22,25] Briefly, it is based on the principle of autofluorescence of lipofuscin, which is located in the RPE cells. This fluorescence is emitted in the 520- to 800-nm spectral range, and it can be excited in vivo between 400 and 570 nm.[26] In the fovea, excitation light within the absorption range of the MP is partially absorbed by the carotenoids, resulting in this area of reduced fluorescence and in the excitation spectrum of foveal lipofuscin being closely related to the absorption spectrum of MP.[22] To measure the MPOD, the AF method compares results from two excitation wavelengths that are differentially absorbed by the MP (488 nm well absorbed and 512 nm minimally absorbed). Quantitative imaging is performed using a modified scanning laser ophthalmoscope (SLO; Heidelberg Retinal Angiography [HRA]; Heidelberg Engineering, Inc., Heidelberg, Germany). The subjects were positioned in front of the SLO camera and instructed to maintain steady fixation straight ahead. After the operator was comfortable with the subject's fixation and the focus of the SLO on the macular region, sequences of 20° images were captured at 488 nm and 514 nm. Macular pigment density maps were generated by digital subtraction of the log autofluorescence images, and mean MPOD values were calculated for circles at specific diameters centered on the fovea. For each eye tested, the average of two good-quality maps was used for the analysis. Criteria for good-quality images included sharp focus and accurate centration within the posterior pole. Results from eyes with poor-quality maps were excluded from the analysis.

Measurements for both methods were made in four corresponding rings around the center of the fovea: 0.25°, 0.5°, 1.0°, and 1.75°. The MPOD profiles of each eye were derived for both techniques. An additional measurement was taken with the HFP at 7°, an area without optically detectable MP, as a baseline for calculating MPOD at the different retinal eccentricities. Because the goal of this study was to analyze the agreement between measurements of two devices and not to compare a clinical characteristic between different groups of patients or identify a risk factor or effectiveness of a treatment, interocular dependency would not be an influence in the analysis.[27,28] Therefore, when eligible, both eyes of each subject were included in the analysis.

Statistical Analysis

The agreement between MPOD measurements obtained with HRA and HFP methods was evaluated by correlation analysis and Bland-Altman plots. This analysis was performed for each of the four locations (degrees of eccentricity) around the fovea. Means and standard deviations of MPOD taken with both methods at each location were calculated and compared using paired *t*-test. Statistical significance was set at $P < 0.05$.

Results

Nine patients (17 eyes) were included in the study. One patient (two eyes) was excluded from the analysis after testing because of poor-quality HRA images and unreliable measurements on the HFP test. Two patients were men and seven were women, mean age was 30.4 ± 3.6 years, and best-corrected visual acuity was 20/20.

Mean MPOD values at each eccentricity from the center of the fovea using both methods are presented in Table 1. Bland-Altman plots revealed differences in MPOD measurements between HRA and HFP for all four locations analyzed. HRA values were consistently higher than HFP values in all eccentricities. The mean difference between the measurements of the two methods increased as the MPOD values increased. Mean differences (absolute values) in MPOD measurements (95% confidence interval) between both techniques at 0.25°, 0.50°, 1.0°, and 1.75° were 0.173 (0.124–0.222), 0.125 (0.081–0.170), 0.115 (0.056–0.173), and 0.040 (0.022–0.058), respectively.

Table 1. Mean Values of MPOD and Correlation Coefficients between HRA and HFP Measurements

Location (degrees of eccentricity)	HRA (DU)	HFP (DU)	*r*	*P*
0.25°, mean ± SD*	0.54 ± 0.11	0.37 ± 0.07	0.56	0.017
0.50°, mean ± SD*	0.44 ± 0.11	0.32 ± 0.02	0.61	<0.001
1.0°, mean ± SD*	0.32 ± 0.11	0.21 ± 0.06	0.17	0.530
1.75°, mean ± SD*	0.11 ± 0.05	0.07 ± 0.05	0.73	<0.001

DU, density unit.

* Paired *t*-test revealed a significant difference ($P < 0.001$) between HRA and HFP results.

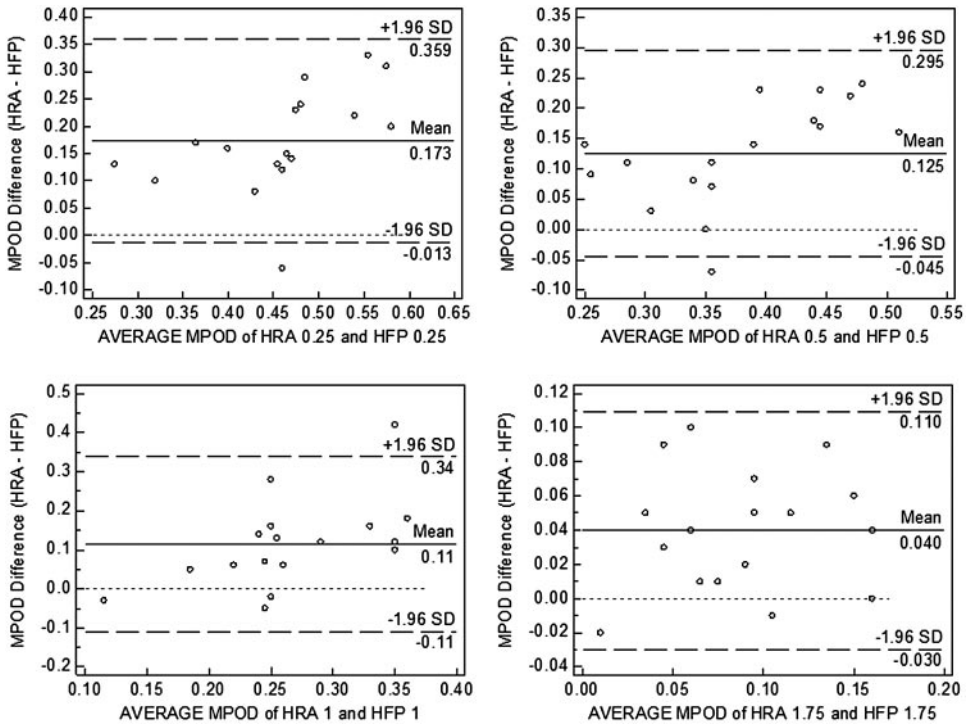

FIGURE 1. Bland Altman-plot comparison of macular pigment optical density at all eccentricities measured with the two methods.

More detailed data are shown in Figure 1. There was a positive correlation between HRA and HFP measurements (Table 1; Fig. 2). The strongest correlation was observed at 1.75° from the center of the fovea ($r = 0.73$; $P < 0.001$), and no significant correlation was found at 1° ($r = 0.17$; $P = 0.530$). Finally, similar curve profiles were found with both methods, as demonstrated in Figure 3.

DISCUSSION

In the present study, a consistent and significant correlation was found between MPOD values obtained with HRA and HFP in almost all eccentricities tested. HRA values were consistently higher than those from HFP in all locations analyzed, and mean differences between the two methods increased as the MPOD values increased. The strongest correlation between the two methods was observed at 1.75° from the center of the fovea.

Numerous studies have mapped the distribution of MP in the human retina, showing it to be nonuniform, including in the vicinity of the fovea where it is visually discernible.[3,17,29] Moreover, other studies have demonstrated a wide variability among patients[3,30] and among different techniques.[31,32] In the present study, although similar curve profiles were found with both methods, different mean values were obtained from each, with HRA mean values consistently greater than HFP values in all retinal locations. Moreover, the present results show that

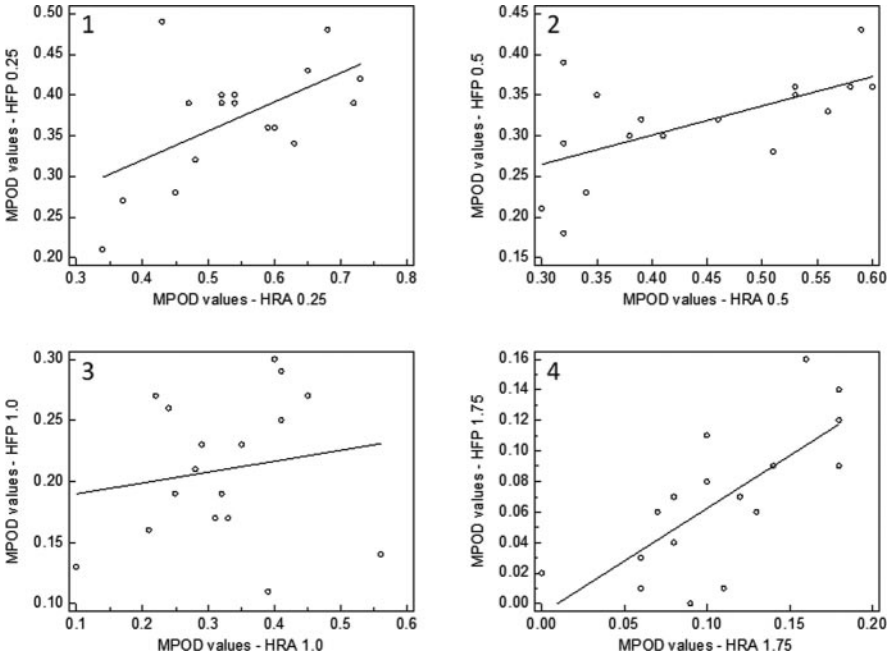

FIGURE 2. Correlation plots between values of macular pigment optical density obtained with the two methods.

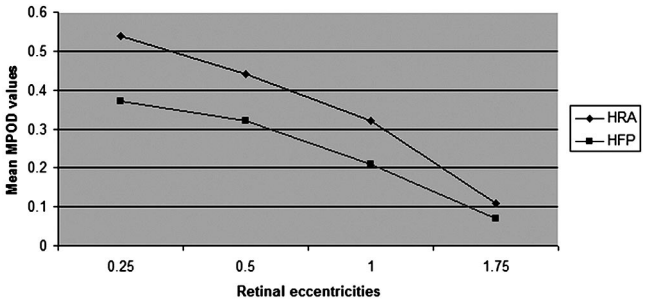

FIGURE 3. Mean macular pigment optical density values at each retinal eccentricity for both tests.

the strongest correlation between the techniques is at 1.75° from the center of the fovea, probably because of lower and closer values with the two techniques in this location.

Previous studies have compared some of the techniques available for the measurement of MP, but little information in the literature regarding the relationship between values from HRA and HFP in either normal or clinical populations can be found. Delori et al.[22] demonstrated that the estimates for MPOD for the same population using the AF spectrometry technique are highly correlated with other tests such as HFP, despite the fact that systematic differences in the estimated density values exist between them. They studied healthy subjects in a wide age range and found a higher average value for MPOD measured with the AF method and a high individual variability for all the methods tested. Based on statistical analysis, the authors found some explanations for discrepancies between the two methods, such as differences in the test field size, self-screening of visual pigment, and the presence of RPE melanin, blood, and secondary fluorophores. Berendschot et al.[33] could demonstrate that MPOD estimates obtained with different objective techniques, including AF, were all age independent in a healthy population. However, using the HFP method, MPOD values showed a small but significant decrease with age. In agreement with our findings, the authors found significant correlation among all methods analyzed. Finally, another study found that although the measurements were correlated, there was a significant difference between values obtained with AF and HFP in patients with type 2 idiopathic macular telangiectasia.[34]

Data from a previous study using HFP indicates that MPOD is determined at the outer edge of the stimulated retinal area rather than the average value over the center of the stimulated retinal area.[3] This effect undoubtedly breaks down, however, with larger stimuli (>2°). The absolute precision of the edge effect for smaller stimuli has not yet been carefully evaluated.[31] Although these results were not consistent with those of more recent studies, one possible explanation may be related to fixation.[32] If the center of a circular stimulus is poorly fixated because of random eye movements, the retinal image will spend only a portion of the time centered on the fovea and the rest of the time off center, where MPOD is lower. Thus, the measured MPOD would be expected to be lower than that obtained with good fixation. In the present study, lower mean values were found with the HFP in all retinal eccentricities when compared with the HRA, despite the fact that a normal population with similar baseline characteristics and with theoretically good fixation was included. Additionally, considering the absolute mean values, the mean difference between MPOD measurements with the two methods increased as MPOD values increased. This fact could be explained by the normal MP curve profile, with higher values closer to the center and lower values more distant from the center of the fovea.

Currently, HFP is the most widely used method for MPOD measurement, and it has been demonstrated as a valid method for subjects without retinal disease.[35] Validity assessments have not been made for subjects with retinal disease despite the use of HFP on these groups, including age-related macular degeneration and choroideremia.[6,36–38] The primary difficulty when using most of the current psychophysical methods relates to task performance. Very young or very old patients and some patients with cognitive or physical impairments simply cannot complete the flicker, threshold, or matching tasks. Psychophysical methods also require usable visual fields and sufficient visual acuity to allow completion of the task.[31]

Fundus AF or reflectance imaging using a two-wavelength method appears suitable for measuring MPOD in retinal and macular diseases, as previously described. Its principle is based on the spectral energy of lipofuscin fluorescence. This fluorescence, however, is based on the type of chromophores that compose lipofuscin, and the question of whether these chromophores change within this area is unknown, particularly for subjects with retinal disease.[34] AF testing represents an objective and rapid method that requires minimal active patient participation, other than steady fixation, unlike psychophysical methods. Most of the physical or optical methods, including AF, are influenced by optical interference from structures either anterior (crystalline lens, inner limiting membrane) or posterior (photopigments and melanin), or possibly both, to the MP. Based on the higher-density estimates obtained with the AF method, Delori et al.[22] hypothesized that it may be applicable in situations in which detailed comparisons are desired and to monitor MPOD changes after nutritional manipulation, particularly in patients with low concentrations of MP. However, this application will require further investigation for validation.

Although subjects for this study were selected prospectively for inclusion, the results should be interpreted with caution because of the small sample size. It is noteworthy, however, that distribution curves for the MPOD from this study are similar to those found in previous studies.[1,2,9,32] Examinations were repeated more than once in each eye, and only good-quality images were used for analysis, but measurements were not repeated on different days as a means of checking reproducibility. Finally, comparison between MPOD values obtained from our study and those reported in some previous studies that have used the AF method is limited because of differences regarding devices and retinal locations at which MPOD was measured. Regarding previously reported MPOD values in healthy patients using the AF method, Delori et al.[22] found higher average values for MPOD measured with this technique (0.48 ± 0.16 density unit [DU] for a 2°-diameter test field) compared with HFP, but a different device was used in that study. Wustemeyer et al.,[19] using the same HRA device we used, demonstrated higher MPOD values calculated from AF images (0.22 ± 0.07 DU) than from reflectance images for a 2°-diameter circle centered on the fovea. Trieschmann et al.[25] found a mean MPOD of 0.12 DU (range, −0.02 to 0.28) at an eccentricity of 2° from the center of the fovea using a dual-wavelength AF imaging.

In summary, the good agreement between the two methods demonstrated in the present study suggests that the modified-HRA AF method for measurement of MPOD is a suitable substitution method for patients unable to perform HPF. The consistency of results also suggests that this method is an appropriate first choice for MPOD assessment. After taking into account the small offset resulting from the higher values of the AF method, it appears that studies conducted with the two methods are essentially comparable. Future trials in clinical populations may provide more opportunity to match the tech-

niques and to develop a better understanding of their correlation in eyes with pathologic conditions.

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