Experimental Eye Research 87 (2008) 445–453

Contents lists available at ScienceDirect

Experimental Eye Research

journal homepage: www.elsevier.com/locate/yexer

The utility of using customized heterochromatic flicker photometry (cHFP) to measure macular pigment in patients with age-related macular degeneration

J.M. Stringham [a,d], B.R. Hammond [a], J.M. Nolan [b,d], B.R. Wooten [c,1], A. Mammen [d], W. Smollon [c], D.M. Snodderly [d,e,*]

[a] Vision Science Laboratory, University of Georgia, Athens, GA, USA
[b] Department of Chemical and Life Sciences, Waterford Institute of Technology, Cork Road, Waterford, Ireland
[c] Walter S. Hunter Laboratory, Brown University, Providence, RI, USA
[d] Department of Ophthalmology, Medical College of Georgia, Augusta, GA, USA
[e] Department of Nutritional Sciences, Institute for Neuroscience, and Center for Perceptual Systems, University of Texas, Austin, TX, USA

ARTICLE INFO

Article history:
Received 27 January 2008
Accepted in revised form 1 August 2008
Available online 20 August 2008

Keywords:
macular pigment
heterochromatic flicker photometry
lutein
zeaxanthin
psychophysics
retina
age-related
macular degeneration

ABSTRACT

The purpose of this study was to assess the utility and validity of using customized heterochromatic flicker photometry (cHFP) to measure macular pigment optical density (MPOD) in patients with intermediate stages of age-related macular degeneration (AMD). The measurement procedure was optimized to accommodate individual differences in temporal vision related to age, disease, or other factors. The validity criteria were based on the similarity of the spectral absorption curves to *ex vivo* curves of lutein and zeaxanthin and the similarity of spatial density profiles to those measured in subjects without retinal disease. Macular pigment optical density (MPOD) spatial profiles were measured with an LED-based macular densitometer; spectral absorption curves were measured with a 3-channel Maxwellian view system including a monochromator. All patients were characterized via clinical exams and all but 2 subjects from whom data were obtained had masked grading of color fundus photographs using the Wisconsin Age-Related Maculopathy Grading System. Most of the patients were in AREDS category 2 (27%) or 3 (57%). Patients with visual acuity as poor as 20/80 were included, and could perform the task as long as they could see the stimulus. Eighty-one percent of the patients screened were able to perform the cHFP task, and data were obtained from 30 AMD patients. Spatial profiles of MPOD were measured in 19 subjects who could see the stimulus at all tested loci. These profiles were highly similar to those that have been measured with HFP in subjects without retinal disease. The average shape of the spectral absorption curves for the AMD subjects corresponded well to an *ex vivo* template. These data support both the utility and validity of the cHFP method for measuring MPOD in subjects with intermediate stages of AMD. The ability to measure the retinal response to nutritional intervention is of practical importance for monitoring patients being supplemented with lutein and zeaxanthin in hopes of retarding visual loss and/or disease progression.

1. Introduction

Age-related macular degeneration (AMD) is caused by slow degeneration of the retina and retinal pigment epithelium (RPE). Although a number of factors contribute to the progression of this degeneration, a central theme appears to be oxidative stress (e.g., Cai et al., 2000; Beatty et al., 2000). The role of oxidative stress is of particular interest because of its potential modifiability. By making healthy life style choices, oxidative stress to the retina and RPE may be lowered, and the probability that an individual will develop AMD could be reduced. One approach to combating oxidative stress, examined in the Age-Related Eye Disease Study (AREDS, 2001), is increasing the dietary intake of antioxidants. For subjects with intermediate stages of AMD, AREDS found that high-dose antioxidant supplementation (including zinc) reduced the risk of disease progression by 25% and the loss of visual acuity by 19%. However, the AREDS supplement did not include lutein (L) and zeaxanthin (Z), the components of the macular pigment, as part of their intervention. A growing body of evidence (e.g., Nolan et al., 2007; Whitehead et al., 2006) has suggested a role for retinal L and Z (L&Z) in protecting the retina and RPE from oxidative damage both by absorbing actinic short-wave light and through antioxidant mechanisms. Moreover, there is evidence that supplementing L&Z

* Corresponding author. Department of Nutritional Sciences Institute for Neuroscience and Center for Perceptual Systems, 1 University Station/A2700, University of Texas, Austin, TX 78712, USA. Tel.: +1 512 232 3307; fax: +1 512 471 5844.
E-mail address: max.snodderly@mail.utexas.edu (D.M. Snodderly).
[1] Proprietary interests. Dr Wooten is a principal of Macular Metrics Inc.

0014-4835/$ – see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.exer.2008.08.005

can improve visual performance for subjects with atrophic AMD (Richer, 1999; Richer et al., 2004). Based on current evidence, one arm of the follow-up AREDS II trial will test whether supplementation with L&Z, along with other nutrients, can retard vision loss and disease progression in subjects with intermediate stages of AMD.

Because subjects vary greatly in the ease with which L&Z is accumulated in the retina as macular pigment (e.g., Hammond et al., 1997a,b, Aleman et al., 2007; Bhosale et al., 2007; Richer et al., 2007) interpreting the outcome of supplementation trials in patients with AMD would be greatly facilitated by measuring the amount of the supplement that accumulates in the retina. If patients benefit from supplementation, one could gain insight into how much macular pigment is needed to derive a benefit. If patients fail to benefit from supplementation, it would be possible to distinguish whether (1) the lack of benefit was due to the failure of L&Z to be incorporated into the tissue, or (2) L&Z were incorporated into the retina but they were not effective in modifying the course of the disease at this stage. If supplementation did not increase macular pigment, it would be appropriate to study ways to improve L&Z uptake in the digestive system and L&Z accumulation in the retina, perhaps by modifying fat intake or lipoprotein profiles (e.g., Wang et al., 2007; Snodderly et al., 2004b). If outcome 2 occurred, effort could be concentrated on other stages of the disease or on evaluation of alternative therapies. Obviously, there could be individual differences, with some patients benefiting and others not. Knowing their macular pigment status might help to determine why individuals differed in their response to the intervention.

The study of L&Z as a potential nutritional therapy represents an unusual opportunity. Unlike most nutrients in tissues, L&Z can be measured as macular pigment using non-invasive *in vivo* methods. In this study, we evaluate whether heterochromatic flicker photometry (HFP), the most common non-invasive means of measuring macular pigment, is useful for patients with intermediate stages of AMD. For this method, the subject must judge the presence or absence of flicker when a properly configured stimulus alternates between a wavelength that is absorbed by MP and one that is not. An eccentric location outside the fovea, where MP levels are generally considered to be optically undetectable, serves as a reference (see Snodderly and Hammond, 1999). The validity of the HFP method in both young and older subjects has been the subject of several recent papers (Snodderly et al., 2004a; Hammond et al., 2005; Wooten and Hammond, 2005; Gallaher et al., 2007). These papers have provided strong evidence that the technique is both highly reliable and valid in normal subjects across the lifespan. Although HFP and a number of other methods have been used to measure MP in AMD patients, until now none of the MP measurement methods have been validated on patients, who have altered retinas and often have cloudy ocular media.

The advantages of HFP (when customized to provide optimal accuracy) include the following: (1) Pupil dilation is not required. (2) High light levels are not required. (3) The results are relatively insensitive to the state of the ocular media (e.g., Wooten et al., 1999; Ciulla et al., 2001; Gallaher et al., 2007; Ciulla and Hammond, 2004). (4) The results are not confounded by head movements (e.g., Wooten et al., 1999). (5) Testing can be optimized to control for individual differences in temporal vision. Optimization of temporal parameters is especially important in AMD patients, who tend to have decreased sensitivity to higher temporal frequencies (e.g., Falsini et al., 2000; Phipps et al., 2004). (6) Measurements can be integrated with other tasks in a single study visit. Elderly participants can complete a standard measurement at one eccentricity in 20–30 min (e.g., Snodderly et al., 2004a; Gallaher et al., 2007) and this location (0.5° eccentricity) captures most of the variance between subjects (e.g., Snodderly et al., 2004a; Mares et al., 2006).

The most serious limitation of HFP is that it requires the active participation of the subject, who must make accurate perceptual judgments of the presence or absence of flicker. For normal older subjects tested with optimized protocols, more than 80% of naïve subjects can perform the procedure (e.g., Snodderly et al., 2004b; Iannaccone et al., 2007). In the largest study on naïve subjects to date ($n = 1158$), comparison of data collected at two wavelengths confirmed that the values were consistent with the spectral absorption curve of MP (Snodderly et al., 2004b). In the present paper, we evaluate the utility of a particular implementation of HFP for measuring MP in patients with AMD. To distinguish our approach from other variants of HFP we refer to it as "customized HFP (cHFP)" because, among other refinements, the flicker frequency is individually customized to the visual capabilities of each subject. Using cHFP, we find that patients with AMD are about as proficient as normal elderly subjects in performing the flicker task, as long as the stimulus is clearly visible to them. Furthermore, their data are consistent with spectral and spatial criteria for a valid measurement of macular pigment (e.g., Snodderly, et al., 2004a; Hammond et al., 2005).

2. Methods

2.1. Subjects

MPOD data were obtained from 28 subjects who were recruited in and around Augusta, GA, USA, and tested at the Medical College of Georgia. Two additional subjects were recruited at other sites, one at the Schepens Eye Research Institute in Boston, MA, and one at Brown University in Providence, RI, USA, making a total of 30 patients from whom data were obtained. The study followed the Tenets of the Declaration of Helsinki. All procedures were approved by the institutional review boards at the respective sites and informed consent was obtained from each subject.

All subjects underwent careful ophthalmic examination prior to testing. The most detailed information was collected for the GA subjects. Personal and clinical data for them are provided in Tables 1 and 2. Information about the 2 subjects from the other sites are provided in the legends for Figs. 3 and 4 where their spectral data are presented. The GA subjects were clinically characterized using masked grading of color stereo fundus photographs based on the Wisconsin Age-Related Maculopathy Grading System (Age-Related Eye Disease Study Research Group, 1994). Intraocular pressure was measured by Goldman tonometry. Subjects were excluded if they had diabetic retinopathy or advanced cataract (3+ or above on a scale of 1 to 4). Best corrected visual acuity after standardized refraction was determined with ETDRS charts, using logMAR distance visual acuity as the criterion. Subjects were excluded if ETDRS acuity was worse than 20/80. Subjects with localized scotomas that did not include the center of the macula, or with glaucoma but no visual field defects, were included. Based on the inclusion criteria, 37 GA subjects were screened as potential participants. Six of the subjects had difficulty seeing the small (5′ diameter) fixation point, and had to be removed from the subject pool. One subject was excluded from the study due to cognitive impairment, resulting in a sample of 28 GA subjects available for our study.

All of the subjects tested in GA completed a personal data questionnaire about health and personal history, including smoking. Iris and skin color were assessed by questionnaire. A crude indicator of dietary intake and bioavailability of L and Z was computed, based on the frequency of consumption of 5 food items (dark green leafy vegetables, colored fruits and vegetables, eggs, fish, and overall fat intake) with examples given. The frequency of consumption was scored as follows: 0, less than once a week; 1; once a week; 2, 2–3 times per week; 3, 4–6 times per week; 4, once

Table 1
Personal characteristics of GA subjects

Subject number	Sex	Age	Smoking frequency (pack years)[a]	Iris color	Diet score[b]	Supplement use
1	Male	74	0	Blue	5	No
2	Male	81	0	Light-hazel	12	O (no L)
3	Female	65	0	Blue-green	6	No
4	Male	74	0	Blue	10	No
5	Male	71	0	Blue	11	No
6	Male	70	~50	Green	2	O, L (5–6 years)
7	Female	80	~55	Green	8	O, L
8	Male	77	~30	Gray	2	L (~2 years)
9	Female	69	~51	Brown	7	O, L (~ 2 years)
10	Female	68	~10	Blue	11	P (no L) (7 years)
11	Female	63	0	Hazel	8	No
12	Female	71	0	Brown	13	L, P (2 years)
13	Female	73	~40	Hazel	9	P (no L) (10 years)
14	Female	71	~20	Gray	7	O, L (2–3 years)
15	Female	73	0	Black	13	No
16	Female	57	0	Green	7	L (2 years)
17	Male	72	~10	Hazel	10	O, L (1.5 years)
18	Male	71	~50	Brown	2	No
19	Female	70	~12.5	Brown	7	O, L (>5 years)
20	Male	68	~4	Blue	5	O, L (3 years)
21	Female	65	~36	Blue	8	No
22	Female	82	0	Gray	11	No
23	Male	75	~20	Hazel	3	No
24	Female	55	0	Green	3	No
25	Female	60	~12	Brown	8	No
26	Female	64	~32	Hazel	3	L (2 months)
27	Female	67	~25	Brown	6	No
28	Female	61	~82	Brown	7	No
29	Male	72	0	Brown	6	P (no L) (3 years)
30	Female	81	~20	Blue	8	O (no L) (1–1.5 years)

L, Lutein; O, Ocuvite; P, Preservision.
[a] Pack years: calculated by multiplying the number of packs of cigarettes smoked per day by the number of years the person has smoked.
[b] Diet score: determined by frequency of consumption of 5 food items (dark green leafy vegetables, colored fruits, eggs, fish, and overall fat intake).

a day; 5, more than once a day. Dietary fat intake (e.g. fried foods, snack foods, cheese, foods cooked in butter) was assessed due to its role in facilitating carotenoid absorption from the gut (Roodenburg et al., 2000; Unlu et al., 2005; fat intake was scored from 1 to 5 as outlined above), whereas fish intake was assessed (scored from 1 to 5, as outlined above) due to its high concentration of *n*-3 docosahexaenoic acid, which has been shown to influence MP concentration (Johnson et al., 2008). In this way, a cumulative score for all food items was assigned to each person that could range from 0 to 15.

2.2. Measurement of macular pigment optical density using cHFP

The eye with better vision was chosen for measurement. At the GA site, 19 of the 28 subjects were able to complete full MP spatial density profiles consisting of measurements at 6 retinal loci (Fig. 1). MPOD at 0.5° eccentricity and at two wavelengths (460 and 500 nm) was assessed in all 28 of the GA subjects (Fig. 2). For the GA subjects, MPOD spatial profiles were measured using a macular densitometer (Macular Metrics Corp., Providence, RI) slightly modified from the one described by Wooten et al. (1999). The modified instrument and the principle of HFP have been fully described in earlier publications (Snodderly and Hammond, 1999; Snodderly et al., 2004a,b). There are, however, some methodological considerations crucial to the accurate measurement of MPOD with HFP worth emphasizing here. Because of individual differences in temporal sensitivity, light transmission of the ocular media, and perceptual differences at different loci in the foveal region, best results are achieved by customizing the HFP task for each subject. As mentioned in Section 1, we have termed this "cHFP," for customized HFP, and it includes the following considerations. First, individual selection of the best flicker frequency for the stimuli enables one to account for the range of normal variation in flicker sensitivity (e.g., Falsini et al., 2000; Tyler, 1989). If flicker

Table 2
Clinical description of GA subjects

Subject	Snellen acuity	IOP	PG	Cataract status	AREDS category
1[a]	20/60 −2	9	Yes	2+ NS	3
2	20/60 +1	14	No	Pseudophakic	3
3	20/30	11	No	Pseudophakic	2
4	20/30 −2	15	Yes	–	2
5	20/30	16	No	Pseudophakic	3
6	20/40 −1	16	Yes	1+ NS	3
7	20/50 −1	14	Yes	1+ NS	3
8	20/50	11	Yes	Pseudophakic	2
9	20/50	23	No	1+ NSC	2
10[a]	20/30 −1	12	Yes	Pseudophakic	1
11	20/30	12	No	–	1
12	20/60 −2	9	No	Pseudophakic	4
13	20/80 +1	18	Yes	2+ NS	3
14	20/50 −1	13	No	1+ NS	3
15	20/30 −2	19	Yes	2+ NSC	3
16[a]	20/40	15	No	–	3
17	20/25	11	Yes	Pseudophakic	2
18	20/50 +1	11	No	1+ NS	2
19	20/30 −2	17	No	Pseudophakic	3
20	20/25 −2	10	Yes	2+ NS	1
21	20/25 −1	17	Yes	1+ NS	3
22	20/60 −1	15	Yes	1+ NS	3
23	20/30	22	No	1+ NS	3
24	20/25 −1	13	Yes	–	3
25	20/25	13	Yes	–	2
26	20/80 −1	22	Yes	1+ NSC	3
27	20/25	11	No	NSC	3
28	20/50 +1	15	Yes	Pseudophakic	1
29	20/30 −2	19	Yes	1+ NS	2
30	20/30 −2	19	Yes	Pseudophakic	3

All descriptions pertain to the study eye. IOP, intraocular pressure; PG, pigmentary changes. Color fundus photographs were obtained for each subject and disease stage was graded by the Wisconsin Reading Center. Lens status was classified as either post-cataract pseudophakes, an intact lens with no overt signs of cataract (indicated by a – sign) or cataractous. Cataract severity is indicated numerically and type is specified as NS, nuclear sclerosis; NSC, nuclear sclerosis cataract.
[a] Subjects 1, 10 and 16 had neovascular AMD in the fellow eye.

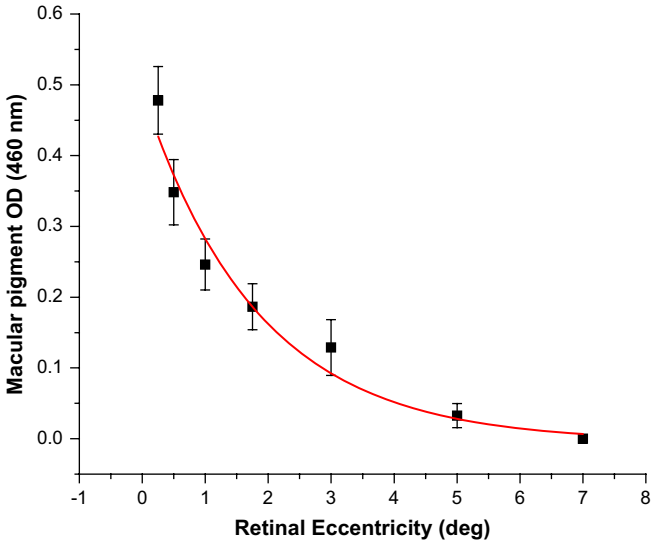

Fig. 1. Peak MPOD versus retinal eccentricity for 19 AMD subjects. The smooth line is a least-squares fit with a first-order exponential ($y = 0.64^{-x/1.4}$; $r^2 = 0.99$). Error bars represent standard error of the mean.

sensitivity is not accounted for and a fixed flicker frequency is used, a subject with low flicker sensitivity (low critical flicker fusion frequency (CFF)) will not see flicker over a wide range of settings and will experience a large null flicker zone. Although the subject will be able to complete the task by eliminating flicker from the test target, settings will be variable, and subjects may exhibit systematic bias toward one end of the null range, resulting in either over- or underestimation of MPOD. Alternatively, a subject with a high CFF may not be able to eliminate flicker from the test target, making the task difficult to complete. As implemented by Snodderly et al. (2004a,b) the flicker sensitivity issue can be addressed by utilizing a CFF task involving a single wavelength (570 nm) outside the absorption band for MP. Based on the subject's CFF, the optimal HFP flicker frequency, which facilitates good subject performance and reduces measurement error, can be estimated. Additionally, an algorithm (described later) can be developed to estimate optimal HFP flicker frequencies for each retinal locus, including the reference locus. The optimization of HFP flicker rate is particularly

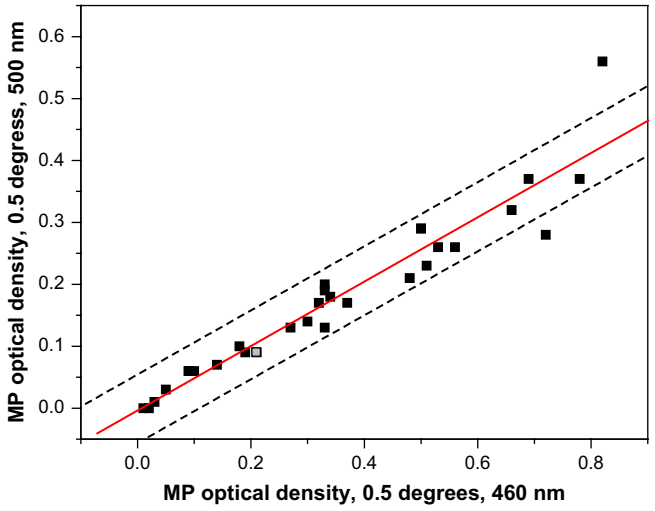

Fig. 2. MPOD measured with a one-degree diameter test stimulus at 460 nm and 500 nm The solid line is the least-squares line bracketed by dashed lines representing ± 0.05 OD from that line. Subject S12, with category 4 AMD is indicated by the gray box.

important for older subjects and for subjects with AMD who often demonstrate significant slowing in their temporal vision (e.g., Falsini et al., 2000; Phipps et al., 2004).

The second methodological consideration involves a test stimulus configuration in which the radiance of the short-wave test component absorbed by macular pigment is inverse-yoked with the mid-wave reference component. In other words, when a subject increases the short-wave component to account for macular pigment absorbance, the radiance of the mid-wave component is commensurately decreased, and vice versa. This procedure keeps the brightness of the test stimulus relatively constant. We regard this change as an improvement because subjects, otherwise, could confuse changes in brightness with changes in flicker (e.g., the dimmer settings could be confused with less flicker).

2.2.1. Procedure

The procedure used in the present study was essentially identical to that described in Snodderly et al.'s (2004a,b) CAREDS publication, including the viewing of a training video, determination of the subject's CFF, and development of an algorithm to predict flicker frequency values appropriate for the different loci tested under cHFP conditions. To familiarize subjects with the cHFP task and to collect data regarding macular pigment spectral absorption, subjects were first tested at a wavelength of 500 nm, presented in square-wave counterphase with 570 nm, a task that subjects found relatively easy to perform. Then subjects were presented with the 460 nm light, in square-wave counterphase with a 570 nm light to measure MPOD at its peak absorption, which provides the best signal-to-noise ratio.

For the 500 nm condition, an MPOD measure was obtained only at 30′ eccentricity, relative to a reference location at 7° eccentricity. For the 460 nm condition, measures at 15′, 30′, 1°, 1.75°, 3°, and 5° eccentricities were obtained along the horizontal meridian of the temporal retina (for the right eye) or nasal retina (for the left eye), relative to a reference location at 7° eccentricity. For the measures at 15′ and 30′ eccentricities, test stimuli were solid disks with those radii, with a small black fixation dot in the center. With these stimuli, when the flicker frequency is optimized as described below, the edge of the stimulus has been shown to determine the no-flicker threshold (e.g., Hammond et al., 1997a,b; Hammond and Caruso-Avery, 2000; Werner et al., 1987; Smollon et al., 2005). For the 1° and 1.75° eccentricities, centrally fixated, 20′ wide annuli with mean radii corresponding to those eccentricities were used. At eccentricities further from the fovea, the test stimulus was viewed eccentrically, using a small red light-emitting diode as a fixation target. The red fixation diode was 5′ in diameter and it was set at a luminance of approximately one log unit above the detection threshold for normal subjects. For the 3° locus, a solid disk with 1°-diameter was centered 3° from fixation, and for the 5° locus and 7° reference target, a 2°-diameter solid disk centered on those loci was used. The order of stimulus presentation was as follows: 30′, 7°, 1°, 1.75°, 3°, 5°, and 15′.[2] The HFP flicker algorithm (based on a subject's CFF) produced the following values: 30′ eccentricity = CFF − 6, 7° = CFF − 12, 1° = CFF − 6, 1.75° = CFF − 7, 3° = CFF − 7, 5° = CFF − 11, and 15′ = CFF − 7. For each stimulus condition/location, the CFF was measured as the average of three determinations.

[2] Note that the smallest stimulus was presented last. This is because subjects, particularly those with eye disease, have the greatest difficulty with the smallest stimuli. Since for some subjects this can be frustrating and fatiguing, we elected to present this stimulus last. Based on similar logic, we presented the conventional relatively easy stimulus with 30′ radius and the 7° reference stimuli first. If subjects could not perform the task at the reference locus, there was no reason to continue testing.

Overall, the values produced by the algorithm for the cHFP determination yielded acceptably low variability for 12 of the 19 subjects. For 7 of the subjects, however, the algorithm predicted a frequency for the HFP measurements that was either too high ($n = 2$) or too low ($n = 5$) by about 1–2 Hz. In these situations the experimenter adjusted the frequency of the flickering stimulus, in steps of 1 Hz, either increasing it (if the subject could not eliminate flicker) or decreasing it (if the subject exhibited a wide range of null flicker values). The range of null flicker was considered too wide if the subject provided values that were separated by more than ~15% radiance units (~0.05 MPOD).

Test stimuli were presented in natural view near the center of a 6-degree, 2.75 cd/m^2, 470-nm circular background. The test stimulus was alternately composed of a 458-nm or a 500-nm measuring field (the peak and shoulder of macular pigment absorbance) and a 570-nm reference field (minimal macular pigment absorbance). Light for the measuring and reference fields and the background was produced by 20-nm half-bandpass light-emitting diodes (LEDs) with peak energy at 458, 500, 570, and 470 nm, respectively (Nichia Corp., Mountville, PA). The radiance of the LEDs was controlled by constant current, high frequency electronic pulses. The measuring and reference fields were superposed and presented out of phase with an alternation rate optimized (as described above) for each subject and for each condition. Once optimized, subjects adjusted the radiance of the 458-nm or 500-nm measuring field (which was counterbalanced with the 570-nm reference in order to maintain constant luminance) until a no-flicker point was achieved. This measurement was done in the fovea (where macular pigment is most dense) and 7-degrees in the parafovea (where light absorption by macular pigment is negligible). The OD of MP is calculated as:

$$\mathrm{OD}_{\lambda} = \log\left(R^{\mathrm{f}}_{\lambda_s}/R^{\mathrm{p}}_{\lambda_s}\right) - \log\left(R^{\mathrm{f}}_{\lambda_\gamma}/R^{\mathrm{p}}_{\lambda_\gamma}\right) \quad (1)$$

where R=radiance of the light, λ_s refers to a short-wave light in the absorption band of MP, e.g., 460 nm, λ_γ refers to a long-wave light outside the absorption band of MP, e.g., 530 nm, f refers to a retinal locus containing a significant concentration of MP, and p refers to a parafoveal locus containing negligible, MP OD (e.g., 7°, in our case).

As a further evaluation of the validity of the cHFP method for use in AMD patients, we derived macular pigment spectral absorption curves for 7 subjects, 5 of whom were selected from the GA sample. These subjects had MPOD at 30′ eccentricity of at least 0.30 at 460 nm, were available for repeat testing, and performed well on the densitometer. Spectral curves were derived for a centrally fixated test stimulus with a 30′ radius using a 3-channel Maxwellian-view optical system diagrammed in Wooten et al., 1999. The Maxwellian-view system was used because it allowed a full spectral density curve to be measured; the densitometer has a limited set of LEDs and therefore measures MPOD only at 460 nm and 500 nm. Care was taken to use the same stimulus conditions and protocol at all three sites for all subjects.

One channel of the Maxwellian view system provided a 15°, 460 nm background (2.2 log Trolands) that passed through an interference filter with half-bandpass of 7 nm. Two other channels were combined to form the test stimulus, which consisted of a variable-wavelength measuring field, alternating with a fixed-wavelength reference field. The flickering test stimulus was always presented in the center of the background field. The wavelength of the measuring field (420–550 nm) was determined using a 500 mm grating monochromator with a 6 nm half-peak bandwidth (Bausch and Lomb, Inc.) in conjunction with blocking filters to eliminate higher order spectra and stray light. The fixed wavelength of the reference beam was selected by an interference filter with 7 nm half-bandpass. The energy output of the system was checked daily using a calibrated radiometer (United Detector Technology Corp). The measuring and reference fields were superposed and presented in square-wave alternation using a sectored mirror. As with the densitometer, the flicker rate was carefully optimized for each subject so that flicker could be completely eliminated with a narrow null zone (about 0.07 OD). An achromatizing lens was placed at a point conjugate with the subject's pupil in order to minimize differences in the effects of chromatic aberration between the measuring and reference fields. An auxiliary channel with a calibrated reticle was used to align the subject's pupil, and to ensure that the image of the xenon arc light source was centered within the subject's pupil and in focus in the plane of the pupil. A dental-impression bite-bar and adjustable forehead rest was used for head stabilization. The procedure used to measure MPOD was nearly identical to that described above for the densitometer.

3. Results

The personal characteristics for each subject are listed in Table 1. About half of the subjects were taking supplements that contained L and Z. The subjects that supplemented, however, did not have significantly higher MPOD levels as a result ($p < 0.90$). A short clinical description for each subject is provided in Table 2. Most subjects were well described by the age-related eye disease study (AREDS) categories 2 and 3 (category 1, no ARM with the exception of a few small drusen; category 2, extensive small drusen, pigment abnormalities, or at least 1 intermediate size drusen; category 3, extensive intermediate drusen, geographic atrophy not involving center of macula or at least 1 large drusen; category 4, advanced AMD).

We used two main approaches to assess the validity of the cHFP method for measuring MPOD on these subjects. The first approach was to measure the spatial distribution of MPOD of the GA subjects using the macular densitometer. Nineteen of the 28 GA subjects were able to provide data for all tested eccentricities. The remaining 9 subjects were all able to perform the task with the centrally fixated 1°-diameter (30′ radius) target but were unable to do the task at one or more of the other standard eccentricities because of compromised vision at some loci. For the full group of 28 subjects, the mean MPOD value at 460 nm and 0.5° eccentricity was 0.37, SD = 0.24. Table 3 shows MPOD values measured at the spatial peak, and at 0.5° eccentricity, as well for all subjects who could provide a measurement at 15′ eccentricity. In addition, the half-widths of the spatial profile derived from a piece-wise linear fit and from an exponential fit to the data are listed. Fig. 1 shows the average of the 19 complete spatial density profiles. The mean data are graphed along with the best-fitting exponential decay with eccentricity, which explains most of the variance ($r^2 = 0.99$). As shown in Table 3, a first order exponential function explains most of the variance for individual subjects as well for the mean (range of $r^2 = 0.90$–0.999).

The second approach we used to assess validity was to compare the obtained spectral absorbance curves to *ex vivo* measures (from Table 1 of Hammond et al., 2005). As a first step, for the GA subjects we measured MPOD at two spectral points, 460 and 500 nm. These data are shown in Fig. 2. From the known macular pigment spectrum, a proportionality of 0.56 at the two spectral points is predicted (Snodderly et al., 2004a,b), which should be the slope of the line fit to the data. Our slope was 0.52 and the variance around the line was small (the dashed line indicates ±0.05 OD from the regression line).

As a more extensive test, Figs. 3 and 4 present the complete spectral measurements (using a one-degree test at the 0 degree locus) for 7 of the AMD subjects, 5 from GA, and 1 each from Boston and from Brown University. The 5 GA subjects were selected based on having high enough MPOD that a good spectral curve could be

Table 3
Spatial distribution data for AMD subjects

Subject	MPOD (15′ eccentricity)	MPOD (30′ eccentricity)	Variance explained (r^2) by a 1st order exponential decay	Exponential half-width[a]	Linear half-width[b]
1	0.2	0.17	0.98	0.78	1.23
4	0.59	0.51	0.98	1.37	1.67
5	0.69	0.55	0.97	0.58	0.80
7	0.7	0.6	0.93	1.96	2.33
9	0.44	0.38	0.96	1.83	2.37
10	0.24	0.19	0.90	0.48	0.45
13	0.75	0.64	0.95	0.49	0.79
14	0.9	0.75	0.97	0.58	0.94
15	0.41	0.32	0.98	1.54	1.97
16	0.38	0.33	0.93	0.12	0.40
17	0.83	0.71	0.97	0.98	1.22
18	0.51	0.4	0.97	1.81	2.62
21	0.8	0.7	0.99	1.18	1.42
22	0.48	0.38	0.97	0.92	0.85
23	0.89	0.7	0.97	1.07	1.0
26	0.3	0.26	0.98	0.60	0.89
27	0.65	0.54	0.99	0.91	0.95
29	0.6	0.47	0.94	0.88	0.98
30	0.49	0.43	0.98	0.76	0.95
Mean	0.57	0.47	0.96	0.99	1.25

[a] The exponential half-width was taken as half the exponential intercept with eccentricity based on the best-fitting 1st order exponential decay function.
[b] The linear half-width is based on the peak density (based on the 15-min MPOD value) with the eccentricity based on a linear interpolation between points.

derived, as well as the impression that they performed the cHFP task well. As shown in the figures, the data compare well to the *ex vivo* template (smooth line). The biggest discrepancy was at 420 nm where the data underestimate the *ex vivo* curve by about 0.07 on average.

Finally, for 4 of the AMD subjects in GA, we evaluated the time necessary to complete a complete measurement protocol. This included the instructional video, assessment of their critical flicker fusion threshold, all spatial points, and two spectral points. On average, these subjects took 27 min to complete the assessment.

4. Discussion

The results from this study demonstrate that valid macular pigment measurements can be obtained in a reasonable time using carefully customized HFP (cHFP) in patients with intermediate AMD, as long as they have visual function sufficient to clearly see the stimulus. This conclusion is based on the similarity of the spectral curves to the *ex vivo* spectrum and the concordance of the MPOD spatial distributions of AMD patients with distributions measured in normal subjects (Table 3 and Fig. 1). Like the current results, past studies using HFP on normal subjects (e.g., Hammond et al., 1997a,b; Wooten and Hammond, 2005) have shown that the macular pigment distribution of most subjects can be fit by a first order exponential decay with eccentricity that captures most of the variance. Furthermore, the lateral extent of the macular pigment distribution of the AMD patients was similar to data from subjects without retinal disease. For example, Hammond et al. (1997a,b) reported an average exponential and linear half-width of about 0.95 and 1.03 degrees, respectively for normal subjects, which is similar to our values for AMD patients (0.99 and 1.25, respectively). As Alexander et al. (1987) originally suggested, disease state can cause morphological disturbances in cone axons and this could conceivably change MPOD spatial profiles. Our data suggest that, at

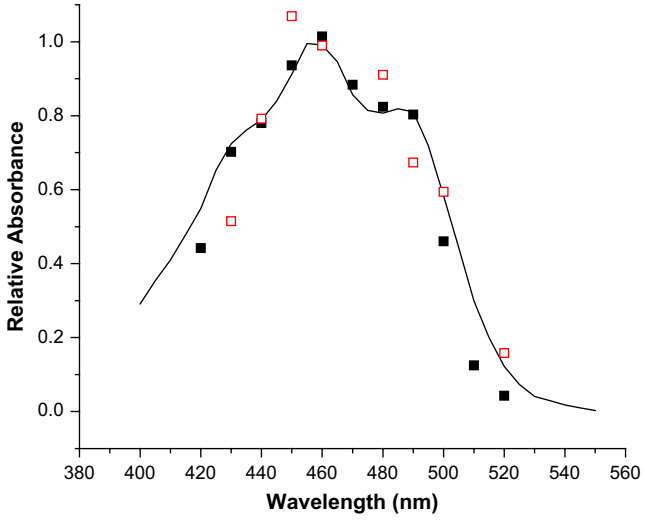

Fig. 3. The macular pigment absorption spectrum as measured in two subjects with early geographic AMD. One 61-year old female with early geographic AMD assessed in Boston (solid squares). One 51-year old female with early geographic AMD assessed at Brown University in 2001. The latter patient first presented with multiple yellow drusen in the macula of both eyes in 1985 that persisted and were enlarged when examined again in 1987 and 1989. The drusen appeared similar at the last examination in 2001. The smooth line represents the *ex vivo* spectrum of L and Z from Table 1 of Hammond et al. (2005).

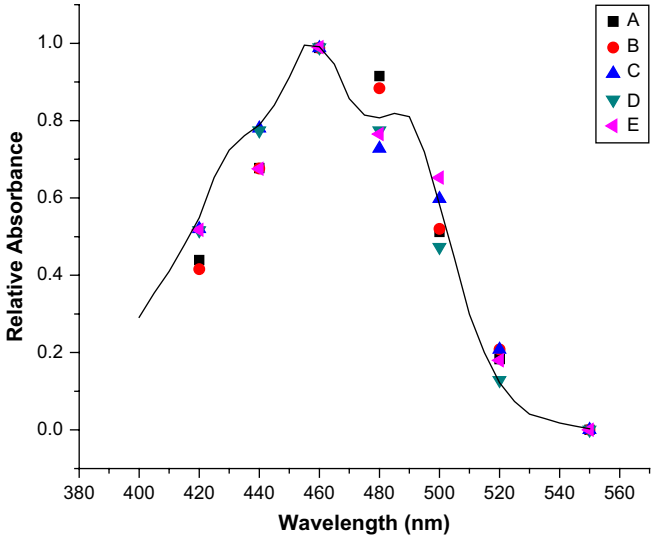

Fig. 4. The macular pigment absorption spectrum as measured in 5 subjects with early to moderate stages of AMD. Letter symbols on this graph correspond to fundus photographs presented in Fig. 5A–E. Brief clinical histories are outlined in the legend of Fig. 5.

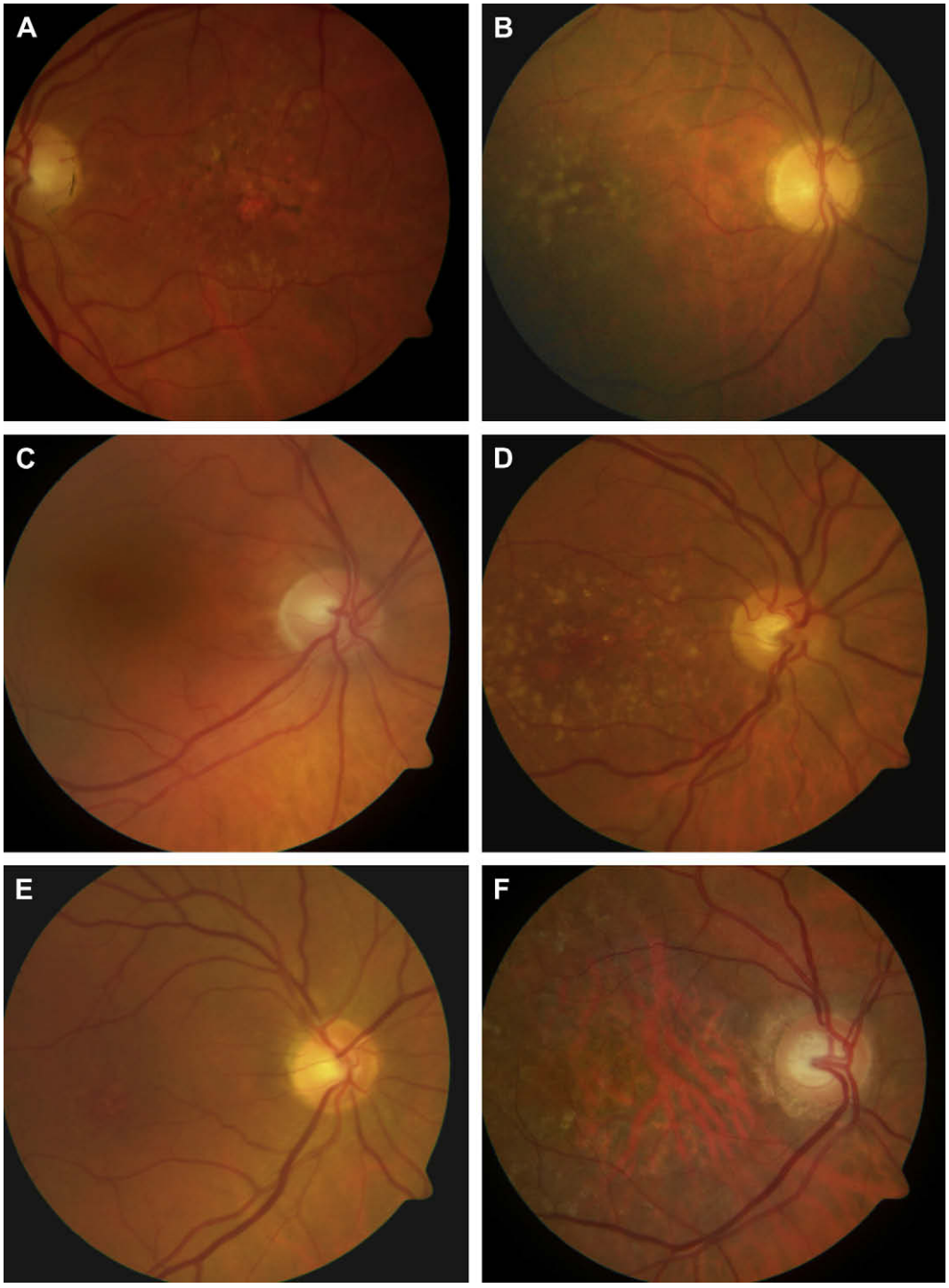

Fig. 5. Fundus photographs of selected subjects included in this study. (A–E) Photographs from subjects who provided the spectral data in Fig. 4. (A) 74-year-old male (S1, AREDS 3) with geographic atrophy characterized by some central RPE depigmentation and increased pigment and multiple soft and indistinct drusen. (B) 81-year-old male (S2, AREDS 3) with geographic atrophy characterized by multiple large indistinct drusen outside of the macula. (C) 65-year-old female (S3, AREDS 2) with geographic atrophy characterized by multiple small hard drusen, some in the macula. (D) 74-year-old male (S4, AREDS 2) with geographic atrophy characterized by RPE depigmentation and increased pigment, as well as large indistinct drusen; both involving the macula. (E) 71-year-old male (S5, AREDS 3) with multiple soft indistinct drusen outside of the macula. (F) Subject S12 whose results are also indicated by the gray box in Fig. 2. This patient performed well on the cHFP task in spite of having AREDS category 4 AMD.

least in intermediate stages of AMD, morphological changes are not yet severe enough to strongly alter macular pigment spatial profiles.

A limitation of our study is the small number of spatial points that were sampled. Although there are clearly individual deviations from the simple exponential function (e.g., flanking shoulders, Hammond et al., 1997a,b; Delori et al., 2006), more detailed analyses would be necessary to establish their characteristics in subjects with AMD.

We also measured MPOD at 460 and 500 nm on a sample of 28 AMD subjects with no psychophysical experience. Our results can be directly compared to the outcomes in the CAREDS study of older healthy adults (Snodderly et al., 2004a,b), using the same model of macular densitometer that we used. That study found that a strong

relation ($r^2 = 0.80$) between MPOD measured at the two spectral points with the ratio (slope = 0.60) close to that predicted from the *ex vivo* spectrum of L and Z (slope = 0.56). For our AMD subjects, with a similar average age, we found an even stronger relation ($r^2 = 0.92$) with proportionality similarly close to the predicted value (0.52). These data suggest that the AMD subjects were performing as well as subjects without retinal disease but similar in age and psychophysical experience. This consistency was striking since some of our subjects had relatively severe disease. For example, subject S12 (see Fig. 5 for the fundus photograph) had category 4 AMD yet accomplished the cHFP task with accuracy (indicated by the gray box symbol in Fig. 2).

To further test the validity of our measurements, we measured 7 AMD subjects at multiple wavelengths in three different laboratories (all using Maxwellian-view systems and similar stimulus conditions and procedures). These data, along with the accompanying fundus photographs, are shown in Figs. 3 and 4. There is a very close correspondence between the spectral curves measured with cHFP and the extinction spectrum of L&Z. The largest consistent discrepancy appears to be at the shorter wavelengths. For example, an average underestimation of about 0.07 was found at 420 nm. This is very similar to the underestimation found by Wooten and Hammond, 2005 for normal subjects without retinal disease. Wooten et al. suggest that the underestimation might be due either to fluorescence of the lens or fundus (essentially adding to the signal) or additional absorbance by the ancillary pigments P410 and P435 identified by Snodderly et al. (1984). In either case, this underestimation appears to be a characteristic of the HFP method as opposed to being specific to the AMD sample.

In conclusion, our data suggest that the cHFP method yields valid results for subjects with intermediate AMD and with visual function that is adequate to see the stimulus. It appears that the method can be utilized for most patients, as long as a moderate level of acuity remains. For example, two of our subjects (see Table 3) had relatively poor acuity (20/80) but could still perform the task and yielded valid data according to our criteria. In particular, they had maximum MPOD at the fixation locus, which is consistent with using the center of the fovea to fixate. Of course, patients who have central scotomas and/or use a nonfoveal fixation locus (Schuchard, 2005) would need to have MPOD measured using a different technique. In such cases, the MPOD values would need to be independently referenced to retinal landmarks.

Our pilot study ($n = 4$) showed that the entire measurement procedure could be completed within about 30 min for naïve AMD subjects. This result obviates a common concern when considering use of this method – namely, that the measurement procedure takes too long. Consistent with this conclusion, Iannaccone et al. (2007) used HFP with 157 subjects with an average age of about 79 years and obtained a standard MPOD measure (1-degree test in one eye) with a mean time of 20 min.

Another major concern is what fraction of AMD patients can be studied with cHFP. Based on the 37 patients we screened, our experience indicates that useful information can be obtained from about 80% of patients with intermediate AMD. This is about the same success rate as found for normal older subjects tested with optimized protocols (Snodderly et al., 2004b; Iannaccone et al., 2007). For cHFP, the factors that limit applicability are primarily compromised visual or cognitive function of the patient. It would be useful to have comparable estimates of success rates for optical methods for measuring MPOD, which are more likely to be limited by inadequate pupillary dilation, deterioration of the visual optics, or concerns about high light exposures.

The ability to assess MPOD in patients with intermediate AMD offers the opportunity to investigate many interesting questions regarding the efficacy of dietary intervention for this group. For instance, does the retinal degeneration of patients with higher levels of MPOD progress at the same rate as patients with similar characteristics (age, sex, iris color, etc.) but lower levels of macular pigment? Richer et al. (2004, 2007) have shown that supplementing with L&Z can improve visual function in elderly veterans with AMD. Is this improvement mediated through an optical mechanism and/or by improved functional status of the retina? If the macular pigments can improve vision through an optical mechanism (e.g., by reducing photostress and glare discomfort or disability; Stringham and Hammond, 2008) then supplementation might be valuable even if it did not slow the disease progression. On the other hand, the elderly veterans studied by Richer et al. may represent a group with particularly deficient diets for whom supplementation corrected frank nutritional deficiencies and slowed disease progression. The ability to obtain accurate macular pigment measures in AMD subjects will help to inform these and other issues.

Acknowledgements

Supported by grants from Gustavus and Lousie Pfeiffer Research Foundation and Fight for Sight, Research to Prevent Blindness (PD04042). We thank Julian J. Nussbaum for access to patients with AMD, and the Fundus Photography Reading Center of the University of Wisconsin, Madison for grading fundus photographs of the patients.

References

Age-Related Eye Disease Study Research Group, 1994. Wisconsin Age-Related Maculopathy Grading System; AREDS Summary Grading Protocol, AREDS Manual of Operations, Appendix 15B. The EMMES Corporation, 11325 Seven Locks Road, Suite 214, Potomac, MD 20854.

Age-Related Eye Disease Study Research Group, 2001. A randomized, placebo-controlled, clinical trial of high-dose supplementation with vitamins C and E, beta carotene, and zinc for age-related macular degeneration and vision loss. AREDS Report No. 8. Arch. Ophthalmol. 119, 1417–1436.

Aleman, T.S., Cideciyan, A.V., Windsor, E.A.M., Schwartz, S.B., Swider, M., Chico, J.D., Sumaroka, A., Pantelyat, A.Y., Duncan, K.G., Gardner, L.M., Emmons, J.M., Steinberg, J.D., Stone, M., Jacobson, S.G., 2007. Macular pigment and lutein supplementation in ABCA4-associated retinal degenerations. Invest. Ophthalmol. Vis. Sci. 48, 1319–1329.

Alexander, K.R., Kilbride, P.E., Fishman, G.A., Fishman, M., 1987. Macular pigment and reduced foveal short-wavelength sensitivity in retinitis pigmentosa. Vision Res. 27, 1077–1083.

Beatty, S., Koh, H.-H., Henson, D., Boulton, M., 2000. The role of oxidative stress in the pathogenesis of age-related macular degeneration. Surv. Ophthalmol 45, 115–134.

Bhosale, P., Zhao, D.Y., Bernstein, P., 2007. HPLC measurement of ocular carotenoid levels in human donor eyes in the lutein supplementation era. Invest. Ophthalmol. Vis. Sci. 48, 543–549.

Cai, J., Nelson, K.C., Wu, M., Sternberg, P., Jones, D.P., 2000. Oxidative damage and protection of the RPE. Prog. Retin. Eye Res. 19, 205–221.

Ciulla, T., Hammond, B.R., 2004. The relation between aging and macular pigment density, assessed in the normal elderly, and subjects with cataracts and age-related macular degeneration. Am. J. Ophthalmol 138, 582–587.

Ciulla, T.A., Hammond, B.R., Yung, C.W., Pratt, L., 2001. Macular pigment optical density before and after cataract extraction. Invest. Ophthalmol. Vis. Sci. 42, 1338–1341.

Delori, F.C., Goger, D.G., Keilhauer, C., Salvetti, P., Staurenghi, G., 2006. Bimodal spatial distribution of macular pigment: evidence of a gender relationship. J. Am. Optom. Assoc. A 23, 521–538.

Falsini, B., Fadda, A., Iarossi, G., Piccardi, M., Canu, D., Minnella, A., Serrao, S., Scullica, L., 2000. Retinal sensitivity to flicker modulation: reduced by early age-related maculopathy. Invest. Ophthalmol. Vis. Sci. 41, 1498–1506.

Gallaher, K.T., Mura, M., Todd, W.A., Harris, T.L., Kenyon, E., Harris, T., Johnson, K.C., Satterfield, S., Kritchevsky, S.B., Iannaccone, A., for the Health ABC Study, 2007. Estimation of macular pigment optical density in the elderly: test-retest variability and effect of optical blur in psuedophakic patients. Vision Res. 47, 1253–1259.

Hammond, B.R., Caruso-Avery, M., 2000. Macular pigment optical density in a Southwestern sample. Invest. Ophthalmol. Vis. Sci. 41, 1492–1497.

Hammond, B.R., Wooten, B.R., Snodderly, D.M., 1997a. Individual variations in the spatial profile of macular pigment. J. Am. Optom. Assoc. A 14, 1187–1196.

Hammond, B.R., Johnson, E.J., Russell, R.M., Krinsky, N.I., Yeum, K.J., Edwards, R.B., Snodderly, D.M., 1997b. Dietary modification of human macular pigment density. Invest. Ophthalmol. Vis. Sci. 38, 1795–1801.

Hammond, B.R., Wooten, B.R., Smollon, B., 2005. Assessment of the validity of in vivo methods of measuring human macular pigment optical density. Optom. Vis. Sci. 82 (5), 387–404.

Iannaccone, A., Mura, M., Gallaher, K.T., Johnson, E.J., Todd, W.A., Kenyon, E., Harris, T.L., Harris, T., Satterfield, S., Johnson, K.C., Kritchevsky, S.B., 2007. Macular pigment optical density in the elderly: findings in a large biracial Midsouth population sample. Invest. Ophthalmol. Vis. Sci. 48, 1458–1465.

Johnson, E.J., Chung, H.Y., Caldarella, S.M., Snodderly, D.M., 2008. The influence of supplemental lutein and docosahexaenoic acid on serum, lipoproteins, and macular pigmentation. Am. J. Clin. Nutr 87, 1521–1529.

Mares, J.A., LaRowe, T.L., Snodderly, D.M., Moeller, S.M., Gruber, M.J., Klein, M.L., Wooten, B.R., Johnson, E.J., Chappell, R.J., CAREDS Macular Pigment Study Group and Investigators, 2006. Predictors of optical density of lutein and zeaxanthin in retinas of older women in the Carotenoids in Age-Related Eye Disease Study, an ancillary study of the Women's Health Initiative. Am. J. Clin. Nutr 84, 1107–1122.

Nolan, J.M., Stack, J., O'Donovan, O., Loane, E., Beatty, S., 2007. Risk factors for age-related maculopathy are associated with a relative lack of macular pigment. Exp. Eye Res. 84, 61–74.

Phipps, J.A., Dang, T.M., Vingrys, A.J., Guymer, R.H., 2004. Flicker perimetry losses in age-related macular degeneration. Invest. Ophthalmol. Vis. Sci. 45, 3355–3360.

Richer, S., 1999. Part II: ARMD- Pilot (Case Series) environmental intervention data. J. Am. Optom. Assoc. 70, 24–36.

Richer, S., Stiles, W., Statkute, L., Pulido, J., Frankowski, J., Rudy, D., Pei, K., Tsipursky, M., Nyland, J., 2004. Double-masked, placebo-controlled, randomized trial of lutein and antioxidant supplementation in the intervention of atrophic age-related macular degeneration: the Veterans LAST study (Lutein Antioxidant Supplementation Trial). Optometry 75, 216–230.

Richer, S., Devenport, J., Lang, J.C., 2007. LAST II: Differential temporal responses of macular pigment optical density in patients with atrophic age-related macular degeneration to dietary supplementation with xanthophylls. Optometry 78, 213–219.

Roodenburg, A.J., Leenen, R., van het Hof, K.H., Weststrate, J.A., Tijburg, L.B., 2000. Amount of fat in the diet affects bioavailability of lutein esters but not of {alpha}-carotene, {beta}-carotene, and vitamin E in humans. Am. J. Clin. Nutr 71, 1187–1193.

Schuchard, R., 2005. Preferred retinal loci and macular scotoma characteristics in patients with age-related macular degeneration. Can. J. Ophthalmol 40, 303–312.

Smollon, W.E., Wooten, B.R., Hammond Jr., B.R., 2005. Stimulus edge effects when measuring macular pigment using heterochromatic flicker photometry. Invest. Ophthalmol. Vis. Sci. 46 E-Abstract 4569.

Snodderly, D.M., Hammond, B.R., 1999. *In vivo* psychophysical assessment of nutritional and environmental influences on human ocular tissues: lens and macular pigment. In: Taylor, A.J. (Ed.), Nutritional and Environmental Influences on Vision. CRC Press, Boca Raton, FL, pp. 251–273.

Snodderly, D.M., Brown, P.K., Delori, F.C., Auran, J.D., 1984. The macular pigment. I. Absorbance spectra, localization, and discrimination from other yellow pigments in primate retinas. Invest. Ophthalmol. Vis. Sci. 25, 660–673.

Snodderly, D.M., Mares-Perlman, J.A., Wooten, B.R., Oxton, L., Gruber, M., Ficek, T., for the CAREDS Macular Pigment Study Group, 2004a. Macular pigment measurement by heterochromatic flicker photometry in older subjects: the Carotenoids and Age-related Eye Disease Study. Invest. Ophthalmol. Vis. Sci 45, 531–538.

Snodderly, D.M., Mares, J.A., Wooten, B.R., Gruber, M., Moeller, S., LaRowe, T., Klein, M., Chapell, R., 2004b. Macular pigment density of women in the Carotenoids in Age-Related Eye Disease Study (CAREDS). ARVO Abstract, IOVS.

Stringham, J., Hammond, B.R., 2008. Macular pigment and visual performance under glare conditions. Optom. Vis. Sci. 85, 82–88.

Tyler, C.W., 1989. Two processes controlling life span variations in flicker sensitivity. J. Optom. Soc. Am. A 6, 481–490.

Unlu, N.Z., Bohn, T., Clinton, S.K., Schwartz, S.J., 2005. Carotenoid absorption from salad and salsa by humans is enhanced by the addition of avocado or avocado oil. J. Nutr 135, 431–436.

Wang, W., Connor, S.L., Johnson, E.J., Klein, M.L., Hughes, S., Connor, W.E., 2007. Effect of dietary lutein and zeaxanthin on plasma carotenoids and their transport in lipoproteins in age-related macular degeneration. Am. J. Clin. Nutr 85, 762–769.

Werner, J.S., Donnelly, S.K., Kliegl, R., 1987. Aging and human macular pigment density. Appended with translations from the work of Max Schultze and Ewald Hering. Vision Res. 27, 257–268.

Whitehead, A.J., Mares, J.A., Danis, R., 2006. Macular pigment: a review of current knowledge. Arch. Ophthalmol 124, 1038–1045.

Wooten, B.R., Hammond, B.R., 2005. Spectral absorbance and spatial distribution of macular pigment using heterochromatic flicker photometry. Optom. Vis. Sci. 82, 378–386.

Wooten, B.R., Hammond, B.R., Land, R., Snodderly, D.M., 1999. A practical method of measuring macular pigment optical density. Invest. Ophthalmol. Vis. Sci. 40, 2481–2489.