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Concordance of macular pigment measurements obtained using customized heterochromatic flicker photometry, dual-wavelength autofluorescence, and single-wavelength reflectance

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ABSTRACT

This study compares *in vivo* measurements of macular pigment (MP) obtained using customized heterochromatic flicker photometry (cHFP; Macular Metrics Densitometer™), dual-wavelength fundus autofluorescence (Heidelberg Spectralis® HRA + OCT MultiColor) and single-wavelength fundus reflectance (Zeiss Visucam® 200). MP was measured in one eye of 62 subjects on each device. Data from 49 subjects (79%) was suitable for analysis. Agreement between the Densitometer and Spectralis was investigated at various eccentricities using a variety of quantitative and graphical methods, including: Pearson correlation coefficient to measure degree of scatter (precision), accuracy coefficient, concordance correlation coefficient (ccc), paired *t*-test, scatter and Bland–Altman plots. The relationship between max MP from the Visucam and central MP from the Spectralis and Densitometer was investigated using regression methods. Agreement was strong between the Densitometer and Spectralis at all central eccentricities (e.g. at 0.25° eccentricity: accuracy = 0.97, precision = 0.90, ccc = 0.87). Regression analysis showed a very weak relationship between the Visucam and Densitometer (e.g. Visucam max on Densitometer central MP: $R^2 = 0.008$, $p = 0.843$). Regression analysis also demonstrated a weak relationship between MP measured by the Spectralis and Visucam (e.g. Visucam max on Spectralis central MP: $R^2 = 0.047$, $p = 0.348$). MP values obtained using the Heidelberg Spectralis are comparable to MP values obtained using the Densitometer. In contrast, MP values obtained using the Zeiss Visucam are not comparable with either the Densitometer or the Spectralis MP measuring devices. Taking cHFP as the current standard to which other MP measuring devices should be compared, the Spectralis is suitable for use in a clinical and research setting, whereas the Visucam is not.

1. Introduction

Macular pigment (MP) is composed of the yellow carotenoid pigments lutein (L), zeaxanthin (Z), and *meso*-zeaxanthin (MZ). MP is found at the macula, the specialized part of the retina that mediates fine central and color vision (Hirsch and Curcio, 1989). Its unique anatomic location (Snodderly et al., 1984), short-wavelength (blue) light filtering properties (Bone et al., 1992), and antioxidant properties (Li et al., 2010; Sujak et al., 1999; Wrona et al., 2004), make this pigment important for visual function.

Indeed, in the non-diseased retina (normal subjects), MP has been shown to enhance visual function by reducing the effects of light scatter (thereby reducing glare disability) (Hammond, et al., 2013; Loughman et al., 2012; Stringham et al., 2011; Stringham and Hammond, 2007, 2008; Yao et al., 2013) and chromatic aberration (thereby optimizing contrast sensitivity) (Hammond et al., 2013; Loughman et al., 2010a, 2012; Nolan et al., 2011; Renzi and Hammond, 2010; Richer et al., 2011; Sasamoto et al., 2011; Yao et al., 2013), via its light-filtering (optical) properties (Hammond and Fletcher, 2012; Loughman et al., 2010b; Wooten and Hammond, 2002). Moreover, MP is also postulated to protect against age-related macular disease, particularly age-related macular degeneration (AMD) (Gale et al., 2003; Snodderly, 1995), the developed world's leading cause of age-related blindness (Bressler, 2004; Resnikoff et al., 2004). This putative protection is likely due to the pigment's optical and antioxidant properties (Sabour-Pickett et al., 2011). Of interest, it has been shown that established risk factors for AMD (i.e. age, family history of disease and cigarette

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smoking) (Beatty et al., 2001; Hammond et al., 1996; Kirby et al., 2010; Nolan et al., 2007a) are associated with low levels of MP. However, carotenoid supplementation studies have demonstrated that serum carotenoid concentrations and MP optical density (MPOD) can be increased through dietary modification (Hammond et al., 1997) or supplementation (Bone et al., 2003; Bone and Landrum, 2010; Garcia-Layana et al., 2013; Huang et al., 2013; Koh et al., 2004; Landrum et al., 2012; Murray et al., 2013; Nolan et al., 2011; Richer et al., 2007; Stringham and Hammond, 2008; Tanito et al., 2012; Weigert et al., 2011; Yao et al., 2013), with good results achieved when the formulation used contains all three of the macular carotenoids (L, Z and MZ) (Bone et al., 2007; Connolly et al., 2011; Loughman et al., 2012; Meagher et al., 2012; Nolan et al., 2012).

The typical profile of MP has a central peak, which decreases in concentration with retinal eccentricity (similar to that of a peaked mountain, e.g. Mount Everest). In addition, atypical spatial profiles of MP, containing "ringlike" structures, secondary peaks or plateaus also exist (Berendschot and van Norren, 2006; Delori et al., 2006; Kirby et al., 2009). Little investigation has been done involving MP spatial profiles; however, it has recently been reported that about 12% of the population has an atypical MP profile, characterized by a central plateau or central dip in the pigment profile (e.g. Mount Kilimanjaro). Of note, such atypical central dips have been found to be more common in subjects at increased risk of AMD (Kirby et al., 2010); but it has been recently shown that a central dip in MP's spatial profile can be normalized following supplementation with a formulation containing the centrally dominant macular carotenoid, MZ (Nolan et al., 2012).

Given the importance of MP for vision, and its potential role in preventing and/or reducing risk of AMD development and/or its progression, there is a clear need to measure this pigment with accuracy *in vivo*. Moreover, it is important to be able to measure patient response to supplement formulations containing the macular carotenoids at the target tissue (i.e. the macula).

There are a variety of methods currently in use that claim to measure MPOD. However, researchers have been debating the advantages and limitations of these techniques for over 20 years (Bernstein and Gellermann, 2003; Hammond et al., 2005; Hammond and Wooten, 2006). These methods are divided into psychophysical (sometimes referred to as subjective) and physical (sometimes referred to as objective). The psychophysical techniques available include color matching (Davies and Morland, 2002), motion photometry (Moreland, 2004), heterochromatic flicker photometry (Bone and Landrum, 2004), and customized heterochromatic flicker photometry (cHFP) (Stringham et al., 2008). Of these psychophysical techniques, HFP and cHFP are the most widely used. With HFP, the subject is required to make isoluminance matches between two flickering lights: a green light (not absorbed by MP) and a blue light (maximally absorbed by MP). The log ratio of the amount of blue light absorbed centrally, where MP peaks, to that absorbed at a peripheral retinal locus (the reference point), where MP is assumed to be zero, gives a measure of the subject's MPOD. Customized HFP optimizes the HFP technique by customizing the procedure for each subject (see below). Importantly, HFP has been validated by measuring its absorption spectrum *in vivo* and comparing it to the *in vitro* spectral absorption curve of the macular carotenoids (Bone et al., 1992; Stringham et al., 2008; Wooten and Hammond, 2005), and it is therefore our view that cHFP, an optimized form of HFP, represents a reference standard to which other MP measuring techniques should be compared. Validation of MP measurement techniques has been the subject of lively debate (Gellermann and Bernstein, 2006; Hammond et al., 2005).

Physical techniques currently used for measuring MP include resonance Raman spectroscopy (Bernstein et al., 1998, 2002), fundus reflectance (Berendschot and van Norren, 2004), and fundus autofluorescence (Delori, 2004). However, none of these physical techniques have yet been properly validated (Hammond et al., 2005).

Fundus autofluorescence (AF) uses a confocal scanning laser ophthalmoscope (cSLO) (Delori et al., 2011) or fundus camera (Spaide, 2003). AF exploits the fluorescent properties of lipofuscin present in the retinal pigment epithelium (RPE) (Sparrow, 2007). RPE lipofuscin is a fluorophore that accumulates over time from the phagocytosis of photoreceptor outer segments. Lipofuscin is excited *in vivo* between 400 and 590 nm (peak excitation at 490–510 nm) and emits AF at 520–800 nm (peak emission at 590–630 nm) (Delori, 2004). MP, which is located anterior to the RPE, absorbs light of 400–550 nm (peak absorption at 460 nm). Therefore, AF at the macula is attenuated by MP if the excitation wavelength falls within that of the absorption spectrum of MP.

Fundus reflectance, which quantitatively measures the light reflected from the retina and choroid using a reflectometer (Kilbride et al., 1989), a fundus camera (Chen et al., 2001), or a cSLO (Brindley and Willmer, 1952), has also been widely used for the measurement of MP. The reflectance method calculates MP in one of two ways; either by comparing the light reflected at the macula, some of which will be absorbed by the MP, to the light reflected at the peripheral areas, where there is minimal MP present to attenuate the reflectance; or by a spectral analysis of the reflected light (Berendschot and van Norren, 2004).

This current study was designed to compare *in vivo* measurements of MP obtained using cHFP (Macular Metrics Densitometer™), dual-wavelength fundus autofluorescence (Heidelberg Spectralis® HRA + OCT MuliColor) and single-wavelength fundus reflectance (Zeiss Visucam® 200), and also reports on the intra-session repeatability of these devices.

2. Methods

2.1. Subjects

62 subjects were recruited into the study, of which 49 (79%: mean age = 49 ± 13 years) were included in the final analysis. Eight subjects (13%) were excluded due to presence of ocular disease (e.g. AMD, diabetic retinopathy) and five subjects (8%) were excluded because they were not able to perform cHFP reliably (i.e. sd > 10%). The eye with best corrected visual acuity (BCVA) was selected as the study eye (27 OD and 22 OS).

An ancillary study was conducted to assess concordance between MP spatial profile as recorded on the Densitometer and Spectralis, and this additional study included the 49 subjects from the primary analysis, and a further 15 subjects recruited specifically for this purpose ($n = 64$; mean age = 48 ± 13 years; 38 male, 26 female).

This study was approved by the Research Ethics Committee of the Waterford Institute of Technology and was conducted in accordance with the tenets of the Declaration of Helsinki.

2.2. Order of testing

First, BCVA was measured using a computerized Snellen Chart (Test Chart 2000 Xpert; Thomson Software Solutions). Following this, the first measure of MPOD was performed using cHFP. Subjects were then dilated with one drop of tropicamide 1% (Bausch + Lomb) before MPOD measurements were performed on the Spectralis and Visucam devices.

2.3. Customized heterochromatic flicker photometry

The Macular Metrics Densitometer™ (Macular Metrics, Rehoboth, MA, USA) was used in this experiment to measure MP by

cHFP. The device and method are described in detail elsewhere (Loane et al., 2007; Wooten et al., 1999). The Densitometer cHFP method utilizes a light stimulus of alternating blue (460 nm) and green (550 nm) wavelengths. Subjects are required to make isoluminance matches between the two wavelengths by adjusting the radiance of the blue light, until the perceived flicker is minimized or a point of "null" flicker is reached. The customization feature of cHFP is achieved by optimization of the flicker frequency and using a yoked method to determine at the isoluminance point.

Optimization of flicker is important because it calculates the flicker frequency at which the subject can perform the measurement best (i.e. the flicker rate at which the subject can clearly identify a narrow null zone), and results in least variation. This was achieved as follows: the flicker frequency was set for each target using age-guided predictions (based on experience) to estimate the optimal flicker frequencies for that subject (e.g. for a 30 year old subject the flicker frequency was set at 15 HZ for 0.25° eccentricity). If the variance was too great, the frequency was reduced by 2 HZ stepwise until the subject was at his/her optimal flicker frequency. If the subject could not identify a null zone, the flicker frequency was increased by 1HZ stepwise until the subject was at his/her optimal flicker frequency and could identify the narrow null zone.

The yoked function of the Densitometer is important because it reduces brightness (luminance) change of the target that may otherwise be mistaken as flicker change (we believe this to be an issue with other HFP devices that do not employ the yoked function). With this method, the luminance of the blue and green light emitting diodes (LEDs) used in the device are adjusted in an inverse-yoked manner so that when the luminance of one wavelength increases, the luminance of the other wavelength decreases. The result is that the overall luminance of the target stays relatively stable.

In this experiment, MP was measured at four different retinal eccentricities: 0.25°, 0.5°, 1°, and 1.75°, with a reference point at 7°. The targets and fixation points used for each retinal eccentricity were as follows: the 0.25° and 0.5° eccentricities were measured using a 0.5° and 1° diameter disc, respectively, with a 5′ black fixation point at the center; the 1° and 1.75° eccentricities were measured using a 20′ wide annuli with mean radii corresponding to those eccentricities, with a central 5′ black fixation point. The 7° reference measurement was a 2° diameter disc located 7° peripherally with reference to a 5′ red fixation point. Subjects were required to perform at least six null flicker matches per target. Radiance values of acceptable null flicker matches fell within a standard deviation of 0.1 (i.e. 10% variance). MPOD was calculated using a log ratio of the foveal to parafoveal luminance values.

2.4. Fundus autofluorescence

The Heidelberg Spectralis® HRA + OCT Multicolor (Heidelberg Engineering GmbH, Heidelberg, Germany) was used in this experiment to measure MP. The Spectralis utilizes cSLO imaging with diode lasers and uses dual-wavelength AF for measuring MP. Dual-wavelength AF in this devise uses two excitation wavelengths, one that is well-absorbed by MP (488 nm, blue) and one that is not well absorbed by MP (518 nm, green). The excitation spectrums of the two different AF images are then compared, and, along with a parafoveal reference point, are used to calculate an MP density profile.

During the measurement, the subject's head was aligned using a head-chin strap and he/she was instructed to fixate on an internal fixation target. Initial camera alignment, illumination and focus were done in infrared (IR) mode. Once the image is evenly illuminated, the camera mode is switched to simultaneous blue AF and green AF imaging (BAF + GAF) mode for MP measurement acquisition. After additional adjustments to illumination and focus to ensure optimal image quality, a 30 s video is recorded. This additional adjustment time (10 s or more) in BAF + GAF mode also allows time for the intense light used during image capture to bleach the photoreceptors, ensuring minimal absorption by these structures (Delori et al., 2011). A second video was taken within the same session to test repeatability.

The images in the video are aligned and averaged using Heidelberg Eye Explorer software (HEYEX, version 1.7.1.0), and an MP density map is created. For analysis, the plateau (equivalent to the reference point) was set to 7° to correspond to the reference point used in cHFP. The average MPOD at radii corresponding to the eccentricities measured with cHFP were recorded (0.23, 0.47, 0.98, 1.72° eccentricity).

2.5. Fundus reflectance

The Zeiss Visucam® 200 (Carl Zeiss Meditec AG, Jena, Germany) was used to measure MP by fundus reflectance. The Visucam is a fundus camera which uses narrow-band wavelength (480–500 nm) reflectance for measuring MP. The acquisition module uses a fixed analysis area of 3.5° eccentricity and a reference area located at 4–7.5° eccentricity. Determination of MPOD is done by comparing the reflectance at the macula with the reflectance at the parafoveal reference area. MPOD is automatically calculated by software to give the mean and maximum amount of MP over the 3.5°.

Subject head alignment was maintained with a head-chin strap, and an internal fixation target was used for image alignment. An MP image was acquired on MPOD capture mode at a field angle of 30°. A second MP image was taken to test repeatability.

2.6. MP spatial profile classification

For the purpose of MP spatial profile comparison between the Densitometer and Spectralis, subjects were classified as having either typical or atypical MP spatial profiles. Spatial profile type was determined by examination of the MP spatial profile generated by each device. Subjects with MP spatial profiles containing a secondary peak or plateau were classed as atypical, while those with a Gaussian distribution were classed as having a typical spatial profile.

2.7. Statistics

Statistical analyses were conducted using SPSS® 19.0 (SPSS, Inc., Chicago, IL, USA) and the statistical programming language R (R Foundation for Statistic Computing) (R Development Core Team, 2009). We report three indices of agreement for comparing MP measurements from two devices: 1. precision, the Pearson correlation coefficient, interpreted as measuring the degree of scatter when MP measurements from the two devices are plotted against each other, with values close to 1 indicating closeness to the ordinary least squares regression line (and hence small scatter); 2. accuracy, constructed from the means and standard deviations of the MP measurements from each device, with values close to 1 indicating that the two means are close to each other and that the two standard deviations are close to each other; and 3. concordance correlation coefficient (CCC), obtained as the product of the other two coefficients. The CCC, in effect, measures closeness of the points in the scatterplot to the line $y = x$, and is probably the best single measure of agreement (Lin et al., 2012). Lower confidence limits for concordance, precision, and accuracy coefficients were obtained from R code supplied with Lin et al. (2012) and R Development Core Team (2009). We also tested for bias (non-zero mean difference of measurements) using the paired t-test.

Table 1
Agreement indices for MPOD measurement between the Densitometer and the Spectralis at each eccentricity.

Eccentricity	CCC[a]	Precision	Accuracy
0.25°	0.872 (0.81)	0.895 (0.83)	0.974 (0.94)
0.50°	0.846 (0.78)	0.882 (0.82)	0.959 (0.91)
1.00°	0.714 (0.59)	0.77 (0.65)	0.928 (0.84)
1.75°	0.461 (0.30)	0.588 (0.41)	0.783 (0.65)

For each coefficient, the 95% lower confidence limit is shown in brackets.
[a] CCC = Concordance correlation coefficient.

Graphical methods included ordinary scatterplots with the line $y = x$ superimposed, and Bland–Altman plots of difference in measurements versus mean measurements.

Power calculations were done using software package PASS 2008 (NCSS, LLC. Kaysville, Utah, USA). We present some illustrative power calculations for a sample of size 49. These calculations are for the CCC, and are based on the (somewhat arbitrary) assumption that the minimum acceptable CCC for comparing two MP measuring devices must exceed 0.80. If the actual CCC exceeds 0.80, we want the probability (power) to be high that we will end up rejecting the null hypothesis that CCC $\leq$ 0.80. At the standard 5% level of significance: (a) if the actual CCC is 0.95 for the two MP measuring devices, then the power is 0.9996 that a sample of 49 will reject the null hypothesis that CCC $\leq$ 0.80; (b) if the actual CCC is 0.90 then the power is 0.83, still acceptable (c) if the actual CCC is 0.85 then the power drops to 0.29. In short, a sample of this size has ample power to detect CCC's which are well above the threshold of 0.80, but does not have sufficient power for detecting CCC's which are only marginally above this threshold.

Agreement between the Densitometer and Spectralis was investigated by comparing MPOD at each eccentricity measured on the Densitometer to the MPOD measured at corresponding eccentricities on the Spectralis (circa 0.25, 0.50, 1.00, 1.75° eccentricity). The relationship between the Visucam and each of the other devices (Spectralis and Densitometer) was investigated using regression methods, because data at different eccentricities is not provided from the Visucam, which just reports the maximum and mean value.

The same statistical methods (precision, accuracy, and CCC) were used to assess repeatability of measurements obtained from each of the Spectralis and Visucam devices.

Agreement between MP spatial profile classification generated by the Densitometer and Spectralis was investigated by cross-tabulation. Gender and smoking status (i.e. current, past, or never smoker) was assessed for a possible relationship with MP spatial profile classification using crosstabulations. The relationship between MP spatial profile classification and age, MPOD at different eccentricities (0.23°, 0.47°, and 0.74°), and mean MPOD was investigated using independent-samples *t*-test.

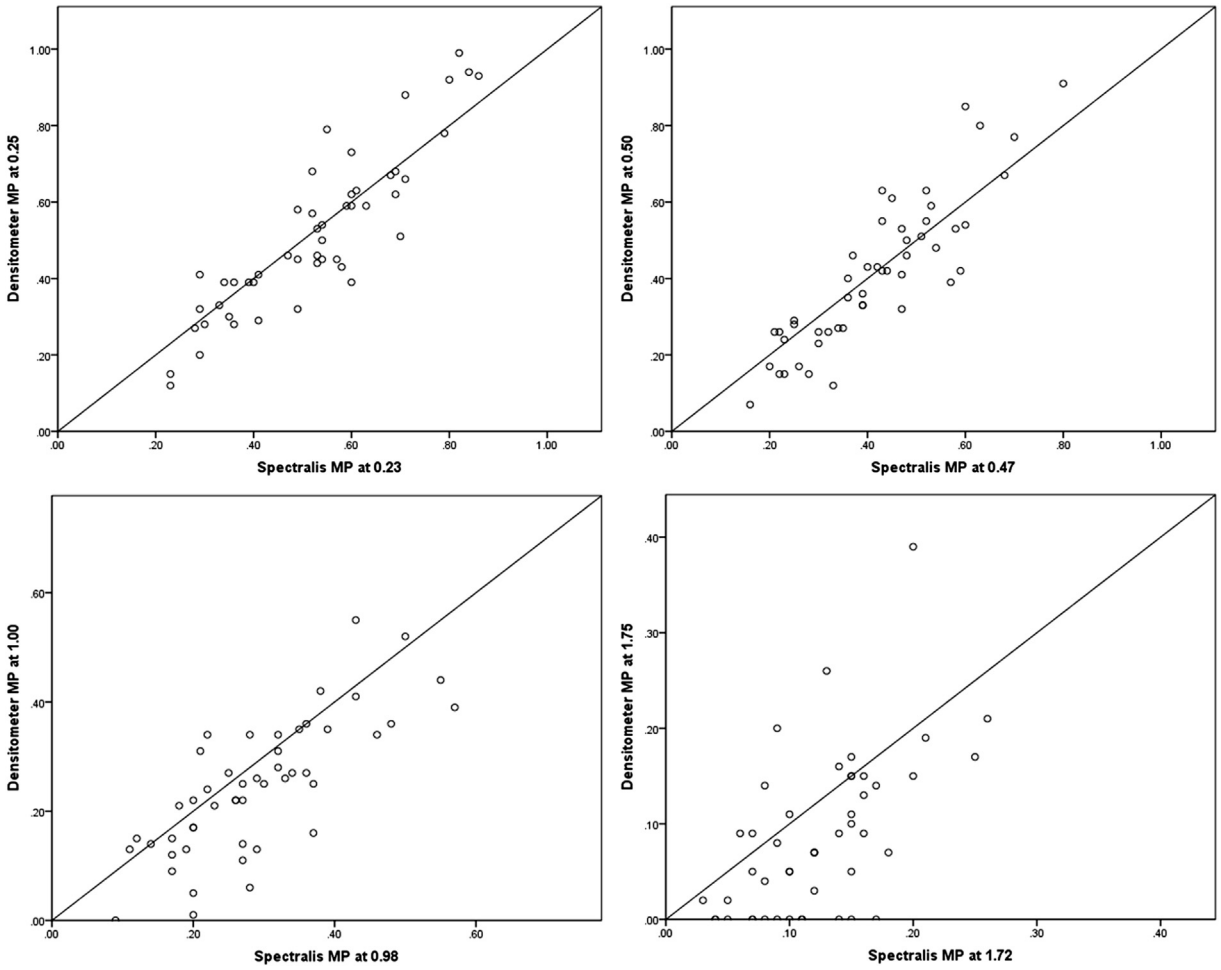

Fig. 1. Scatterplots of the MP values obtained with the Densitometer and the Spectralis at circa 0.25°, 0.50°, 1.00°, and 1.75° eccentricity, with the line $y = x$ superimposed. Any negative values obtained were plotted as zeroes.

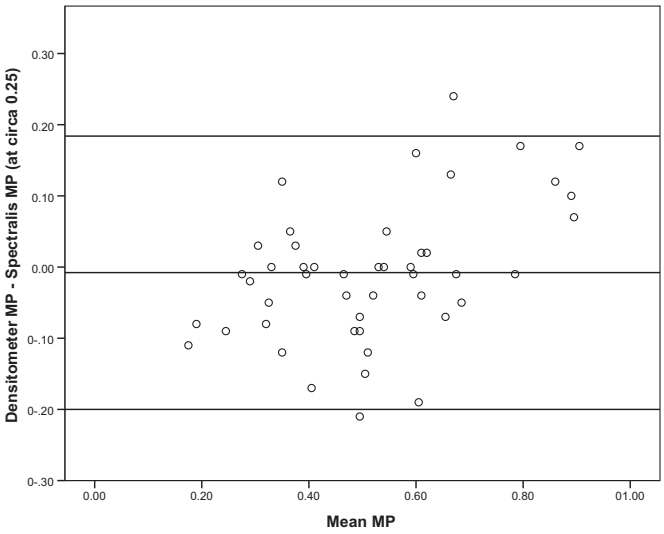

Fig. 2. Bland–Altman plot of the difference in MPOD values at 0.25° eccentricity obtained with the Densitometer and the Spectralis versus the mean MP measured with both devices.

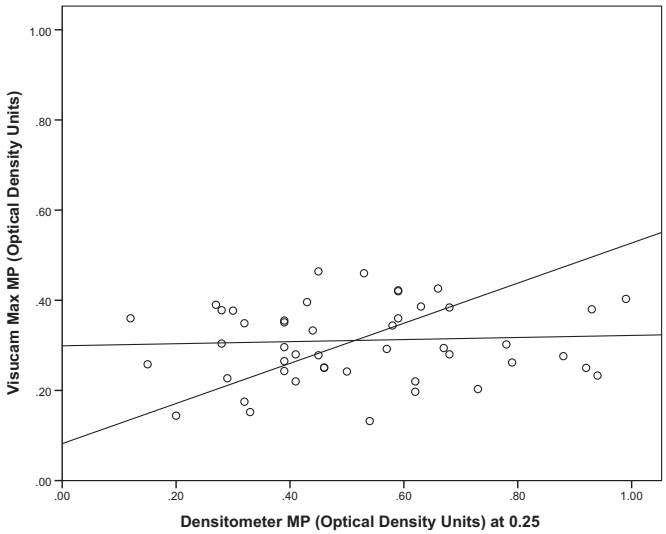

Fig. 3. Scatterplot of Densitometer MP values at 0.25° eccentricity and max MP values from the Visucam. The lines displayed are the ordinary least squares ($y = 0.299 + 0.023x$) and ordinary least products regression ($y = 0.082 + 0.445x$) lines.

3. Results

3.1. Densitometer versus Spectralis

Agreement was strong between the Densitometer and Spectralis (Table 1), especially at central eccentricities (0.25° and 0.50°) and for subjects with midrange MP values (e.g. 0.20 to 0.60 optical density units at 0.25° eccentricity). Precision (degree of scatter) ranged from 0.895 at 0.25° to 0.588 at 1.75°. Accuracy ranged from 0.974 at 0.25° to 0.783 at 1.75°. CCC ranged from 0.872 at 0.25° to 0.461 at 1.75°.

Paired *t*-test analysis demonstrated that there was no significant difference between mean MP measured on both devices at 0.25° and 0.5° (mean difference = 0.0078, $p = 0.575$, and mean difference = 0.0055, $p = 0.690$, respectively); however, there was a statistically significant difference between mean MP measured at 1° and 1.75° (mean difference = 0.0449, $p < 0.001$, and mean difference = 0.0384, $p < 0.001$, respectively), representative of a slight bias at these eccentricities, with the Spectralis giving marginally higher readings than the Densitometer. These findings suggest that agreement between MP measurements taken on these two instruments diminishes with increasing eccentricities.

Scatterplots of MPOD readings obtained from the Spectralis and Densitometer at four eccentricities all show good agreement (Fig. 1). It is notable that Densitometer readings tend to be higher than Spectralis readings for subjects with high MP at central eccentricities (e.g. > 0.8 optical density units at 0.25°). A Bland–Altman plot of the difference in MPOD values obtained with the Densitometer and the Spectralis versus the mean MP measured with both devices is presented in Fig. 2; this plot conveys much the same information as the scatterplots of Fig. 1, but the limits of agreement (upper and lower horizontal lines) convey the additional information that, for 95% of subjects, the maximum difference in MP at 0.25°, from the two devices, will not exceed about 0.2.

We found no effect of age (range from 21 to 70 years) on the difference in MP measurements between the Densitometer and the Spectralis (e.g. simple linear regression of the difference in MP measurements on the Densitometer and the Spectralis at 0.25° eccentricity to age gave an R^2 value of 0.002, $p = 0.735$).

3.1.1. MP spatial profile comparison

In terms of MP spatial profile classification, there was agreement between the two devices in 51 of 64 subjects (80%). Twelve subjects (19%) were classed as atypical on the Spectralis, but typical on the Densitometer. One subject was classed as atypical on the Densitometer, but typical on the Spectralis.

Of the 64 MP spatial profiles measured on the Spectralis, 25 subjects (39%) were classed as atypical, and no significant correlation was found between MP spatial profile classification and age ($p = 0.995$), gender ($p = 0.138$), or smoking status ($p = 0.104$).

However, a typical MP spatial profile, as measured on the Spectralis, was positively and significantly related to MPOD at 0.23° eccentricity ($p = 0.009$), but not related to MPOD at other eccentricities ($p = 0.053$ and 0.452 for 0.47° and 0.74°, respectively), or to mean MP ($p = 0.252$).

3.2. Densitometer versus Visucam

No relationship was found between the Densitometer and Visucam MP measurements. In the absence of data at different eccentricities from the Visucam, which ruled out measuring for agreement at specific eccentricities, we switched to a regression approach in order to compare Visucam and Densitometer measurements of MP. We investigated the relationship of max MP from the Visucam to MP at the two central eccentricities (jointly) from the Densitometer (0.25° and 0.50°). We also investigated the relationship of mean MP from the Visucam to MP at all eccentricities (jointly) from the Densitometer. Very weak relationships were found in both cases. Multiple regression of max MP from the Visucam on Densitometer MP at eccentricities 0.25 and 0.50° had an associated R^2 value of just 0.008 and was not statistically significant ($p = 0.843$). Multiple regression of mean MP from the Visucam on Densitometer MP at all eccentricities had an associated R^2 value of 0.095 and was not statistically significant ($p = 0.345$).

Regression results were also poor when ordinary least products regression was used in place of ordinary least squares (Ludbrook, 2010). To illustrate, Fig. 3 shows both the ordinary least squares and ordinary least products regression lines for the regression of max MP from the Visucam on Densitometer MP at 0.25° eccentricity. It is clear from this figure that neither regression line fits the data. It is also notable that no subject yielded an MPOD value

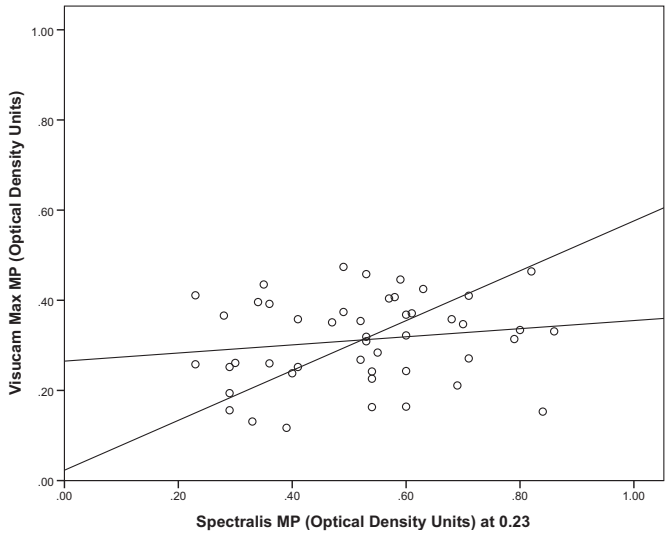

Fig. 4. Scatterplot of Spectralis MP values at 0.23° eccentricity and max MP values from the Visucam. The lines displayed are the ordinary least squares ($y = 0.265 + 0.090x$) and ordinary least products regression ($y = 0.023 + 0.553x$) lines.

greater than 0.5 optical density units on the Visucam, despite many subjects exhibiting MPOD readings greater than this value, as recorded on the Densitometer.

3.3. Visucam versus Spectralis

No relationship was found between the Visucam and the Spectralis MP measurements. Multiple regression of max MP from the Visucam on Spectralis MP at eccentricities 0.23° and 0.27° had an associated R^2 value of just 0.047 and was not statistically significant ($p = 0.348$). Multiple regression of mean MP from the Visucam on Spectralis MP at various eccentricities ranging from 0.23° to 1.76° had an associated R^2 value of 0.005 and was not statistically significant ($p = 0.634$) (Fig. 4).

3.4. Repeatability

Densitometer repeatability has previously been investigated by Kirby et al. (2009). Their study found very good repeatability with respect to the cHFP technique, reporting intraclass correlations (ICC) ranging from 0.93 to 0.96 at 0.25, 0.5, and 1° eccentricity.

Repeatability in the present study, results given in Table 2, was excellent for the Spectralis and very good for the Visucam.

4. Discussion

Interest in the macular carotenoids, L, Z and MZ, and their composite at the macula (MP), amongst scientists, clinicians and the general public, continues to grow. However, valid and reliable measurement of tissue concentration of these nutrients at the macula is needed in order to study (in both research and clinic settings) their potential for vision and their contribution (if any) to the natural history of macular disease. Of note, there are many commercially available MP measuring devices which claim to measure MP accurately and reproducibly, but not all of these devices have been validated. One way to test the validity of new technologies is to compare values obtained from such devices with those of a validated instrument (such as the Densitometer). This study was conducted to assess concordance between three devices, namely the Macular Metrics Densitometer™ (taken as the standard for MP measurement in this experiment), the Heidelberg Spectralis® HRA + OCT MultiColor, and the Zeiss Visucam® 200. Of note, this is the first study to measure MP using the new acquisition module of the Heidelberg Spectralis® HRA + OCT MultiColor.

Table 2
Repeatability of the Spectralis and Visucam MPOD measurements.

Eccentricity	CCC	Precision	Accuracy
Spectralis			
0.23°	0.988	0.992	0.996
0.47°	0.996	0.998	0.998
0.98°	0.995	0.997	0.998
1.72°	0.988	0.992	0.996
Visucam			
Mean	0.873	0.873	0.991
Max	0.832	0.84	0.999

The literature on statistical methodology for measuring agreement is confusing, and particularly challenging to the unfamiliar investigator. Practitioners may use correlation, regression (both ordinary least squares and ordinary least products) and intraclass correlation (ICC) to measure agreement between two quantitative variables, as well as measures arising from graphical methods, such as the limits of agreement from the Bland–Altman approach (Bland and Altman, 1986). For the most part, in this study, we followed the approach of Lin et al. (2012), and have calculated their suggested measures for our data. Thus, we report three indices of agreement: precision, accuracy, and CCC (defined above). The CCC, in effect, measures closeness to the line $y = x$, and is probably the best single measure of agreement. It is identical to one version of the ICC, which is why the ICC is not also reported in this study. However, all correlation measurements (and hence our precision and CCC measurements) are affected by the range of the data being compared, and this should be borne in mind when interpreting our findings. The lower agreement indices that we obtained at greater eccentricities may reflect, in part, the smaller ranges of MP with increasing eccentricity.

As mentioned above, in this experiment we use the Densitometer as the standard for measurement of MP. We justify this decision given the extensive validation undergone by this MP measuring flicker device. Of note, the Densitometer is currently being used at over 40 research centers around the world, including the National Institute of Health (NIH), and the data generated by this instrument has been published in over 100 peer-reviewed scientific papers. Notably, this MP-measuring device has been validated fully and correctly (i.e. by comparing and matching the data it generates with the *in vitro* spectral absorption curve of the macular carotenoids; by assessing the relationship between MP measured using this device and its constituent carotenoids in diet and serum; by confirming test-retest repeatability) (Nolan et al., 2007b; Stringham et al., 2008; Wooten et al., 1999).

In this experiment we found strong agreement (for most subjects; e.g. Fig. 2 shows that measurements differed by more than 0.2 for only two individuals) between the Densitometer and the Spectralis, and we found no relationship between the Visucam and either of the other two devices. Of course, each of these devices uses different measuring techniques, but all claim to measure MP. However, it is important to point out that it is difficult to directly compare our findings to previously published studies using these methodologies, given the inter-study variations of technique and given the differences with respect to the apparatuses and protocols used by other investigators.

With respect to statistical methodology, comparisons between the current and previous studies are also difficult, as previous reports have tended to emphasize only one aspect of agreement, namely precision as measured by the correlation coefficient; also,

the reported p-values for such correlations were testing closeness to zero, whereas closeness to one is the real issue. We present below (with caution, given the differences between devices and protocols used) some of the observations from previous reports.

In 2001, Delori et al. (2001) compared MP measured using HFP, dual-wavelength (470 and 550 nm) AF spectrometry, and dual-wavelength (470 and 550 nm) reflectance, and found the AF technique correlated well with HFP and the reflectance method used in their study ($r = 0.77$ and 0.73, respectively). They also reported that the HFP and reflectance techniques correlated well ($r = 0.61$). Of interest, and consistent with our findings, no subject in their study measured above 0.40 optical density units on the reflectance method, while some subjects yielded MP values of 0.90 (and greater) optical density units using the HFP and AF methods. It appears, therefore, that the reflectance method used here and elsewhere underestimates MP values.

In another study, Berendschot and van Norren (2005) compared MPOD of subjects measured using HFP (device designed by Mellerio et al.) (Mellerio et al., 2002), dual-wavelength AF (custom-built SLO) (Ossewaarde-Van et al., 2002), dual-wavelength reflectance (custom-built SLO), and broad spectral reflectance (Foveal Reflection Analyzer [FRA] 1 [420–790 nm] and FRA 2 [400–950 nm]) (Zagers et al., 2002). In brief, they reported that all techniques used in their study correlated well (significantly) with each other ($r = 0.42$ to 0.94, $p < 0.05$, for all). However, they concluded that the correlation between HFP and all other methods was lowest ($r = 0.42$–0.59). Of note, the HFP technique used in their study was not customized and, used a reference point of only 5° eccentric to the fovea (Berendschot and van Norren, 2005), which has been shown to underestimate MPOD in older subjects and in subjects with high MPOD (Loane et al., 2007).

In another MP measurement comparison study, Canovas et al. (2010) compared MPOD measured in nine subjects using dual-wavelength AF with the Heidelberg Retina Angiograph (HRA) to MPOD measured with cHFP (Densitometer) and reported good (statistically significant) correlations between readings yielded on these two devices, with the strongest correlation at 1.75° ($r = 0.73$; $p < 0.001$). This is not consistent with the current study, which found that the correlation between the Densitometer and the Spectralis was weaker for measurements performed away from the center of the fovea (i.e. with increasing eccentricity from the foveal center, the agreement between the devices lessened), whereas the strongest correlation in the current study was found at the epicenter (i.e. 0.25°).

Of note, none of the previous studies investigating concordance between MP measuring techniques reported the strong concordance that we observed between the Spectralis and the Densitometer. Indeed, and although each of these technologies is designed to measure MP, each device employs a different methodological approach, and each method has its own inherent advantages, assumptions and limitations (Howells et al., 2011). Additionally, MP spatial distributions generated by the Spectralis and Densitometer correlated well between these two techniques (i.e. in 80% of subjects). Discrepancy between these two devices in MP spatial profile classification is mainly due to secondary peaks or plateaus around 0.75° eccentricity, an eccentricity not measured by the Densitometer. Therefore, the Densitometer may not detect such atypical profiles.

The advantages of the cHFP method (using the Densitometer) include its proven validity, reliability, and reproducibility (Hammond et al., 2005; Kirby et al., 2009; Wooten and Hammond, 2005). The Densitometer obtains a parafoveal reference value (unique to each subject) to calculate MPOD. This parafoveal measure is essential to the measurement of MP as it accounts for light absorption and scatter within the eye (factors known to be influenced by cataract and age) (Gaillard et al., 2000; van den Berg et al., 2010). On the other hand, the Densitometer assumes that there is virtually no MP present at 7° of eccentricity (Snodderly et al., 1984; Stabell and Stabell, 1980). Also, this method requires the subject to be able to fixate on the targets presented, and follow operator instructions, rendering this method unsuitable for persons with learning difficulties, memory problems, or young children. Also, when measuring the spatial profile of MP (as performed in this experiment), this technique can take up to 30 min per eye, and can be difficult for some subjects to complete. Indeed, five (8%) of the 62 subjects recruited into this study could not complete the MP measurement using cHFP.

Physical methods are typically faster, and involve little participation from the subject. However, these methods require pupil dilation, expose subjects to bright lights, and are expensive. Also, physical methods are potentially vulnerable to “noise” attributable to a variety of assumptions relating to intraocular light scatter, light absorption, excitation spectrums, emission spectrums, and reflectance spectrums (Delori et al., 2001; Howells et al., 2011).

The dual-wavelength AF method utilized by the Heidelberg Spectralis for MP acquisition requires little subject compliance, and takes just several minutes to perform. This new Spectralis® HRA + OCT MultiColor device acquires blue and green AF images simultaneously. This results in exact pixel to pixel alignment and equal illumination of both images. Earlier studies using cSLOs for AF measurement of MP acquired blue and green AF images sequentially, with risk of consequential eye movement artifacts (Wustemeyer et al., 2002). The Spectralis® HRA + OCT MultiColor also has the benefit of providing an MP profile that yields MPOD across all eccentricities (i.e. a full spatial profile of MP is obtained). With the AF method, assumptions include: 1. the responsivity of the retina to light is the same across this tissue; 2. the fluorophores are the same across the retina; 3. the emission spectrum of the fluorophores is the same throughout the retina; 4. each wavelength used is subject to the same amount of intraocular light scatter. However, it has been claimed that the use of two-wavelengths, confocal optics, and complex algorithms account and control for these assumptions. Also, it should be noted that we found no effect of age on the difference in MP measurements between the Densitometer and the Spectralis, which suggests that age (and maybe lens density, which is known to increase with age) does not influence MP measurement when using the Spectralis’ AF method. However, further investigation in patients with cataracts is needed to fully understand the effect of lens absorption and scatter when using this device.

The Zeiss reflectance method is quick and requires minimal subject involvement. The technique used applies mathematical models which, the manufacturers claim, correct for the many assumptions inherent in their method. These assumptions include: 1. the reflection spectrum of the retina is homogenous across the retina; 2. intraocular light scatter can be corrected using mathematical models (however, these models are not well described and contain many free and undefined parameters); 3. distribution of RPE melanin and other light absorbers is the same throughout the retina; 4. there is negligible MP at the parafoveal reference area. As mentioned earlier, the reflectance technique can measure MP either by comparing the light reflected at the macula to the light reflected at a peripheral (reference) area, or by a spectral analysis of the reflected light (Berendschot and van Norren, 2004). The former method can also be performed using one or two wavelengths. The benefit of having two wavelengths, as opposed to one, is that the lens absorbance can be estimated and controlled for (Berendschot and van Norren, 2004). The Zeiss Visucam® 200 (the reflectance device used in this study), however, only uses one wavelength (a narrow-band wavelength: 480–500 nm), and therefore cannot

account for lens absorption. We see this as a serious weakness in the Zeiss method.

In conclusion, MP values obtained using the Heidelberg Spectralis are comparable to MP values obtained using the Densitometer. In contrast, MP values obtained using the Zeiss Visucam are not comparable with either the Densitometer or the Spectralis MP measuring devices, and the Zeiss Visucam appears to underestimate MP measurement. The Densitometer and Spectralis are suitable for measuring MP in both the clinical and research settings, whereas the Visucam is not. Further study using Heidelberg Spectralis is required to assess its suitability in patients with ocular disease, including cataract and AMD.

Disclosures

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