EXTENDED REPORT

In vivo macular pigment measurements: a comparison of resonance Raman spectroscopy and heterochromatic flicker photometry

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Aim: To investigate whether two methods of measuring macular pigment—namely, heterochromatic flicker photometry (HFP) and resonance Raman spectroscopy (RRS)—yield comparable data.

Methods: Macular pigment was measured using HFP and RRS in the right eye of 107 participants aged 20–79 years. Correlations between methods were sought and regression models generated. RRS was recorded as Raman counts and HFP as macular pigment optical density (MPOD). The average of the top three of five Raman counts was compared with MPOD obtained at 0.5° eccentricity, and an integrated measure (spatial profile; MPODsp) computed from four stimulus sizes on HFP.

Results: The coefficient of variation was 12.0% for MPODsp and 13.5% for Raman counts. MPODsp exhibited significant correlations with Raman counts ($r=0.260$, $p=0.012$), whereas MPOD at 0.5° did not correlate significantly ($r=0.163$, $p=0.118$). MPODsp was not significantly correlated with age ($p=0.062$), whereas MPOD at 0.5° was positively correlated ($p=0.011$). Raman counts showed a significant decrease with age ($p=0.002$) and were significantly lower when pupil size was smaller ($p=0.015$).

Conclusions: Despite a statistically significant correlation, the correlations were weak, with those in excess of 90% of the variance between MPODsp and Raman counts remaining unexplained, meriting further research.

Macular pigment is composed of the oxycarotenoids lutein and zeaxanthin. These pigments are potent antioxidants, which are thought to protect the retina and other adjacent tissues from the damaging effects of oxidative stress and thus limit ageing or other degenerative processes.^{1–6} Two psychophysical methods have been used to measure macular pigment in vivo—namely, heterochromatic flicker photometry (HFP)^{7–8} and minimum motion photometry⁹—as well as several physical methods, such as fundus reflectometry,¹⁰ autofluorescence spectrometry¹¹ and resonance Raman spectroscopy (RRS).¹²

Currently, the most widely used method of measuring macular pigment is HFP. The advantages cited by its proponents are relatively inexpensive instrumentation, absence of necessity for pupillary dilatation, relative robustness to changes in ocular media that accompany increasing age, and the short period required to train operators to achieve reliable results.¹³ However, the patient typically needs to be alert and cooperative, and as central fixation is essential, good visual acuity is required. These disadvantages limit the use of HFP in elderly populations in whom robust reliable estimates of macular pigment are most needed to identify putative links between altered macular pigment and macular disease.

RRS is a more recently developed alternative, and this technique provides an objective, rapid, sensitive, specific and highly reproducible method for estimating macular pigment in patients with a wide variety of macular pathological features as long as they retain central fixation.^{14–16} However, features of normal ageing such as senile miosis and lens opacity are known to degrade the Raman spectra and may also limit its application in cohorts of elderly people.¹⁴ The present study was designed to compare HFP and RRS in healthy adults from a wide age range, and the results of this comparison are presented.

PARTICIPANTS AND METHODS

Participants

All procedures were carried out according to published protocols and none of the authors had any proprietary interest

in either technique. Informed consent was obtained from all participants. The research ethics committee of the Faculty of Medicine, Queen's University of Belfast, Belfast, UK, approved the study design. The study adhered strictly to the tenets of the Declaration of Helsinki on research on human volunteers. Participants were contacted via their general medical practitioners, or by means of posters and leaflets distributed throughout the hospital. Those eligible for screening were men and women aged >20 years, without a history of major ocular disease (uveitis, glaucoma, diabetes, macular degeneration, amblyopia, retinal detachment or ocular trauma). Cataract was not an exclusion criterion, but as the visual acuity cut-off for the study was 0.1 logarithm of the minimum angle of resolution (LogMAR) (20/25), the presence of any major medial opacity in eligible participants was highly unlikely.

Heterochromatic flicker photometry

HFP is based on psychophysical brightness matching principles.^{7–8} It requires the patient to adjust a flickering stimulus to a point of minimum flicker or no flicker, while first fixating foveally and then parafoveally (7°). As macular pigment absorbs blue light, the perception of flicker is altered by pigment concentration, thus allowing an estimate of the macular pigment optical density (MPOD) to be made. The machine used was an early version of the HFP densitometer and was on loan to us from Macular Metrics, Rehoboth, Massachusetts, USA.⁸ The protocol we used was based on the methods developed and validated for the Carotenoids and Age Related Eye Disease Study (CAREDS).¹³ As the instrument on loan differed slightly from that used in CAREDS, minor

Abbreviations: CAREDS, Carotenoids and Age Related Eye Disease Study; HFP, heterochromatic flicker photometry; LogMAR, logarithm of the minimum angle of resolution; MPOD, macular pigment optical density; MPOD0.5, macular pigment optical density measured at the 0.5° arc radius; MPODsp, macular pigment optical density spatial profile; PPS, predicted pupil size; RRS, resonance Raman spectroscopy

changes in the protocol were needed. The instrument was set up to test only the right eye, and hence only right eyes were study eyes.

Four test field configurations were used to enable a spatial profile to be drawn. The first three stimuli were centred on the fovea, and the stimulus appearance and sizes were (a) solid disc 10' arc radius, (b) solid disc 0.5° arc radius, (c) annulus 1° arc radius and (iv) solid disc 0.5° arc radius centred 2° from fixation providing an eccentricity of 2.5°. After several practice runs, four different determinations of the point of minimal flicker were made for each target.

Resonance Raman spectroscopy

The Raman spectroscope was purchased from Spectrotek LC, Salt Lake City, Utah, USA. The principles and protocol for best practice of RRS have been described in detail by Bernstein *et al.*^{14–16} It is a relatively new technique that exploits the inherent property of lutein and zeaxanthin to produce resonance Raman spectra when stimulated using 488-nm argon laser light. Pupillary dilatation was achieved using tropicamide (Minims, Charvin Pharmaceuticals Ltd, UK) 1% for those aged <50 years or a combination of cyclopentolate 1% and phenylephrine 2.5% for those aged >50 years to ensure maximum dilatation.¹⁷ Pupil size was recorded to the nearest 0.5 mm using a millimetre rule after maximum dilatation. Raman signal intensity is expressed as photon counts. To overcome errors introduced by misalignment or blink, the manufacturers advise that the highest three of the five measurements recorded at any one sitting be used for statistical analysis.

For assessment of variability, HFP using all the stimulus sizes and Raman counts were measured on 11 volunteers who attended four times over a 2-week period.

Clinical evaluations

Best-corrected visual acuity was recorded in each eye separately using the Early Treatment Diabetic Retinopathy Study (ETDRS) logarithm of the minimum angle of resolution charts at 4 m, and the anterior segment was clinically assessed by slit-lamp biomicroscopy. The status of the crystalline lens of each eye was recorded after comparison with Lens Opacities Classification System II standards.¹⁸ Iris colour was recorded as brown, intermediate or blue using the European Eye Study (EUREYE) standards.¹⁹ A history of cigarette smoking, which is considered to be a risk factor for low macular pigment, was obtained.²⁰

Photographic and grading procedures

All photographs were taken using the Topcon TRC 50 EX acquisition system with imageNET2000, V.2.53, Digital Imaging systems (Topcon, UK). A single anterior segment image of each eye was taken after full pupillary dilatation. The diameters of the dilated pupil and the cornea were measured using the measurement tool on the imageNET software. For fundus grading, a pair of 35° images of field 2 were captured (Wisconsin Age-Related Maculopathy Grading System).²¹ The fundus images were graded using standardised protocols based on the definitions provided by the Wisconsin Age-Related Maculopathy Grading system.²¹

Definitions and statistical analysis

We used two HFP parameters in the analysis. The first parameter was the MPOD from the 0.5° arc radius (MPOD0.5), which we used because it was shown to have the best within-to-between-subject variation.¹³ The second parameter was the area under the spatial profile curve (MPODsp), which was plotted using the right eye measurements from all four targets where the x axis (angle) was transformed logarithmically to avoid overinfluence of results at large angles.

Calculation of the area under the curve was based on the trapezium rule, with no extrapolation made for any areas beyond that bounded by the measurements.

The diameter of the laser beam at the pupil was 7 mm. To prevent its absorption by the iris, we excluded the data from participants whose pupils were <7 mm. in line with the recommendations of the manufacturers of Raman spectroscopy.^{14–16 22} Pupillary diameter was measured using a millimetre rule, which is a crude measurement. Therefore, we also measured pupillary diameter from the anterior segment image. As image magnification can vary with camera focusing, we derived the pupillary diameter:corneal diameter ratio. Linear regression was undertaken using pupil size measured on the millimetre rule as the dependent variable, and pupillary diameter and pupillary diameter:corneal diameter ratio as independent variables. The unstandardised predicted values from the model were saved and used as the surrogate measurement for pupil size. This method works on the assumption that both techniques of measuring pupillary diameter are prone to measurement error, but that this error may be minimised by using a third variable that is a composite of the two methods. We termed this variable the predicted pupil size (PPS).

Data were analysed using SPSS V.10. The RRS data were found to be skewed and were therefore log transformed to assume a normal distribution and enable comparison with MPOD, which is a logarithmic measure. Data obtained from the 11 participants (7 women, 4 men, age range 21–50 years) who returned four times over a 2-week period were used to construct a components of variance model to calculate coefficients of variation for between-visit repeatability for both methods. The coefficient of variation was expressed as a percentage. Pearson's correlation coefficients were calculated to investigate the relationship between average Raman and the two HFP variables (MPOD0.5, MPODsp). A series of regression analyses were run with average Raman, MPOD 0.5 and MPODsp as the dependent variables and age as the independent variable. Linear regression using backward stepwise elimination was applied to examine relationships between average Raman (dependent variable) and age, sex, iris colour, pupil size, smoking history, lens and age-related maculopathy status as independent variables.

RESULTS

Participant demographics

A total of 107 people (69 women, 38 men), ranging in age from 20 to 79 years (mean 42 years), participated in the study. Data from HFP recordings from four participants were deemed unreliable, as the standard deviation of 0.5° arc radius stimulus determination exceeded 0.20; therefore data from 103 participants were available for univariate analysis. In nine participants pupillary dilatation failed to reach the specified value, and data from these were excluded from all analyses. Raman counts could not be obtained from one participant owing to equipment failure. Therefore Raman counts from 93 participants were available for univariate analysis. After accounting for all exclusions, 93 sets (HFP and Raman counts) were available for the bivariate analysis. Table 1 gives a summary of the demographic findings, participant numbers and the effect of categorisation by sex, smoking status, iris colour, and decade of age on mean MPOD0.5 and average Raman. Mean (standard deviation (SD)) MPOD0.5 was 0.38 (0.18). The mean of the average Raman was 1048 (SD 435).

Repeatability

Of the 11 participants, seven were women and four were men; the average age of the participants was 31.5 (range 21–

Table 1 Average Raman and MPOD0.5 stratified by participant characteristics

	RRS			HFP		
	Participants (n = 97)	AR*	SD	Participants (n = 103)	MPOD0.5†	SD
Overall	97	1048	435	103	0.38	0.18
Men	34	1122	478	37	0.38	0.19
Women	63	1008	408	66	0.39	0.18
Iris						
Blue	66	1033	448	72	0.38	0.18
Intermediate	15	1120	385	15	0.37	0.21
Brown	16	1039	445	16	0.41	0.13
Smoking status						
Current smoker	9	823	396	10	0.34	0.14
Non-smoker	85	1073	434	82	0.38	0.19
Age (years)						
20–29	34	1220	459	35	0.34	0.18
30–39	19	1060	459	20	0.39	0.18
40–49	15	907	456	16	0.36	0.16
50–59	13	1058	347	15	0.41	0.17
60–69	14	784	329	14	0.44	0.20
70–79	2	837	203	3	0.53	0.07
>60	16	791	311	17	0.46	0.17
Lens status						
0	81	1082	451	86	0.38	0.18
1	2	1006	469	2	0.39	0.24
2	8	946	272	8	0.48	0.14
3	6	729	258	7	0.39	0.16
No drusen or just hard drusen	94	1050	438	98	0.38	0.17
Soft distinct drusen	2	953	544	4	0.53	0.25
No drusen, focal pigment present	1	974	0	1	0.34	-

AR, average Raman (the average of the top three counts from each participant); HFP, heterochromatic flicker photometry; MPOD0.5, macular pigment optical density measured at 0.5° arc radius; RRS, resonance Raman spectroscopy.

*Mean Raman counts from groups of participants.

†Data represent the average of four independent readings per participant.

50) years. The components of variance model showed that MPODsp exhibited less between-visit variability (12.0%) when compared with average Raman (13.5%); and the 0.5° arc radius stimuli showed the least variability (11.5%; table 2).

Correlations between MPOD and Raman counts

MPOD0.5 did not significantly correlate with average Raman ($r = 0.163$, $p = 0.118$), whereas MPODsp was significantly correlated ($r = 0.260$, $p = 0.012$). The correlations were weak, with those in excess of 90% of the variance between MPODsp and Raman counts remaining unexplained. A scatter plot of MPODsp and average Raman (fig 1) shows this relationship.

Influence of age on MPOD and Raman counts

Regression analyses with age as the independent variable and each of the two HFP parameters and average Raman entered as

the sole dependent variable into three separate models showed that MPODsp was not significantly correlated with age. MPOD0.5 had a weak significant positive correlation (table 3), and average Raman showed a significant negative correlation with increasing age.

Relationships between putative risk factors and average Raman and HFP parameters

Multiple linear regression with backward stepwise elimination in a model with average Raman as the dependent variable and age, sex, iris colour, PPS, smoking status, lens status and age-related maculopathy status as independent variables yielded age and PPS as the most significant parameters (model 1, table 4). All other parameters were rejected by the model. Apart from age, none of the parameters reached significance when a similar model was run with MPOD0.5 as the dependent variable (model 2, table 4). In model 1, after exclusion of non-significant independent variables, the final model (shown by *) included only age and PPS with an adjusted $R^2 = 0.138$. In model 2, after exclusion of non-significant independent variables, the final model (shown by *) included only age with an adjusted $R^2 = 0.052$.

DISCUSSION

Many studies using HFP have found MPOD to lie in the range 0.21–0.47.^{23–27} The mean MPOD0.5 in our population was within this range and closest to that reported in CAREDS (0.38 in the present study v 0.42 in CAREDS). The mean Raman counts in the present study (1048) are similar to those in another study that used an identical instrument (1118).²⁸

The present study was designed to examine the strength of the relationships between the two methods of measuring macular pigment—namely, HFP and Raman spectroscopy—through undertaking both sets of tests on the same eye of the sample

Table 2 Between-visit repeatability in measurements by resonance Raman spectroscopy and heterochromatic flicker photometry

Variable	Between-visit coefficient of variation (%)
HFP	13.0
10' arc radius	
0.5° arc radius	11.5
1° arc radius	33.0
2.5° arc radius	54.0
Spatial profile	12.0
RRS	
Average Raman	13.5

RRS, resonance Raman spectroscopy; HFP, heterochromatic flicker photometry.

Figure 1 Relationship between heterochromatic flicker photometry and average Raman. Although the scatter plot shows a considerable degree of variation, a significant correlation between the two methods ($R=0.260$, $p=0.01$) is evident.

population on the same day. We found a statistically significant correlation only between the MPODsp HFP parameter and average Raman, whereas the correlation between average Raman and MPOD0.5 was close to significance. It was evident, however, that the relationships were disappointingly weak, with substantial discordance detected between methods. The slightly stronger correlation noted between MPODsp and average Raman is intuitively in keeping with the nature of the RRS signal as it collects the back scatter from the retinal area covered by the 1-mm-diameter laser spot. It is therefore likely to correlate better with the HFP parameter that provides an integrated estimate of the area under the curve reflecting the spatial profile of macular pigment.

The present work is not in accord with another recent study that undertook a similar validation of the RRS against a flicker-based method.²⁸ Neelam *et al*²⁸ reported that MPOD measured by HFP and Raman counts obtained on RRS showed good correlations. By contrast, in the present study, correlations between these methods were poor. The following are the possible reasons for this discrepancy:

1. The maculometer used by Neelam *et al*,²⁸ although based on HFP principles, did not have the ability to optimise the flicker rate which is crucial for accurately defining the null zone and

Table 3 Regression models examining relationships between age and heterochromatic flicker photometry and resonance Raman spectroscopy parameters

Dependent variable	Adjusted R^2	Regression coefficient	t Statistic	p Value
Average Raman	0.091	-0.010	-3.27	0.002
MPODsp	0.025	0.002	1.89	0.062
MPOD0.5	0.052	0.003	2.58	0.011

MPOD0.5, macular pigment optical density measured at the 0.5° arc radius; MPODsp, macular pigment optical density spatial profile.

thus reliability.²³ Critical flicker frequency is influenced by both age²⁹⁻³¹ and MPOD levels,³² necessitating its determination in each participant to prevent confounding.

2. Neelam *et al* based their conclusions on the use of Bland and Altman plots, which simply measure agreement between methods rather than correlations. Notably, correlations can be poor despite good agreement shown by Bland and Altman plots.
3. Neelam *et al* converted Raman counts arbitrarily to optical density measurements to enable comparison with MPOD using Bland and Altman methods. Given the radically different scientific principles that underlie the two methodologies (HFP and RRS), we believe it inappropriate to convert Raman counts to MPOD.

Neelam *et al*²⁸ also state that MPOD and Raman counts in their sample population showed the same relationships with age, sex and serum concentrations of lutein and zeaxanthin, thus authenticating RRS as a valid method of measuring macular pigment. In the present study, with a sample whose upper age limit was 79 years, we found that MPOD and Raman counts showed differential relationships with age. MPOD0.5 showed a small positive correlation with age, MPODsp was unrelated to age and Raman counts reduced significantly with increasing age. Our findings are consistent with the majority of the literature in this discipline, which show that MPOD does not change with increasing age,^{11 23 24} whereas Raman counts decline with age even after participants with insufficient pupil dilatation were excluded.^{16 33} Also supporting an absence of an association between MPOD and age are the findings of biochemical analysis of autopsy samples.³⁴

The correlations between RRS and HFP are notably lower than those found in studies that have compared HFP with other physical methods of measuring macular pigment, where

Table 4 Parameter estimates from two regression models examining the relationships between putative risk factors and the two methods of macular pigment estimation

Independent variables	Model 1: average Raman			Model 2: MPOD0.5		
	Regression coefficient	t Statistic	p Value	Regression coefficient	t Statistic	p Value
Age	-0.010	-2.30	0.02	0.004	2.63	0.01
Sex	-0.043	-0.43	0.67	0.037	-0.67	0.50
Iris colour	0.031	0.50	0.62	0.012	0.51	0.61
PPS	0.224	2.10	0.04	—	—	—
Smoking history	0.092	1.22	0.23	0.046	1.59	0.12
Lens status	0.055	0.761	0.45	-0.025	-0.94	0.35
Age-related maculopathy status	0.163	0.85	0.40	0.042	0.64	0.52
Age*	-0.008*	-2.69*	0.009*	0.002*	2.73*	0.007*
PPS*	0.253*	2.47*	0.015*			

AR, average Raman; MPOD0.5, macular pigment optical density measured at 0.5° arc radius.

*The final model after exclusion of non-significant variables.

intermethod correlations range from 0.42³⁵ to 0.77.¹¹ One possible explanation for the discordant results when comparing flicker-based methods and RRS is the attenuation of the Raman signal by an age-related increase in lens optical density as a result of yellowing.^{36–38} The input and output signals of the RRS laser are within the wavelengths absorbed by the crystalline lens, and individual differences in absorption may differentially attenuate the signal. Although yellowing increases with age,^{36–38} the variation between individuals is substantial, meaning that a simple age-related correction factor would not reliably compensate for the attenuation. Although nuclear colour was evaluated using Lens Opacities Classification System II classification, it is not a reliable measure of yellowing. Therefore, differences in short-wavelength absorption may be present, although the lens may seem clear from a clinical perspective. This important factor that influences Raman signal intensity may account for some of the age-related decline in macular pigment when measured by this method.

Our analysis also showed that the Raman signal was adversely influenced by the pupillary diameter even in eyes with diameters ≥ 7 mm. This finding is not in agreement with that of Neelam *et al.*,¹⁷ who reported that the age-related decline in Raman counts was dramatically attenuated to non-significance on exclusion of participants with a pupil size < 7 mm. The differences between the present study and that of Neelam *et al.*¹⁷ may be related to the technique used to measure pupillary diameter. Although we also used a millimetre rule to determine a cut-off for inclusion or exclusion, we accepted that this is likely to lead to measurement error. Therefore, in the present study, the corneal diameter:pupillary diameter ratio along with the pupillary diameter measured on the millimetre rule was used to derive a more robust measurement, thereby improving the validity and reliability of the data.

Head stabilisation and alignment are also critical for the optimisation of the Raman signal, and may contribute to recording noise and account for the differences in the methods.^{39–40} These factors are particularly pertinent in those participants whose pupil diameter is close to the 7-mm limit, where alignment is much more critical to ensure that the laser beam is not impeded by the iris as it traverses the anterior segment.

In conclusion, although a small significant correlation between MPOD and Raman counts suggests that a relationship exists between measurements by HFP and RRS, our study has shown that these two methods of measuring macular pigment in vivo cannot as yet be used as surrogates for each other.

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