Resonance Raman spectroscopic measurement of carotenoids in the skin and retina

Billy R. Hammond
University of Georgia
Vision Sciences Laboratory
Department of Psychology
Athens, Georgia 30602
E-mail: bhammond@egon.psy.uga.edu

Billy R. Wooten
Brown University
Walter S. Hunter Laboratory
Providence, Rhode Island

Abstract. Carotenoid pigments within the skin and retina are measured using resonance Raman spectroscopy (RS). These RS instruments are unique in that they have been designed to obtain vibrational spectra in normal and diseased subjects using noninvasive procedures. Raman spectra have traditionally been used as a means of identifying a given chemical within some substrate. The new generation of RS instruments, however, has been designed to quantify the amount of carotenoids within the retina and skin. These amounts are typically reported in nonstandardized units called Raman counts (RC). These RCs are dependent on many factors intrinsic to their measurement, such as the specific optics used for stimulation and acquisition. The question of whether RCs can be used to derive valid quantitative measures of the carotenoid pigments *in vivo* is discussed. [DOI: 10.1117/1.2116767]

Keywords: Raman spectroscopy; carotenoids; macular pigment; lutein; zeaxanthin.

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1 Introduction

A recent series of articles[1–20] (e.g., 19 articles in the last few years by one research group) described the ongoing development of Raman spectroscopy (RS) for the noninvasive measurement of specific carotenoids in the skin and retina. This development reflects a real need to quantify carotenoids (antioxidant pigments that may prevent a number of degenerative diseases[21]) as noninvasive biomarkers for epidemiological research and clinical study. For these quantities to be meaningfully interpreted, however, a number of measurement issues must be considered. These measurement issues center on the issue of calibration.

Unlike all other quantitative methods of measuring carotenoids *in vivo*, Raman values are reported in units that are defined according to the specific testing conditions used in the experiment. In brief, these values, called Raman counts (RCs), are derived by measuring backscattered light using a unique optical arrangement and analysis program designed to extract a signature intensity peak (at around 527 nm) from a broader light signal composed mostly of fluorescent light. For RCs to be translated into numbers that can be externally referenced (e.g., optical density units), a realistic calibration procedure is needed. Unlike some methods of measuring carotenoids such as high-performance liquid chromatography (HPLC), an internal standard cannot be used; therefore, an external calibration is necessary to supply the necessary conversion factors. Such a calibration must precisely account for all relevant variables that affect the relation between the *in situ* value of the carotenoids being measured and the response of the detector. Average corrections are insufficient, since these variables can vary widely between individuals. We review the existing data regarding measuring carotenoids in the skin and retina using RS from a critical perspective, and discuss the variables that must be evaluated for external calibration models to be valid. We also consider difficulties in applying such calibrations to actual individual measurements.

2 Issues Using Raman Spectroscopy to Measure Carotenoids within the Skin

There are two major methods used to assess carotenoids noninvasively by measuring scattered light: reflectometry and RS. The former measures elastic or Rayleigh scatter, whereas the latter measures inelastic scatter (light that has changed frequency because of interaction with the molecule). In a typical measuring scenario, the skin is exposed to an intense laser light (488 and/or 514 nm), which illuminates carotenoid molecules found primarily within the stratum corneum.[22] The laser light induces a large fluorescence signal and a much weaker but detectable Stokes line. This Stokes line is usually detected at around 530 nm (this value being slightly different for different carotenoids). Using baseline correction algorithms, the Stokes line can be extracted and peak heights can be used to derive RCs. The question of how accurately these scattered light signals reflect individual differences in carotenoid concentrations within the palm was originally addressed by Hata et al.[22] These authors, however, measured carotenoid concentrations in just three samples of abdominal skin obtained from a tissue bank. These samples were analyzed for carotenoid content by HPLC, and then the values were compared to RC counts. Of the three data points, one was assumed to be zero, since no signal could be detected. The overall correlation was nonsignificant ($p < 0.41$). To date,

Address all correspondence to Billy Hammond, Psychology Dept., University of Georgia, Baldwin, Athens, GA 30602. Tel: 706–542–4812. Fax: 706–542–3275. E-mail: bhammond@egon.psy.uga.edu

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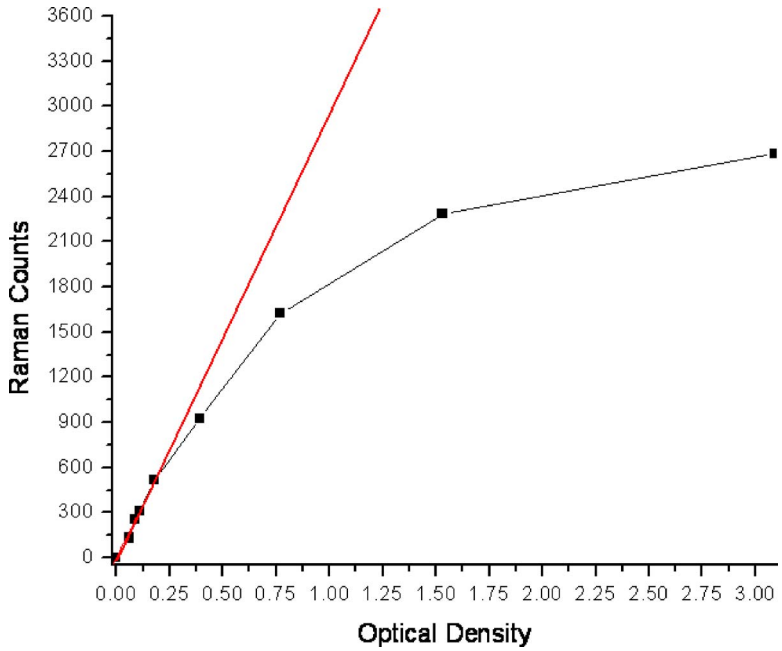

Fig. 1 The relation between Raman counts and OD values. These data were derived from the *in vitro* calibration procedure described in Bernstein et al.[19] using their Fig. 2. The straight line represents the least-squares linear fit to the lowest five data points after the Raman data had been normalized to reflect an origin of zero (so that a RC of zero was equivalent to an OD of zero).

these are the only validation data available. Based on these results, Hata et al.[22] nonetheless concluded that their method is "precise, accurate, specific, and sensitive." The question of whether this limited preliminary data proves the validity of their method is, however, clearly open to question. The thin, bloodless, postmortem-abdominal skin from three subjects, for instance, probably does not reflect the kind of variations one would see in the thicker palmar skin of living subjects. Obviously, what is needed is more direct comparisons (e.g., doing the comparisons at the same site) on a large diverse group.

In the absence of direct data, the authors[23] argue that the method is valid based on a wide variety of indirect evidence. For example, Gellerman, Ermakov, and Bernstein[23] report that in a study of 1266 healthy volunteers, they found a significant positive relation between palmar RCs and self-reported fruit and vegetable intake, and a significant negative relation to smoking behavior. Since fruit and vegetable intake and smoking are expected to raise and lower skin carotenoid concentrations, respectively, the finding that RCs vary in the expected direction is argued as evidence that the method is valid. Again, however, these data (and therefore their conclusions) are nearly impossible to evaluate. The work by Gellerman, Ermakov, and Bernstein[23] contains no details regarding how the data were collected. Instead, the authors cite another paper by different authors (Ref. 17 in Smidt and Shieh). The paper by Smidt and Shieh is listed as a submitted manuscript that, as of this date, has not yet been published.

The ability to critically analyze the results presented by Gellerman, Ermakov, and Bernstein[23] is especially important, since the results they report are unusual as well. For example, the subjects with the highest fruit and vegetable intake (six or more servings per day) still have relatively low palmar RCs (mean=26342) when compared to the range of RCs reported in Fig. 1 of their paper. This range was 15,000 to 55,000 and related very highly to total carotenoids in the serum, which ranged from about 0.40 to 3.4 μg/ml. These observations imply that the "pronounced" relation reported by the authors is actually quite weak (the correlation was not given and the data are unpublished, so a complete analysis is not possible). From a statistical perspective, the relations are hard to evaluate, since the groups the authors compared are such different sample sizes. For example, 30 subjects with high fruit and vegetable intake (>6 servings per day) were compared with 534 subjects with low fruit and vegetable intake (1 or less serving per day), and 32 heavy smokers (>5 cigarettes per day) were compared to 1047 nonsmokers.[24]

Gellerman, Ermakov, and Bernstein[23] also contend that hemoglobin and melanin within the skin do not confound the Raman measurements. Melanin does absorb the Raman input and output wavelengths, and is found at the measuring sites within the stratum corneum.[25,26] These pigments would therefore have to confound the Raman signal. The real question is what is the magnitude of the confound. It is possible, for instance, that the effect is sufficiently small so that the resultant RCs are not affected for most individuals. Gellerman, Ermakov, and Bernstein[23] argue that "when comparing statistically significant groups of Caucasians and African Americans, we find an insignificant difference in Raman levels." This result, however, is based on a comparison of only 8 African Americans with 1111 Caucasians.[27] Moreover, no information is available regarding the measurement conditions, the method used to quantify differences in melanin content, etc. This type of information is needed to determine whether the groups were sufficiently matched (e.g., equating a light-pigmented and dark-pigmented group with regards to dietary intake would be insufficient). To directly determine whether

melanin is confounding the Raman signal (e.g., by absorbance of the stimulating beam), carotenoids and RCs should be directly measured at the same site in a number of subjects with varying amounts of melanin.

Gellerman, Ermakov, and Bernstein[23] note that individual RC values could be corrected for confounds such as melanin, thickness differences, etc., if these variables were also measured. The authors, however, have never applied such corrections to their own data or published results that could be used to accurately derive corrections based on mathematical averages. In any event, average corrections would be inconclusive, since individual variation is high (e.g., the oxygenation state of hemoglobin changes its absorption spectrum). To make individual corrections would require individual measurements (e.g., of melanin content), which would require additional equipment and time. Gellerman, Ermakov, and Bernstein suggest that corrections for all of the confounds could be made by measuring absorption and scatter within the tissue. This would be difficult, since the correction would have to precisely match the tissue being measured using the Raman stimuli (e.g., both would have to penetrate to the same depth).

They also argue that morphological changes within the stratum corneum do not influence RCs (even in diseased skin). The basis for this contention is unclear, since the authors apparently have not made morphological measurements. Rather, the authors point out that they have made qualitative inspections ("squamous cell carcinoma tissue sites … appeared optically quite similar upon visual inspection to adjacent healthy tissue sites").[23] Without actual measurements, however, morphological changes cannot be determined.

Gellerman, Ermakov, and Bernstein[23] note that making a direct HPLC/RC correlation is "of course desirable, but they are difficult to perform on a large group of healthy normal subjects in view of the extreme invasiveness of the HPLC method, which typically requires prohibitively large tissue samples." Such measures, however, have been done. For example, Peng et al. (1995)[28] obtained HPLC measures of carotenoids within the skin of 96 subjects using 3-mm punches. Similar measures are needed before the Raman method is mass marketed as an "objective portable device" and before it can be used as a tool for assessing biomarkers in cancer research.

A direct comparison is essential. The indirect comparisons the authors report cannot be used to assess the quantitative validity of the Raman method for measuring carotenoids in the skin. For example, Smidt, Gellerman, and Zidichouski[29] provide data on 104 subjects showing the relation between RCs measured in the palm and total serum carotenoid concentrations. The authors show a strong correlation between the two variables ($r=+0.78$), concluding that RCs are able to predict variation in fasting total serum carotenoids within ±10%. This result was replicated in 372 subjects when subjects were tested multiple times ranging over several days (correlation coefficients ranged from 0.78 to 0.82).[30]

If one assumes that their results are accurate, and one assumes that RCs are an extremely valid measure of carotenoids within the skin, then correlation coefficients this high for this many subjects suggest that there is a nearly perfect relation between circulating carotenoids within the serum and skin concentrations. The strength of any correlation is attenuated by the between-session reliability of the variables under consideration (the coefficient is less than or equal to the product of the square root of the reliability of the two measures).[31] For example, if one assumed that the test-retest correlations for total carotenoids within the serum and within palmar RCs (measured across days) was 0.80 (the authors note intraindividual variability of about 10% for the serum values and Raman measures), a perfect correlation between the two variables would yield an r of about 0.80. Thus, a correlation of 0.78 could only be obtained by chance or if the true correlation between the two variables is nearly perfect. Such a result is inconsistent with past data (e.g., Peng et al.[28]). For example, Peng et al. measured fasting carotenoid levels several times for 96 subjects over the course of one month to get a very stable baseline measure of serum carotenoid concentrations. Peng et al. then compared these measures to individual carotenoids measured directly using HPLC within well-defined areas of skin taken from the thigh (a tissue less subject to mechanical and actinic stress than the palm). Peng et al. found an average correlation of 0.57 between carotenoids in the serum and in the skin (with correlations ranging from 0.26 to 0.75). For example, the relation between the levels of skin lycopene and lutein and serum lycopene and lutein was 0.52 and 0.51, respectively. The correlation between lutein and zeaxanthin in the retina (measured using another method based on measuring scattered light, reflectometry) and blood is about 0.30.[32] Since the authors have presented only the results and not the details of their method, it is difficult to evaluate the discrepancy between the author's results and past data.

It should be noted that the authors[23,33] have reported "strongly disagreeing" with our critique of their method and noting that Raman spectroscopic analysis of skin is "specific, sensitive, and precise," and stating that to their knowledge "there are no serious confounding factors for the technology." We would like to stress that we are *not* arguing that their method is invalid. Rather, we disagree with the authors' continued argument that the method *is* valid. This is not a matter of opinion. There is currently no published data that adequately addresses the validity of the method. The argument that the method is specific and precise is based on the fact that Stokes lines are exactly determined by the configurational characteristics of carotenoid molecules (and therefore they are very specific and precise). The magnitude of these specific signals, however, can be altered in numerous ways. For example, both the input and output Raman wavelengths can be attenuated by numerous light absorbing molecules, and there is currently no evidence regarding the precise degree of these confounds.

3 Issues Using Raman Spectroscopy to Measure Carotenoids within the Retina

Although the carotenoids lutein and zeaxanthin are found throughout the tissues of the eye, they are only dense enough to return a Raman signal in the anterior layers of the central fovea [at this site, termed macular pigment (MP)]. Similar to the skin measurements, these signals are typically reported as Raman intensity (or counts) and reflect the peak height of the backscattered light at the signature frequencies of lutein and zeaxanthin. For these numbers to be interpretable, they must

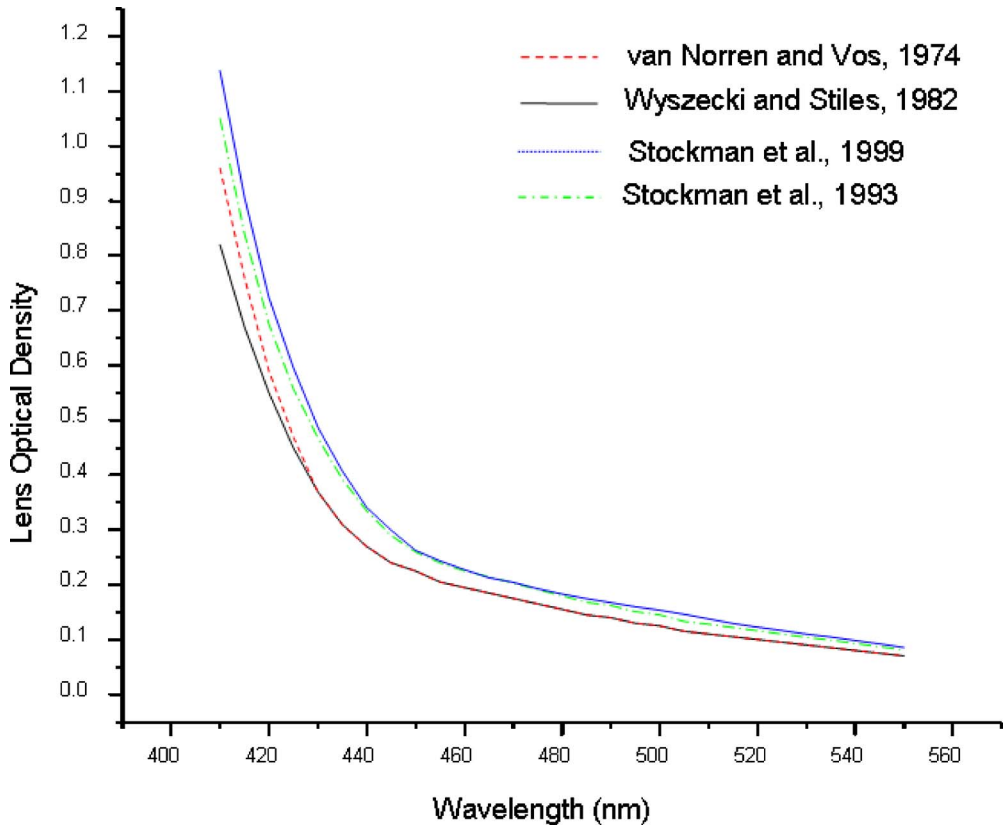

Fig. 2 Absorption spectrum of the human lens as measured using a variety of *in vivo* and *ex vivo* methods.[40–43] The data from van Norren and Vos represents an average based on 13 different studies (their lens calculations being mostly based on derivations from the CIE scotopic spectral sensitivity curve from a standard rhodopsin spectrum). The data from Wyszecki and Stiles represent an average based on five different studies using both *in vivo* and *ex vivo* techniques.

be translated according to accepted units such as optical density or chemical concentration. This has been done using several models.[2,13,19] For example, these models include an artificial eye consisting of a glass ball, a plastic lens, and a hollow sphere that is the same size as a typical human eye[13] and a 53-diopter lens that forms a 1-mm spot in the plane of the solution.[2] Data from the latter model[19] are shown in Fig. 1[19] (data derived from their Fig. 2). In this figure, we plotted their data only up to a value that represents a reasonable peak density for MP (an OD of ~1.6). We also adjusted their Raman values so that a zero RC was equivalent to zero OD (in the original figure, zero OD is equivalent to an RC of about 64). Using this origin, the bottom five data points are well described by a best-fitting linear curve (r^2=0.99). Using the zero origin, the sixth point clearly falls below the best-fitting linear line and defines the beginning of the curvilinear nature of the empirical relation. As seen in Fig. 1, the underestimation of the empirical points relative to the linear fit is clear and increases strongly with increasing density. For example, a peak MP optical density of 1.0 is relatively common, and it is not uncommon to have a MPOD approaching 1.0 at 30-min eccentricity. As shown in Fig. 1, however, the empirical points from the model underestimate the linear fit at 1.0 by about 38%. The curvilinear nature of this curve is based on the decreasing ability of the laser to penetrate pigments of increasing density. Of course, this penetration depth (and thus, the translation of the values to OD) is dependent on the intensity of the laser at the level of the reflecting material. With the model eyes, the incident laser and the returned Raman signals are always constant. With real eyes, the intensity of the laser and the magnitude of the returned Raman signal is influenced by many properties that vary widely across individuals and change systematically with age.

The accuracy of the calibration is particularly important for the Raman method, which is fundamentally different than most other existing methods for measuring MP. The primary difference is that the Raman method measures absolute signal strength arising from a specific retinal locus. In contrast, most other methods are relative and use a peripheral reference to control individual differences in the anterior ocular media (the one other exception is a new imaging method based on spectral analysis of fundus reflectance[34]). By using an absolute signal, any factor that alters that signal (either attenuating or magnifying it) directly influences RCs. Thus, the validity of RCs (signal strength) as a quantitative measure of MP relies on the assumption that there is no significant degradation by the ocular media (or other intermediaries such as spectacle lenses[10]) that would influence across-subject comparisons. The questions that must be addressed to assess the validity of RS are whether there are any factors that might be reasonably expected to influence the input and output beams (488 and 527 nm, respectively) used in Raman assessments.

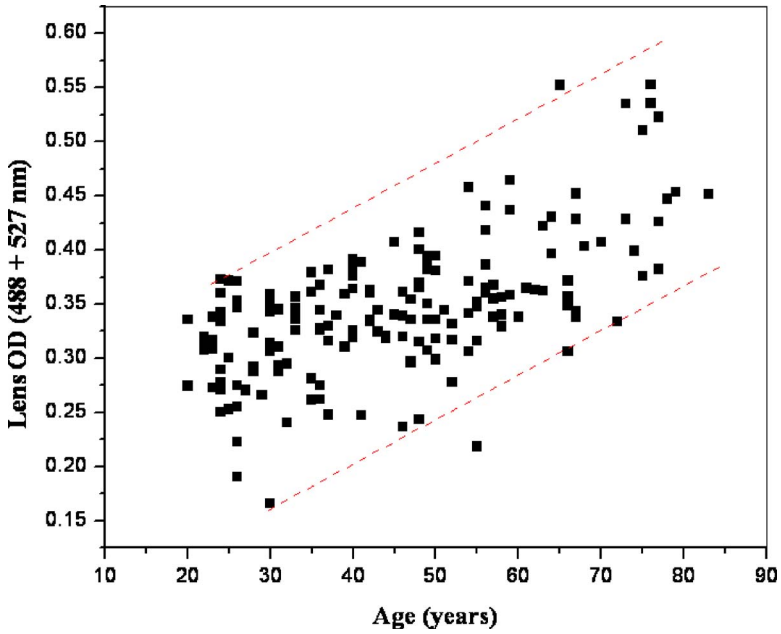

Fig. 3 Lens OD at 488 and 527 nm derived from data from Hammond et al.[44] Lens OD values at 410 nm were adjusted to 488 and 527 nm based on the assumption of an invariant spectral template, as shown in Fig. 1.

In a recent article, Zhao et al.[18] used resonance RS to measure macular pigment (MP) in normal subjects and patients with retinitis pigmentosa (RP), choroideremia (CHM), and Stagardt's macular dystrophy. This study found that there was no difference in the RCs measured in their patients compared to their matched controls. As noted, for the results of the Zhao et al. study to be valid, one must assume that there are no differences between the normal subjects and their retinal patients in the absorption and scatter of 488 and 527 nm light by the anterior media, extent of pupil dilation, etc. Since the overall average Raman counts for RP, CHM, and Stagardt's patients, as well as age-matched normals was low (ranging from RCs of 178 to 684, which, based on their calibration procedure, translates to an OD of about 0.03 to 0.14)[2], even small differences could confound the results.

There is a large body of empirical data comparing the anterior media of RP and CHM patients with age-matched normal controls. For example, several studies have measured intraocular scatter (which is wavelength independent)[35] in patients with RP and CHM, and found that even patients with relatively clear ocular media tend to have substantially higher scatter (by a factor of 2 to 3) compared to age-matched controls.[36–38] The authors[18] do state that they excluded subjects with "significant cataracts," because cataracts are a common complication of hereditary retinal degenerations such as RP. Of course, even subjects without visibly significant cataract can have higher than normal lens density and intraocular scatter. For example, RP patients tend to have subtle lens opacities that significantly interfere with their visual performance.[39] Grover et al.[37] noted that even CHM patients with "no clinically apparent changes in the lens" had much higher levels of intraocular scatter when compared to age-matched controls. Thus, there is substantial evidence suggesting that the anterior media of the clinical population studied by Zhao et al. differ from normal age-matched controls, making a direct comparison between these two groups problematic. A similar analysis could be applied to the results of Bernstein et al.[2] Bernstein et al. measured patients with age-related macular degeneration (AMD) and compared their RCs to age-matched controls. The authors report a 32% difference (RCs of 148 and 219) that they suggest was "striking and highly significant." When the nominal RC values, however, are translated into real numbers (using the author's calibration curve shown in Fig. 1), the actual difference is equivalent to an optical density of 0.02. This translates to an absorption difference of less than 3%. Such a small difference could easily be explained by differences in the characteristics of the anterior optics between the two groups.

Of course, the differences in anterior media would only be problematic if lens optical density (OD) and intraocular scattering of 488- and 527-nm light were of high enough magnitude to actually significantly affect Raman signals. Zhao et al.[18] state that their 76 normal subjects had "natural clear lenses," implying that OD at 488 and 527 nm was minimal Natural lenses, however, absorb a substantial amount of light at 488 and 527 nm. This effect is illustrated in Figs. 2 and 3.[40–43] Figure 2 shows the relative absorbance of the lens by wavelength. As can be seen in the figure, the shape of the absorbance profile is very similar across studies and methods, and that the magnitude of absorbance declines sharply with increasing wavelength. For the young eyes shown in this figure, total lens optical density (OD) at 488 and 527 nm averages about 0.25. This average OD increases strongly with age (see Fig. 3). Perhaps even more significant when trying to interpret individual Raman scores is the high individual variation in lens OD. This is illustrated in Fig. 3. In this figure, we used the spectral template shown in Fig. 2 to adjust the lens data originally presented in Hammond et al.[44] Hammond et al. only measured lens OD at 410 nm. Based on strong evidence showing an invariant spectral

template,* however, allows us to adjust these data to reflect absorbance at other wavelengths. This was done and is shown in the figure. As can be seen in the figure, lens OD both increases with age and varies significantly throughout life (i.e., independent of age). Although average corrections could be applied to group data, individual Raman values will be directly impacted by these kinds of individual variations in lens OD that are manifest throughout life.

Gellerman et al.[13] acknowledged that increases in lens density would cause an average 38% drop in the Raman signal when comparing 20 and 60 year olds. In a later publication, however, Bernstein and Gellerman[49] stated, "We agree that it might be useful to incorporate a lens correction factor for each subject, but we doubt that it would change our findings appreciably." More recently, the authors (Bernstein et al.[19]) have stated that, "It will also be useful to concurrently measure lens optical density objectively in the 488- to 527-nm range in a wide range of subjects in order to incorporate appropriate correction factors for age-dependent changes in the crystalline lens." Individual differences in the optical density of the anterior media would only be insignificant with the Raman technique if the media were essentially transparent at 488 and 527 nm for all subjects young and old. This possibility was argued by Bernstein and Gellerman[50] in a follow-up letter, but is inconsistent with the bulk of the published data using a variety of *in vivo* and *ex vivo* methods (see Wooten and Hammond).[51,52] The author's argument that lens optical density does not represent a significant confound is largely based on their finding that the RCs of elderly pseudophakes is only slightly higher than the RCs of age-matched controls (e.g., age range=65 to 76 yrs, RCs=281 and 175 for psuedophakes and phakes, respectively, p value=0.013[13]). The authors[13] argue that since the lens density of phakic subjects is so much higher than pseudophakic subjects, similar RC scores between the two groups suggest that lens density is not overly influencing the Raman method. Such a conclusion, however, relies on the assumption that the only factor that differs between the phakic and psuedophakic subject is lens density. This is not the case. Empirical data suggest that intraocular scatter is as high or higher in pseudophakic subjects compared to matched controls (see Wooten and Hammond).[52] Such scatter would also significantly reduce the Raman signal.

Light that is misdirected out of the stimulus image due to aberrations and scatter (together termed diffusion) will reduce the Raman signal as a direct function of how much light is lost. As noted, empirical measurements have shown that intraocular scatter is higher in patients with RP and CHM. Intraocular scatter also appears to vary dramatically across normal individuals and age. Westheimer and Liang (1994)[54] have examined individual differences and age effects on intraocular scatter using a double-pass method and calculating a "diffusion index," defined as the ratio of light energy falling in an annular zone between 14 and 28-min arc radius to the light energy in the central zone of 14-min arc radius. (Thus, a value of zero means no diffusion; the higher the index, the more the diffusion.) We have plotted their results (from their Table 2) in Fig. 4.[53] Notice that, although they had only 13 healthy subjects, diffusion clearly increases with age (r^2=0.64). In addition, the relevant literature shows (and Fig. 4 verifies) that diffusion is highly variable between individuals across age. For example, a factor of 1.52 comparing young with older eyes is equivalent to a relative reduction of 34%, but the estimated range at each age group (as shown by the dashed lines in Fig. 4) is a factor of 1.65, which is equivalent to a range of 26 to 44%.

Although the results of Westheimer and Liang show a dramatic increase in the diffusion index with age (a factor of 4.3 comparing the interpolated values of the 20 and 70 year olds), they cannot be used to quantitatively evaluate the corresponding reduction in RC. For that, an absolute point spread function (PSF) is needed to account for the actual distribution of light across the 3-deg stimulus and across the entire visual field. Vos, Walraven, and van Meeteren[55] originally linked the two methods by attaching a value to the double-pass PSF (for stimuli up to 7-min arc radius), so that when combined with the glare-determined PSF for large angles, the total amount of light under the curve adds up to 100%. Westheimer and Liang[56] and Liang and Westheimer[54] enhanced the procedure by applying improved double-pass and glare techniques to the same subjects. We should emphasize that the results from the glare method are taken as absolute and the results from the double-pass technique (which are always normalized) are adjusted so that all of the light is accounted for. In this case, therefore, the fact that phase information is lost in the double-pass method (rendering the exact shape and height of the central PSF impossible to determine)[57,58] is irrelevant. Westheimer and Liang used these results in conjunction with the concept of the Strehl ratio (the ratio of the radiance at the peak of a real PSF to the radiance at the peak of a diffraction-limited image with the same aperture) to determine an absolute ocular PSF with which they could examine the aging process. They found that the Strehl ratio for the healthy 69-year old was reduced by approximately a factor of 4.5, which compares favorably with the increase in the diffusion index of 4.3 for the same age range. Using the absolute PSF for the young and old eyes, a simple convolution with the 3-deg diameter Raman stimulus shows that, based on diffusion alone, the older eye's RC would be 34% less than that of a young eye.

A recent study by Neelam et al.[20] identified another factor in the anterior portion of the eye that can potentially attenuate Raman signals. Because the standard exit pupil in the Raman instrument is about 7 mm, the pupil must be dilated wider

*Two groups[45,46] have questioned the generally accepted assumption that the spectral template of the lens is invariant with age. These authors have argued that psychophysical techniques of measuring lens OD overestimate density increases with age, particularly at longer wavelengths. These arguments are largely based on the question of whether the major assumption of the psychophysical techniques is correct: namely, that the lens accounts for nearly all of the deviation between the normal scotopic sensitivity curve and the pure rhodopsin spectrum. Although fully addressing this issue is beyond the scope of this work, we would simply note that most of the *ex vivo* data also support substantial age-related increases in lens OD at the Raman wavelengths of 488 and 527 nm. For example, the Wyszecki and Stiles template[47] shown in Fig. 1 is based on both data derived from aphakic comparisons and direct measurement of extirpated lenses. More recently, Dillon et al.,[48] using an intraocular probe inserted through the posterior sclera (thus measuring light transmission through the anterior segment), also showed relatively high and variable absorbance for older subjects at 488 and 527 nm. The idea that the normal elderly lens is essentially transparent between 450 to 550 nm also seems inconsistent with age-related anatomical (e.g., oxidative modifications) and physiological changes within the lens itself. For example, changes in lens OD at 490 nm can be closely predicted by physiological changes within the lens, such as the relative cation permeability of lens membranes.[46] Based on such data, we conclude that the basic template shown in Fig. 1 is a good first-order description of the spectral characteristics of the normal lens across age.

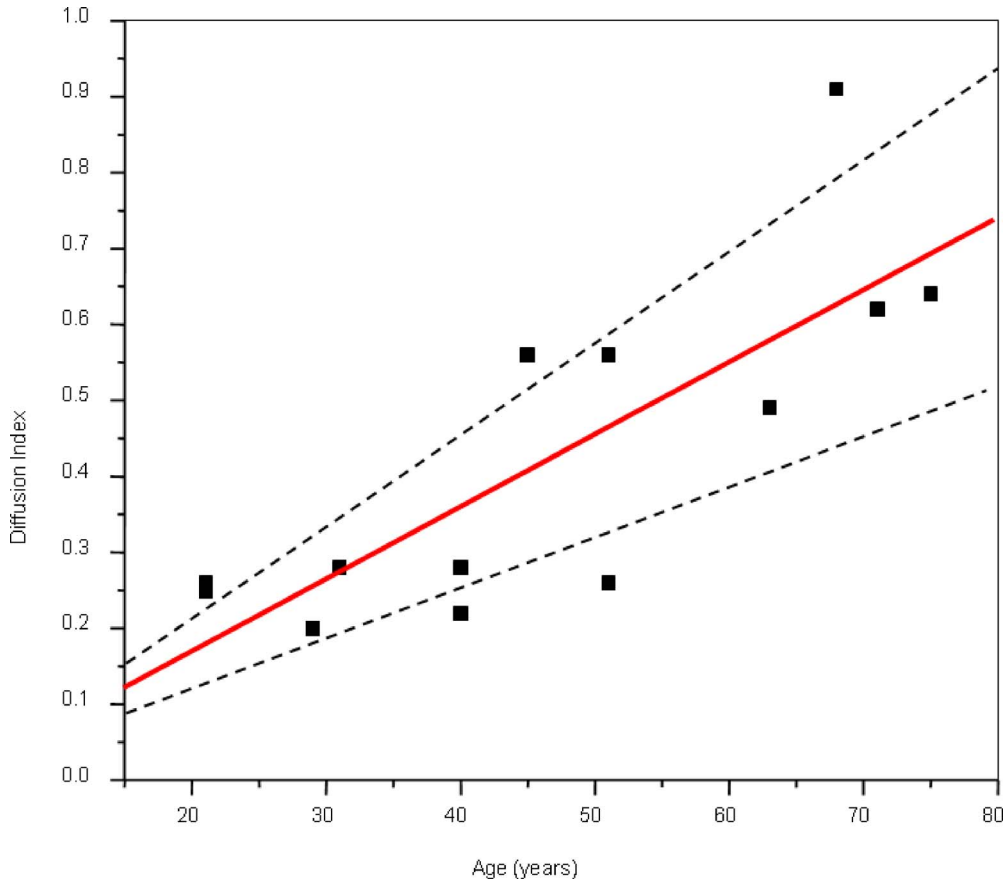

Fig. 4 The relation between age and light diffusion (r^2=0.64). Data were derived from Liang and Westheimer.[54]

than this area for the iris to avoid occluding the Raman signals. If, for example, the pupil is dilated to exactly 7 mm, any pupil displacement caused by small head movements will directly reduce RCs. For example, the dramatic age decline in RCs found by Neelam et al. (between the ages of 20 to 60 years) was not statistically significant when only subjects with pupils that could be dilated to larger than 7 mm were analyzed. The primary reason for using a large aperture is to maximize the collecting optics to increase the detectability of the relatively weak Raman signal. This signal could be increased, however, if the intensity of the incident laser was increased. As noted by Gellerman et al.,[13] "under the typical experimental conditions (exposure time 0.5 s, laser power 0.5 mW, spot size 1 mm), a safety factor of ~19 is realized." Since this is well below American National Standards Institute (ANSI) safety standards, the exit pupil could be reduced significantly (thereby reducing occlusions by the iris) by increasing the intensity of the stimulating beam. This change to the instrument would largely remove the confounding influence of iris occlusions.

Since absorption, diffusion, and iridial occlusions result in reduced Raman signals, and since these factors are strongly associated with age, age would be expected to result in strong losses in the Raman signal. This effect is clearly seen when examining the Raman data shown in Fig. 5, which relates age to RC for normal subjects (taken from Table 1 in Zhao et al.[18]). As shown in this figure, when comparing subjects at an average age of 26.5 years with subjects whose average age is 54.1 years, RCs decline by 65% and variability in RC declines by 70%. A similar but even more dramatic age effect was reported by Gellerman et al.[13] on a different population.[†] The relation between MP density and age has been examined in at least 12 other studies using a variety of methods (psychophysics, autoflourescence, reflectometry, and biochemical analysis of donor retinas). An analysis of the extant literature (see Wooten and Hammond[51,52]) using both *in vivo* and *ex vivo* methods, shows that MP does not decline with age in terms of absolute density or variability across subjects. This is illustrated in Fig. 6,[59–61] which shows data on 1151 subjects obtained using the HFP technique on several large samples. This type of *in vivo* data are consistent with the most direct *ex vivo* data available (HPLC data on donor retinas),[62,63] showing that older subjects have average levels and ranges of MP that are comparable to younger subjects. The large age decline, and

[†]Both Gellerman et al.[13] and Zhao et al.[18] argue that their finding of a decline in RCs with age is not due to an insufficiently dilated pupil. In contrast, Neelam et al.[20] found that a large portion of the age decline in their data was due to pupil sizes dilated to diameters smaller than the aperture of the collecting detector of their system (7 mm). This discrepancy may be due to Gellerman et al. and Zhao et al. achieving a more fully dilated pupil than Neelam et al. It is also possible, however, that the latter authors underestimated the influence of iridial occlusions on their results. For example, Zhao et al. conclude that "once the pupil size exceeds 6 mm, a maximum Raman signal is acquired." This conclusion implies that pupil sizes between 6 and 7 mm would not influence the Raman signal. This is inconsistent with the results of Neelam et al.[20] It also seems clear that the pupil must be dilated to a size larger than the aperture (by a margin that allows for a reasonable amount of pupillary displacement due to head movements) of the collecting detector to avoid occlusions.

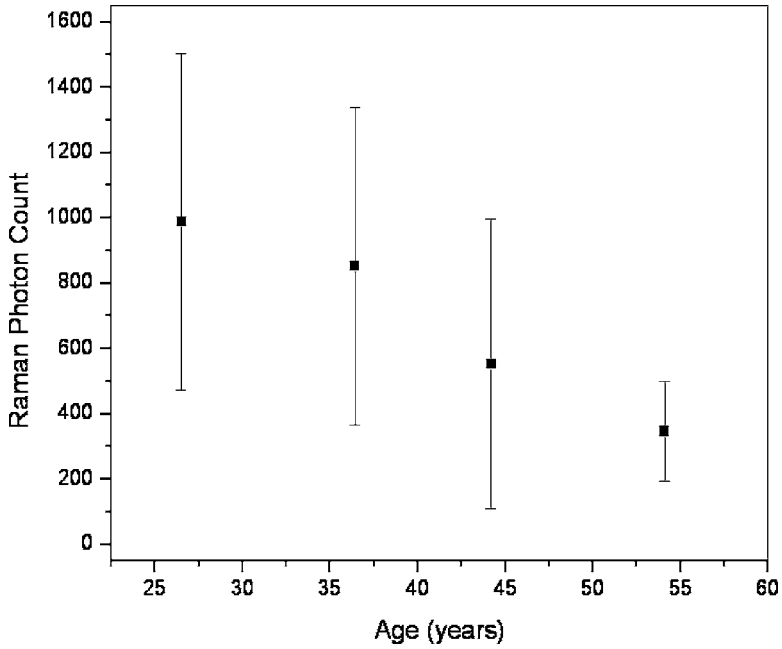

Fig. 5 The relation between age and RC for normal subjects (from Table 1 of Zhao et al.).[18]

drastic reduction in the range of MP with age, is unique to the studies that have used Raman methods. The Raman method relies on collecting an absolute signal that is quite clearly nearly disappearing with age, but not as the authors allege due to a decline in macular pigmentation.

Gellerman, Ermakov, and Bernstein, in a long series of articles, have disagreed with this conclusion and argued that the Raman method is an accurate and objective measure of MP density that is better suited than other *in vivo* methods for measuring a wide variety of subjects, particularly elderly subjects with ocular pathology. The authors provide no direct data, however, to support this conclusion. The authors do supply some indirect data. For example, Gellerman et al.[13] measured RCs in monkeys and then compared those values to HPLC analysis of carotenoids within the monkey's retina and found a correlation of $r=0.68$ (the slope of the regression line was about 0.70). The average RC of the monkey eye (4928),

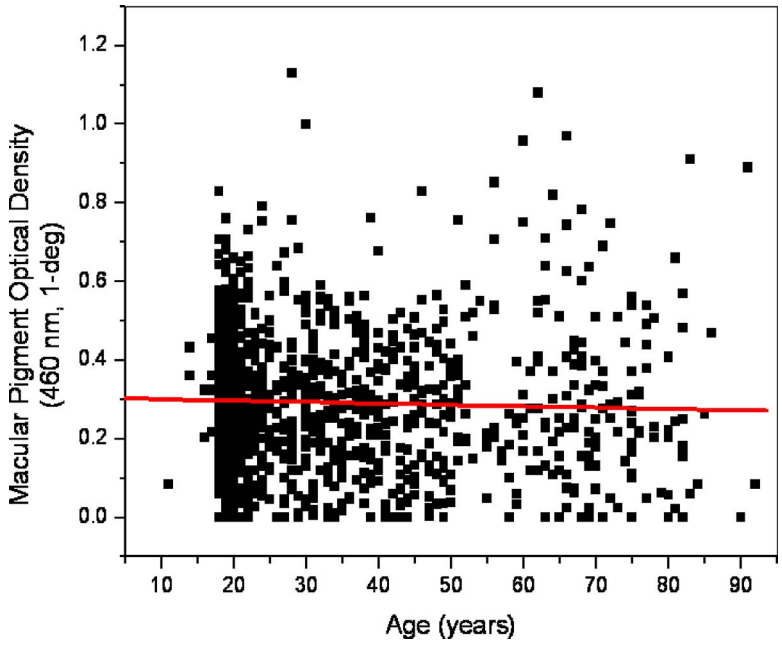

Fig. 6 MP density (at 460 nm using a 1-deg test stimulus) versus age ($n=1151$, $r=-0.04$). Data were aggregated based on published data on normal subjects only collected in the Southwestern,[59] Northeastern,[60] and Midwestern[61] United States geographical areas. Unpublished data ($n=451$) from the Southeastern region (Athens, Georgia) was also included (using similar data collection procedures and equipment).

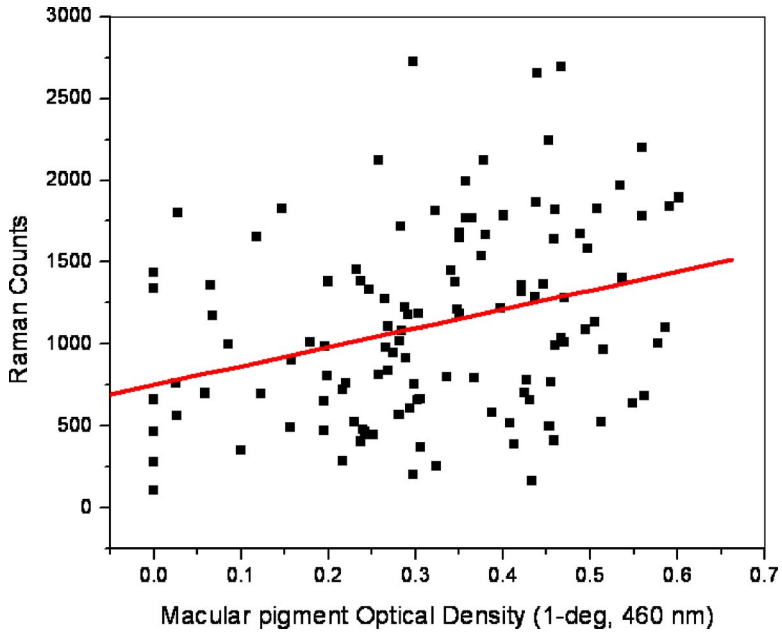

Fig. 7 MP values (n=118) as determined by HFP and RS (data obtained from Fig. 4 in Neelam et al.[20]). These data are based on the values from the right eye only. The line represents the least-squares fit (r^2=0.10).

however, was about seven times higher than the much larger (n=212) sample of human subjects that were tested. Moreover, according to their calibration curve (shown in Fig. 1) a RC of 4928 corresponds to a MP OD that far exceeds the point where their calibration curve plateaus (an OD of about 3.0). The results from their monkey experiment therefore cannot be used to support the validity of the method in humans, particularly in subjects with eye disease.

The only published data that currently exists on the validity of using any *in vivo* method of measuring MP in patient populations is data using HFP on cataractous subjects. Ciulla et al.[64] showed that HFP measures of MP were similar before and after cataract extraction, suggesting a minimal effect of lens opacity on the HFP measures. Ciulla et al., however, did not provide spectral absorption curves; therefore, his data could only speak to the confounding influence of the lens, not to whether the numbers accurately quantified lutein and zeaxanthin within the retina. For this conclusion to be made convincingly, any given technique should be able to generate an MP absorbance curve that matches the unique spectral signature of L and Z as measured *ex vivo*. Such a comparison has been done using numerous methods with nondiseased subjects. For example, Werner, Donnelly, and Kliegl,[65] using HFP, provided spectral curves on 12 subjects that closely matched the *ex vivo* curve of lutein and zeaxanthin.

Since the HFP technique is known to be valid when used on normal subjects, one means of assessing the validity of the Raman method (which cannot generate spectral curves) on normal subjects is by comparing the results of the two methods. Such a comparison has been done by three independent groups. Wintch et al. (r^2=0.13, $p<0.02$) and Hogg et al. (r^2=0.06) reported significant, but low, correlations between the methods.[66,67] Neelam et al.[20] also recently compared the two methods, and their data (derived from their Fig. 4) are shown in Fig. 7. As seen in the figure, although the relation is statistically significant, the overall correlation is low (r^2=0.10). Neelam et al. concluded that the methods "demonstrated good correlation." We would argue, however, that although the relation is statistically significant, the magnitude and nature of the relation is insufficient to conclude that the two methods yield equivalent results (e.g., when measuring the same variable on the same subjects, the results from one method explain only about 10% of the variance in the other method). For example, the relation between HFP values and dietary intake of L and Z is also highly significant ($p<0.0005$).[68] No one would claim, however, that questionnaires are a valid estimate of MP density. To truly determine whether HFP and the Raman method yield similar results, one would need to test subjects across the life span and determine whether the relation was reasonably described by a slope of one and an intercept of zero. When the RCs in Fig. 7 are translated to OD values according to the calibration curve shown in Fig. 1,[19] the slope and intercept are actually 0.08 and 0.28, respectively.

With regards to testing patients with RP and CHM, the Raman and HFP method[69,70] both conclude that MP density in these patient populations does not differ from normal subjects. Zhao et al. argue that their findings "confirm the results of [these] previous studies in an objective and specific manner." Indeed, the similarity in results could be used as evidence that the Raman method provided valid data for these populations (although the HFP method has also not been validated on these patient populations). One difficulty in comparing these results, however, is that absorption, pupil size, diffusion, and head movements bias the Raman method toward giving lower values. Thus, the results from the two methods are not actually very similar. For example, the RCs of their patients (using a least square regression on data taken from their Table 2) declined by over 60% when comparing patients in their 20s

and 50s, with a reduction in range of about 60%. Aleman et al.[69] and Duncan et al.[70] did not report age declines or a restricted range in the MP values of older patients. The floor effect evident in the Raman method could easily obscure any differences in MP density, particularly for the older subjects. Thus, the question of whether a true difference existed in the actual MP density in the patient population studied by Zhao et al. cannot be determined based on their RC data.

When comparing their results to the psychophysical data, Zhao et al.[18] refer to psychophysical methods as "subjective." This is probably in reference to the fact that successful task performance is necessary to obtain accurate data (e.g., subjects with poor acuity cannot complete the tasks). To a large extent, however, this limitation is true of most physical methods as well. For example, RS measurements require accurate fixation that rely on reasonable acuity. Given the difficulties with the method, it is important that the Raman technique be validated on both normals and patients with ocular disease. Referring to psychophysical methods as subjective and the Raman method as objective in this case is incorrect usage (see Vogt).[71] Subjective methods are those "based on the researcher's feelings or intuitions about the topic being studied." In contrast, objective methods are those that are independent of "the beliefs and desires of researchers or subjects." As currently conceived, and as long as good scientific practices are followed, all of the methods of measuring MP are properly considered objective methods.

4 Conclusion

Carotenoids are believed to play important roles in helping to prevent a number of chronic diseases. Noninvasive measures of these pigments within tissues allows study of their protective potential often at the precise site where the disease is manifest (e.g., measuring carotenoids within the macula to assess their role in macular degeneration). The validity of the Raman method for qualitative identification of specific molecules, including carotenoids, has been historically well established. The frequency shifts that occur during Raman scattering are precisely determined by the vibrational/rotational energy transitions of carotenoid molecules (e.g., the precise number of conjugated double bonds causing predictable shifts in the peak). Measuring these shifts therefore provides an accurate means of identifying specific carotenoids. The current version of the method cannot, however, be used to quantify the amount of carotenoids within the retina or the skin of an actual subject unless all of the confounding variables are independently measured using other time-consuming methods. For example, a high Raman count in the eye could mean a number of very different things: high MP density, very low lens density, a very low diffusion index, or some combination of these factors. On the other hand, a low Raman count could mean low MP density, high lens density, insufficiently dilated pupil, poor head position, inaccurate fixation, a high diffusion index, or (almost certainly) some combination of all of these factors. Obviously, most of the confounds bias the method toward giving low values, especially as the eye ages. Simply put, an *in vivo* RC from skin or retina reflects the unknown weighted influence of many factors. Based on current measurement practices, the actual value of the carotenoid component is simply one of the unknowns.

RS has been used to great advantage in the qualitative analysis of chemicals embedded within some substrate. To utilize RS as a quantitative tool, however, additional development is needed. For example, we suggest the following as a means of improving RCs as a quantitative measure of MP:

1. The measurement and analysis conditions should be standard across laboratories. RCs are determined by the specific arrangement of stimulating and collecting optics, laser intensity and duration, head stabilization, etc. Such factors should be held constant across laboratories. For example, Bernstein et al.[19] used a 0.5-mW 488 nm laser for 0.5 s, Neelam et al.[20] used a 1-mW 488 nm laser for 0.25 s.

2. Lens OD and diffusion at 488 and 527 nm need to be measured directly in each subject and RCs need to be corrected for that value.

3. The effects of photopigment OD on the RCs for subjects with low MP density should be assessed (as originally suggested by Wooten and Hammond).[51] This could be tested by simply measuring RCs at various bleaching levels.

4. The confounding influence of iridial occlusions must be minimized by either reducing the size of the exit pupil or ensuring that all subjects are dilated to at least 8 mm. The effects of pupil displacement could be reduced by more effective head stabilization. This could be accomplished by using a bite bar in conjunction with the head-and-forehead rest.

5. For any method, one must assume that the observed value of MP is a weighted sum of the true value accompanied by some level of measurement error. Further, one must assume that the fraction of the observed value explained by measurement error varies across trials. To avoid introducing bias, most researchers use a nondirectional statistical criterion for discarding outlying values. In contrast, the RS method routinely discards the two lowest out of five values for a given subject. These low values are removed based, presumably, on the assumption that they are low due to some confound (most often head movement, which causes pupil displacement). The effect of that same confound on the other three measures is simply accepted as an unknown. This practice is arbitrary (why not discard just one value or three?). Moreover, there is currently no evidence that it actually reduces bias due to measurement error. Without direct knowledge of the distribution and nature of the error, a better and more defensible approach would be to simply use all values in the calculation. By adding more trials, a better average could be obtained and the distribution of error could be more adequately evaluated.

6. The nonlinear nature of the Raman response should be considered when interpreting RCs, particularly when they are used to evaluate the MP spatial distribution. For example, when the Raman method is used in the imaging mode,[14,15] RCs are collected across the central retina and a spatial density map can be generated. These maps have often shown dips at the central MP peak and are typically more Gaussian than exponential. This result, however, may be due to the fact that the Raman method is measuring different proportions of the pigment based on location. For example, Hammond et al.[72] report a subject (JR) whose central MP density was 1.63 OD (based on an extrapolation from the measured point of 1.35 at a radius of 6 min) and whose MP density at 3 deg was 0.30. Based purely on the calibration curve shown in Fig. 1, the 3-deg point would not be underestimated by the Raman method, but the central point would be underestimated by

53%. Even if all of the other confounds were addressed and the calibration curve was an accurate simulation of the eye, the nonlinear nature of the Raman effect would cause distortion in MP estimates that would also need to be corrected.

Like the authors, we recognize the potential value of applying Raman methods for the quantitative measurement of carotenoids within the skin and retina. There are risks, however, to advancing technology for pure or clinical research without proper validation. Future research efforts in this area should be aimed at validation so that results obtained with the method can be properly interpreted.

References

1. P. S. Bernstein, M. D. Yoshida, N. B. Katz, R. W. McClane, and W. Gellerman. "Raman detection of macular carotenoid pigments in intact human retina," *Invest. Ophthalmol. Visual Sci.* **39**, 2003–2010 (1998).
2. P. S. Bernstein, D. Y. Zhao, S. W. Wintch, I. V. Ermakov, R. W. McClane, and W. Gellerman, "Resonance Raman measurement of macular carotenoids in normal subjects and in macular degeneration patients," *Ophthalmology* **109**, 1780–1787 (2002).
3. P. S. Bernstein. "New insights into the role of the macular carotenoids in age-related macular degeneration. Resonance Raman studies," *Pure Appl. Chem.* **74**, 1419–1425 (2002).
4. I. V. Ermakov, R. W. McClane, W. Gellermann, and P. S. Bernstein, "Resonant Raman detection of macular pigment levels in the living human retina," *Opt. Lett.* **26**, 202–204 (2001).
5. I. V. Ermakov, M. R. Ermakova, R. W. McClane, and W. Gellermann, "Resonant Raman detection of carotenoid antioxidants in living human tissues," *Opt. Lett.* **26**, 1179–1181 (2001).
6. I. V. Ermakov, M. R. Ermakova, and W. Gellermann, "Non-invasive laser Raman detection of lycopene and β-carotene antioxidants in skin," *Proc. SPIE* **4967**, 117–128 (2003).
7. I. V. Ermakov, M. R. Ermakova, and W. Gellermann, "Compact single-channel Raman detector for macular pigments," *Proc. SPIE* **5314**, 150–160 (2004).
8. I. V. Ermakov, M. R. Ermakova, and W. Gellermann, "Raman measurement of carotenoid composition in human skin," *Proc. SPIE* **5326**, 30–40 (2004).
9. I. V. Ermakov, M. R. Ermakova, W. Gellermann, and J. Lademann, "Noninvasive selective detection of lycopene and β-carotene in human skin using Raman spectroscopy," *J. Biomed. Opt.* **9**, 332–338 (2004).
10. I. V. Ermakov, M. R. Ermakova, W. Gellermann, and P. S. Bernstein, "Macular pigment Raman detector for clinical applications," *J. Biomed. Opt.* **9**, 139–148 (2004).
11. W. Gellermann, I. V. Ermakov, M. R. Ermakova, R. W. McClane, S. W. Wintch, D. Y. Zhao, and P. S. Bernstein, "Raman detection of carotenoid pigments in the human retina," *Proc. SPIE* **4245**, 146–157 (2001).
12. W. Gellermann, I. V. Ermakov, M. R. Ermakova, and R. W. McClane, "Noninvasive laser Raman detection of carotenoid antioxidants in living human skin," *Proc. SPIE* **4244**, 36–44 (2001).
13. W. Gellerman, I. V. Ermakov, M. R. Ermakov, R. W. McClane, D. Y. Zhao, and P. S. Bernstein, "*In vivo* resonant Raman measurement of macular carotenoid pigments in the young and the aging human retina," *J. Opt. Soc. Am. A* **19**, 1172–1186 (2001).
14. W. Gellermann, I. V. Ermakov, R. W. McClane, and P. S. Bernstein. "Raman imaging of human macular pigments," *Opt. Lett.* **27**, 833–835 (2002).
15. W. Gellermann, I. V. Emakov, and R. W. McClane, "Raman imaging of carotenoid pigments in the human retina," *Proc. SPIE* **4611**, 197–204 (2002).
16. W. Gellermann, M. Sharifzadeh, M. R. Ermakova, I. V. Ermakov, and P. S. Bernstein, "Resonant Raman detectors for noninvasive assessment of carotenoid antioxidants in human tissue," *Proc. SPIE* **4958**, 78–87 (2003).
17. W. Gellermann and P. S. Bernstein, "Noninvasive detection of macular pigments in the human eye," *J. Biomed. Opt.* **9**, 75–85 (2004).
18. D. Y. Zhao, S. W. Wintch, I. V. Ermakov, W. Gellerman, P. S. Berstein, K. Neelam, N. O' Gorman, J. Nolan, O. O' Donovan, H. B. Wong, K. G. Au Eong, and S. Beatty, "Resonance Raman measurement of macular carotenoids in retinal, choroidal, and macular dystrophies," *Arch. Ophthalmol. (Chicago)* **121**, 967–972 (2003).
19. P. S. Bernstein, D. Y. Zhao, M. Sharifzadeh, I. V. Ermakov, and W. Gellermann, "Resonance Raman measurement of macular carotenoids in the living human eye," *Arch. Biochem. Biophys.* **430**, 163–169 (2004).
20. K. Neelam, N., O'Gorman, J. Nolan, et al., "Measurement of macular pigment: Raman spectroscopy versus heterochromatic flicker photometry," *IOVS* **46**, 1023–1032 (2005).
21. E. J. Johnson, "The role of carotenoids in human health," *Nutr. Clin. Pract.* **5**, 56–65 (2002).
22. T. R. Hata, T. A. Scholz, I. V. Ermakov, R. W. McClane, F. Khachik, W. Gellerman, and L. K. Pershing. "Non-invasive Raman spectroscopic detection of carotenoids in human skin," *J. Invest. Dermatol.* **115**, 441–448 (2000).
23. W. Gellerman, I. V. Ermakov, and P. Bernstein, "Author response," *Invest. Dermatol.* **122**, 546–548 (2004).
24. C. R. Smidt, "Clinical screening study: Use of the Pharmanex® biophotonic scanner to assess skin carotenoids as a marker of antioxidant status," see http://dwp.bigplanet.com/breenl40/nss-folder/ramanfolder/scanner_clinical.pdf (Dec. 2004).
25. M. Ferguson-Pell and S. Hagisawa. "An empirical technique to compensate for melanin when monitoring skin microcirculation using reflectance spectrophotometry," *Med. Eng. Phys.* **17**, 104–110 (1995).
26. H. A. D. Lu, S. Pearse, S. Gaskell, and R. Marks, "A quantitative assessment of melanin content in surface corneocytes from subjects of varying phototypes," *J. Eur. Acad. Dermatol. Venereol* **5**, S43 (1995).
27. C. R. Smidt, "Biophotonic scanner: Clinical applications," see http://www.youcan2.com/nss-folder/scannerpresentations/0207131Smidt.ppt (Dec. 2004).
28. Y. M. Peng, Y. S. Peng, Y. Lin, T. Moon, D. J. Roe, and C. Ritenbaugh, "Concentrations and plasma-tissue-diet relationships of carotenoids, retinoids, and tocopherols in humans," *Nutr. Cancer* **23**, 233–245 (1995).
29. C. R. Smidt, G. Gellerman, and J. A. Zidichouski, "Non-invasive Raman spectroscopy measurement of human carotenoid status," *FASEB J.* **18**, A480 (2004).
30. J. A. Zidichouski, S. J. Poole, W. Gellerman, and C. R. Smidt. "Clinical validation of a novel Raman technology to non-invasively assess carotenoid status in humans," *J. Am. Coll. Nutr.* **23**, 468 (2004).
31. E. E. Ghiselli, J. P. Campbell, and S. Zedeck, "Measurement theory for the behavioral sciences," pp. 289–291, W.H. Freeman and Co., San Francisco (1981).
32. W. M. P. Broekmans, T. T. J. M. Berendschot, I. A. A. Klopping-Ketelaars, A. J. de Vries, R. A. Goldbohm, L. B. Tijburg, A. F. Kardinaal, and G. van Poppel, "Macular pigment density in relation to serum and adipose concentrations of lutein and serum concentrations of zeaxanthin." *Am. J. Clin. Nutr.* **76**, 595–603 (2002).
33. W. Gellerman and P. Bernstein. "Letter to the editor: Raman detection of carotenoid antioxidants in human tissue," *Carotenoid News* **14**, 5–7 (2004).
34. T. T. J. M. Berendschot and D. van Norren, "Objective determination of the macular pigment optical density using fundus reflectance spectroscopy," *Arch. Biochem. Biophys.* **430**, 149–155 (2004).
35. B. R. Wooten and G. Geri, "Psychophysical determination of intraocular light scatter," *Vision Res.* **27**, 1291–1298 (1987).
36. K. R. Alexander, G. A. Fishman, and D. J. Derlacki, "Intraocular light scatter in patients with retinitis pigmentosa," *Vision Res.* **36**, 3703–3709 (1996).
37. S. Grover, K. R. Alexander, D. M. Choi, and G. A. Fishman, "Intraocular light scatter in patients with choroideremia," *Ophthalmology* **105**, 1641–1645 (1998).
38. S. Grover, K. R. Alexander, G. A. Fishman, and J. Ryan. "Comparison of intraocular light scatter in carriers of choroidseremia and X-linked retinitis pigmentosa," *Ophthalmology* **109**, 159–163 (2002).
39. H. Jackson, D. Garway-Heath, R. Rosen, A. C. Bird, and S. J. Tuft. "Outcome of cataract surgery in patients with retinitis pigmentosa," *Br. J. Ophthamol.* **85**, 936–938 (2001).
40. G. Duncan, K. R. Hightower, S. A. Gondolfi, J. Tomlinson, and G. Maraini, "Human lens membrane cation permeability increases with age," *IOVS* **30**, 1855–1859 (1989).
41. D. van Norren and J. J. Vos, "Spectral transmission of the human ocular media," *Vision Res.* **14**, 1237–1244 (1974).
42. A. Stockman, D. I. A. MacLeod, and N. E. Johnson, "Spectral sen-

sitivities of human cones," *J. Opt. Soc. Am. A* **10**, 2491–2521 (1993).
43. A. Stockman, L. T. Sharpe, and C. C. Fach, "The spectral sensitivity of the human short-wavelength cones," *Vision Res.* **39**, 2901–2927 (1999).
44. B. R. Hammond, J. Náñez, C. Fair, and D. M. Snodderly, "Iris color and age-related changes in lens optical density," *Ophthalmic Physiol. Opt.* **20**, 381–386 (2000).
45. G. L. Savage, C. A. Johnson, and D. L. Howard, "A comparison of noninvasive objective and subjective measurements of the optical density of human ocular media," *Optom. Vision Sci.* **78**, 386–395 (2001).
46. N. P. A. Zagers and D. van Norren, "Absorption of the eye lens and macular pigment derived from the reflectance of cone photoreceptors," *J. Opt. Soc. Am. A* **21**, 2257–2268 (2004).
47. G. Wyszecki and W. S. Stiles, *Color Science*, 2nd ed., Wiley, New York (1982).
48. J. Dillon, L. Zhenga, J. C. Merriama, and E. R. Gaillard, "Transmission of light to the aging human retina: Possible implications for age related macular degeneration," *Exp. Eye Res.* **79**, 753–759 (2004).
49. P. Bernstein and W. Gellerman, "Author response: Assessment of the Raman method of measuring human macular pigment," *Invest. Ophthalmol. Vis. Sci.*, see http://www.iovs.org/cgi/eletters/39/11/2003.
50. P. Bernstein and W. Gellerman, "Author response: Assessment of the Raman method of measuring human macular pigment (II)," *Invest. Ophthalmol. Vis. Sci.*, see http://www.iovs.org/cgi/eletters/39/11/2003.
51. B. R. Wooten and B. R. Hammond, "Assessment of the Raman method of measuring human macular pigment," *Invest. Ophthalmol. Vis. Sci.*, see http://www.iovs.org/cgi/eletters/39/11/2003.
52. B. R. Wooten and B. R. Hammond, "Assessment of the Raman method of measuring human macular pigment (II)," *Invest. Ophthalmol. Vis. Sci.*, see http://www.iovs.org/cgi/eletters/39/11/2003.
53. G. Westheimer and J. Liang, "Evaluating diffusion of light in the eye by objective means," *Invest. Ophthalmol. Visual Sci.* **35**, 2652–2657 (1994).
54. J. Liang and G. Westheimer, "Optical performance of human eyes derived from double-pass measurements," *J. Opt. Soc. Am. A* **12**, 1411–1416 (1995).
55. J. J. Vos, J. Walraven, and A. van Meeteren; "Light profiles of the foveal image of a point source," *Vision Res.* **16**, 215–219 (1976).
56. G. Westheimer and J. Liang, "Influence of ocular light scatter on the eye's optical performance," *J. Opt. Soc. Am. A* **12**, 1417–1424 (1995).
57. P. Artal, S. Marco, R. Navarro, and D. R. Williams, "Odd aberrations and double-pass measurements of retinal image quality," *J. Opt. Soc. Am. A* **12**, 195–201 (1995).
58. P. Artal, I. Iglesias, and N. Lopez-Gil, "Double-pass measurements of the retinal-image quality with unequal entrance and exit pupil sizes and the reversibility of the eye's optical system," *J. Opt. Soc. Am. A* **12**, 2358–2366 (1995).
59. B. R. Hammond and M. Caruso-Avery, "Macular pigment optical density in a Southwestern sample," *Invest. Ophthalmol. Visual Sci.* **41**, 1492–1497 (2000).
60. B. R. Hammond, J. Curran-Celentano, S. Judd, K. Fuld, N. I. Krinsky, B. R. Wooten, and D. M. Snodderly, "Sex differences in macular pigment optical density: Relation to serum and dietary patterns," *Vision Res.* **36**, 2001–2012 (1996).
61. T. Ciulla and B. R. Hammond, "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 (2004).
62. R. A. Bone, J. T. Landrum, L. Fernandez, and S. L. Tarsis, "Analysis of the macular pigment by HPLC: Retinal distribution and age study," *Invest. Ophthalmol. Visual Sci.* **29**, 843–849 (1988).
63. G. J. Handelman, E. A. Dratz, C. C. Reay, and F. J. G. M. van Kuijk. "Carotenoids in the human macula and whole retina," *Invest. Ophthalmol. Visual Sci.* **29**, 850–855 (1988).
64. T. A. Ciulla, B. R. Hammond, C. W. Yung, and L. Pratt. "Macular pigment optical density before and after cataract extraction," *Invest. Ophthalmol. Visual Sci.* **42**, 1338–1341 (2001).
65. J. S. Werner, S. K. Donnelly, and R. Kliegl, "Aging and human macular pigment density," *Vision Res.* **27**, 257–268 (1987).
66. S. W. Wintch, D. Y. Zhao, I. V. Ermakov, R. W. McClane, W. Gellermann, and P. S. Bernstein, "Evaluation of two macular carotenoid measurement methods: Resonance Raman spectroscopy and heterochromatic flicker photometry," see http://www.arvo.org (2003).
67. R. E. Hogg, M. R. Stevenson, U. Chakravarthy, and R. S. Anderson, "Comparison of heterochromatic flicker photometry and resonance raman spectroscopy as methods of measuring macular carotenoids *in vivo*," see http://www.arvo.org (2004).
68. J. Curran-Celentano, B. R. Hammond, T. A. Ciulla, D. A. Cooper, L. M. Pratt, and R. B. Danis, "Relation between dietary intake, serum concentrations, and retinal concentrations of lutein and zeaxanthin in adults in a Midwest population," *Am. J. Clin. Nutr.* **74**, 796–802 (2001).
69. T. S. Aleman, J. L. Duncan, M. L. Bieber, et al., "Macular pigment and lutein supplementation in retinitis pigmentosa and Usher syndrome," *Invest. Ophthalmol. Visual Sci.* **42**, 1873–1881 (2001).
70. J. L. Duncan, T. S. Aleman, L. M. Gardner, E. Castro, et al., "Macular pigment and lutein supplementation in choroideremia," *Exp. Eye* **74**, 371–381 (2002).
71. J. Vogt, *Dictionary of Statistics and Methodology*, 2nd ed., Sage Publishers, Thousand Oaks, CA (1999).
72. B. R. Hammond, B. R. Wooten, and D. M. Snodderly, "Individual variations in the spatial profile of macular pigment," *J. Opt. Soc. Am. A* **14**, 1187–1196 (1997).