

## CORRESPONDENCE

### ASSESSMENT OF THE VALIDITY OF *IN VIVO* METHODS OF MEASURING HUMAN MACULAR PIGMENT OPTICAL DENSITY

Recently, Hammond et al. published a manuscript in *Optometry and Vision Science* entitled “Assessment of the Validity of *in Vivo* Methods of Measuring Human Macular Pigment Optical Density.”<sup>1</sup> The authors claim to assess the validity of various optical methods used for the *in vivo* measurement of human macular pigment (MP). Specifically, they attempt to compare the MP measuring method of heterochromatic flicker photometry (HFP)—a psychophysical method that is their specific field of expertise—with several other emerging objective methods that are based on reflection, fluorescence, and resonance Raman spectroscopy.

We feel obliged to point out a string of shortcomings, misrepresentations, and flawed conclusions of this manuscript, especially because a large portion of the manuscript provides a duplicative critique of our resonance Raman method for measurement of macular pigment *in vivo*. The authors recapitulate the points that they have already published as lengthy Letters to the Editor of the journals *Investigative Ophthalmology and Visual Science*<sup>2,3</sup> and *Carotenoid News*.<sup>4</sup> Disappointingly, however, the authors fail to include any of our rebuttals to their arguments, as provided in detail in earlier published letters<sup>5,6</sup> and in *Carotenoid News*.<sup>7</sup> We highlight a few of the more important points of contention subsequently.

1. HFP is a *subjective* method because a subject uses his or her own photoreceptors to measure MP. Like with any psychophysical method, extreme care must be taken to ensure that the subject understands the task and performs it properly and reproducibly. *Obviously, HFP cannot be externally validated because it is impossible for excised tissue to participate in an HFP measurement.* In the absence of this possibility, it may provide comfort to the authors that HFP can generate spectral stimulation spectra that to some extent can follow the *shape* of absorption spectra measured for excised tissue or a model system

with another method, i.e., absorption spectroscopy. A calibration to the absorption *strength* of the excised tissue, however, is *fundamentally impossible for HFP*. A spectral match of HFP stimuli to absorption shapes of external tissue alone is not sufficient. A very important aspect, of course, is the correlation of HFP perceived MP levels with levels existing in the tissue. In particular, one wonders about the linearity in this regard. Certainly, at some MP concentration level, the HFP measurements must start to saturate. However, because a calibration of concentration levels is fundamentally impossible with this technique, saturation effects cannot be taken into account. By contrast, *objective* optical spectroscopic techniques require no subject cooperation beyond fixation on a target (which can be monitored by a video camera), and validation can be performed on animal or model eyes. For the Raman method, we validated the technique by measurements in living monkey eyes followed by high-performance liquid chromatography measurements of MP levels after enucleation.<sup>8</sup>

2. In their discussion of the influence of ocular media on MP Raman measurements, the authors fail to cite recently published work by Savage et al.<sup>9</sup> and Zagers et al.<sup>10</sup> questioning the validity of previously published crystalline lens density functions at wavelengths longer than 450 nm. If these more recent results had been used for their calculations, their putative reduction factors would have been much lower in magnitude.

3. Our finding that MP levels remain low in elderly pseudophakic eyes is *not* explainable by scattering at the intraocular lens interface with the posterior capsule as they assert. At the University of Utah Moran Eye Center, only modern silicone and acrylic lens are implanted during cataract surgery. These lenses are designed for high optical clarity above 450 nm, and they are shaped for a tight fit with the posterior capsule to prevent lens epithelial migration that can cause light scattering. In situations when posterior capsule opacification does eventually occur, the ophthalmologist routinely performs a YAG laser capsulotomy to clear the visual axis. All subjects enrolled in our studies had a dilated eye

examination by a board-certified ophthalmologist to confirm that no media opacities were present that could attenuate the MP measurements.

4. The authors have never used our Raman instrumentation as evidenced by their erroneous statement that no chin rest is used during measurements, yet they assert that elderly subjects would be unable to hold fixation steady for the time period of a measurement (0.25–0.5 second). Clinical practice clearly contradicts their assertion. Elderly patients with impaired visual acuity routinely hold steady fixation under bright light conditions for much longer periods during therapeutic and diagnostic procedures such as ocular photodynamic therapy and video angiography.

5. HFP measures the perceived MP only at the edge of the test stimulus. Although the authors recognize correctly that autofluorescence and Raman methods are based on a signal that is integrated or averaged over the entire stimulus area, they state incorrectly that this is only an *assumption* that has not been tested. The authors completely ignore our published correlation results between *in vivo* Raman MP levels and direct high-performance liquid chromatography measurement levels in the same primate eyes.<sup>8</sup> Similar correlation results could never be obtained for a psychophysical test such as HFP.

6. The authors state incorrectly that the Raman method does not provide spectral profiles. In fact, the intensity of resonance enhancement closely follows the spectral absorption shape. At any resonant excitation wavelength, the molecules yield very sharp, highly distinctive vibrational peaks, a spectral “fingerprint” of the molecules of interest. Contrary to the authors’ assertions, these Raman signals can be readily quantified, and unlike HFP, they can be calibrated against the concentration of external standards.

7. The authors fail to cite our published correlation results for Raman- and HFP-derived MP levels.<sup>11</sup> Similar correlation results have also been reported recently by another group.<sup>12</sup> The correlations are statistically significant and would likely improve if HFP

experiments were to include measurements at varying eccentricities.

8. HFP-derived MP measurements often differ substantially from MP levels derived by other techniques. We caution against the authors' portrayal of HFP as a standard against which these other techniques should be judged. Besides the calibration issue (see previously), there are other problems. The authors seem to believe, for example, that the HFP method is not influenced by confounding influences from ocular structures anterior and/or posterior to the MP. Apparently, it is assumed that these ocular effects cancel out when referencing HFP-perceived MP levels to peripheral retinal locations. This assumption is not generally valid. The stimulating light used in HFP has to traverse all anterior media besides the MP-containing retinal layer before it is viewed with the subject's photoreceptors. Certainly, any absorption or scattering of the ocular media would affect the shape of the light stimulus, its eccentricity, and therefore the derived results. Furthermore, care has to be taken in the choice of the peripheral reference location. In cases of wide pigment distributions, a reference point chosen too closely to the fovea leads to an underestimation of MP levels or even to negative MP levels. In the past, these same authors have used peripheral reference points of as little as 4°. It is now generally recognized that this distance is far too close to the fovea to provide reliable data.

9. HFP is not amenable to imaging of the MP spatial distribution. At best, it can attempt a crude point-by-point approximation using cumbersome repeated measurements at a few discrete eccentricities. It is therefore missing spatial asymmetries, side peaks, and central depletions of the MP distributions easily observable with optical imaging techniques. Based on reflectometry, autofluorescence, and Raman spectroscopy, these imaging methods are currently under intense development. They yield objective information about the *concentrations of MP levels as well as their spatial distributions*, and it is now apparent that it will be possible to achieve this through undilated pupils within a fraction of a second per measurement. Already, these imaging approaches have clearly demonstrated that MP distributions can vary widely in concentrations, spatial extent and shape, including distributions with ring-like patterns and pronounced shoulders. Certainly, older HFP results that extrapolate MP levels from a single measurement at an arbitrary eccentricity are seriously flawed, and we continue to object to Hammond and Wooten's citations of these

flawed findings as evidence against Raman-derived results.

In summary, accurate noninvasive measurement of MP could have important use in furthering our understanding of carotenoids' roles in the prevention of AMD. We are fortunate that a number of techniques are available, each with its own strengths and weaknesses, and a well-crafted and balanced review of the methods could have great value to investigators interested in the field. Unfortunately, it is abundantly clear that Wooten et al. have a strong and unyielding bias against the use of Raman spectroscopy to quantify MP *in vivo*, as evidenced by their incessant Letters to the Editor and misleading pejorative statements such as, "As currently conceived, the Raman method of measuring MP can only be used as a qualitative indication that MP is present in the central retina." At least part of this bias undoubtedly derives from the fact that at least one of the authors has a commercial interest in flicker photometry (Macular Metrics Corp., Rehoboth, MA).<sup>13</sup> Although disclosure of commercial interests by publishing authors is an explicit policy of *Optometry and Vision Science*, we see no indication that this has occurred.

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## Authors' Response

We are pleased to respond to Gellerman and Bernstein's (G&B) critique of our review. Unlike many fields, science is self-corrective and largely succeeds because of its openness to criticism. We applaud G&B's efforts to develop a useful device for the important purpose of measuring macular pigment (MP) noninvasively. We were disappointed, however, that G&B feel that we have an "unyielding bias against the use of Raman spectroscopy to quantify MP *in vivo*..." We would criticize any method that made claims regarding these important issues that were not supported by reasonable evidence. In our review, we critically analyzed both psychophysical and physical methods and noted shortcomings and strengths. We pointed out that "as currently conceived," the Raman method of MP optical density measurement is invalid. In a recent review,<sup>1</sup> however, we provide many suggestions for how the method could be improved. An acceptable response to our criticisms would be to restrict counterclaims to those supported by reasonable evidence or, even better, to actually test the critical assumptions. For example, one could measure lens density and diffusion at 488 and 527 nm, pupil size, head movements, and so on, on a sample of varying age and quantify the effects of these variables on the Raman signal. If these factors really do have minimal influence as G&B continue to assert, our questions, which are legitimate despite their protestations, could be easily addressed.

Providing solid evidence and/or reasoned arguments is undoubtedly a better scientific practice than simply responding negatively and, personally, to being critiqued. Readers can, after all, evaluate the arguments and evidence and come to their own conclusions. What we hope is obvious to all readers is that personal motivations, even if they do exist, are completely irrelevant. For example, G&B suggest that our critique of their method is biased because of a commercial interest by one of the authors. They also describe our review as a "string of shortcomings, misrepresentations, and flawed conclusions." Such charges do not address content; they simply seek to discredit the source.

G&B also suggest that our critique simply duplicated our earlier published corre-

spondence on these issues. It is, however, legitimate to raise similar issues in different papers (G&B, however, often duplicate text and graphs verbatim, which is not an accepted practice). When there was sufficient overlap, we simply referenced earlier sources. Our primary goal with the Raman section of our review was to quantify the confounds that we had identified in earlier papers. G&B also note that our review fails to include any of their rebuttals to our arguments. However, we were careful to note that there is debate regarding these issues and refer the readers to the relevant literature. Most of our criticism of the Raman method is organized around addressing the points that G&B have made in their many publications using the Raman device (often quoting the points G&B made directly). It is, of course, proper scientific practice to cite debate when discussing an issue for which significant debate exists. This is a practice G&B do not follow, e.g., see the most recent papers by Bernstein et al., Ermakov et al., and Ermakov et al.<sup>2-4</sup>

We have been motivated to write about the Raman method because of the importance of this area and the dramatic claims made by G&B. For example, recently Ermakov et al.<sup>3</sup> wrote "The ease and rapidity of Raman MP measurements, the simplicity of the instrumentation, the high accuracy of the measurements, and the lack of significant systematic errors should make this technology attractive for widespread clinical research." As long as G&B continue to assert validity claims in the absence of evidence, they should expect "incessant" criticism of such claims to continue. This is simply a manifestation of the rigor of our discipline.

Subsequently, we respond to G&B's major points of contention in the same order they were presented in their response letter.

1. The validity of a method has nothing to do with its assumed subjectivity or objectivity. One could, for example, argue that the Raman method has an important subjective component because it relies on a subject's own perception of whether they are, or are not, properly aligned. According to Vogt,<sup>5</sup> subjective methods are based on the feelings and intuitions of the researcher. Objective methods, in contrast, are based on the "object," i.e., they are independent of the feelings, beliefs, or desires

of the researcher or subject. Based on this definition, all methods currently used to measure MP are objective. Referring to psychophysical methods as subjective seems to reflect a general bias based on the idea that if a test requires a subject's response, it is inherently less accurate despite means for controlling bias, validating the response, and so on. A similar general bias appears to apply in the opposite direction. To some, direct physical methods appear inherently more accurate and less subject to bias despite free parameters, questionable assumptions, a lack of validity testing, and so on. Such biases have no bearing on the actual validity of a method. Validity has to be determined based on a specifiable and reasonable criterion. G&B take some issue with our criterion: matching spectral absorbance profiles with *ex vivo* curves. This, however, is a common criterion for determining the *identity* of a signal (i.e., if the spectral curves match, one can be confident that they are measuring carotenoids). George Wald, for instance, originally used this method to identify MP as composed of xanthophylls.<sup>6</sup>

G&B are, of course, correct in noting that one cannot determine wholly from spectral curves whether they have correctly measured absolute quantity. Essentially, G&B argue that the HFP method yields values that cannot be *directly* evaluated against known standards and is, therefore, not validated (This argument is, of course, the very one that we have made about the Raman method and has not been refuted by G&B.). When applied to HFP, however, the argument is specious. HFP relies on a derivation strategy rather than a calibration-against-a-standard procedure as is possible in bench scenarios. This derivation is based on a double normalization procedure using wavelength (i.e., relative sensitivity at two wavelengths, those absorbed by MP, e.g., 460 nm, and a reference wavelength that is not, e.g., 560 nm) and retinal site comparisons (two sites, one where MP is present, e.g., the foveal center, and one where MP is known to be optically undetectable, e.g., 7° off the fovea). These normalization procedures are based on the well-established extinction spectrum of MP and the near identity (when using appropriate stimulus conditions) of the relative spectral sensitivity of the response mechanisms at the retinal sites of comparison. The rationale for these assumptions is

based on an extensive and well-established literature that is covered in our review.

G&B are incorrect in their notion that HFP would be nonlinear as a result of saturation effects. In fact, the inherent perfect linearity of HFP as a measure of MP is an important strength of the method. This is because HFP relies on quantal absorption in outer segments, which are, of course, located behind the MP. Thus, the MP is treated as a filter that merely attenuates the light reaching the receptors. The receptors merely act like photocells responding to quanta. Furthermore, HFP uses a *criterion* response rather than response amplitude. In other words, the null flicker response is achieved whenever the quantal rate of the variable short-wave component results in a magnitude of response equal to that of the fixed midwave standard. Thus, independent of the amount of MP, the response that determines the match point is of fixed amplitude. The subject merely adjusts the amount of short-wave light until the null point is achieved. A departure from nonlinearity at high OD levels would only result from such extreme light levels as to cause anatomic damage to the eye, an absurdity because such light levels would correspond to an MP OD many orders of magnitude above observed values. As we note within our review, linearity is, of course, a central issue. In comparison even to other physical methods, the Raman method fares particularly poorly in this respect because the signal is returned directly from the molecules of MP. Thus, the deeper layers are screened by the shallower layers. For lower OD levels (approximately <math>0.30</math>),<sup>1</sup> the response could be considered acceptably linear. This range of acceptable linearity could, theoretically, be increased by increasing the intensity of the stimulating laser, but then safety issues would become a serious concern. Using current intensity levels, the response is increasingly nonlinear past 0.30 and this problem cannot be corrected, adjusted, or calibrated away.

It is fair to question whether a method designed to measure MP levels returns accurate quantitative estimates for individual subjects. In our review, we discuss this issue with respect to all of the *in vivo* methods. The Raman method fails seriously even at low levels where the method might be considered acceptably linear. For example, imagine an individual has a true MP

OD of 0.30. The Raman signal could be as high as 1500 if none of the signal is lost, or the signal could be near zero if lens absorbance is as high as 0.30 at 488 and 527 nm and scatter is large (such individuals have been identified in the empiric literature, especially at older ages). This permits a range of error that makes interpretation of RCs (Raman count) impossible even on an ordinal scale. In this example, a subject could appear to have either zero MP or average MP density with no way of knowing the true values.

2. G&B note that we did not cite Savage et al.<sup>7</sup> and Zagers et al.<sup>8</sup> and conclude that “if these more recent results had been used for their calculations, their putative reduction factors would have been much lower in magnitude.” We estimated that the amount of reduction in the Raman signal resulting from lens absorbance between the ages of approximately 20 and 60 was 41%. As we note in our review (footnote h), this estimate is quite similar to that made by G&B,<sup>9</sup> which was 38% between the ages of 20 and 60 years. If our “putative reduction factor would have been much lower in magnitude,” it would then be much lower than the estimate made by G&B themselves. Lens absorbance could be easily corrected for if it was simply the *average* error introduced by the lens that was important. Unfortunately, to interpret *individual* RCs, knowing the amount of attenuation resulting from absorption by an individual lens is critical. Despite their own estimate of a systematic age-related error of 38%, Ermakov et al.<sup>3</sup> wrote that there were no “significant systematic errors” with the method. In another recent paper, Ermakov et al.<sup>4</sup> wrote that “To our knowledge there are no serious confounding factors for the technology. . .” These assertions directly contradict G&B’s own analysis and conclusions (and inspire our “incessant letters”).

The Zagers et al.<sup>8</sup> paper was not available when we wrote our review, which was submitted in March 2003. We have since discussed the Zagers et al. and Savage et al. papers in a more recent review<sup>1</sup> pointing out that all of the *ex vivo* data and all of the other *in vivo* data also support the idea that lens OD at the Raman wavelengths of 488 and 527 nm is significant, increases with age, and is quite variable across subjects at all ages. The fact that two papers conclude that lens absorbance is not as high at the

Raman wavelengths as all other papers (>20 other papers using both *ex vivo* and *in vivo* methods) find is missing the point. Unless the majority of empiric papers on this topic are incorrect and the lens is perfectly transparent at the relevant wavelengths for all subjects irrespective of age, it will represent an unknown source of error. Although G&B persist in simply claiming that this error is insignificant, that determination cannot be made in the absence of evidence. This is not a “misleading, pejorative statement” that reflects an “unyielding bias.” Based on the author’s own conclusions, this is a legitimate question. In fact, confounding by lens absorption alone would invalidate their method, e.g., it could introduce approximately 30% to 40% error of unknown direction.

3. In our review, we cite a number of studies that show that modern intraocular lenses do produce substantial scatter *in situ*. It is not adequate for G&B to simply assert that their patients are different. In the absence of evidence, we can only rely on the empiric peer-reviewed results that have been published and are cited in our review. If G&B feel that diffusion does not confound their measures, they should demonstrate this by controlled study. Their continued assertions that it does not matter cannot be considered evidentiary.

4. In this section, we were specifically referring to the Gellerman et al.<sup>9</sup> study, which we carefully referenced. In the schematics of the device shown in that paper, only an eyepiece is shown and the text states that subjects rest their forehead against the device and must move their heads to achieve alignment. More recent Raman studies have abandoned this procedure and now use a chin and forehead rest. This would help, but not eliminate, problems with head movements (as shown by the data from Neelam et al.).<sup>10</sup> We did not assert that “elderly subjects would be unable to hold fixation steady for the time period of a measurement (0.25–0.5 s).” The fixation accuracy of most elderly subjects is known to be quite good. Our point was that because the standard exit pupil of the device is approximately 7 mm, the eye pupil must be dilated wider than this area to avoid occlusions of the illuminating beam and the signal by the iris. Thus, even relatively small head movements, probably more likely in the elderly, would result in occlusions. Neelam et al.<sup>10</sup> recently completed a study in which they measured MP

*in vivo* using a Raman device supplied by G&B. They note that, despite “maximum pupillary dilation,” nearly half of their RCs were confounded by iris occlusions. These occlusions were presumably caused by small head movements. It is also worth noting, that like B+G, Neelam et al. routinely rejected 40% of their measured RC values (the lowest values) as a result of misalignment or blinking. According to measurement theory, routinely rejecting data based on presumed confounds (that the authors tend to deny or minimize) is a questionable practice. For example, the choice of 40% rejection is arbitrary and assumes (without justification) that the remaining 60% is untainted. A more defensible practice would be to obtain more trials so that the actual distribution of error could be evaluated.

5. We did not ignore the results obtained by G&B in their primate study (e.g., see footnote f). We did, however, feel that the results could not be used to draw any meaningful conclusions. To quote our review, “the authors conducted a control study comparing Raman counts with carotenoid concentrations measured in the macula of six monkeys. The average Raman count of their monkeys was approximately 4900, which is approximately seven times higher than their average human values. The interpretation of these RCs, however, is difficult because the authors show that, in their model calibrations, their method nearly completely plateaus above a RC of approximately 2750 (see Fig. 4 in Bernstein et al. 2004).”

6. The Raman method does not provide spectral absorption profiles that can be compared with extinction spectra in the same manner as other methods of measuring MP. We did not mean to imply, however, that this was a weakness of the Raman method. As G&B note, and we agree, analyzing the vibrational peaks produced with their technique is an elegant means of identifying the source of a signal. G&B also note that “contrary to the authors’ assertion, these Raman signals can be readily quantified, and unlike HFP, they can be calibrated against the concentration of external standards.” Again, we did not mean to imply that the Raman signal could not be quantified. Instead, we argued that the resulting number can not be used as a valid estimate of MP within the retina. What G&B seem to miss here is that external calibrations are only valid to the extent that

they accurately model the *in vivo* situation. We have argued that their static model-eye calibrations are not valid representations of the dynamic living eye that differs both across individuals and age. The inadequacy of this calibration we feel explains their unique finding in Gellerman et al.<sup>9</sup> that nearly all subjects over approximately 60 years of age have virtually no MP (a result at odds with over 20 other empiric studies). Again, the burden of proof rests with the authors to show that their model eye accurately captures the individual differences and properties of the actual eye across age.

7. The comparison of HFP and Raman results, cited in the review by Bernstein et al.,<sup>2</sup> would have been difficult to evaluate (even if their paper would have been available when writing our review) because virtually no details were provided regarding the comparison. In a more recent review,<sup>1</sup> we do discuss the available data regarding the comparison between HFP and the Raman method. The only study that has been published as a full manuscript (including full detail regarding the methods and procedures) is Neelam et al.<sup>10</sup> Neelam et al. found that the relation between HFP and RCs was described by an  $r^2$  of approximately 0.10. The fact that the methods are measuring the same variable in the same individuals at the same time and only explain approximately 10% of the variance is not a strong argument that the methods agree (particularly if one tried to expand the sample to include the elderly). True agreement would also have to be reflected in the ability to translate the scores such that the relation could be described with an intercept near zero and a slope close to one.

It is worth noting also that G&B continue to tout the significance of the correlation between HFP and Raman as evidence for the validity of their method because they argue that HFP is not particularly reliable and may not be valid (see their points 1 and 8). For example, Ermakov et al.<sup>3</sup> recently wrote “As a precaution, we avoid media-opacity-related problems by limiting the measurements to subjects with visual acuity better than 20/80. A correlation of our MP Raman responses with data derived by high-performance liquid chromatography and flicker techniques is proof that these precautions are adequate.” We assumed that the authors evaluated the validity of the HFP technique before citing it as evidence. With respect to the former

statement, excluding subjects based on visual acuity will not reduce confounds based on individual differences in the lens density of normal subjects because the two have not been shown to be related.

8. G&B disagree with the argument that using a parafoveal reference removes confounding by optical structures anterior and posterior to the MP. The nature of the argument, based on *ex vivo* data, is that MP is essentially zero (i.e., optically undetectable) at the reference location (e.g., 7°). All methods that use a peripheral reference (e.g., HFP, motion photometry, color matching, autofluorescence, and reflectometry) are based on a similar strategy. B+G are correct in noting that MP has not, in fact, reached a zero asymptote for some subjects at the reference location for many of the earlier studies, some of which used closer reference sites (this is why the reference was moved in later studies). We have noted this point ourselves in a number of publications. As we discuss in our review, there is one other aspect of effective univariance that for the HFP method is not entirely correct. There can be a very small difference between the measurement and reference locations that is attributable to self-screening. All of these effects, however, are quite small as demonstrated by our empiric data, which we cite within our review (on the order of 0.01–0.05). We have also studied, empirically, the possibility of confounding by anterior structures and found no effect. For example, in Wooten et al.,<sup>11</sup> we measured the lens density of 10 subjects and evaluated their effects on HFP results (based both on Newtonian and Maxwellian view) and found no relation. We also simulated the effects of varying lens density by varying retinal illuminance for three subjects and found no effect on the HFP results. In a later study,<sup>12</sup> we measured MP using HFP in subjects with cataracts so severe they required extraction. We then measured MP in the same subjects approximately 2 months after surgery and found that average MP levels did not change (preoperative MP,  $0.206 \pm 0.13$ ; postoperative MP,  $0.18, \pm 0.12$ ).

9. A number of studies have used HFP to map MP spatial profiles. These studies (e.g., Hammond et al., Wooten et al.)<sup>13,14</sup> have produced very well-defined spatial distribution profiles. These psychophysical studies have also identified perturbations in the MP profile. For example, Hammond et al.<sup>13</sup> notes that approximately 40% of the subjects they tested show flank-

ing peaks and shoulders in their MP distributions. Psychophysics has a very long and respected tradition in vision science and, when done correctly, is not generally regarded as “crude” or “cumbersome.” In fact, G&B have cited results based on HFP mapping as evidence for the congruent validity of their own method.<sup>15</sup>

It is certainly true, however, that many of the physical methods have shown much more pronounced deflections than is generally seen in the HFP data. How these types of deflections reflect the underlying biology of the retina is an interesting question. It is therefore important to confirm that these topographic irregularities are real. In some cases, for instance, they may reflect artifacts with the method. For example, G&B, when using the Raman method in the imaging mode, report on an individual that they observe had a “central hole in the MP distribution.” Based on what is known regarding the underlying anatomy, it is hard to imagine an actual hole in the center of the MP distribution of a normal healthy individual. Unusual results require careful scrutiny. As we note in a recent review,<sup>1</sup> the inherent nonlinearity of the Raman signal could create distortions when used to map MP spatial profiles (as a result of different proportions of the pigment being measured based on location). For example, Hammond et al.<sup>13</sup> provide MP spatial density data for a subject whose central MP peak was 1.63 OD (based on an extrapolation from the measured point of 1.35 at a radius of 6 minutes) but whose MP density at 3° was 0.30. Using the Raman calibration curves, the central peak for such a subject would be underestimated by 53%, whereas the 3° site would not be underestimated. In this instance, artifacts with the method would create distortions in the profile that did not, in truth, exist.

All areas of science tend to share a focus on methodology. We agree with G&B that the pursuit of multiple methods of measuring MP *in vivo* is extremely beneficial, including resonance Raman spectroscopy. G&B have claimed that our review of their methodology is biased as a result of the commercial interest of one of the coauthors. One of the

coauthors, Dr. Wooten, has built a relatively small number of HFP-based macular densitometers for fellow researchers and colleagues. Our review, however, was in no way designed to promote this instrument nor was the instrument even mentioned. We do not hold a patent or claim ownership of the HFP technique, in specific, or obviously psychophysics, in general. Many laboratories have used HFP to measure MP and results have been published using the method before our laboratory (e.g., Bone).<sup>16</sup> Ultimately, critical evaluation must stand on its own merit, irrespective of the author, and is a central feature of our field and an obligation of all scientists. If researchers in the area of macular pigment do not critique their own work, then who will?

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*Billy Wooten, a coauthor of this letter and the original article, is a principal in Macular Metrics Corp. (Providence, RI), which manufactures densitometers. These densitometers use heterochromatic flicker photometry and are used to measure MP.*

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