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REVIEW ARTICLE

Measuring macular pigment optical density in vivo: a review of techniques

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Abstract
Background Macular pigment has been the focus of much attention in recent years, as a potential modifiable risk factor for age-related macular degeneration. This interest has been heightened by the ability to measure macular pigment optical density (MPOD) in vivo.
Method A systematic literature search was undertaken to identify all available papers that have used in vivo MPOD techniques. The papers were reviewed, and all relevant information was incorporated into this article.
Results Measurement of MPOD is achievable with a wide range of techniques, which are typically categorized into one of two groups: psychophysical (requiring a response from the subject) or objective (requiring minimal input from the subject). The psychophysical methods include heterochromatic flicker photometry and minimum motion photometry. The objective methods include fundus reflectometry, fundus autofluorescence, resonance Raman spectroscopy and visual evoked potentials. Even within the individual techniques, there is often much variation in how data is obtained and processed.
Conclusion This review comprehensively details the procedure, instrumentation, assumptions, validity and reliability of each MPOD measurement technique currently available, along with their respective advantages and disadvantages. This leads us to conclude that development of a commercial instrument, based on fundus reflectometry or fundus autofluorescence, would be beneficial to macular pigment research and would support MPOD screening in a clinical setting.

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Introduction

Macular pigment is the collective name for three carotenoids, lutein, zeaxanthin and meso-zeaxanthin, which are found at higher concentrations in the retina than anywhere else in the body, and to the exclusion of all other carotenoids [1]. They are only accessible to the body by dietary intake of foodstuffs or supplements containing them [2, 3], with high levels being found in certain fruits and vegetables, such as kiwi fruit, corn and spinach, as well as egg yolks [4].

Analysis of donor maculae is possibly the most unequivocal approach for assessing the distribution of macular pigment in the retina, and pioneering work by Snodderly and colleagues in the 1980s achieved this [5, 6]. Using primate monkeys and the technique of microdensitometry, it was confirmed, as expected, that macular pigment reaches its peak in the centre of the retina. There was then a sharp decline to negligible levels at approximately 1 mm (4°) from the central fovea. In 1988, Bone et al., using high-performance liquid chromatography (HPLC), investigated the spatial distribution of macular pigment in human donors; in this case, it was found to reach negligible levels at 7° eccentricity [7]. Within the retinal layers, macular pigment is primarily located in the photoreceptor axons and to a lesser extent in the inner plexiform layer [6, 8].

The macular carotenoids have an absorption spectrum of 400–540 nm, peaking at approximately 460 nm [9]. This

spectral peak, along with the spatial distribution and retinal layer localization of macular pigment contribute to its proposed function as a blue-light filter. Short-wavelength (blue) light is more damaging to the retina than longer-wavelength light [10], so by attenuating the amount of blue light reaching the photoreceptors, macular pigment may protect the macula from this photo-damage; the higher the density of macular pigment (macular pigment optical density, or MPOD), the greater the amount of blue-light filtering that will occur [11, 12]. A second proposed function of macular pigment is that it protects the macula against oxidative stress by acting as an antioxidant [13]. These blue-light filter and antioxidant functions have led to the school of thought that having a high MPOD could help to protect against the eye disease age-related macular degeneration (AMD), the most prevalent cause of severe visual impairment in Western society [14–16]. As a result, there have been a multitude of studies investigating possible links between MPOD and AMD, using a variety of measurement techniques. Some of these studies have supported an MPOD–AMD association [e.g., 17–19] and some have not [e.g., 20, 21]. This inconsistent evidence is not too surprising, given the apparent multifactorial nature of AMD. What's more, it is highly likely that an individual's MPOD is equally multifactorial, but as one of the few potentially modifiable risk factors for AMD, its continued investigation is extremely important.

Macular pigment optical density may be measured in vitro or in vivo. In vitro measurement involves the techniques of HPLC [e.g., 22, 23] or microdensitometry [e.g., 24]. However, they can only be performed on excised retinas, and so are clearly not suitable for widespread use. This review therefore details the most common techniques currently used to measure MPOD in vivo. These in vivo techniques are noninvasive, and are normally categorized under one of two headings: psychophysical (requiring a response from the subject) or objective (requiring minimal input from the subject). Together, these techniques have established that MPOD varies widely between individuals, from virtually no macular pigment to greater than 1 log unit optical density, with average levels ranging from 0.16 [25] to 0.69 [26], depending on the method and/or the study population.

Method

A systematic literature search was conducted using ISI Web of Knowledge and PubMed. Key words and their combinations used for the search included 'macular pigment', 'macular pigment optical density', 'lutein', 'zeaxanthin', 'heterochromatic flicker photometry', 'motion photometry', 'reflectometry', 'autofluorescence', 'Raman', 'electrophysiology', and 'macular degeneration'. Further searches were undertaken for key researchers in the field such as Beatty, Berendschot, Bernstein, Bone, Delori, Gellermann, Hammond, Landrum, Moreland, Nolan, Robson, Snodderly, Stringham, Trieschmann, van de Kraats, and Wenzel. Further papers were obtained from the references of the retrieved articles. All the articles were reviewed and relevant information was incorporated into the manuscript.

Results of review

Psychophysical techniques

Psychophysical techniques of measuring MPOD include the following:

Threshold spectral sensitivity [e.g., 27–31].
Colour matching [e.g., 32–35].
Dichroism-based measurements [e.g., 9, 36].
Minimum motion photometry and apparent motion photometry [e.g., 37–41].
Heterochromatic flicker photometry [e.g., 42–53].

The first three of the psychophysical methods have now been largely superseded by heterochromatic flicker photometry (HFP) and, to some extent, minimum motion photometry. This is in part due to their increased level of difficulty and/or the longer time needed to perform them [54]. This review will therefore focus on the latter two methods. For information regarding the threshold sensitivity, colour matching and dichroism techniques, the reader is directed to the referenced studies, along with a validity review by Hammond, Wooten and Smollon [54].

Heterochromatic flicker photometry

Developed by Ives in the early 1900s (cited by Viner [55]), HFP has so far been the most commonly used of all the techniques for measuring MPOD. As such, it is often used as a standard against which other techniques are validated [e.g., 56–60], although at present there is no true 'gold-standard' in vivo measure of MPOD.

The use of HFP to measure macular pigment levels was first described over 30 years ago by Werner and Wooten [61] but the technique wasn't elaborated on until 1987, in a key paper by Werner, Donnelly and Kliegl [42]. Since then, HFP has been developed and used by numerous research groups investigating macular pigment. Key papers incorporating detailed descriptions and variations of the technique include Hammond and Fuld [43], Hammond et al. [44], Landrum et al. [45], Wooten et al. [46], Beatty et al. [47], Mellerio et al. [48], Bone and Landrum [49], Snodderly et al. [50], Tang et al. [62], Iannaccone et al.

[51], Stringham et al. [52], and van der Veen et al. [53]. All other studies using HFP to measure MPOD tend to use the instruments originally designed or developed by these investigators.

Procedure In conjunction with many of the MPOD techniques, HFP exploits the spectral absorption properties and retinal location of macular pigment. Essentially, HFP determines MPOD by presenting a light stimulus of two alternating wavelengths at the fovea and at a parafoveal area. The wavelengths are chosen such that one is a short wavelength blue light that is maximally absorbed by macular pigment, and the other is a longer wavelength green to yellow light that is not absorbed by macular pigment [6]. If the colours are alternated at an appropriate frequency and the luminance of the two colours is not perceived to be equal by the subject, the stimulus will appear as a flickering light; the perceived colour of this light will be an amalgamation of the two source colours [47, 49, 50, 63]. Typically, the radiance (often also termed intensity) of the blue light is adjusted by the subject until the observed flicker is minimized [e.g., 44–46, 48, 50, 52, 62]. This occurs when there is an equiluminance match between the blue and green lights [63, 64]. The procedure is then repeated at a parafoveal locus where macular pigment is negligible [65]. Since more blue light will be absorbed by macular pigment at the fovea than the parafovea, a greater radiance of blue light will be required at the fovea to appreciate minimal flicker. The log ratio of the radiance of blue light needed at the fovea compared with that needed at the parafovea gives a measure of peak MPOD (Formula 1), although whether this is truly the peak value is subject to discussion (see 'The edge hypothesis of heterochromatic flicker photometry').

$$\mathrm{MPOD} = \log\left(R^{f}_{\lambda s}/R^{p}_{\lambda s}\right) - \log\left(R^{f}_{\lambda \gamma}/R^{p}_{\lambda \gamma}\right)$$

Formula 1. Example calculation for macular pigment optical density, from Stringham et al. [52]. $R^{f}_{\lambda s}$ = radiance of a peak macular pigment absorption wavelength, e.g., 460 nm, measured at a foveal location. $R^{p}_{\lambda s}$ = radiance of a peak macular pigment absorption wavelength, measured at a parafoveal location, e.g., 7°. $R^{f}_{\lambda \gamma}$ = radiance of a negligible macular pigment absorption wavelength, e.g., 570 nm, measured at a foveal location. $R^{p}_{\lambda \gamma}$ = radiance of a negligible macular pigment absorption wavelength, measured at a parafoveal location.

Instrumentation Several variations on MPOD measurement by HFP have been developed since its first use in the 1970s. Traditionally, Maxwellian view devices have been used [e.g., 42, 43, 45]. These are complex optical systems that are not easily portable and which require the use of a dental bite bar. The bite bar keeps the subject's head stable so that their eye is correctly aligned with the incoming light beam [46, 65]. Given the complexity of these devices, operators need a significant amount of training [46]. As a result, several research groups have simplified the optics and allowed the use of a free view, or Newtonian view, setup [e.g., 46–48, 62]. This negates the need for a bite bar, making the procedure more comfortable for the subject. Free view optical systems are also cheaper, easier to operate, and more portable (if not completely portable) than their Maxwellian counterparts [46, 47, 62]. Wooten et al. [46] demonstrated a strong correlation for mean and individual MPOD calculated between their free view system and an established Maxwellian view system (r= 0.95, no p-value provided). This showed that using the free viewing technique does not affect the accuracy of the derived result. Figures 1 and 2 depict a typical Maxwellian view optical system and free view optical system respectively, whilst Table 1 summarizes their differences. It should be noted that slight variations in these differences do occur. For instance, the instrument developed by Beatty and co-workers [47] uses both a quartz halogen and light-emitting diode (LED) light source.

Test fields

The test fields are viewed at a near working distance, e.g., 33 cm [48]. Most devices use a central stimulus that corresponds to a visual angle of 1° as standard, although there are exceptions; Landrum's 1.5° [45] and Werner's 0.70° [66], for instance. Moreover, many studies have used smaller test stimuli such as 12′ or 30′ when mapping the spatial profile of macular pigment [42, 44, 49, 50, 52, 60, 64, 67–79].

The wavelength chosen for the blue light has varied between researchers from 458 nm [e.g., 11, 46, 52, 75, 80, 81] to 476 nm [17], and from 530 nm [e.g., 43, 82–85] to 575 nm [62] for the green light. Where the blue wavelength does not coincide exactly with the peak of macular pigment absorption, this should be accounted for in the final calculation of MPOD. This is of greater importance in the objective techniques where wavelengths are often further from the peak than for those used in HFP. MPOD should also be adjusted according to the bandwidth of the light source; the narrower the bandwidth, the more accurately the measurement reflects MPOD at the particular wavelength [54]. For the HFP device first described by Wooten et al. [46], the LED with peak

energy at 458 nm has a half-bandwidth of 20 nm. As a result, MPOD must be increased by a 15% constant to correct for this [20, 50, 54, 77].

The peripheral reference measurement is usually made using the same test stimulus as used for the central measure, but the subject's gaze is directed to an eccentric fixation point. An exception to this is the Maculometer (developed by Mellerio et al. [48]) which instead turns the central 1° field into a fixation point and presents an annular test field at 5.5° from fixation. The subject therefore fixates centrally throughout the procedure. The authors reported that many subjects found this easier than maintaining an eccentric fixation. The parafoveal location used in different HFP apparatus varies from 4° from the central fovea [e.g., 11, 43, 46, 62, 71, 80, 81, 86] to 10° [72, 76] or 12° [66]. Similarly, the location of the peripheral point on the retina varies from temporal, nasal or superior retina, depending on instrument type.

Flicker rates

The rate at which the blue and green lights are alternated is a difficult decision for researchers to make, since flicker sensitivity can vary between observers [65]. Ideally, the flicker rate should allow for a suitable amount of null or minimal flicker to be achieved when adjusting the radiance of the test stimulus. If the flicker frequency is too low for an individual, they will have difficulty obtaining a point of null flicker. Conversely, if the flicker frequency is too high for an individual, they will have a wide range of null flicker, leading to variation in measurements [50, 52, 63, 75]. There is also the need for rod and short-wavelength cone suppression to consider (see 'Assumptions' lower in this section). Until recently, most investigators have used set flicker frequencies that have varied from 11 Hz [e.g., 11, 57, 80, 81, 87, 88] to 30 Hz [e.g., 49, 68, 89, 90] in the fovea and from 6 Hz [e.g., 11, 46, 57, 80, 81, 87, 88] to 25 Hz [e.g., 17, 47, 91] in the parafovea.

Background fields

Like test fields, the backgrounds upon which they are presented can also vary in size and wavelength between equipment. Sizes have ranged from 4° in diameter [e.g., 43, 82] to 30° [53]. The colour of the background is invariably a blue wavelength or white. The purpose of these colours is discussed under 'Assumptions' lower in this section.

Recent developments

In 2001, Moreland et al. [38] described a novel method of measuring MPOD by HFP. They used the blue and green phosphor emissions of a colour computer monitor as stimuli for flicker minimization. Although perceptually distinct as blue and green, the broadband emission spectra of the phosphors resulted in a 50% underestimation of MPOD. This was correctable with a model that incorporated these emission spectra and therefore allowed a way of calibrating the monitor. Bone and Landrum [49] questioned whether the retinal illuminance provided by the instrument was high enough to avoid rod intrusion. Apart from further use by Robson et al. [92] and Robson and Parry [41], this method of MPOD determination does not appear to have been widely used.

Snodderly et al. [50] paved the way for a new *customized* approach to HFP when they established a standardized protocol for measuring MPOD. The device used was a modified version of the one described by Wooten and colleagues in 1999, and included the addition of optimizing the flicker frequency for each individual. This was achieved by working out each subject's critical flicker frequency at the fovea and parafovea, then using an algorithm to determine the appropriate flicker rate to use when measuring MPOD. This procedure has since been adopted in other MPOD research [e.g., 51, 52, 64, 71, 72, 75, 76, 78, 79, 86, 93].

A new HFP technique called the macular assessment profile (MAP) test has been described by Rodriguez-Carmona et al. [94] and Kvansakul et al. [95]. The principle appears similar to the method detailed by Moreland et al. [38] and described above, in so far as the broadband phosphors of a visual display are employed and again require a correction model for this. The authors state that the test is a 'rapid and convenient' way of measuring a subject's macular pigment profile up to 8° from the fovea, taking advantage of the ability of visual displays to produce stimuli of different sizes at randomized locations [94]. Although the test is said to have been validated, there does not appear to be any formally published data on this.

A further development for customized HFP was briefly described by Engles et al. [93] and elaborated upon by Nolan et al. [75] and Stringham et al. [52]. This involves the inverse-yoking of the radiances of the blue and green stimuli so that the overall luminance of the test field remains constant, i.e., when the radiance of the blue stimulus is increased, the radiance of the green stimulus is proportionately decreased. As a result, potential distractions by changes in perceived brightness for the subject are avoided.

An entirely different approach to measurement of MPOD by HFP has been adopted in a new commercially available device which is described in detail by van der Veen et al. [53]. Instead of the subject responding to minimal or no flicker, they respond to the *appearance* of flicker as the alternation rate is decreased at 6 Hz per sec from a starting level of 60 Hz.

This is above the critical flicker fusion frequency for the test conditions, and therefore subjects do not perceive any flicker initially. Rather than the radiance of one wavelength being adjusted by the observer, a sequence of blue–green ratios is used. These are inverse-yoked to ensure that overall luminance stays the same. With similarities to Snodderly et al. [50], the instrument determines each observer's sensitivity to flicker prior to the main part of the test. The technique also offers the possibility of estimating MPOD from a central measure alone, the peripheral measure being estimated from the age of the subject and their expected level of lens yellowing. A comparison between central and peripheral derived MPOD and estimated (central only) MPOD in 5616 eyes revealed a very close correlation (r=0.92, no p-value provided) [96].

Most recently, a further development on customized HFP and inverse yoking has been described by Connolly et al. [78] and Nolan et al. [97]. Whilst sticking with the traditional method of HFP MPOD determination, i.e., the radiance of blue light being increased/decreased until the point of minimal flicker, it takes on a more automated approach, as per the device of van der Veen et al. [53]. The instrument's electronics increase/decrease the amount of blue in the stimulus at a set rate. This removes any inter-individual variability in the speed at which the blue–green ratio is adjusted, therefore improving the accuracy of the MPOD value.

Assumptions The HFP measurement of MPOD relies on several assumptions. Many of these assumptions are largely accepted because of the close relationship between HFP-derived macular pigment spectral absorption curves and spectral curves derived in vitro. Nevertheless, some of the main assumptions are described:

1. Absorption or scattering properties of the ocular media being accounted for through use of a parafoveal locus [47, 49, 56, 98]. Essentially, this means that the amount of yellowing in the media (e.g., the crystalline lens) would influence the measured MPOD value, but the reference measure outside of the fovea cancels this effect [96]. This is demonstrated by Formula 2, below. Evidence that this assumption is correct comes from real and simulated data. For instance, Ciulla et al. [81] measured MPOD in 24 patients before and after cataract surgery. No significant difference in MPOD pre or post surgery was found, indicating that varying degrees of crystalline lens absorption does not affect macular pigment measurement when the HFP method is used. Wooten et al. [46] simulated clear and dense lenses by incrementally altering the background field radiance of their free view device. No significant differences in MPOD were found. Most recently, Makridaki et al. [96] demonstrated on a new HFP instrument [53] that lens yellowing, whether simulated or real, had no effect on the measured MPOD.

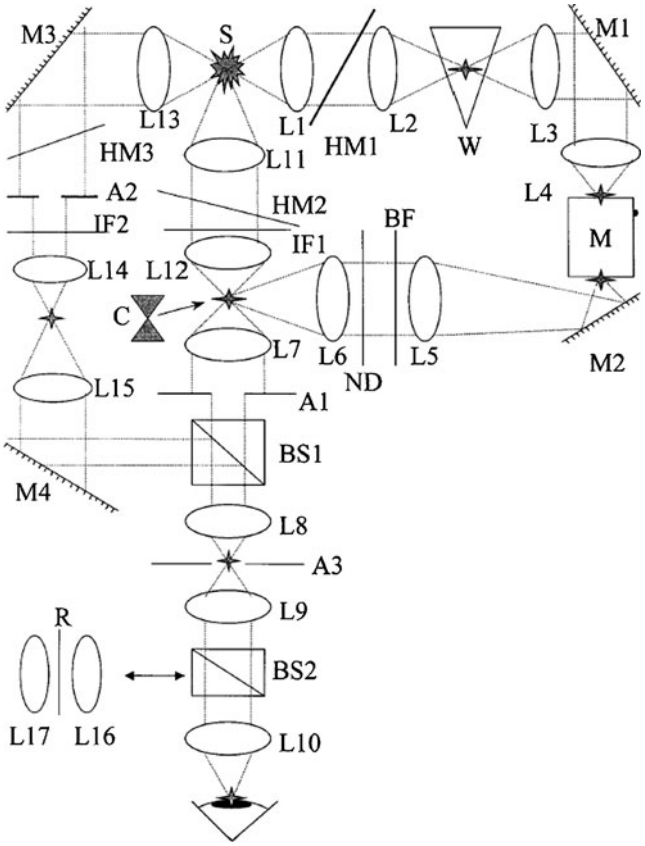


Fig. 1 Schematic diagram of a Maxwellian view optical system, as used by Wooten et al. [46]. *A1–A3*=apertures 1–3. *BF*=blocking filter (removes stray light). *BS1* and *BS2*=beam splitters 1 and 2. *C* = flicker vanes with a first surface mirror (produces alternation of the test and reference lights). *HM1–HM3*=hot mirrors 1–3 (reduce heat transfer). *IF1* and *IF2*=interference filters 1 and 2. *L1–L17*=lenses 1–17 (achromatic, planoconvex). *M* = monochromator (produces the test wavelength). *M1–M4*=mirrors 1–4 (right angle, first surface). *ND* = neutral density filter (together with interference filters, produces the reference and background wavelengths). *R* = reticle. *S* = source light, in this case a xenon arc lamp. *W* = wedge (used to adjust the radiance of the test light). (Reprinted from Wooten et al. [46], with permission from the Association for Research in Vision and Ophthalmology)

$$\mathrm{B\,fov} \times \mathrm{T_{lens}} \times \mathrm{T_{MP}} = \mathrm{B\,ref} \times \mathrm{T_{lens}} \quad (1)$$

$$\mathrm{T_{MP}} = \mathrm{B\,ref}/\mathrm{B\,fov} \quad (2)$$

$$\mathrm{MPOD} = \log 1/\mathrm{T_{MP}} = \log(\mathrm{B\,fov}/\mathrm{B\,ref}) \quad (3)$$

Formula 2. Macular pigment optical density derivation, from Snodderly and Hammond [65]. In

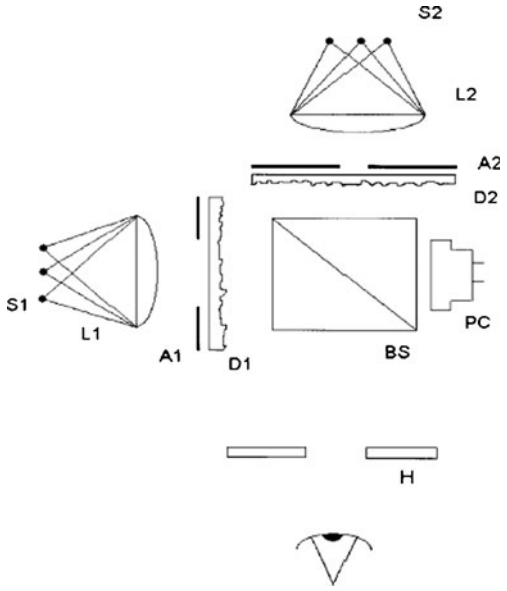


Fig. 2 Schematic diagram of a free view optical system, as used by Wooten et al. [46]. *A1* and *A2*=apertures 1 and 2. *BS* = beam splitter. *D1* and *D2*=optical diffusers (increase transmission efficiency). *H* = hole (1-inch circular viewing hole). *L1* and *L2*=lenses 1 and 2 (achromatic, planoconvex). *PC* = photocell. *S1* and *S2*=source lights 1 and 2 (3×470 nm LEDs for S1, i.e., background field, and 2×458 nm plus 1×570 nm for S2, i.e., test field). (Reprinted from Wooten et al. [46], with permission from the Association for Research in Vision and Ophthalmology)

addition to the transmission of blue light through the macular pigment (T_{MP}), the transmission through the lens (T_{lens}) is taken into account. B fov and B ref are the radiances of blue light needed to minimize flicker at the fovea and reference point respectively. Since T_{lens} is assumed to be the same at both the fovea and reference, it is removed from equation (1). The final equation (3) is a simplified version of Formula 1.

2. Accurate subject fixation and response. This is partly checked through assessment of instrument reliability. Werner et al. [42] also checked fixation accuracy on four of their subjects (age range 15 to 71) with an additional test; all subjects were able to accurately fixate to within ±1.00° of the foveal and parafoveal stimuli, or better, providing further evidence for this particular assumption.
3. Equal spectral sensitivity and distribution of photoreceptors across the retina, such that the difference ratio between the foveal and parafoveal locations is dependent solely on the macular pigment [12, 42, 65]. This assumption is not correct, but is accounted for with the design of HFP instrumentation. Rods and short-wavelength sensitive cones (S-cones) are absent at the fovea, whilst being abundant in the peripheral retina and parafovea respectively [99, 100]. Conversely, medium-wavelength cones (M-cones) and long-wavelength cones (L-cones) are present in much higher concentration in the fovea than elsewhere [101]. However, unlike rods and S-cones, the ratio of M- to L-cones has been shown to remain fairly constant, at least in the central retina [102–104] and, as a result, should not affect the measured MPOD. It is therefore

Table 1 A summary of the main differences between Maxwellian and free view optical systems

Maxwellian view	Free view
Complex optical system.	Simplified optical system.
Test stimuli produced by a single light source, e.g., a quartz halogen lamp, an advantage being that deterioration of the equipment with age or voltage fluctuations alter both stimuli equally and therefore should not affect the results [49].	Test stimuli produced by light-emitting diodes (LEDs) with appropriate peak wavelengths.
Light from the lamp is rendered into the appropriate monochromatic wavelengths using filters.	LEDs have the advantage of producing near monochromatic light, i.e., no need for filters [48].
Test and reference lights alternated by a rotating chopper [e.g., 49] or mirror [e.g., 43–46], with the radiance of the test light adjustable by a 'highly linear, compensated, neutral density wedge' [49].	Alternation of the test and reference lights is achieved by square wave current pulses [48]. The use of LEDs also permits the possibility of adjusting the test light radiance directly through voltage, thereby simplifying the use of the instrument and avoiding any need for rotating mirrors and neutral density wedges (see Wooten et al. [46]).
Narrow beam light stimuli must enter the eye precisely in the centre of the pupil, which requires head stabilization with a dental bite bar [46, 65].	Wider beam light stimuli can enter the eye through the whole pupil, so head stabilization with a bite bar is not necessary [46, 65].
Not easily portable.	Portable.
Expensive.	Relatively inexpensive.
Considerable training required to operate correctly.	Simpler to operate than Maxwellian systems, therefore less training required.

generally accepted that removing the rod and S-cone contributions is of greater importance. To do this, investigators have designed their HFP apparatus accordingly. The background field is often blue to suppress the S-cone population [e.g., 42–44, 46, 48, 50–52, 62, 105] or bright white to provide photopic conditions and hence suppress the rod population [e.g., 45, 49, 53]. The flicker frequency is chosen so that it is high enough to further exclude rods and S-cones. This is achieved because the flicker rate is above the critical flicker fusion frequency (the alternation rate at which a flickering light is no longer resolvable by the visual system and thus appears steady to an observer) of rods and S-cones, but is still lower than the critical flicker fusion frequency of M- and L-cones [65, 106, 107].

4. The peripheral reference locus having a negligible level of macular pigment. Some studies have questioned this assumption [39, 94, 108–112], particularly when eccentricities as little as 4° from the fovea have been used [11, 20, 43, 46, 62, 71, 80, 81, 86–88, 113]. HFP spatial distribution plots of macular pigment (see 'Validity and reliability') have gone some way to disproving any concerns, such that for most individuals, the assumption holds.

Validity and reliability The validity and reliability of a technique are two important but quite different issues. Reliability, as described by Gallaher et al. [114], refers to the 'ascertainment of the reproducibility of a given measurement on the same subject at two distinct points in time'. Although this is a very important aspect of any instrument, it does not automatically imply that the instrument is valid. This was well-illustrated by Snodderly et al. [50], where it was pointed out that one observer had a repeatable negative value of MPOD. The measure was therefore reliable but was nevertheless of questionable validity. For in vivo measurement of MPOD, validity is demonstrated by showing a matching comparison with the known spectral and spatial properties of macular pigment in vitro. Some studies have done this indirectly by comparing the measurements from a new device with those of an older, more established device [46, 56–59].

Validity As mentioned above, the validity of MPOD measurement by HFP can be assessed in two main ways [52, 71]; either by deriving the spatial profile of macular pigment across the fovea [e.g., 42, 44, 50, 52, 53, 67, 70, 71, 75] and comparing that to in vitro knowledge of macular pigment distribution [e.g., 5, 7, 24, 115], or by deriving the spectral absorption profile of macular pigment [17, 42, 43, 47, 49, 52, 71] and comparing that to the shape of a known in vitro absorption curve of macular pigment. The latter method is considered to be more robust than the former, since spatial profile can vary between individuals [44, 50, 59, 64, 71, 75, 78, 116, 117].

Spatial profiles of MPOD are achieved by altering the size and/or the eccentric position of the test stimulus, thus producing a curve that can be used to describe the change in MPOD with increasing eccentricity from the central fovea. An example is shown in Fig. 3.

Spectral absorption profiles of MPOD are obtained by systematically altering the wavelength of the test stimulus. The procedure is the same as that used to determine peak MPOD, except that a number of light wavelengths must be alternated with the reference stimulus until minimum flicker is accomplished, instead of the blue light alone. The extra measurements increase the duration of the test but permit a curve of spectral absorption to be plotted (see Fig. 4). The in vivo HFP-generated curve is simultaneously compared with the shape of an in vitro spectral curve of lutein and zeaxanthin. The choice of which in vitro curve to use is not an easy one, since the ideal comparison of in situ spectral data from human retinas does not currently exist [54]. Consequently, different researchers have used different data. These include: (1) Wyszecki and Stiles' composite data curve[1] [118], used by Werner et al. [42], Hammond and Fuld [43], Beatty et al. [47], and Beatty et al. [17], (2) Snodderly and colleagues' microspectrophotometry-derived data from primate monkeys [6], used by Hammond and Fuld [43], and (3) spectral measures of lutein and zeaxanthin dissolved in olive oil [24] or incorporated in liposomes [9]. These last two in vitro spectrums have more recently been used in combination when assessing validity [52, 54, 71].

The outcomes of these validity tests have shown a good correlation with in vitro MPOD distribution and absorption [e.g., 71], and therefore attest to the validity of HFP as a measurement method for MPOD.

Reliability Numerous researchers, through test–retest checks, have assessed the reliability of HFP measurement of macular pigment. The statistical descriptors used to evaluate reliability vary between studies. Table 2 is a summary of all available information on HFP test–retest reliability indicators. It shows that for most subjects HFP provides repeatable measures of MPOD, and is therefore a reliable technique. Interestingly, the two most recent

[1] (The data from Wyszecki and Stiles [118] is actually a weighted composite curve of six *psychophysical* methods that had been used up to that time, rather than in vitro data.

evaluations of HFP appear to give the weakest indication of its reliability [119, 120]. These studies were independent of each other but used the same HFP device, one of the first that has been designed for use in a clinical setting rather than a research setting. The results suggest that further developments may be required for this particular HFP instrument in order to verify its suitability to accurately assess MPOD.

The edge hypothesis of heterochromatic flicker photometry As mentioned previously, most instruments use a stimulus size of 1° to measure MPOD. One might reasonably assume that this measures the total amount of macular pigment across the whole 1° area, or the peak level of macular pigment. However, many investigators disagree with both these assumptions, and instead believe that the level of calculated MPOD is mediated by the edge of the stimulus [e.g., 11, 42, 44, 48, 50, 52, 121], so that for a 1° stimulus, the recorded MPOD corresponds to the macular pigment level at 0.5° from the centre of the fovea. Werner et al. [42] were the first to suggest this theory, and it gained momentum when Hammond et al. [44] found a very high correlation (r=0.91, p<0.00001) between MPOD measured with a 1° test stimulus and a point test stimulus of 12′ placed at 0.5° (see Fig. 5). Their data also indicated that MPOD at 0.5° (as measured with a 1° stimulus) is an estimated 69% of the true peak MPOD. For example, an MPOD value of 0.4 measured with a 1° test would indicate a peak macular pigment density of 0.58.

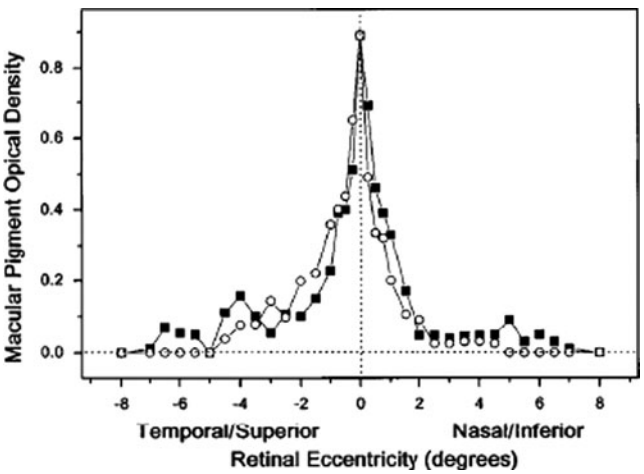


Fig. 3 A detailed spatial profile of one subject's macular pigment density, obtained using heterochromatic flicker photometry. Here, measurements have been taken along the horizontal (*filled squares*) and vertical (*open circles*) meridians of the retina, demonstrating a rapid and symmetrical decline in macular pigment with increasing eccentricity (Reprinted from Hammond et al. [44], with permission from the Optical Society of America)

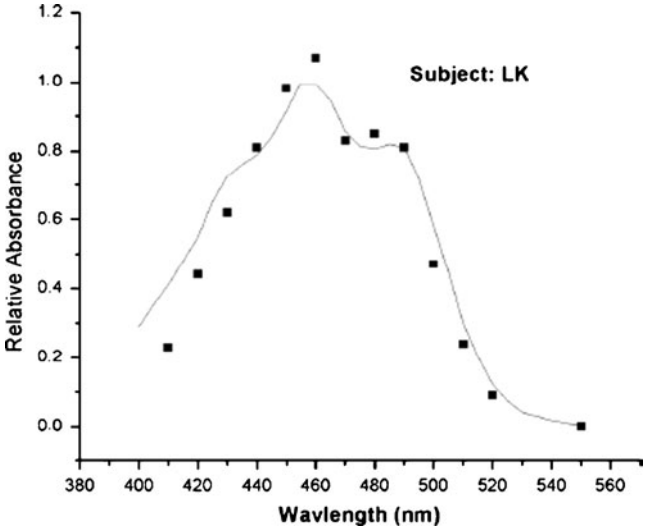


Fig. 4 The relative macular pigment spectral absorption profile of one subject (*filled squares*), as derived by heterochromatic flicker photometry. The continuous curve is an in vitro combination template as described in the text. Small deviations from the template at wavelengths below about 440 nm are typical. The reason for this is not clear, and several theories have been proposed [see, for example, 54, 66, 71]. However, above 440 nm, the in vivo and in vitro methods are in very close agreement, so measurements of peak macular pigment optical density should remain accurate. (Reprinted from Wooten and Hammond [71], with permission from the American Academy of Optometry)

The 'edge hypothesis' has been questioned, however, most notably by Bone et al. [68]. In contrast to Hammond et al. [44], they found that the measured MPOD corresponds to the level of macular pigment at approximately 50% of the stimulus radius (Fig. 6). Further evaluation indicated that this equated closely to the average amount of macular pigment over the whole stimulus area [68]. Nevertheless, in spite of this conflicting evidence, most researchers have continued to assume the edge hypothesis in their HFP work [e.g., 60, 63, 64, 73–75, 78, 122] and this would appear to be a reasonable decision, with further evidence for it coming most recently from van der Veen et al. [121].

Advantages and disadvantages of HFP Advantages include: 1) no pupil dilation required, 2) inexpensive equipment relative to objective techniques, 3) independence from absorption and scattering properties of the ocular media, 4) good test-retest reliability on many subject populations, and 5) proven validity. Disadvantages include: 1) some subjects finding HFP difficult to carry out, especially the peripheral task, which is subject to Troxler's effect — a perceptual fading of peripheral stimuli [123]; Troxler's effect also becomes more distracting the more eccentric the peripheral target is, making the use of

eccentricities certain to have no macular pigment difficult to record accurately, 2) a long testing time if complete spectral and/or spatial distribution is required, and 3) unsuitability for some individuals, such as young children, people with learning difficulties or people with insufficient visual acuity or visual fields.

Motion photometry

The minimum motion paradigm was initially described by Stumpf [124], although this went largely unnoticed until its translation into English by Todorović in 1996 [40, 125]. With parallels to HFP, it refers to the perceived reduction in motion of a moving square or sine wave grating as equiluminance of the colours involved is reached. The concept was taken up for use in photometry by both Moreland [126, 127] and Anstis and Cavanagh [128], but in subtly different ways. This then led to the use of minimum motion photometry for in vivo measurement of macular pigment [e.g., 33, 37].

Procedure Many of the principles described for HFP also apply for motion photometry measurement of MPOD, i.e., a wavelength of light at the peak of macular pigment absorption is compared with a wavelength of light not absorbed by macular pigment, at central and parafoveal locations. Moving square wave gratings are used, with the bars being alternately illuminated by the two light wavelengths. The radiance of the longer wavelength stimulus is adjusted until the motion appears to slow down or change direction, depending on the method being employed [126–128]. The slowing down of the grating is *minimum* motion photometry, whereas the reversal of grating movement is known as *apparent* motion photometry [40]. As with HFP, different radiances of the test wavelength will be required for equiluminance at the foveal and parafoveal positions, on account of the higher levels of macular pigment at the fovea. A log ratio of these radiances provides a measure of MPOD [37–41, 92, 129].

Instrumentation The motion photometry technique for measurement of MPOD has not been as widely used as HFP. Apart from the fundamental differences between minimum motion photometry and apparent motion photometry (explained below), there is little variation in the instrumentation that has been adopted.

Minimum motion photometry

Minimum motion photometry for MPOD, as described and used by Moreland, Robson and colleagues [e.g., 37–41, 92, 129], employs a Moreland anomaloscope (traditionally used for colour vision assessment) that is adapted to produce a moving square wave grating with a spatial frequency of 0.38 cycles per degree. A rotating spiral mirror generates the grating which, when viewed through a circular or annular stop, appears to move horizontally across the visual field. The bars of the grating are alternately illuminated with two narrow-band interference filters from a single tungsten–halogen lamp. The interference filters typically provide wavelengths of 460 nm (blue — maximal absorption by macular pigment) and 580 nm (yellow — negligible absorption by macular pigment). Luminance matched filters of 450 nm are added to the grating bars to create a background pedestal that saturates S-cones (see ‘Assumptions’ lower in this section). The grating moves at a constant velocity of 14 Hz; this also rules out any rod or S-cone contribution.

Unlike HFP, it is the norm with motion photometry for the spatial profile of macular pigment to be plotted, rather than peak MPOD alone. Consequently, the test fields comprise up to two central, circular stops of 0.8-0.9° (visual angle) and 2.2°, and 11 annular stops placed eccentrically from 0.8° to 7.5° in the superior visual field. The setup is illustrated in Fig. 7. The minimum motion technique described here uses a Maxwellian view system but does not require a bite bar; rather, an adjustable chin rest (vertically and horizontally) is used for pupil centration [40].

Apparent motion photometry

Apparent motion photometry, based on the phenomenon detailed by Anstis and Cavanagh [128], has been developed into a commercially available device for MPOD assessment by West and Mellerio [130]. However, there do not appear to be any peer-reviewed studies on the instrument. The instrumentation details given below are therefore based on information from the Cambridge Research Systems (CRS) website (http://www.crsltd.com/catalog/metropsis/MP.html).

Instead of a Moreland anomaloscope, a cathode ray tube (CRT) monitor is used. Four square wave gratings on a blue background are presented sequentially, 90 degrees out of phase with each other. The first and third gratings are made up of blue and red bars, produced by the blue and red phosphors of the CRT; blue for maximal macular pigment and red for negligible macular pigment absorption. The second and fourth gratings are achromatic, being composed of light and dark grey bars, i.e., luminance gratings. The apparent motion paradigm dictates that when the luminance of the red bars is greater than the blue, the red bars appear to jump rightwards to the light grey bars in the luminance grating, and when the luminance of the blue bars is greater than the red, the blue bars appear to jump

Table 2 A list of all heterochromatic flicker photometry (HFP) studies to date that have given statistical data on test-retest (inter-session) reliability

Study/year	Number of subjects	Age range; eye health status; experience with HFP	Reliability statistics
Hammond and Fuld/ 1992 [43]	10	19–42; normal; seven novices.	Five sessions on different days. Intersession correlation 0.85 (Cronbach's α). Mean absolute difference between 1st and 2nd session 0.05±0.03 (16% of mean MPOD, as cited in Delori et al. [56]); Y=0.87X+0.03, r=0.94, p<0.0005.
Hammond, Fuld and Curran-Celentano/ 1995 [82]	20	19–22; normal; all novices.	Five sessions on different days. Intersession correlation 0.68 (Cronbach's α). Mean absolute difference between 1st and 2nd session 0.10±0.10 (33% of mean MPOD, as cited in Delori et al. [56]); Y=0.56X+0.12, r=0.73, p<0.0005.
Hammond, Wooten and Snodderly/1997 [44]	32	21–63; normal; 29 novices.	Two sessions on different days. Mean absolute difference between sessions 0.08±0.08; Y=0.92X+0.07, r=0.91, no p-value given.
Hammond et al./1997 [67]	13	30–65; normal; not reported.	Two sessions. Mean absolute difference between sessions 0.08±0.07 (23% of mean MPOD, as cited in Delori et al. [56]); Y=0.83X+0.03, r=0.72, no p-value given.
Hammond, Wooten and Snodderly/1998 [105]	10+27	24–36 & 60–84; some older subjects with mild RPE changes and two with early cataract; not reported.	Two sessions. Mean absolute difference between sessions 0.08±0.09; Y=0.90X+0.06, r=0.91, p<0.0000001.
Wooten et al./1999 [46]	4	30 subjects in full study, 16–60; not reported; not specifically reported.	Ten sessions over 2–4 weeks. Intersession correlation 0.89 (Cronbach's α).
Hammond and Caruso-Avery/2000 [11]	8	217 subjects in full study, 17–92; normal; all novices.	Ten sessions over 2–4 weeks. Intersession correlation 0.97 (Cronbach's α). Average range over sessions=0.166 (best to worst 0.07– 0.27). Mean absolute difference between two sessions 0.04±0.04 (18% of mean MPOD, as cited in Delori et al. [56]).
Aleman et al./2001 [189]	58 'eyes'	58 'patient' subjects in full study, 11–59, age unreported for 29 'normal' subjects in full study; 38 eyes RP or Usher syndrome, 20 eyes normal; not reported.	Two sessions on different days. Mean absolute difference (at 0.5°) between sessions 0.04±0.04 (RP/Usher) and 0.035±0.04 (normal).
Ciulla et al./2001 [81]	24	48–82; pre-post cataract; all novices.	Mean absolute difference between 1st (pre cataract op) and 2nd (post cataract op) session 0.085±0.08; Y=0.53X+0.07, r=0.58, p<0.001. Excluding two outliers strengthens the relationship to Y=0.69X+0.03, r=0.75, p<0.0001.
Duncan et al./2002 [190]	5	13 'patient' subjects in full study, 15–65, age unreported for 40 'normal' subjects in full study; 3 CHM, 2 normal; not reported.	Two sessions < 1 month apart. Mean absolute difference (at 0.5°) between sessions 0.05±0.06 (CHM) and 0.02±0.01 (normal).
Mellerio et al./2002 [48]	3	124 subjects in full study, 18–84; normal; not reported.	Four sessions on successive days. Coefficients of variation=8.6%, 5.1% and 6.7% for subjects 1, 2 and 3 respectively.
Bernstein et al./2004 [110]	40	< 60; normal; not reported.	Three sessions over 1 month. Mean intersession variability 10.1%.
Koh et al./2004 [91]	7+6	58–81; seven early ARM, six normal; not reported.	Two sessions 1-4 weeks apart. Mean absolute difference between sessions 0.063±0.054 (normal eyes), 0.037±0.02 (ARM eyes) and 0.041±0.027 (fellow eyes); Y=0.95X+0.019, r=0.88, no p-value given.
Nolan et al./2004 [107]	100	22–60; normal; not reported.	Two sessions at least 90 mins apart. Mean difference between sessions −0.01±0.08.
Snodderly et al./2004 [50]	48	50–79; 15 with self-reported eye disease; all novices.	Two sessions on different days. Test–retest correlation at 0.5° RE was r=0.90, no p-value given; Y=0.94X+0.02. Mean absolute test–retest difference (as a percentage of mean density)=17%. Coefficient of repeatability=0.19.[a]
Tang et al./2004 [62] (1)	6	68 subjects in full study, 22–23; normal; not reported.	Three sessions, each separated by a week. Coefficient of variation=8.0% and coefficient of repeatability=0.12.
Tang et al./2004 [62] (2)	1	45; normal; not reported.	Five sessions on consecutive days. Coefficient of variation=7.2%.
Hammond and Wooten/ 2005 [86]	8	20–30; normal; novices.	Ten separate sessions over 2–4 weeks. Intersession correlation 0.97 (Cronbach's α).
Lam et al./2005 [70]	9	Average age 31.2; normal; novices.	Two sessions on different days. Test–retest correlation at 0.5° was r=0.68, no p-value given.
Liew et al./2005 [163]	17	150 twin pairs in full study, 18–50; normal; not reported.	Two sessions on different days. Mean difference between sessions 0.10±0.11. Mean coefficient of variation=17.6% (± 16.5%).
Gallaher et al./2007 [114]	40	69–84; not specifically reported; not reported.	Two sessions, 1 week to 20 months apart. Mean difference between sessions −0.01±0.16. Test–retest correlation was r=0.734, no p-value given. Mean coefficient of variation=18.4%. Intraclass correlation=0.96.
Hogg et al./2007 [176]	11	21–50; normal; novices.	Four sessions over 2 weeks. Mean coefficient of variation between sessions= 11.5% at 0.5°.

Table 2 (continued)

Study/year	Number of subjects	Age range; eye health status; experience with HFP	Reliability statistics
Johnson et al./2008 [122]	49	60–80; normal; not reported.	Two sessions 1-4 days apart. Test–retest correlation was r=0.90, no p-value given.
Kirby et al./2009 [64]	16	Not reported; normal; experienced.	Three sessions on different days. Intraclass correlations 'in the range' 0.93–0.96 at 0.25°, 0.5° and 1° retinal eccentricity.
Van der Veen et al./ 2009 [53]	11	26 subjects in full study, 22–64; normal; not reported.	Two sessions on different days. Mean difference between sessions 0.0195± 0.047; Y=1.2X − 0.05, r=0.97, p<0.001. Mean test–retest difference (as a percentage of mean density)=11.7%.
Bartlett, Acton and Eperjesi/2010 [119]	38	19–46; normal; novices.	Two sessions 1 week apart. Test–retest coefficients of repeatability were 0.45 (operator 1) and 0.58 (operator 2).
Hagen et al./2010 [120]	24	38.1±10.6; normal; novices.	Three sessions over 11±6.9 days. Coefficients of variation were 36.1% and 23% for right and left eyes respectively.

[a] Snodderly et al. [50] also calculated the equivalent values for the left eye, which were r=0.86, 22% test–retest difference and a coefficient of repeatability of 0.21.

ARM = age-related maculopathy, the general term for degeneration of the macular region of the retina, which is normally separated into the two categories of early and late ARM, with the latter also being called age-related macular degeneration [188].

CHM = choroideremia.

MPOD = macular pigment optical density.

RE = right eye.

RP = retinitis pigmentosa.

RPE = retinal pigment epithelium.

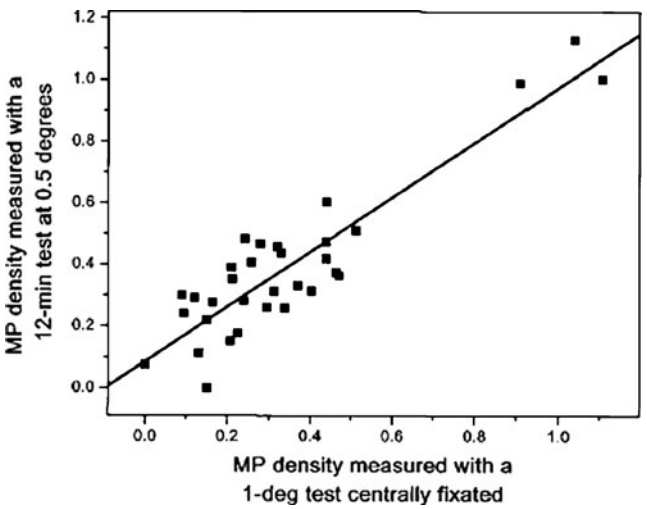


Fig. 5 A high correlation (r=0.91, p<0.00001) between macular pigment optical density measured with a centrally positioned 1° test stimulus and a 12′ test stimulus positioned 0.5° from the central fovea lends support to the edge hypothesis of heterochromatic flicker photometry. (Reprinted from Hammond et al. [44], with permission from the Optical Society of America)

leftwards to the light grey bars in the luminance grating (Fig. 8, left). The subject therefore sees movement in one of two directions. The setup of West and Mellerio's device results in these directions being up or down (Fig. 8, right). The red luminance is adjustable, and at the point of red–blue equiluminance, the direction of motion becomes 'ambiguous'. A two-alternative forced choice procedure is used to determine equiluminance (and subsequent MPOD) at central and eccentric locations. The grating alternation frequency must be optimized, usually between 8 and 20 Hz, for each subject for reliable results.

Like minimum motion, the apparent motion photometer for MPOD routinely plots the spatial distribution of macular pigment. Two central vertical strips (0.3°×1.25°) are located at 0° and 1°, whilst six 45° annular test fields are located from 2° to 7° from fixation. A shorter three-location test is also available.

With a CRT monitor comes the problem of spectral overlap of the red, green and blue phosphors, similar to the problems of the colour monitor for HFP discussed in HFP 'Recent developments' [38, 41, 92]. The apparent motion photometer overcomes this with

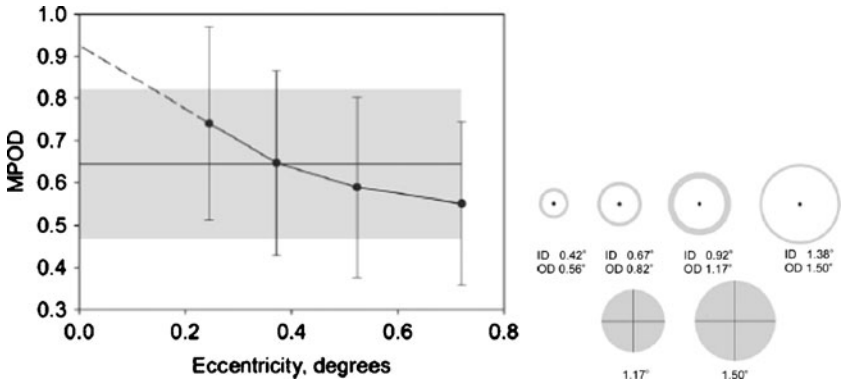


Fig. 6 *Left panel*: Macular pigment distribution in the central 1.5°, as determined by Bone et al. [68], using heterochromatic flicker photometry. The horizontal line is the average macular pigment optical density (MPOD) for ten subjects, calculated using a centrally positioned 1.5° circular test stimulus (*grey area*=± 1 standard deviation). The *filled circles* depict the average MPOD at various eccentricities (*bars*=± 1 standard deviation), determined using a number of annular stimuli with central fixation marks (*right panel*). Whereas Hammond et al. [44] used a very small stimulus placed at the required retinal eccentricity, Bone and colleagues have used annular stimuli. This is an alternative method of knowingly measuring MPOD at a retinal eccentricity equivalent to the stimuli radii. The intersection point at 0.38° is the position at which MPOD appears to be measured when using a 1.5° test stimulus, i.e., 51% of the stimulus radius. *Right panel*: The four annular stimuli and two circular stimuli (1.17° circular stimulus not used in this graph). *ID* = inner diameter, *OD* = outer diameter. (Reprinted from Bone et al. [68], with permission from Elsevier)

the use of an optical filter that blocks light between 460 and 640 nm, thus avoiding a significant underestimate of MPOD.

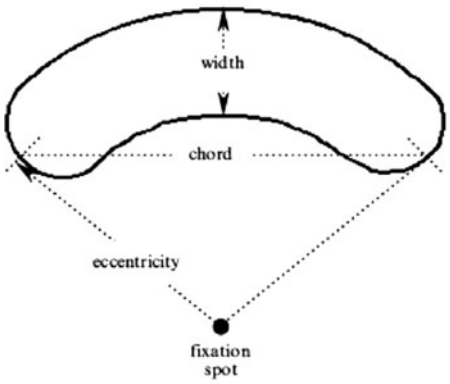
Assumptions The same assumptions apply to minimum motion photometry as for HFP, namely: (1) absorption or scattering properties of the ocular media being accounted for through use of a parafoveal locus, (2) accurate subject fixation and response, (3) equal spectral sensitivity and distribution of L- and M-cones across the retina, and (4) the peripheral reference locus having a negligible level of macular pigment.

The apparent motion device offers an interesting solution to accurate fixation by using a video gaze tracking system that inhibits stimulus presentation whenever fixation is not maintained to a sufficient level (within±0.5° of target). This offers an improvement over unmonitored free view techniques whilst avoiding the need for a Maxwellian view system. With respect to L- and M-cone distribution, Robson et al. [129] used a 460/550 nm combination as well as the customary 460/580 nm to see if there was any variation in MPOD measurements. The correlation was extremely strong (slope= 1.00, r=0.99), indicating that even if the distribution does vary with eccentricity, the effect is likely to be very small [129]. Like HFP, motion photometry also uses a blue background and a suitable temporal frequency to ensure rod and S-cone suppression. The assumption of the peripheral reference eccentricity having negligible macular pigment should hold for most individuals, because spatial distribution is always plotted out as far as 7 or 8 degrees with motion photometry.

Validity We could find no evidence of motion photometry being used to derive spectral profiles of macular pigment. As a result, it is difficult to know the true validity of the technique, although average MPOD values and spatial profiles are in line with HFP, and consistent correlations with the autofluorescence method of macular pigment measurement [39, 129] and two HFP devices [38, 48] have been found. Nevertheless, questions regarding the validity of motion photometry for measurement of macular pigment have been raised [54].

Reliability The reliability of minimum and apparent motion photometry has not been as vigorously assessed as it has been with HFP. The average of five readings is taken at each location [37, 39, 41, 92, 129] and repeated measurements have been incorporated in averaged results [39]. However, there does not appear to be any published statistical data on test–retest reliability.

The non-edge hypothesis of motion photometry It was explained earlier that most researchers using HFP assume an edge hypothesis with MPOD. In minimum motion photometry, data analysis has led to the support of a *non-edge* hypothesis [34, 39, 40]. To reiterate, this would mean that when a circular, foveal stimulus is used, the measured MPOD would not represent the amount of macular pigment at the stimulus radius. In the case of motion photometry, researchers believe that the measured MPOD actually represents the amount of macular pigment at approximately 70% of the stimulus radius [34, 40]. Subsequently, the 'peak' MPOD value using the smaller 0.8–0.9° central stimulus has been plotted at 0.3° eccentricity from the fovea [e.g., 39, 41, 129].



Foveal field/deg		0.9
Diameters/deg		2.2
Annuli		
eccentricity/°	chord/deg	width/deg
0.8	1.8	
1.2	2.1	0.8
1.7	2.8	
2.3	3.8	
2.8	4.7	
3.3	5.2	
3.9	6.1	1.1
4.4	6.0	
5.3	6.0	
6.6	5.9	
7.5	6.0	

Fig. 7 Test configuration used in minimum motion photometry (*left panel*), with the associated stimuli dimensions given in the table (*right panel*). (Reprinted from Robson et al. [129], with permission from Pion Limited)

Advantages and disadvantages of motion photometry As a psychophysical technique, motion photometry has much in common with HFP in terms of pros and cons. There is no need for pupil dilation, any interference from the ocular media is accounted for, and it is a relatively straightforward test for subjects to partake in. On the down side, Troxler's effect remains a problem for some individuals, and good comprehension of the task is required, so it is not suitable for everyone. No information is provided in any motion photometry studies regarding the level of visual acuity of subjects; therefore, it is unknown whether reliable MPOD measurements are achievable on subjects with lower than normal acuity.

Objective techniques

The objective techniques for measuring MPOD are:

- Fundus reflectometry
- Fundus autofluorescence
- Resonance Raman spectroscopy
- Electrophysiology using visual evoked potentials

Fundus reflectometry

Quantitative measurement of light reflected from the fundus is known as fundus reflectometry (FR), and the researchers Brindley and Willmer [131] were the first to adopt this technique. Their aim was to estimate MPOD in vivo by comparing light reflected at the macula with light reflected from a peripheral area of retina. Since then, FR has gone on to become the most widely used of the objective methods for MPOD measurement, although many improvements and variations have been developed along the way.

Procedure When light enters the eye it has many structures to pass through, including the cornea, the crystalline lens, the retina and the choroid. Some of these structures (and their components) will reflect a small part of the light, whilst others will absorb part of it. Through measurement of reflected light from the retina and choroid, FR is able to assess several ocular features, including macular pigment [109]. A thorough history of FR is provided by Berendschot, DeLint and van Norren [132]; this review will be limited to the use of FR in measuring MPOD.

Although there are several variations on the reflectometry procedure, there are two methods that predominate. The first is a comparison technique, similar to that used in HFP. Light reflected from the fovea is compared with light reflected from an eccentric retinal area, using two wavelengths (one absorbed by macular pigment and one not) or using a spectrum of wavelengths. Since macular pigment absorbs rather than reflects certain wavelengths, there will be a difference in the observed reflectances at the fovea and periphery, owing to the assumed lack of macular pigment at the eccentric site. Researchers who have used this method include Brindley and Willmer [131], van Norren and Tiemeijer [133], Delori and Pflibsen [134], Elsner et al. [135], Berendschot et al. [136], Delori et al. [56], Bour et al. [137], Wüstemeyer et al. [98], and Cardinault et al. [138].

The second core technique is known as a spectral analysis [109]. As the name suggests, this involves the

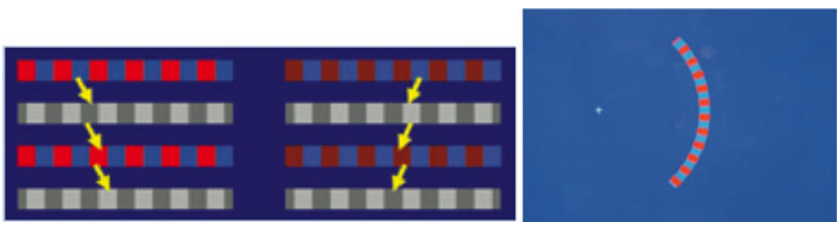


Fig. 8 Apparent motion photometry. *Left panel*: apparent movement to the right when the red bars are brighter than the blue, and vice-versa. *Right panel*: the test configuration for a parafoveal target [130]

analysis of a spectrum of reflected light from a spot of light on the retina. To achieve this, a detailed optical model of the pathways of light in the eye is required. A number of optical models of increasing complexity have been proposed over the years, from van Norren and Tiemeijer [133] through to van de Kraats and van Norren [139]. Probably the most familiar optical model is that derived by van de Kraats, Berendschot and van Norren [140], which has been used to work out MPOD in several studies [e.g., 58, 136, 141–144]. In essence, the density of macular pigment is determined using its known spectral characteristics, and by taking into account the amount of light reflected at the internal limiting membrane, the photoreceptor discs and the sclera [58, 136, 140, 141, 143]. The densities of the lens, melanin and blood are likewise calculated.

Even with these two quite separate forms of FR, there is often an overlap between the two, as demonstrated in the following section.

Instrumentation Many instruments have been used for FR, and it is beyond the scope of this review to explain them all. Consequently, only the more recent fundus reflectometers will be described in any detail. However, common to most methods of FR is the need for pupil dilation, a bleaching of the visual pigments prior to measurement, and some form of head stabilization, either with a bite bar or with a chin rest and temple pads.

Equipment for reflectometry can be broadly categorised into modified fundus cameras, purpose-built reflectometers and modified scanning laser ophthalmoscopes.

Fundus cameras

Many investigators have used modified fundus cameras to measure MPOD, including van Norren and Tiemeijer [133], Delori and Pflibsen [134], Chen et al. [108], Bour et al. [137], Chang et al. [145], Neuringer et al. [3], and Bone et al. [59]. Of these, some have used the comparison technique, some a spectral analysis, and others a mixture of the two. Chen et al. [108], for example, used an optical model as per the spectral analysis technique, but only two wavelengths (460 nm and 560 nm) rather than a full spectrum of wavelengths. In brief, their setup, like several others, consisted of a fundus camera connected to a cooled CCD (charge-coupled device). The filter normally used to take red-free photographs was replaced with narrow-band interference filters of 460 and 560 nm, i.e., maximal and minimal macular pigment absorption. Following pupil dilation, the subjects were instructed to fixate a dim red dot with the eye not being tested whilst the measured eye was slowly light-adapted in order to bleach nearly all the photoreceptor pigments [56, 108, 146]. Two fundus pictures, one taken at each of the two wavelengths, were manually aligned using retinal landmarks. Using a chosen optical model, MPOD at each pixel point in the retina was calculated. In this way, Chen et al. were also able to plot the spatial distribution of macular pigment across the central retina.

Recently, Bone et al. [59] described a modified fundus camera that does not require pupil dilation or bleaching of the photoreceptor pigments.

Purpose-built reflectometers

The most recent 'purpose-built reflectometers' are the foveal reflection analyzer (FRA), originally developed by Zagers et al. [146] and the macular pigment reflectometer (MPR), originally developed by van de Kraats et al. [58].

The diagrammatic setup of the FRA is shown in Fig. 9. After dilation, subjects fixate a central cross-hair (Fig. 9b). In the original instrument (FRA 1), light from a halogen lamp is directed into the eye as a Maxwellian view system with an entrance pupil of 0.8×1.2 mm. This illuminates a 2.8° spot on the central fovea, of which the middle 1.9° is used for analysis. A video observation channel of the pupil and retina helps alignment as well as allowing monitoring of subject fixation. An imaging spectrograph collects the reflected light from the 1.9° area and focuses it onto a cooled CCD camera. The spectrograph has a slit that creates a 0.8×12 mm exit pupil above the smaller entrance pupil, and its spectral range is 420 to 790 nm, thus allowing a spectral analysis of the reflected light using one of the optical models referred to earlier. The FRA 1 has been used to investigate MPOD in studies by Zagers and van Norren [147] and Berendschot and van Norren [141]. Berendschot and van Norren [141] also used a newer version of the device, the FRA 2, which has a number of differences from the first version, including being smaller, which makes it desktop-mountable. Kanis et al. [143, 148] and van de Kraats and van Norren [139] have also used the FRA 2 in their studies.

The diagrammatic setup of the MPR is shown in Fig. 10. Like the FRA, the MPR, as described by van de Kraats et al. [58], involves a 30 W halogen lamp directed into the pupil, a separated exit pupil and a spectrometer, i.e., spectrograph. The spectrometer has a spectral range of 400 to 800 nm. The subject is asked to fixate the centre of a 1° incoming light beam. This incoming illumination forms a 1° spot on the central fovea; the reflected light is also collected over the same 1° area and analyzed using an optical model. The MPR is the first reflectometry device that has a proven ability to measure MPOD through an undilated pupil, provided the pupil is 3 mm or larger; van de Kraats et al. [58] found no significant difference between their MPOD

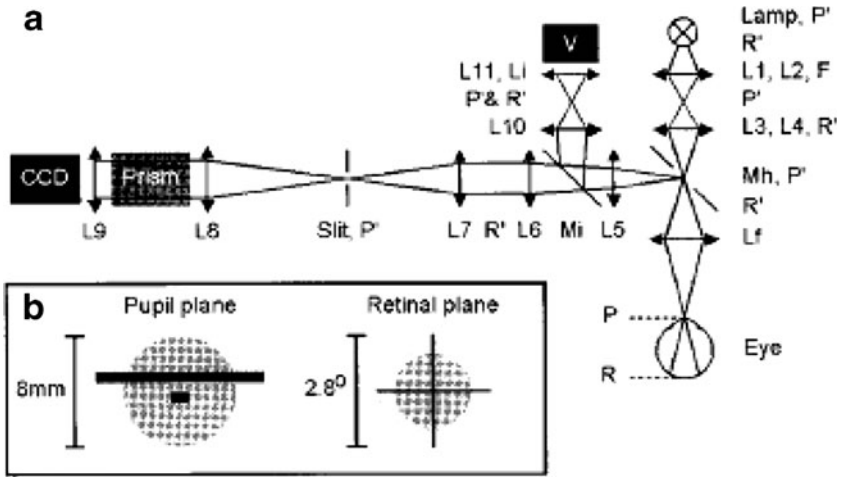


Fig. 9 Schematic diagram of the foveal reflection analyzer 1, from Zagers et al. [146]. In **a**: *F* = spectral filters. *Lamp*=30 W halogen lamp. *L1–L11*=lenses 1–11. *Lf* = front lens. *Li* = insertable lens. *Mh* = mirror with central hole. *Mi* = insertable mirror. *P* = pupil plane. *R* = retinal plane. *P'* and *R'* = planes conjugate to P and R. *V* = video camera. In **b**: the dilated pupil with entrance and exit pupil shown to scale (*left panel*). The illuminated field and the concentric sampled field, with fixation cross hairs (*right panel*). (Reprinted from Zagers et al. [146], with permission from the Optical Society of America)

measurements for dilated and undilated pupils in 20 subjects. A further development of the MPR, recently reported by van de Kraats et al. [26], is the means to measure the individual optical densities of lutein and zeaxanthin, the components of macular pigment.

SCANNING LASER OPHTHALMOSCOPES

Elsner et al. [149] were the first to use a scanning laser ophthalmoscope (SLO) for the purpose of measuring MPOD. Since then it has become a popular FR method for measuring macular pigment [e.g., 25, 98, 116, 136, 141, 150]. Some SLOs have been custom-built for MPOD measurement, and as such are not accessible to most clinicians [135, 136]. However, Wüstemeyer et al. [98] modified a commercially available SLO, allowing reflectance images to be recorded with an argon laser at wavelengths 488 nm and 514 nm, with a fast switch between the two. They used the comparison technique, with an eccentric reference point of 14° from the fovea. MPOD in a 2° central fovea test field was calculated as follows (Formula 3):

$$\mathrm{MPOD} = C_\lambda * [\log(\mathrm{Ref}_{514,\mathrm{foveal}}/\mathrm{Ref}_{488,\mathrm{foveal}}) - \log(\mathrm{Ref}_{514,\mathrm{parafoveal}}/\mathrm{Ref}_{488,\mathrm{parafoveal}})]$$

Formula 3. Calculation of macular pigment optical density, from Wüstemeyer et al. [98]. C_λ = constant, dependent on the absorption coefficients of macular pigment. Ref_{514} and Ref_{488}=reflectances measured at 514 and 488 nm.

Using a foveal and parafoveal comparison is not common to all SLOs when measuring MPOD. Berendschot and van Norren [116, 141], for instance, used the same two wavelengths as Wüstemeyer et al. [98] but did not use any specific eccentric reference point, and therefore produced density maps of the sum of both the lens and macular pigment.

One of the main advantages of using a SLO over other FR techniques is its confocal optics, which help minimize stray

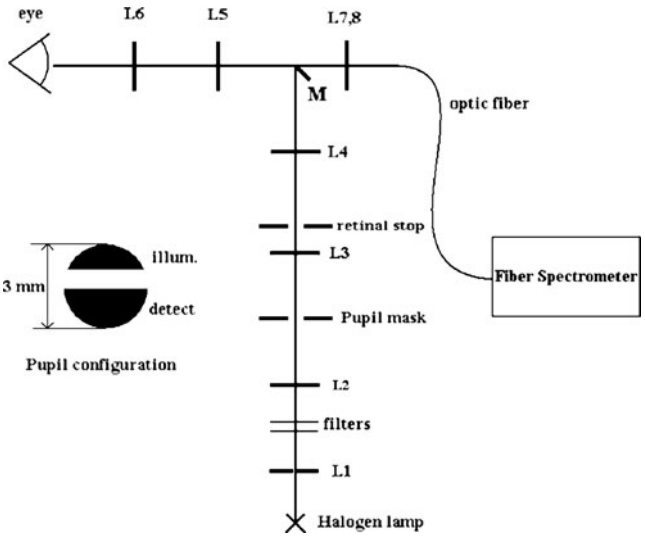


Fig. 10 Schematic diagram of the macular pigment reflectometer, from van de Kraats et al. [58]. *L1–L8*=lenses 1–8. *M* = mirror. (Reprinted from van de Kraats et al. [58], with permission from SPIE)

light scatter, the biggest hindrance in FR. This will be elaborated on in 'Assumptions' below.

Test field variations

The size of the detection field chosen to measure peak MPOD varies not only between the three categories of reflectometer but also within the categories, from 0.5° [116, 141] to 2° [25, 56, 98, 138] and 2.5° [133]. Likewise, when two wavelengths corresponding to high and low macular pigment absorption are used, as is often the case for modified fundus cameras, the chosen wavelengths differ slightly between equipment, as they do in HFP devices. In SLOs, the two wavelengths are always 488 and 514 nm, because these are the two most appropriate argon laser lines. The deviation of these laser lines from the true maximum and minimum of macular pigment absorption (460 and >530 nm respectively [54]) requires that a correction is made to account for this in the final MPOD estimation [116, 136, 141, 151], although it is not clear whether all research groups actually do this. For the comparison technique when a peripheral reference point is used, the chosen eccentricity has ranged from as little as 4° from the central fovea [137] up to 14° [98, 136].

Assumptions The assumptions for FR are not as openly explained in the literature as for HFP. Nonetheless, several of the more commonly noted assumptions are highlighted below:

1. Homogeneity of fundus tissues. The spectral characteristics, absorption, reflection and scattering properties of the various retinal tissues (e.g., melanin) are assumed to be homogenous across the areas being assessed. Gellermann and Bernstein [111], among others, point out that this is a simplification. However, most researchers [e.g., 58] do not consider this to be a problem, and with good reason it would seem. For instance, the effect of irregular RPE melanin distribution on measured MPOD was investigated by Delori et al. [56]. They concluded that it had no strong effect on MPOD as measured with their reflectometry technique.
2. Bleaching of photoreceptor pigments. It has been established that 93–99% of cone photopigment and 59-85% of rod photopigment is bleached as a result of the level of illumination used prior to measurement, depending on the particular reflectometry method used [56, 58, 108, 137]. Bleaching is important to avoid light absorption by the pigments and their subsequent interference with MPOD. It is assumed that any remaining unbleached photopigment, particularly rhodopsin (the pigment in rods), has a minimal effect. This has been investigated by Chen et al. [108], Delori et al. [56] and Bour et al. [137], and proven to be the case.
3. Light scatter accounted for. If reflectance from pre-retinal and intra-retinal structures are not controlled for, the measured MPOD can be artificially low [56, 109, 141]. This is because the reflectance method works on the principle that all the incident light is reflected after passing through the macular pigment. If some light is reflected before it reaches the macular pigment, e.g., by the crystalline lens, then this will be collected as reflected light, but it won't actually have been affected by macular pigment absorption, thus leading to an erroneously low MPOD [25]. Most reflectometry devices do aim to eliminate this problem, although it would appear that some are more successful than others, judging by the lower than 'average' MPOD estimates found in some studies [e.g., 25, 56, 98, 137]. Methods used to allow this assumption to hold as far as possible include separating the entrance and exit pupils; using confocal optics as found in SLOs; and the incorporation of stray light into optical models. In addition, for the comparison technique of FR, the use of a peripheral reference should account for crystalline lens scatter [56].
4. Negligible macular pigment at a peripheral reference site. If the comparison method is used, the same rules apply as per HFP; provided the peripheral locus is eccentric enough to exclude any macular pigment contribution, the assumption will hold. Choosing a point far enough away from the fovea is easier with FR, since there is little participation required by the subject. Delori et al. [56] commented that the use of a peripheral reference in FR is enough to reduce the influence of the ocular media on the MPOD measurement. Hammond et al. [54], however, argue that regardless of this, as an objective technique FR will suffer from a loss of signal as a result of increased lens scatter and density in some subjects.
5. MPOD measured over the entire stimulus area. Whereas with the psychophysical methods there is some disagreement regarding which part of the macular pigment distribution is actually being measured with the test stimulus, with FR there is a general consensus that the MPOD is the mean amount over the chosen detection field [56, 98, 136, 141]. However, this assumption has not been verified [54].

Validity Like HFP, the validity of FR for measurement of MPOD can be assessed by plotting spectral and spatial profiles of macular pigment and comparing these with in vitro data. It was argued by van de Kraats et al. [58] that any technique demonstrating an increase in MPOD following increased lutein intake is also an indication of its

validity. Several reflectometry studies have shown such an increase [3, 26, 136].

Spectral profiles of macular pigment have not been generated to the extent that they have in HFP studies. In fact, there appears to be only one study that has comprehensively investigated this aspect of validity. Delori et al. [56] used the comparison technique, and measured reflectance at wavelengths of 430, 450, 470, 490, 520 and 550 nm. Their results from 147 subjects accurately matched the spectral curve of macular pigment in vitro, albeit with some small systematic deviations such as lower values at 430 nm (see Fig. 12). The investigators point out, however, that the deviations altered depending on which in vitro curve they chose for comparison, highlighting the point that the true macular pigment absorption spectrum is not known with enough certainty to assume that the reflectometry deviations are an inaccuracy. It is also interesting to note that lower values at the short wavelength end of the spectrum are also a common finding in HFP spectral profiles (see Fig. 4), perhaps lending further support to there being a genuine difference between the in vivo and in vitro macular pigment spectral profile.

The plotting of 'macular pigment maps' to assess macular pigment spatial profiles has become reasonably commonplace in FR studies [e.g., 59, 108, 116, 135–137]. For SLOs, this generally involves a digital subtraction of the images obtained at two wavelengths (maximal and minimal macular pigment absorption). Chen et al. [108], used a modified fundus camera (the method is described above) and obtained spatial distributions for 54 subjects of various ages. These distributions, divided into three age groups, are shown in Fig. 11. The decline in macular pigment from the fovea is rapid and symmetrical, very similar to the decline in macular pigment expected from in vitro knowledge and also from HFP-derived plots of MPOD (see Fig. 3).

Chen et al. [108] looked at the half width of macular pigment distribution (HWMPD) for each of the age groups, and found a significant increase (i.e., widening) with age. With further analyses they also noted that 'shoulders' of varying types were present in the MPOD profile of all subjects. Small irregularities in the otherwise undisturbed decline of macular pigment with eccentricity have been reported in other studies [5, 44, 59, 64, 71, 75, 115–117, 152, 153], both in vivo and in vitro, and are the subject of much ongoing discussion [e.g., 154].

Recently, the MPR [58] has been used to investigate MPOD distribution, or more specifically, to plot the individual distributions of lutein and zeaxanthin [26]. Rather than macular pigment maps, reflection spectra were taken at a variety of eccentricities up to 8° from the fovea, in a similar manner to HFP.

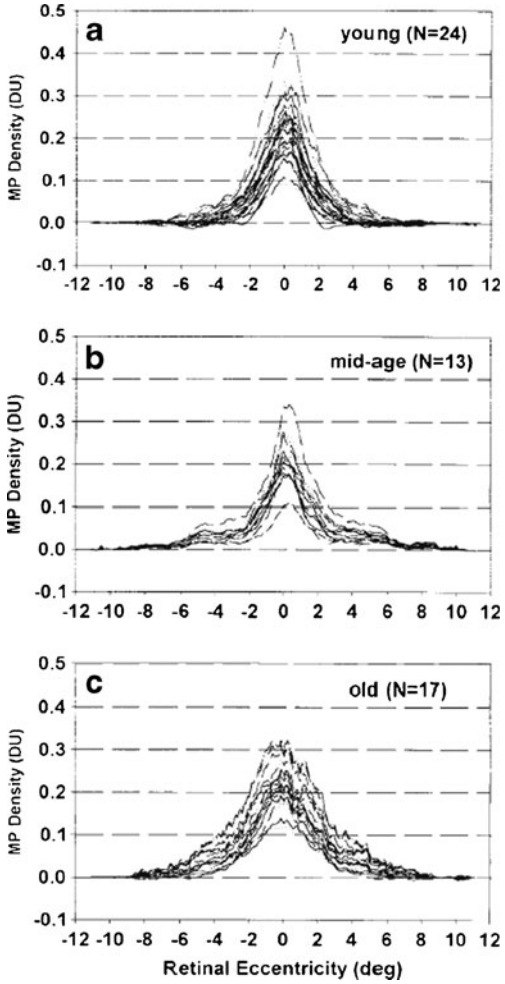


Fig. 11 Spatial profiles of macular pigment density in three age groups, obtained by fundus reflectometry: **a** young (24.8±2.6 years), **b** mid-age (40.2±8.3) and **c** old (67.5±7.1). (Reprinted from Chen et al. [108], with permission from Informa Healthcare)

An indirect way of demonstrating validity is to compare results with those of a technique with established validity, such as HFP. This has been done in several FR studies with fairly good results [56, 58, 59, 141].

Reliability With so many different instruments being used to measure MPOD by FR, there ought to be an abundance of reliability data available. However, few studies have assessed the inter-session reliability of their devices, although more have assessed within-session reliability. This is perhaps because, unlike HFP, the actual measurement time in FR is short and does not demand too much effort from the subject; hence it's more convenient to take repeat measurements within the same session. That said, Zagers et al. [146] believed the variability in their intra-session MPOD results was the result of fixation errors, with the less experienced subjects showing greater variability. Nonetheless, as Snodderly and colleagues point out, inter-

session reliability is really more valuable than intra-session reliability [50]. Since results generally show higher variability between sessions, this is a more robust test for an instrument. Table 3 outlines the reliability indicators provided in FR studies with regard to instrument reliability. It shows that for the studies with published data on reliability, the results are good and comparable with HFP.

Advantages and disadvantages of FR Advantages include: 1) as an objective method it requires minimal effort from the subject, 2) it has a quick measurement time, 3) density maps of macular pigment distribution can be plotted quickly, 4) reliability appears to be good in several instruments, and 5) it is suitable for many subject populations, including children. Disadvantages include: 1) pupil dilation normally required, 2) the need for precise alignment before measurements, 3) unpleasant light levels because of the requirement for photopigment bleaching, 4) the need to control for light scatter, which can include considerable modeling, and 5) costly and complicated instruments, although attempts are being made to produce less expensive reflectometers using commonly available equipment [59, 98, 137].

Fundus autofluorescence

One of the newer ways for measuring MPOD in vivo relies on the intrinsic fluorescence, or *autofluorescence* (AF), of lipofuscin in the retinal pigment epithelium (RPE). Lipofuscin in the RPE is a waste product of photoreceptor outer segment phagocytosis and it accumulates with age [155–157]. When excited with light wavelengths of 400 to 590 nm, lipofuscin fluoresces, emitting light in the wavelength range 520–800 nm [158].

Delori [155] was the first to develop a technique for fundus AF with the primary aim of measuring lipofuscin. Further studies by Delori et al. [156] and von Rückmann et al. [157] provided evidence for lipofuscin being the main fluorophore in AF. It was their observations of a decrease in AF at the macula that lead to the use of AF as a means for measuring MPOD. For an in-depth look at fundus AF and its application, see Schmitz-Valckenberg et al. [159]. The current review will concentrate on the use of AF in macular pigment measurement.

Procedure To recall, the absorption spectrum of macular pigment is in the range 400–540 nm [6] and the absorption spectrum of lipofuscin is in the range 400–590 nm [158]. Since macular pigment is located anterior to lipofuscin, incoming light directed at the fovea will be absorbed by the macular pigment before it reaches the lipofuscin, provided the wavelength of the light is within the absorption range of macular pigment. As a result, there will be an attenuation of lipofuscin fluorescence at the macula; the more macular pigment present, the higher the level of attenuation. By comparing the emitted AF at the fovea and parafovea of two excitation wavelengths, one that is well-absorbed by macular pigment and one that is not, MPOD can be calculated [56].

Two AF procedures exist for measurement of macular pigment. The first is a comparison method as used in HFP and some forms of FR. The emitted fluorescence is collected from a foveal and parafoveal sampling area, and then compared to give a measure of MPOD [e.g., 56]. The second and more common procedure is an imaging method whereby up to 32 images [39, 129] are taken in succession with one or two wavelengths. The images are aligned (manually or using dedicated software) and averaged, then a greyscale index of intensity is used to generate density maps of macular pigment, which includes a measure of peak MPOD. Key studies using the AF imaging technique include those of Wüstemeyer et al. [25], Berendschot and van Norren [141], Delori et al. [117], Liew et al. [160], Trieschmann et al. [161], and Wolf-Schnurrbusch et al. [153].

Instrumentation By far the most commonly used instrument for AF acquisition of MPOD is the confocal SLO, purpose-built [e.g., 116, 141] or a modified version of a commercially available SLO [e.g., 25, 160, 162]. All SLOs use the imaging method of fundus AF. The subject fixes a target whilst multiple AF fundus images, usually taken over a 20° field, are obtained at wavelengths of 488 nm and 514 nm. A barrier filter above or close to the threshold of MP absorption (e.g., 530 nm) is used to ensure that the emitted AF is only collected outside the absorption range of macular pigment, thereby avoiding any further absorption and allowing a single-pass measurement rather than a double-pass as used in reflectometry. All the AF images are aligned and averaged for each wavelength. A computer program (see Trieschmann et al. [161] for details) digitally subtracts the averaged images at the two wavelengths and uses a greyscale index of intensity to create a map of MPOD. A foveal MPOD value is calculated at a point eccentricity [117, 160, 163] or within a certain area centred on the fovea [25, 153, 160, 162]. As in FR, a correction should be made when using SLOs, to account for the argon laser lines not coinciding exactly with the maximum and minimum wavelengths of macular pigment absorption [39, 141, 151, 152, 163, 164].

Other equipment that has been used to assess MPOD using AF includes the fundus fluorometer/spectrophotometer (first described by Delori [155] and used specifically for macular pigment measurement by Delori et al. [56]) and a modified fundus camera [117, 158]. The fundus fluorometer

Table 3 A list of all fundus reflectometry (FR) studies to date that have given statistical data on reliability

Study/year	Number of subjects	Age range; eye health status; inter or intra session reliability	Reliability statistics
Berendschot et al./ 2000 [136]	8	18–50; normal; intra-session.	Two measurements. Within subjects variation and coefficients of repeatability were 10% and 0.17 with a SLO, and 17% and 0.27 with a purpose-built reflectometer.
Delori et al./2001 [56] (1)	9	21–72; normal; both.	Two sessions, four subjects on different days & the other five on the same day. Mean absolute difference between 1st and 2nd session 0.039±0.029. Mean absolute test–retest difference (as a percentage of mean density)= 22%.
Delori et al./2001 [56] (2)	22	22–78; normal; inter-session.	Two sessions, 10-24 months apart. Mean absolute difference between sessions 0.042±0.042. Mean absolute test–retest difference=19%.
Berendschot et al./ 2002 [144]	17	Average age 67.5; six ARM, 11 normal; intra-session.	Two sets of measurements. Coefficient of repeatability 0.11. Mean relative difference between measurements=10%.
Bour et al./2002 [137]	'6 eyes'	23 subjects in full study, 6–20; normal; intra-session.	Two sets of measurements with a fundus camera. Correlation coefficient between measures was r=0.77 (p<0.05).
Wüstemeyer et al./ 2002 [98]	10	16–43; normal; intra-session.	Two sessions with a SLO, no more than 30 mins apart. Mean within subjects coefficient of variation=6.2%. As a percentage of mean density, as used by Delori et al. [56], mean test–retest difference=3.1%.
Zagers et al./2002 [146]	21	18–27 (n=15) & 40–74 (n=6); normal; intra-session.	Twenty-five measurements all in the same sitting with the FRA. Coefficient of repeatability 0.084.
Berendschot and van Norren/2005 [141]	53	19–76; normal; intra-session.	Five measurements (same sitting). Mean within subjects variation and coefficients of repeatability were 5.5% and 0.078 with the FRA 1, and 7.0% and 0.09 with the FRA 2.
van de Kraats et al./ 2006 [58]	10	20 subjects in full study, 18–79; normal; both.	Two sessions on different days with the MPR. Test–retest correlation was r=0.94 (p<0.001). Five spectra measured in each test condition (intra-session), gave a mean within subjects variation of 'typically' 7%.
Bone, Brener and Gibert/2007 [59]	22	18–24; normal; inter-session.	Six to eight sessions at ~2-weekly intervals with a fundus camera. Standard deviation of the set of measurements was 'typically' ≤0.05.

ARM = age-related maculopathy.

FRA = foveal reflection analyzer, a purpose-built reflectometer.

MPR = macular pigment reflectometer, a purpose-built reflectometer.

SLO = scanning laser ophthalmoscope.

employs the comparison method. A number of different wavelengths are directed at a 3° retinal area, and the fluorescence is collected from a 2° sampling field concentric within the 3° area [56]. The subject is asked to fixate centrally or at 7° in order to obtain emission data from the fovea and parafovea. MPOD is then calculated using the foveal and parafoveal AF information at excitation wavelengths of 470 nm and 550 nm. In contrast, the modified fundus camera employs the imaging method. The camera is coupled to a cooled CCD camera and takes pictures of a 15° retinal field using wavelengths of 470 and 545 nm. At this point the technique becomes very similar to that of SLO AF imaging, i.e., image alignment and analysis by a computer program, thus providing macular pigment density maps, including a measure of peak MPOD [117, 158].

Common to all forms of AF instrumentation is the need for bleaching of the visual pigments prior to measurement (see 'Assumptions' below) and pupil dilation, although a non-mydriatic version has been described [152].

One-wavelength versus two-wavelength AF The vast majority of AF-based macular pigment studies have used two wavelengths (corresponding to high and low macular pigment absorption) to derive MPOD. There have been a handful of studies, however, that have used only the high absorption wavelength [19, 39, 129]. It is then presumed that any reduction in AF across the imaged area is due entirely to the presence of macular pigment [19]. Whilst some good correlations between MPOD measured with one-wavelength AF and minimum motion photometry have been found [39, 129], certain criticisms have been leveled at the one-wavelength method. Principally, the problem lies with the assumption that the fluorophores, i.e., lipofuscin, are distributed evenly across the imaging field. This is not the case [165] and, as a result, any attenuation in AF could be due to the presence of macular pigment but may also be a consequence of a lower level of lipofuscin in that area [19, 158, 161]. The use of a second, longer wavelength that is minimally absorbed by macular pigment eliminates this

issue. Trieschmann et al. [161] compared the one and two-wavelength methods on 120 subjects. They concluded that one-wavelength AF is acceptable as a screening method, particularly in view of its widespread availability in SLOs, whilst two-wavelength AF should always be used for precise MPOD assessment. The same conclusion was reached by Sharifzadeh et al. [152] using a CCD camera-based AF device.

Assumptions Macular pigment assessment using AF assumes the following:

1. There are no fluorophores anterior to the macular pigment. Delori et al. [56] did find evidence of such a fluorophore, and noted that it would cause a small underestimation of MPOD. This underestimation is minimized by detecting AF at a longer wavelength — 710 nm [56].
2. Lipofuscin at the fovea has the same excitation spectrum as lipofuscin in the surrounding retina. It is unknown whether this is entirely correct, but according to Delori and colleagues [56], any differences are not big enough to affect the measured macular pigment spectral curve, as determined by their technique. The total amount of lipofuscin is known to vary across the retina, but this is accounted for as long as the two-wavelength method is being used.
3. Any foveal–perifoveal differences in absorbers other than the macular pigment — retinal blood, photoreceptor pigments and RPE melanin — have a negligible effect on the measured MPOD. Delori et al. [56] investigated these assumptions in detail and found that retinal blood differences had virtually no effect, and photoreceptor bleaching meant there was very little error in terms of photopigment differences. They did, however, find that RPE melanin slightly overestimated MPOD.
4. Negligible macular pigment at any peripheral reference site. This is particularly important when the comparison technique is adopted but should be a true assumption, because an adequate eccentricity is more easily accomplished with objective than subjective MPOD techniques.

Validity The only study to date that has investigated the validity of AF in terms of a spectral comparison with in vitro MPOD data is that by Delori et al. [56]. Excitation wavelengths of 430, 470, 510 and 550 nm were used on 147 healthy-eyed subjects, plus 450, 490 and 530 nm on two of these subjects. The resultant spectral profiles for seven subjects are shown in Fig. 12, along with the equivalent spectral profiles using a FR technique. The curves are in very good agreement with the chosen in vitro macular pigment curve, attesting to the validity of the AF method of MPOD measurement, in healthy eyes at least.

Spatial profiles (or maps) of MPOD are the norm for AF imaging. In terms of validity, Robson et al. [39] demonstrated the symmetrical nature of macular pigment distribution (Fig. 13), in line with findings using HFP and FR. Also like HFP and FR, however, an array of inter-individual macular pigment distributions have been found. Figure 14, for instance, shows two distinct MPOD spatial profiles from a study by Wolf-Schnurrbusch et al. [153]. The top image is the classic distribution of a central peak in macular pigment, with a rapid decline as distance from the fovea increases. The bottom image has a central peak in macular pigment followed by a decline, and there is also a secondary peak (a 'parafoveal ring') before further decline. In this particular study, the average eccentricity of the parafoveal ring was 0.66° from the fovea, which is in line with several other studies [59, 116, 117]. Many researchers now propose that the total complement of macular pigment, rather than the peak amount, may better represent an individual's risk for, or protection from, AMD (see review by Bernstein et al. [154]).

In several studies, AF has been compared with other techniques of MPOD assessment, including HFP, using the same set of subjects [39, 56, 60, 129, 141]. All have shown good correlations, albeit with some systematic differences.

Reliability As with FR, most tests of reliability concerning AF have been carried out within the same session and are

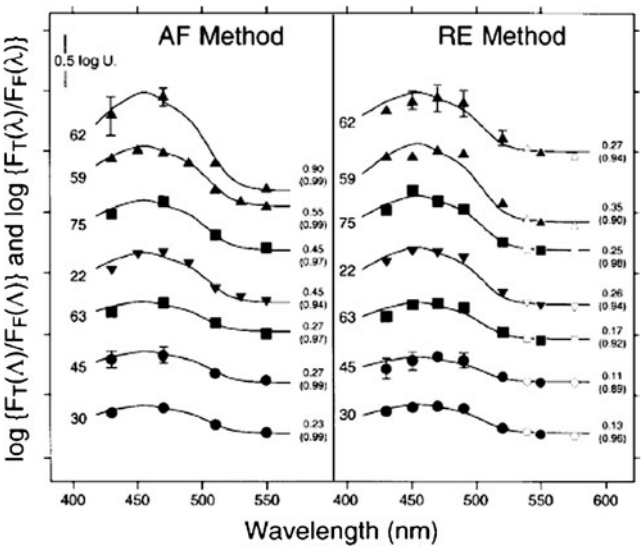


Fig. 12 *Left panel*: Log ratio autofluorescence (*AF*) plotted against wavelength for seven subjects, along with fitted macular pigment spectra (curves). The age of each subject is given to the left of each curve and the derived macular pigment optical density (with r^2 values of the fits) is given to the right. *Right panel*: The equivalent fundus reflectometry (*RE*) results. (Reprinted from Delori et al. [56], with permission from the Optical Society of America)

perhaps, therefore, not as useful as inter-session reliability data. Nevertheless, the results are impressive, indicating similar, if not better, reliability than HFP and FR. Table 4 contains a list of AF studies that have provided information on reliability.

Advantages and disadvantages of AF Advantages include: 1) its objectivity; as an objective test, AF requires no subject participation other than a short period of reasonable fixation, 2) quick measurement time, 3) spatial plots of macular pigment distribution produced as standard in AF imaging, 4) good test–retest reliability, and 5) applicability to many subject populations, including children. Disadvantages include: 1) the need for pupil dilation, 2) equipment expense, 3) a lack of commercially available two-wavelength SLOs, 4) the need for photopigment bleaching and therefore unpleasant light levels, and 5) difficulty obtaining clear images from eyes with lens opacities.

Resonance Raman spectroscopy

With the exception of electrophysiology methods, resonance Raman spectroscopy (RRS) is the most recently developed MPOD technique and, arguably, the most controversial. First described by Bernstein et al. in 1998 [166], RRS takes advantage of lutein and zeaxanthin's ability to exhibit a phenomenon called Raman scattering [167]. Over the last 10 years, the use of RRS to measure MPOD has quickly gained momentum, with many papers published on its use [57, 111, 168–178].

Procedure When monochromatic light is directed at a molecule, some of the light is scattered. Most of the light is scattered elastically (Rayleigh scattering), but a small proportion is scattered inelastically (Raman scattering). When this inelastic back-scattering happens, there is a wavelength shift of the incident light, known as a Raman shift; the shift in wavelength is molecule-specific, and therefore the back-scattered light can be collected and analyzed to identify the molecule in question. Usually the Raman signal is very weak, and as such is not easily identified. However, if the incident wavelength overlaps with the absorption spectrum of the molecule, a large resonance enhancement of the Raman scattered light occurs, and the molecule can be recognized. Carotenoids, including lutein and zeaxanthin, are an excellent example of this. When excited by 488 nm argon laser light, they exhibit a resonance enhancement of up to five orders of magnitude [169], with three characteristic Raman spectral peaks [166, 167, 171], as shown in Fig. 15. The strongest peak is at 1525 cm^{-1}, and this is the Raman line that is subsequently quantified in Raman counts (RCs).

RRS is completely different from almost all other MPOD techniques in that it measures absolute levels of macular pigment in a 1 mm (3.5°) area, with no peripheral consideration at all. The researchers in this field claim that this is acceptable because the signal is derived directly from the pigment itself, rather than relying on light that must travel to deeper layers of the retina [111, 171], and that furthermore, the signal is only strong enough to register at carotenoid concentrations found in the macula, rather than in any other structures such as the cornea and lens [166].

Instrumentation The setup for RRS detection of MPOD is shown in Fig. 16. It consists of a 488 nm argon laser that is directed at the fovea. The returning back-scattered light is filtered so that only Raman shifted light is sent to the Raman spectrograph (via a fibre optic bundle). The spectrograph is linked to a CCD camera, which is in turn linked to a computer that is programmed to subtract the background fluorescence and quantify the intensity of the Raman peaks, in particular the 1525 cm^{-1} line. Optical alignment of the instrument to the fovea is achieved in human subjects by use of a red LED and a small portion of the blue argon laser light. The red LED is visible to the observer as a polka-dot pattern and the laser light as a blue disc. By small head movements along a head rest, the subject lines up the two images, at which point the operator pushes a button to begin the whole procedure. The 488 nm light is directed as a 0.5 mW, 1 mm spot onto the macula for 0.5 seconds [170, 171, 173]. Later studies using RRS have altered the settings slightly by using a 1 mW, 1 mm spot directed at the macula for 0.25 seconds [57, 174, 175, 179]. Five measurements are taken at intervals of 30–180 secs (the time is dependent on afterimage fading), and the original protocol [171] dictated that the three highest recordings are used in the data analysis, to allow for subjects that blink or misalign.

Prior to measurement, pupil dilation to at least 6 mm is necessary [170, 171]; some later studies have found that dilation to at least 7 mm gives more reliable results [57, 174] — see discussion about pupil size in the 'Validity' section below.

Resonance Raman imaging

One limitation of RRS in its classic form is that it is limited to measuring MPOD in a 1 mm (3.5°) area centred on the fovea, rather than being able to produce a spatial profile of macular pigment across a larger retinal area. The technique has therefore been extended into an imaging mode covering a 3.5 mm area; initially using excised donor eyes [172], then more recently, following further modification, using living volunteers [175, 178].

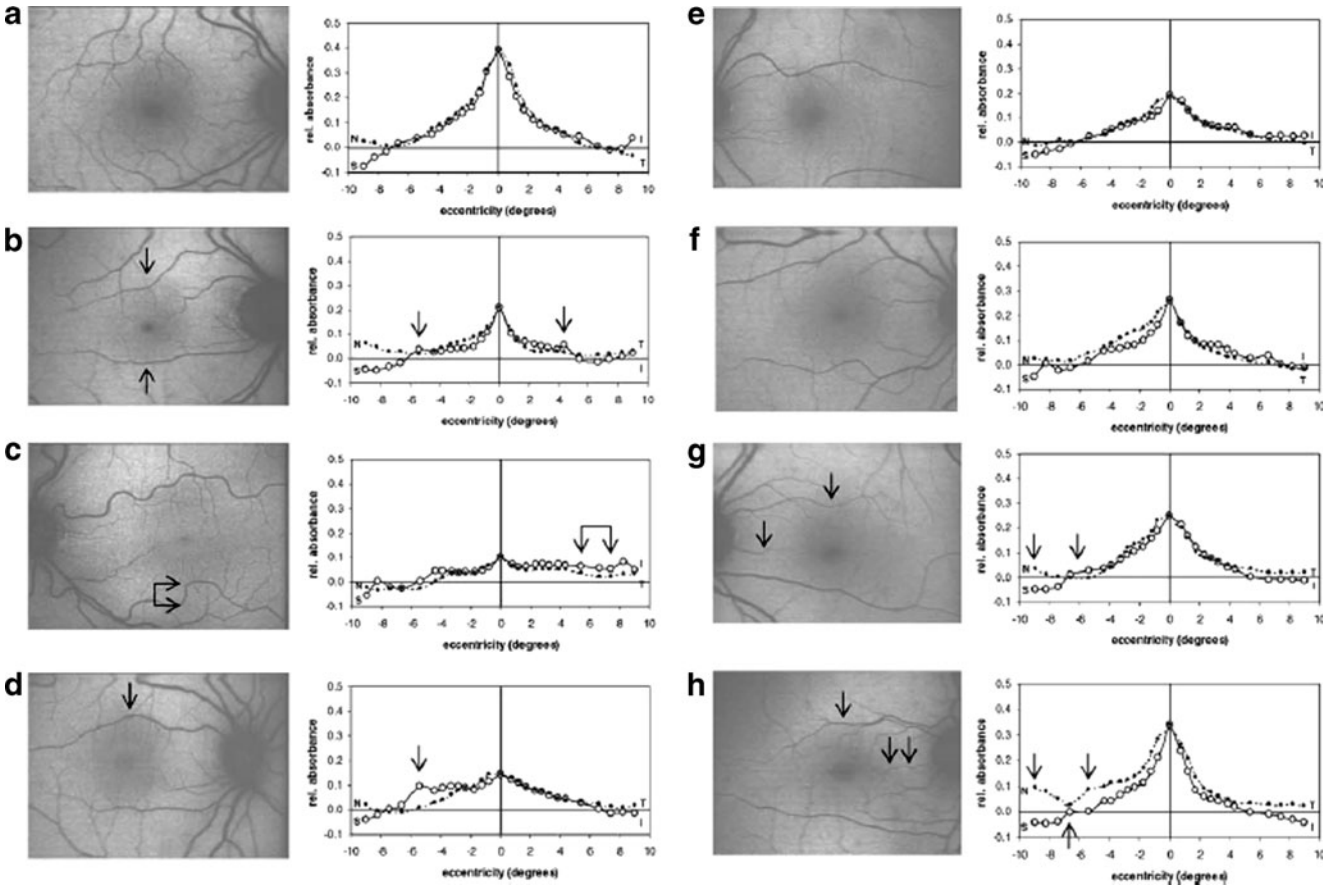


Fig. 13 Autofluorescence images (*first and third columns*) and macular pigment spatial profiles (*second and fourth columns*) for eight subjects (**a**-**h**). *Open circles* indicate the vertical meridian and *filled circles* the horizontal meridian. The *arrows* indicate disruptions due to prominent blood vessels. (Reprinted from Robson et al. [39], with permission from Elsevier)

Assumptions The assumptions associated with RRS are listed below. They are elaborated upon in the following section on validity.

1. Accurate alignment and fixation by the subject, and no significant head movement during image capture.
2. No significant effect on the Raman signal by differing levels of crystalline lens yellowing, i.e., absorption, within and across age groups.
3. No significant effect on the Raman signal by inter-individual differences in the level of lens diffusion (scatter and aberration).

Validity The validity of RRS has been a subject of fierce debate in the literature [e.g., 54, 180–186]. It is beyond the scope of this review to repeat the issues of contention verbatim; rather, the main points are summarized.

The research group that developed RRS for measurement of MPOD has shown that it is very specific and sensitive to measurement of lutein and zeaxanthin [166, 170, 171]. However, unlike the other methods of MPOD measurement, RRS cannot generate spectral absorption curves of macular pigment to be compared with in vitro curves. Instead, the investigators attest to the validity of RRS in several other ways. Firstly, the strength of the Raman signal from donor maculae has been compared with actual macular pigment levels as measured by HPLC (Fig. 17) [166]. The results revealed a highly linear correlation (r=0.94, no p-value provided). Secondly, a model eye containing known amounts of lutein was measured with the Raman device (Fig. 18), and the results again demonstrated a linear correlation (r=0.99, no p-value provided) [171]. Thirdly, the Raman signals from six intact monkey eyes were determined, then compared with their HPLC-measured macular pigment level (Fig. 19) [171]. The correlation between the two was good but not perfect (r=0.68, no p-value provided); the authors attributed this to differences in the detection area between the Raman (1 mm) and the HPLC (5 mm) method, and to difficulties with foveal alignment. Finally, Bernstein et al. [170] found a perfect linear correlation between Raman signals from lutein and zeaxanthin solutions placed in a model eye and their known concentrations (Fig. 20a), up to about 0.35 density units (equivalent in this case to ~1,600 RCs). At higher

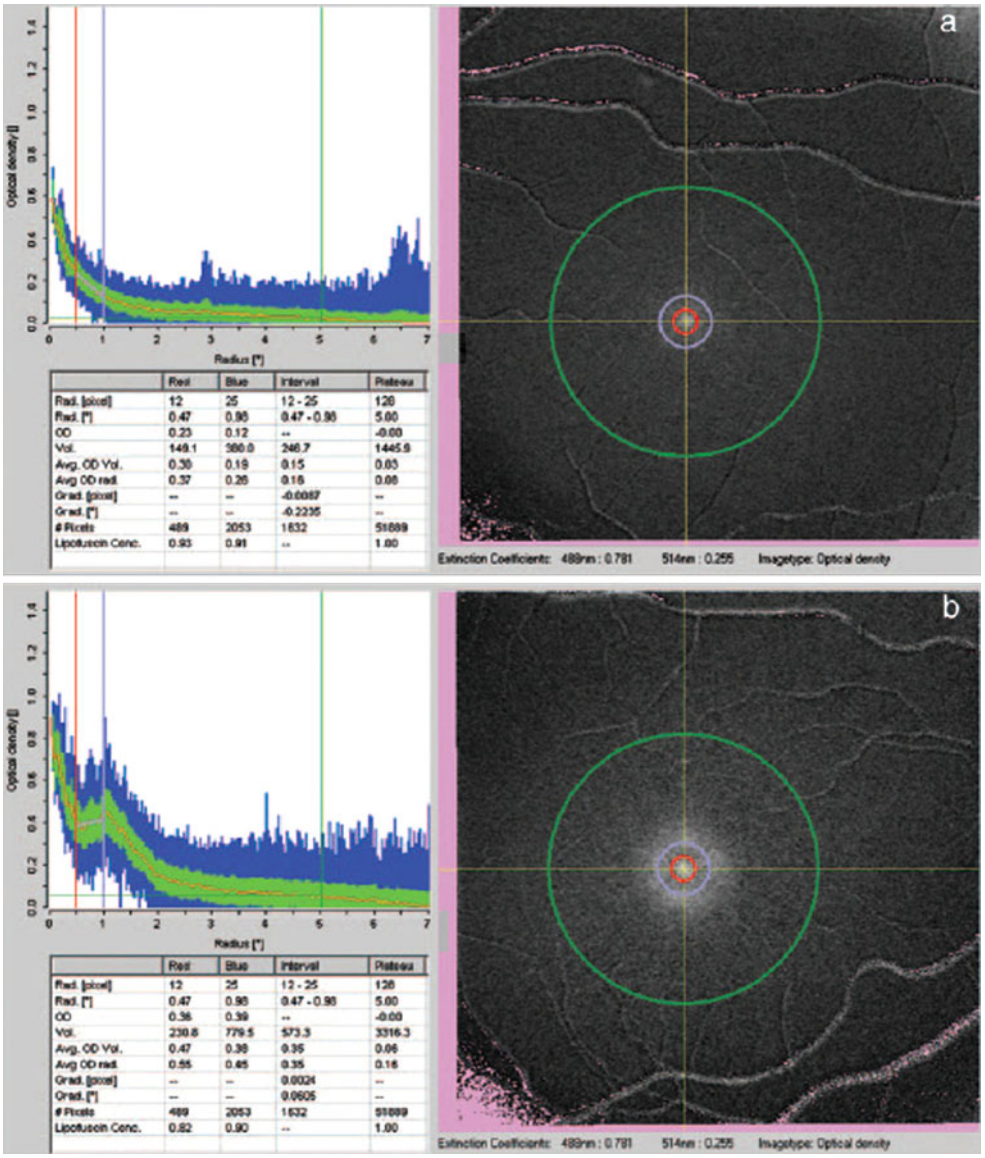


Fig. 14 Two examples of macular pigment spatial profiles with their corresponding autofluorescence images: **a** classic profile — central peak in macular pigment followed by a rapid decline, and **b** parafoveal ring profile — central peak in macular pigment, plus a secondary peak, before further decline. (Reprinted from Wolf-Schnurrbusch et al. [153], with permission from the Association for Research in Vision and Ophthalmology)

concentrations, the Raman response became saturated and therefore non-linear (Fig. 20b), as a result of the 488 nm laser beam being unable to penetrate increasingly dense carotenoid concentrations [170]. Ermakov et al. [174] found a similar linear then non-linear response for various zeaxanthin concentrations, again using a model eye.

Those questioning the validity of RRS as a method of MPOD measurement have made several criticisms of the above calibration procedures. The issues of contention include whether or not the model eye is a true representation of a real in vivo eye; whether or not the increasing underestimation of MPOD at higher concentrations is a problem in ordinary subject populations; and the credibility of using the monkey data as evidence of validity, given that the level of RCs was in general far higher than most human levels, and well above the point at which the plateau occurs in the external calibration curve (see Figs. 19 and 20).

Studies of MPOD using RRS have consistently shown a strong decline in macular pigment with increasing age (see, for example, Fig. 21). Hammond et al. [54] argue that this is another area where the validity of RRS is questionable, since with all other MPOD techniques, there appears to be little or no age-related macular pigment decline [141]. The developers of RRS believe that the decrease is genuine [110, 111, 169–171, 173–175] and not just attributable to increasing lens absorption (yellowing) and diffusion (scatter and aberration) with age, as suggested by Hammond and colleagues [54, 182–184]. An independent study simulating incremental increases in lens yellowing and scatter found that the Raman signal intensity was significantly attenuated as the density of the yellow and scatter filters increased [179]. The authors concluded that when using RRS to assess MPOD, the status of the lens needs to be taken into account. In other words, the large decline in MPOD with age reported in many RRS

Table 4 A list of all autofluorescence (AF) studies to date that have given statistical data on reliability

Study/Year	Number of subjects	Age range; eye health status; inter or intra session reliability	Reliability statistics
Delori et al./2001 [56] (1)	9	21–72; normal; both.	AF comparison method: two sessions, four subjects on different days & the other five on the same day. Mean absolute difference between 1st and 2nd session 0.042±0.019. Mean absolute test–retest difference (as a percentage of mean density)=9.0%.
Delori et al./2001 [56] (2)	22	22–78; normal; inter-session.	AF comparison method: two sessions, 10-24 months apart. Mean absolute difference between sessions 0.053±0.048. Mean absolute test–retest difference=11%.
Delori/2004 [158]	38	24–77; normal; intra-session.	AF imaging: test–retest reproducibility 0.03±0.03.
Berendschot and van Norren/2005 [141]	53	19–76; normal; intra-session.	AF imaging: ten single AF images at 488 nm and 514 nm (without averaging). Mean within subjects variation=17% and coefficient of repeatability=0.13.
Liew et al./2005 [163]	8	150 twin pairs in full study, 18–50; normal; inter-session.	AF imaging: two sessions less than 1 month apart. Mean difference between sessions 0.02±0.02 (range 0–0.05). Mean coefficient of variation=3.3% ± 2.1%.
Delori et al./2006 [117]	37	20–70; normal; intra-session.	AF imaging: Short break between first and second measurement. Mean absolute test–retest difference (as a percentage of mean density)= 6.4% (range 0–34%) for peak (0°) MPOD and 6.4% (range 0–36%) for MPOD averaged over a 1° foveal-centred area.
Sharifzadeh, Bernstein and Gellermann/2006 [152]	1	Age unreported; normal; inter-session.	AF imaging: eight measurements over 4 weeks. Standard deviation of MPOD 2.4%.
Trieschmann et al./2006 [161]	20	120 subjects in full study, 20–86; 15 normal, five AMD; intra-session.	AF imaging: five repeats. 1-λ method at 0.5°: median coefficient of variation=3.6% and reliability ratio=0.97. 2-λ method at 0.5°: median coefficient of variation=6.8% and reliability ratio=0.94.

AMD = age-related macular degeneration.

MPOD = macular pigment optical density.

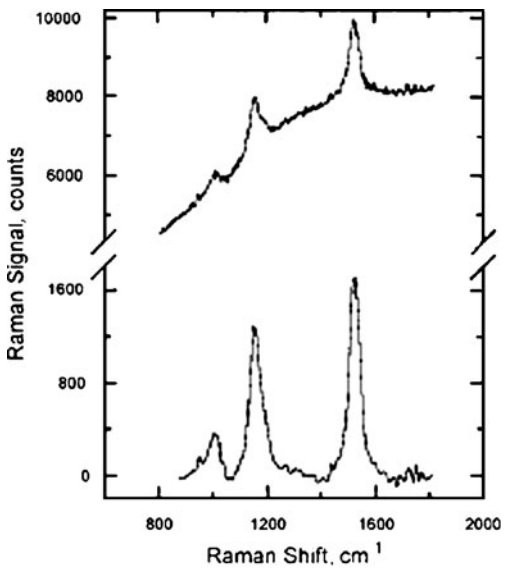


Fig. 15 In vivo Raman spectra of a healthy subject, showing the characteristic peaks of macular pigment. *Top trace* = before subtraction of the background ocular fluorescence. *Bottom trace* = after subtraction. (Reprinted from Gellermann et al. [171], with permission from the Optical Society of America)

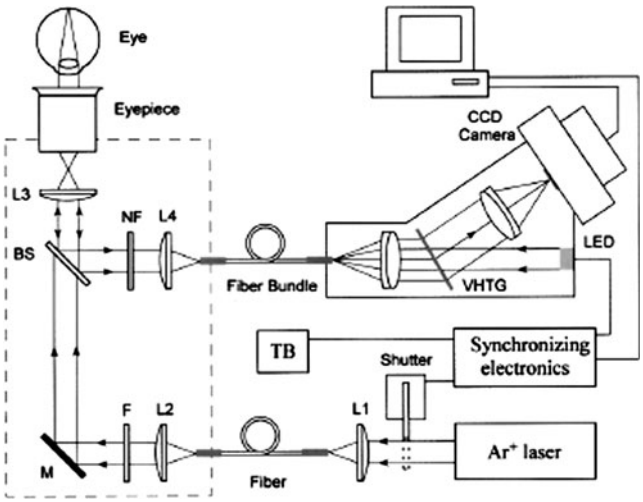


Fig. 16 Schematic diagram of a resonance Raman spectroscopy macular pigment detector. *Ar^+ laser* = air-cooled argon laser. *BS* = dichroic beam splitter. *F* = filter. *LED* = light emitting diode. *L1–L4*= lenses 1–4. *M* = mirror. *NF* = holographic rejection notch filter. *TB* = trigger button. *VHTF* = volume holographic transmission grating. (Reprinted from Gellermann et al. [171], with permission from the Optical Society of America)

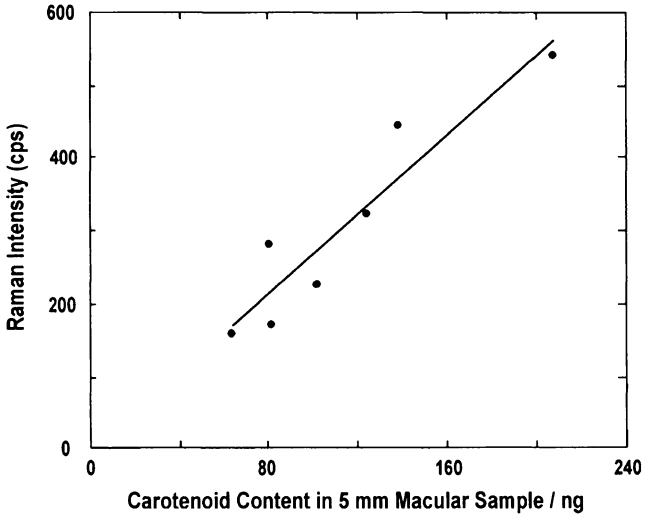


Fig. 17 Macular pigment measurements in seven human donor maculae, measured by high-performance liquid chromatography and resonance Raman spectroscopy. (Reprinted from Bernstein et al. [166], with permission from the Association for Research in Vision and Ophthalmology)

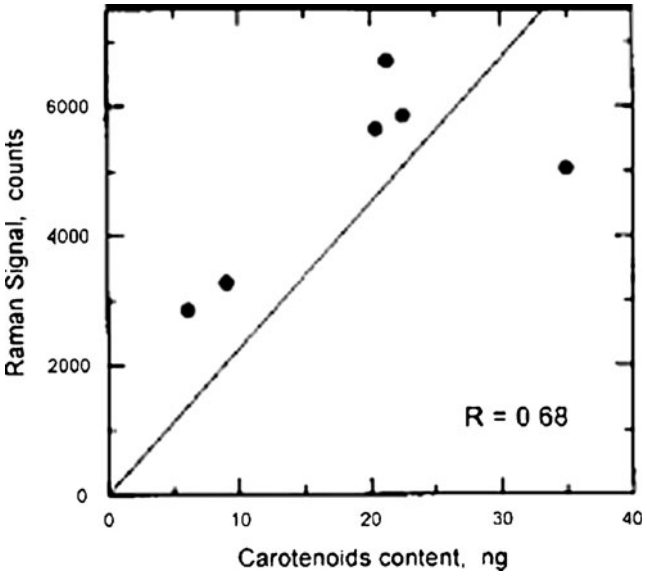


Fig. 19 Macular pigment measurements in six monkey eyes, measured by high-performance liquid chromatography and resonance Raman spectroscopy. (Reprinted from Gellermann et al. [171], with permission from the Optical Society of America)

studies is unlikely to represent a true drop in macular pigment levels, and therefore the validity of RRS in older subjects, at least, is uncertain.

Another reason for the decrease in MPOD with age — as found by RRS — may be due in part to inadequate pupil dilation. Studies have shown that the Raman signal is weakened when pupil diameter is smaller than 7 mm [57, 171, 174]. This is because the entrance/exit pupil of the Raman instrument is also 7 mm, and thus any pupil diameter less than this will result in a loss of signal [171, 174]. Neelam et al. [57] found that the significant age-related decline in RRS-derived MPOD of their subject population was reduced to a non-significant level when subjects with inadequate pupil dilation (< 7 mm) were excluded. It would therefore seem that an inability to sufficiently dilate the pupils of some older individuals might contribute to the decline in MPOD seen with RRS. Further to this, small head movements in subjects whose pupil diameter is at the 7 mm limit could also reduce the Raman signal, regardless of age [54, 176, 182–184, 186], although Bernstein and colleagues may contend that their procedure of taking the three highest RCs of five measurements allows for such head movements.

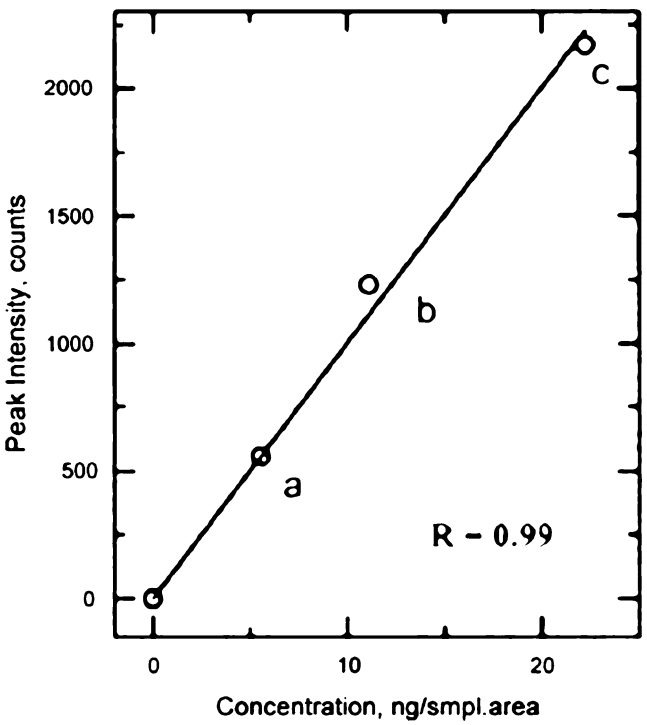


Fig. 18 Macular pigment measurements by resonance Raman spectroscopy of four known lutein concentrations placed in a model eye. (Reprinted from Gellermann et al. [171], with permission from the Optical Society of America)

Widely varying macular pigment spatial profiles have been observed using the RR imaging (RRI) device in living human subjects, including asymmetries and local depletions [178]. Although such distributions are not normally typical of other MPOD techniques, in comparing the integrated macular pigment densities of the entire imaged area with the densities measured over the same area with an AF method, the investigators found a very high correlation in 17 subjects (r=0.89, no p-value provided). Furthermore, the integrated macular pigment levels of 11 donor maculae measured with the RRI instrument were compared with the levels as measured by HPLC. The correlation between the two was very strong (r=0.92, p<0.0001), although the influence of

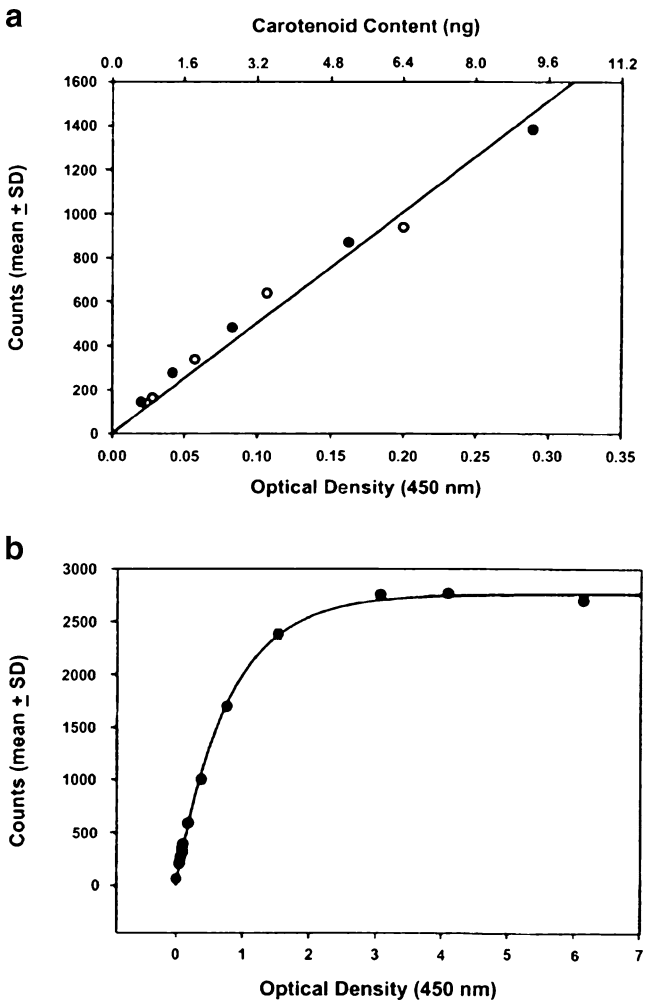


Fig. 20 Macular pigment measurements by resonance Raman spectroscopy of known lutein and zeaxanthin concentrations placed in a model eye: **a** = linear response range (*filled circles* are lutein, *open circles* are zeaxanthin). **b** = linear and non-linear response range. (Reprinted from Bernstein et al. [170], with permission from Elsevier)

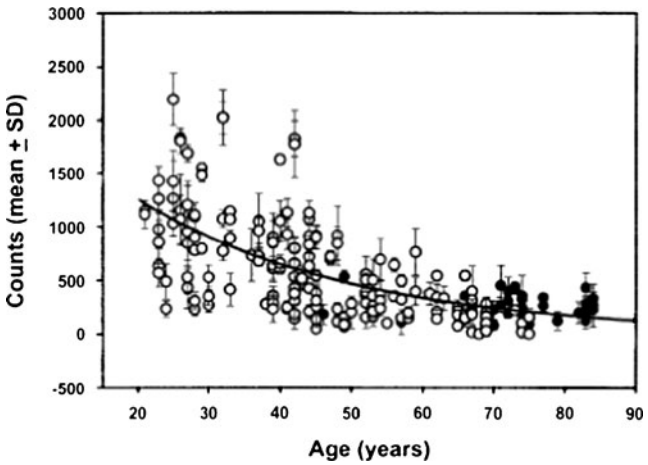


Fig. 21 The decline in macular pigment optical density with age, as measured by resonance Raman spectroscopy. (Reprinted from Bernstein et al. [170], with permission from Elsevier)

the ocular media was removed and therefore may have lead to an erroneously high agreement. Nevertheless, the validity of RRI looks promising.

Indirect methods of assessing validity, mentioned previously, consist of comparisons with more established, validated techniques, and the ability to show rises in MPOD with increased lutein and/or zeaxanthin intake. The latter has apparently been proven, although the study was only briefly described [110]. RRS-measured MPOD has been compared with HFP-measured MPOD in only two, detailed published papers [57, 176]. Neelam et al. [57], using Bland–Altman plots, demonstrated an agreement between the two techniques close to statistical significance. The correlation, as described by Pearson product-moment correlation coefficient, was r=0.32 (derived by Hammond and Wooten [184]). Although statistically significant, this correlation only explains 10% of the variance in the two methods [184]. Hogg et al. [176] also found a weak, albeit statistically significant, correlation between RRS and HFP (r=0.26, p=0.012). They did not feel it was good enough for the two techniques to be interchangeable. Bernstein et al. [110] reported a better correlation (r=0.467, p=0.0024) in 40 healthy subjects, but their study was not described in any detail.

Reliability RRS appears to exhibit good within- and between-session reliability in the majority of studies (Table 5). The high variation in readings experienced in subjects from a study by Obana et al. [177], particularly in individuals with age-related maculopathy (ARM), is a notable exception. This is the only study that has provided any RRS reliability data for subjects with ocular disease and is an indication, perhaps, that more data is needed before evaluating information on MPOD in these populations. That said, 32 out of the 180 eyes with ARM had worse visual acuity than the recommended limit for RRS of 6/24 (20/80). This may have contributed to the variation. In view of the variation, Obana and colleagues chose to accept only the highest of the five Raman readings in all their subjects [177]. This means that 80% of the measurements were rejected, which seems a lot for a technique claiming to be validated. Nevertheless, many authorities have shown that MPOD measurements, as derived by RRS, do have good test–retest reliability (see Table 5).

Advantages and disadvantages of RRS Advantages include: 1) sensitivity and specificity for retinal carotenoids, 2) rapid measurements requiring only momentary fixation from the subject, 3) the possibility of quickly generated, detailed spatial distribution plots of macular pigment, using the RRI method, 4) reasonable reliability, and 5) measurements possible in many individuals, including those with reduced visual acuity, up to 6/24 (20/80). Disadvantages include: 1)

Table 5 A list of all resonance Raman spectroscopy (RRS) studies to date that have given statistical data on reliability

Study/Year	Number of subjects	Age range; eye health status; inter or intra session reliability	Reliability statistics
Bernstein et al./2002 [170]	2	26 and 37; normal; both.	Five sessions over 2 weeks. Variability within and between sessions was 'generally less than±10%'.
Gellermann et al./2002 [171]	2	26 and 37; normal; both.	Five sessions over 2 weeks. Standard deviation within sessions (five readings) was typically less than 15%. Reproducibility between sessions was 'high'.
Bernstein et al./2004 [110]	40	< 60; normal; inter-session.	Three sessions over 1 month. Mean intersession variability 6.4%.
Ermakov et al./ 2004 [174]	2	Age unreported; normal; both.	Subject 1: six measurements at 3-4 minute intervals. Relative standard deviation=5.3%.
			Subject 2: five sessions over 2 weeks. Relative standard deviation=5.1%.
Neelam et al./ 2005 [57]	20	120 subjects in full study, 20–60; normal; both.	Two sessions less than 2 weeks apart. Within-session mean coefficient of variation (using three highest readings)=12.61% ± 9.46% at session 1 and 8.42% ± 7.12% at session 2. Bland–Altman plots for inter-session reproducibility showed that 95% of MPOD readings were within the 95% limits of agreement.
Hogg et al./ 2007 [176]	11	21–50; normal; inter-session.	Four sessions over 2 weeks. Mean coefficient of variation between sessions=13.5%.
Obana et al./ 2008 [177]	197	20–80 & 50–85; 100 normal, 97 ARM/AMD; intra-session.	Coefficient of variation of five readings was 1.0%–69.4% in normal subjects and 0.9%–145.4% in ARM subjects.

AMD = age-related macular degeneration

ARM = age-related maculopathy

MPOD = macular pigment optical density

the need for pupil dilation, 2) the reliance on subjects for accurate alignment, i.e., the lack of an objective alignment procedure, 3) afterimages between measurements, 4) questionable validity, 5) attenuation of the Raman signal with changes in the ocular media and inadequate pupil dilation, 6) instrumentation that is highly specialized and expensive, and 7) RCs not being readily convertible to macular pigment density units, making direct comparisons with other techniques difficult.

Electrophysiology — visual evoked potentials

The first suggestion that visual evoked potentials (VEPs) could potentially be used to detect macular pigment was made over 10 years ago, by Moreland et al. [37]. This was investigated further by Robson et al. [92] some time later. However, it is only very recently that this particular technique has looked like it could be a truly viable method for measuring MPOD. Using steady-state VEPs, Robson and Parry [41] measured MPOD across a range of eccentricities in three subjects. Blue-green gratings on a colour monitor were employed, and these same gratings were also used to measure MPOD with HFP (see 'Recent developments' in HFP section). The VEP and HFP results were compared with each other as well as with the equivalent MPOD as measured by minimum motion photometry. This required a correction factor with regard to the VEP and HFP results, to allow for the overlapping phosphor emissions of the blue and green stimuli. The correlation between all three techniques was excellent ($r \geq 0.94$, $p < 0.0005$, in all cases), suggesting that steady-state VEPs have potential as a valid, objective method for measuring macular pigment and its distribution.

Discussion

There are currently two main psychophysical techniques for measuring MPOD in vivo, and three main objective techniques. All take advantage of the spectral absorption properties of macular pigment, but in very diverse ways. This diversity may be useful for macular pigment research, but it does present difficulties for those wishing to compare MPOD values between techniques. For instance, does the value represent the peak density of macular pigment, the density of macular pigment at a certain point within the fovea, or the total amount within the target area?

If macular pigment measurement is to become commonplace in large populations, then equipment investors will

have an important decision to make with regard to the method they choose to employ. Unfortunately, as each MPOD technique has its own benefits and limitations, there is no clear ideal choice, as highlighted by Beatty, van Kuijk and Chakravarthy [187]. Heterochromatic flicker photometry is probably the most affordable choice. It is also an established, valid and reliable method, particularly when protocols are followed as per 'customized' HFP [50, 52]. There is, however, the problem that some individuals find this task very difficult, and their results cannot necessarily be relied upon. A commercially available objective technique would therefore be desirable, possibly through adaptation of a scanning laser ophthalmoscope or fundus camera. The former is often used in a hospital setting, and the latter is commonly found in optometric practice. A future objective technique could make use of FR or AF to assess MPOD, although AF may be preferred over FR because it is less influenced by light scatter and appears to have better reliability. Both have the facility to measure the spatial distribution of macular pigment, and this seems to be an increasingly useful advantage [8, 39, 74, 108, 116]. Bhosale, Zhao and Bernstein [112] observed elevated lutein levels at the macula and at the peripheral retina in donors known to be using high-dose lutein supplements. Therefore, if non-macula areas are not taken into account, the total complement of macular pigment may be underestimated, particularly with methods such as HFP, where the eccentric references are assumed to have virtually no macular pigment. The main issue associated with macular pigment screening using an objective technique is the need for pupil dilation, although several non-mydriatic devices have now been developed [58, 59, 152].

It is our view that the measurement of MPOD is best conducted using an objective technique based on FR or AF, but we acknowledge that a commercial instrument capable of this is not currently available. The development of such an instrument will aid research in this area and provide a better understanding of the relationship between MPOD and AMD, as well as supporting MPOD screening in a clinical setting.

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