Flicker-photometric electroretinogram estimates of L:M cone photoreceptor ratio in men with photopigment spectra derived from genetics

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Relative proportions of long-wavelength-sensitive (L) to middle-wavelength-sensitive (M) cones were estimated by use of the flicker-photometric electroretinogram (ERG). It has been demonstrated that a major source of error in estimates of cone proportions from spectral luminosity functions is the known variation in the λ_{max} of the photopigments [Vision Res. **38**, 1961 (1998)]. To correct for these errors, estimates of cone proportions were derived by use of individualized L-cone spectral sensitivity curves deduced from photopigment gene sequences from each subject. For some individuals this correction made a large difference in the estimated cone proportions compared with the value obtained when a fixed standard L cone was assumed. The largest discrepancy occurred in a man estimated to have 62% L cones (L:M ratio 1.6:1) when a standard L pigment was assumed but a value of 80% L cones (L:M ratio 4:1) when his individualized L-cone spectrum was used. From repeated measurements made with the ERG, it was determined that individual estimates of the relative L-to-M cone contributions, expressed as %L cones, are usually reliable within ~2%. The average L:M ratio for 15 male subjects was estimated at 2:1 (67% L cones). Previously, a large range of individual variability was reported for L:M ratios obtained from photometry. An unresolved issue concerns how much of the range might be attributed to error. Here efforts have been taken to markedly reduce measurement error. Nonetheless, a large range of individual differences persists. Estimated L:M ratios for individuals ranged from 0.6:1 to 12:1 (40% L to 92% L). © 2000 Optical Society of America [S0740-3232(00)01903-7]

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1. INTRODUCTION

The first images of the arrangement of the short-, middle-, and long-wavelength-sensitive (S, M, and L, respectively) cones in the living human eye were obtained recently by Roorda and Williams.[1] The two male subjects examined had strikingly different proportions of L-to-M cones. One had an L:M ratio near 1:1, the other, near 4:1. This is direct confirmation of what had been deduced previously: that there are large individual differences in the ratio of L-to-M cones in the central retina among individuals with normal color vision.[2–7] The large variation raises a number of particularly engaging questions. For example, what are the implications for visual performance? If someone can have four L cones for every one M cone without an effect on color vision capacity, how many cones of one type are too few to support normal color vision? During embryogenesis and development, which biological mechanisms lay down the cones in the photoreceptor mosaic? Are those mechanisms controlled genetically and, if so, how do the genes exert their actions? Is there selective wiring of the L and M cones or of higher-order neurons that carry chromatic information? Do the neural circuits for color vision adjust to compensate for large biases in cone ratios; if so, is the adjustment accomplished by use of information derived from visual experience or is it genetically specified?

Our goal is to collect information that could be useful in answering these questions; a key objective will be to obtain accurate L:M ratios from relatively larger numbers of subjects. More information about the extent of the variation and its distribution in the population would, by itself, be useful for understanding its significance. A genetic approach might be used as a tool to discover the biological mechanisms that control the relative numbers of cones in the mosaic. Such approaches require measurements of L:M ratio for a relatively larger number of individuals; this is true when it is done by either of the following two methods: following inheritance through families or searching for a correlation between gene ratio variations and sequence variations in candidate genes. Studies designed to determine the consequences of cone ratio variation for visual performance could also benefit from the ability to screen larger numbers of subjects; for example, from a large sample one could find people at the farthest extremes and characterize their vision.

The problem is that imaging the cone mosaic, which is the most direct method for characterizing the L:M cone ratio,[1] does not lend itself to the study of large samples of subjects; this is also true of some of the psychophysical methods.[2,8,9] One method that was recently used on relatively larger sample sizes is to measure the ratio of L:M messenger RNA from excised patches of central retina.[5,10,11] This method yields reproducible measurements that can be assumed to reflect cone ratio to the extent that L and M cones produce the same average amounts of messenger RNA.[11] However, this method is

restricted to use on cadaver eyes, which obviates the possibility of measurement of visual performance on the same subjects.

Heterochromatic flicker photometry is the most widely used method for estimating cone ratios.[12] One obtains estimates by fitting spectral luminosity data with weighted sums of L- and M-cone fundamentals. The relative weighting factors are taken as representing the L:M cone ratio. The psychophysical procedure of measuring spectral luminosity has the advantage of being less time consuming than other psychophysical methods, and the process can be extremely efficient when the relative luminosity is measured for only two wavelengths. However, estimates of cone ratio from psychophysical flicker photometry are severely limited in both their accuracy and their reliability. The accuracy is limited because factors in addition to cone ratio contribute to variations in spectral luminosity; major factors are variations in preretinal absorption and variations in the spectral sensitivities of the cones.[13] The reliability is limited by the fact that large changes in the cone ratio lead to only modest changes in spectral luminosity. For example, a change in L:M ratio from 2:1 to 3:1 predicts an increase in relative sensitivity to long wavelengths of no more than 0.05 log unit. This value is smaller than the usual error of measurement for flicker photometry, as measured by psychophysics, in naïve observers.

The flicker-photometric electroretinogram (ERG) has been used to estimate L:M cone ratios and proves to be an efficient and reliable procedure.[4,14] The measurements have the advantage of being more reliable than is usual for psychophysical flicker photometry, and because electroretinography is an inherently objective procedure, it works well on naïve subjects. The stimuli used in the ERG illuminate a large retinal area. Thus, theoretically, the vast majority of the cones that contribute to the ERG signals are outside the fovea, where the influence of variations in macular pigment is small and the cone outer segments are short, minimizing the effect of photopigment optical-density variations. What remains as a major source of error in the estimates of L:M ratio is the known variation in λ_{max} of the visual pigments. Bieber *et al.* have shown that estimates of cone ratio are highly dependent on variations in the λ_{max} of the L cones.[13] The λ_{max} of the M cones varies less than the L cones,[15,16] and the variations that do occur have much less effect on the estimated ratios.[13]

The purpose of the present investigation was to estimate cone ratios in human males with normal color vision using ERG flicker photometry. Unlike in previous studies, however, here the sequences of the L-cone photopigment genes were determined for each observer. These sequences were used to predict a spectral sensitivity function for the L cones of each subject. The individualized pigment spectra were used to derive estimates of L:M proportions for each subject. Repeated measures were run from which to estimate the experimental error inherent in estimates of L:M-cone proportions from this method. We obtained estimates from a sampling of male subjects to begin to characterize the range of L:M cone proportions estimated with this technique. We were also interested in evaluating the utility of this method for estimating cone proportions for larger numbers of subjects. Because of the low measurement error and because other sources of error including those that arise from variation in the λ_{max} of the L cones are reduced, the range of cone ratio estimates may represent a value close to the true range of variation in the population.

2. METHODS

A. Subjects

Two groups of subjects were recruited, men with normal color vision ($n = 15$) and men with dichromatic color vision ($n = 16$). Color vision was classified based on color-matching performance on a Nagel anomaloscope. Subjects classified as dichromats accepted the entire range of red–green mixture ratios as matching the monochromatic test light. They also were selected for this study because they were found to have a single photopigment gene on the X chromosome (see Subsection 2. B below). Normal observers made a match over a small range of red and green ratios on the anomaloscope. They also made zero errors on standard color vision tests, including the AO-HRR, the Ishihara (38 plates), the Dvorine, and the D-15. Most subjects were native to the midwestern United States. The subjects' ages ranged from 18 to 52 years, with means of 30 years for the normals and 28 years for the dichromats.

B. Genetic Analysis

The subjects' DNA was extracted from blood as previously described[17] or from buccal swabs as described elsewhere.[18] The L and M genes were selectively amplified by long-distance polymerase chain reaction (PCR). The forward primer corresponded to the 5′ end of exon 2 (5′ CCTTCGAAGGCCCGAATTA), which is shared by both L and M genes. The reverse primer corresponded to sequences within exon 5 that encode one of the amino acid differences that are responsible for the majority of the spectral difference between L and M pigments. The L-gene-specific primer is 5′ GCAGTACGCAAAGATCATCACC. The M-gene-specific primer is 5′ AAGCAGAATGCCAGGACCATC. Amplifications were done by use of a hot start with the XL-PCR kit and AmpliWax gems (Perkin-Elmer ABI), following the manufacturer's instructions. The thermal cycling parameters were 94 °C for 5 min for one cycle, followed by 30 cycles of 94 °C for 30 s, 59 °C for 1 min and 72 °C for 5.5 min. The specificity of these primers for L and M genes was shown previously.[17] The PCR product obtained was used in a second round of amplifications to amplify separately exons 2, 3, and 4 of L and of M genes. The primers and thermal cycling parameters for these amplifications were reported previously;[17] the only difference is that here the forward primers were tagged with the M13–21 primer sequences and the reverse primers were tagged with the M13 reverse primer sequence. The resultant PCR products were directly sequenced with the AmpliTaq FS sequencing kit from Perkin-Elmer ABI, and sequencing analysis was done with the ABI 310 genetic analyzer.

C. ERG Flicker Photometry

A three-channel Maxwellian-view optical system (Fig. 1) produced the stimuli for the flicker-photometric ERG.[19]

One beam could be used for adaptation, although it was blocked in this application. The other two beams consisted of a test light and a reference light, each produced by an Osram Xenophot HLX bulb (50 W, 12 V underrun at 11 V). A Varispec liquid-crystal electronically tunable filter (Cambridge Research & Instrumentation) controlled the wavelength of the test light. The half-band pass (7 nm at 550 nm) and the out-of-band transmittance ratio (10^{-4} of peak transmission) were set by the manufacturer. The reference and the test lights were superimposed to illuminate a circular portion of the retina centered on the fovea and subtending 70°. High-speed electromagnetic shutters (Uniblitz, Vincent Associates) were configured to alternate presentation of the reference and the test lights at 31.25 Hz. An OFF period was interposed between presentations of the test and the reference lights such that the stimulus sequence was test light (8 ms), no stimulus (8 ms), achromatic reference light (8 ms), and no stimulus (8 ms). A circular neutral-density wedge was used to control the intensity of the test light. Two electrocardiogram conductive electrodes were applied: a ground electrode just above the right eye, and a reference electrode just below the right eye. The subject's right pupil was dilated with tropicamide 0.5%. A third electrode, made with fiber from the DTL Plus™ electrode, was used as an active corneal electrode. The DTL fiber electrode has been shown in similar applications to yield reproducible and reliable measurements.[20] The details of the signal-processing system are described elsewhere.[14,19] Briefly, the ERG signal that resulted from each stimulus train was filtered and averaged to produce a resultant single-cycle, sinusoidal waveform. The intensity of the reference light remained constant while the neutral-density wedge was used to adjust the intensity of the test light to produce a null of minimum ERG signal amplitude and intermediate phase. This null was taken as the point when the effectiveness of the test light equaled that of the fixed reference light. For all subjects the null point was determined at 10-nm increments over a range of 480–670 nm. However, with all normal subjects and some dichromats, readings at 470 and 680 nm were also obtained. For the dichromats, a few additional recordings at 5-nm increments were taken near the expected peak (530 nm for protanopes and 560 nm for deuteranopes). Spectral sensitivities were determined by the average of two complete runs through all wavelengths. One run tested wavelengths in order from 480 to 670 nm, and a second run tested in reverse order from 670 to 480 nm. Each run was done independently, but at the end of the session the two measurements at each point were compared. If the measurements at any one wavelength disagreed by more than 0.10 log unit, a third null was determined for that wavelength. This was usually true for fewer than two wavelengths in the series. For these wavelength points, either the final value was taken as the average of the three measurements or, if one value was extremely different from the other two, the two closer values were averaged. The final spectral sensitivity values are reported as quantal intensities. Intensities measured at the cornea were corrected for absorption by the lens by use of the age-dependent lens correction of Xu *et al.*[21]

D. Construction of a Visual Pigment Template Expression

A wavelength-shiftable visual pigment template curve was necessary to represent the spectral sensitivities of

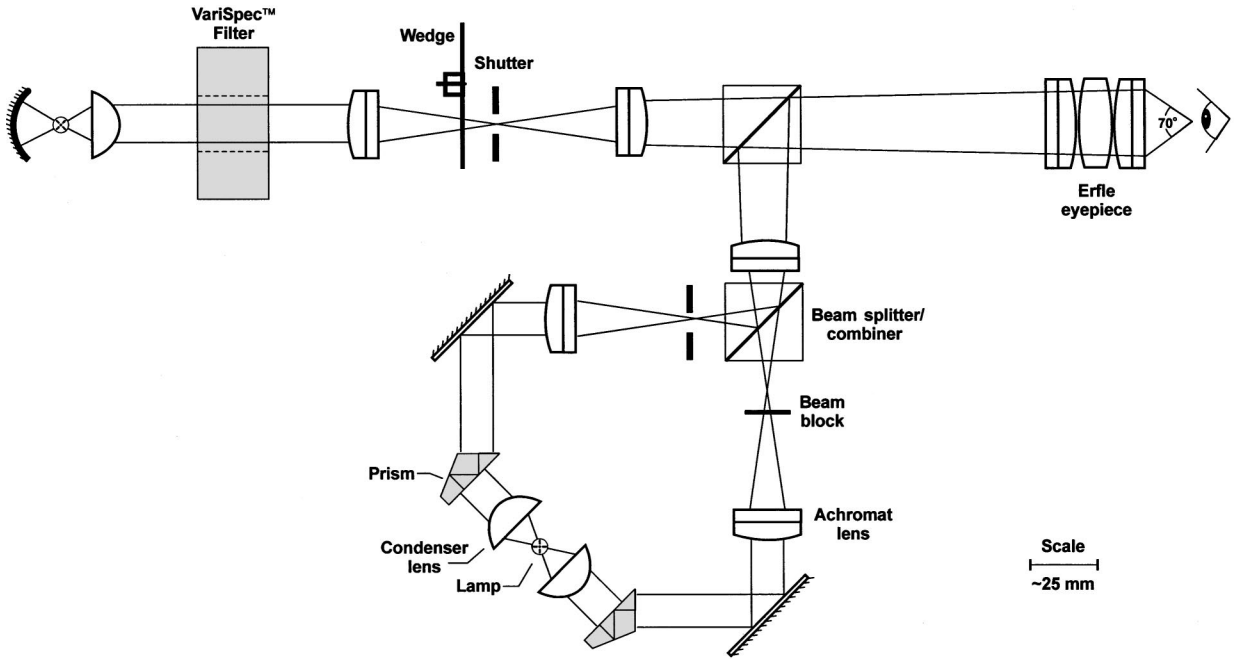

Fig. 1. Monochromatic test lights were obtained with a Varispec liquid-crystal electronically tunable filter incorporated into a compact three-channel Maxwellian-view optical system. The wavelength of the test beam was computer controlled with the tunable filter and could be varied in subnanometer increments over the range 400–700 nm. An achromatic reference light was derived from a second beam. The test and the reference lights were presented alternately with an interposed OFF period through the use of high-speed shutters. A third beam can be used to provide accessory adaptation, but it was blocked in the experiments reported here.

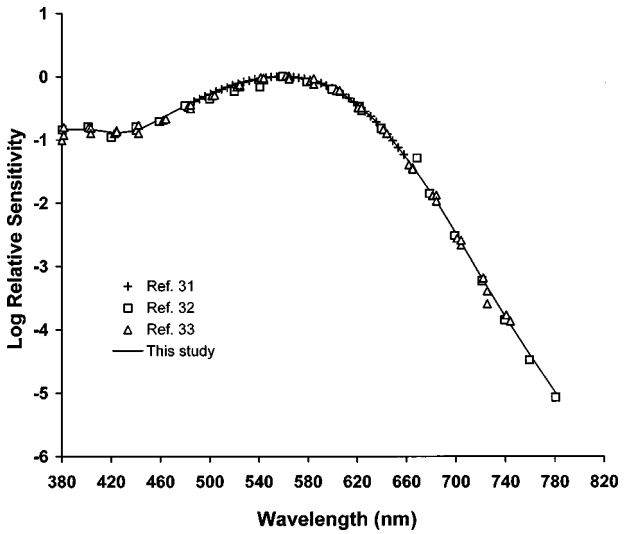

Fig. 2. Wavelength-adjustable expression for use in curve fitting spectral sensitivity data. The expression was designed to correspond to the extinction spectrum of bovine rhodopsin[31] over the top of the curve. Rhodopsin data are indicated by pluses. The spectral limbs were made to fit to spectral sensitivities of L cones measured electrophysiologically (triangles, measurements of Kraft *et al.*[33]; squares, measurements of Schnapf *et al.*[32]). The expression met three additional design criteria: (1) that the curve be described by a single continuous function, (2) that the function be well behaved at all wavelengths from 180 nm below the peak to infinity, and (3) that the long-wavelength side have a linear asymptote with a slope of 30.4 when the data are plotted on a normalized frequency scale.

the L and M cones for individual observers. Because the L and M photopigments are invariant in shape when they are plotted as a function of log wave number $(1/\lambda)$,[22–25] one can represent any photopigment simply by shifting a template curve along a log wave-number axis. Several template expressions have been proposed,[24–30] although none was ideal for our purposes. The standard set by Lamb is that a template curve should fit well near the peak and display the correct asymptotic behavior at long wavelengths.[25] However, Lamb's expression was not designed to represent absorption data on the short-wavelength side of the absorption peak. The experiments reported here required a curve that could accurately represent wavelengths that were significantly shorter than the peak. The available template curve expressions vary in how closely they approximate the parent data set from which they were derived. Any deviations between the template curve and the measured spectra that they represent become errors that could potentially limit the accuracy of the measurements in this study.

The mathematical expression derived here was designed to represent the photopigment extinction spectrum, where the peak optical density (OD) is 0.0. This design allowed changes in spectral shape that come from photopigment self-screening (with increases in OD) to be incorporated as a variable in fitting spectral sensitivity data. The rhodopsin extinction curve measured by Partridge and DeGrip[31] was used to represent the absorption values near the peak. In practice, when λ_{max} is set to the bovine rhodopsin peak of 497.63 nm, the data points from 416 to 604 nm correspond to the region of the curve that was fitted to the bovine rhodopsin spectrum.[31] We derived the values of the spectral limbs by fitting electrophysiological measurements from human L cones,[32,33] with the added constraint that the slope of the long-wavelength asymptote equal 30.41 $\log_{10}$ units per unit of normalized frequency (λ_{max}/λ).[25] Because the light is passed transversely through the cone outer segment in these electrophysiological measurements, the effective OD is expected to be small overall and is probably negligible in the spectral limbs. A curve produced from the derived mathematical expression is shown in Fig. 2,

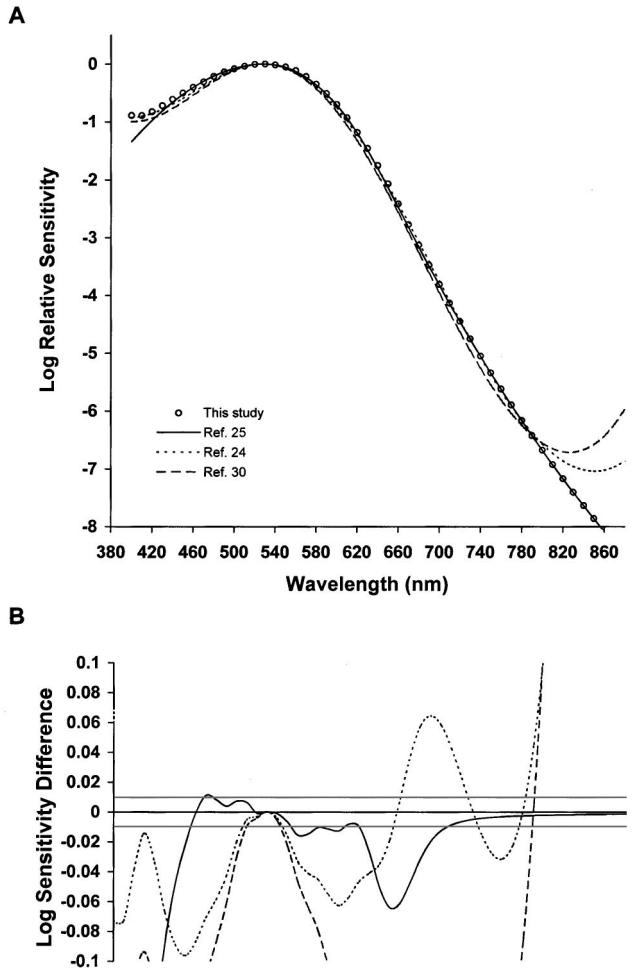

Fig. 3. A, Photopigment template expression used here compared with previously reported expressions. The template curves of both Baylor *et al.*[24] and Sharpe *et al.*[30] deviate from linearity in the very long-wavelength region. Lamb's template expression[25] was not designed to represent the pigment shape in the short-wavelength region. Near its peak, our curve was designed to match the bovine rhodopsin spectrum to extremely close tolerances. In the region near the peak of the curve, mismatches between our template and the earlier expressions closely mirror differences between those templates and the rhodopsin extinction spectrum. The accuracy of our curve near its peak depends on the assumptions that the rhodopsin curve is accurate[31] and that rhodopsin and the M and L pigment spectra are identical when they are translated on a log wave-number scale. B, Difference between our template curve and each of the others. The differences are plotted on a greatly expanded scale. Even though most of the differences are of the order of 0.01–0.1 log unit over the range of visible wavelengths, they are large enough to have a significant effect on estimated cone ratio derived from linear combinations of these curves.

Table 1. L Pigment Sequences and ERG-Derived %L for 15 Normal Male Subjects

Subject	Age (Years)	L Pigments[a] Exon 2	Exon 3	Exon 4	λ_{max}	%L[b]	559.11 %L[b]	Rayleigh Match
003	23	TIS	MVAIA	IAM	556.58[c]	71.73	60.98	40–42
004	30	TIS	LVAIA	IAM	556.58	54.57	46.09	41–42
005	46	TIS	MVVIS	IAV	559.09	70.31	70.22	37.5–38.5
013	19	TIS	LVAIS	IAM	559.11	66.28	66.28	38–43.5
014	49	TIS	MVVIS	IAV	559.09	69.21	69.13	39–41
015	28	TIS	MVVIS	IAV	559.09	92.11	91.97	35–39
023	27	TIS	MVAIA	IAM	556.58[c]	40.13	33.56	41–44
030	25	TIS	LVAIS	IAM	559.11	78.51	78.51	37–40
043	29	TIS	LVAIS	IAM	559.11	58.64	58.64	35–42
056	23	TIS	LVAIA	IAM	556.58	56.83	48.11	42–43
057	24	TVY	MVAIA	IAM	555.08[d]	80.68	62.22	38–45
059	25	TVY	LVAIS	IAM	557.61[d]	65.75	59.77	39–42
067	27	TIS	LVAIS	TSV	555.11[e]	53.50	40.45	39–41.5
075	43	TIS	LVAIA	IAM	556.58	68.28	57.81	41–43
129	30	TIS	LVAIS	IAM	559.11	78.24	78.24	38–40

[a] Amino acid positions: exon 2, 65, 11, 116; exon 3, 153, 171, 174, 178, 180; exon 4, 230, 233, 236.[15]
[b] Average single-gene protanope pigment was used for the M fundamental.
[c] Estimated peak based on the spectral tuning effect of M substitution in exon 3.[16,30]
[d] Estimated peak based on the spectral tuning of VY substitution in exon 2.[30]
[e] Estimated peak based on the spectral tuning effect of TSV substitution in exon 4.[16]

where it is compared with the data that it was designed to represent. In formulating the expression we set the constraint that the error between our candidate template expression and the absorbance data used to derive it could not exceed strict criteria. These criteria ensure that errors in the mathematical representation of the absorption spectrum are smaller than the errors that are inherent in the ERG spectral sensitivity measurements. For example, over the peak, where the expression represents the rhodopsin spectrum, the absolute error at any given point is $<0.01\,\log_{10}$ unit. Furthermore, the average absolute deviation was kept below $0.003\,\log_{10}$ unit over the same range. The expression is compared in Fig. 3 with others previously reported. We were unable to discover a simple and elegant mathematical expression that would produce a template curve that conformed to the constraints described above. Therefore the expression that met the criteria is somewhat cumbersome and is not given here; it can be found at http://www.mcw.edu/cellbio/colorvision. The file can be read with Microsoft Excel 97 or later versions of this software.

3. RESULTS AND DISCUSSION

A. Dichromats

For the dichromats, flicker-photometric ERG spectral sensitivities were measured and the nucleotide sequences of the X-encoded visual pigment genes were examined. The ERG spectral sensitivities of the protanopes ($n = 10$; mean age, 28 years) were averaged. The parameters that provided the best fit to the template expression were a λ_{max} of 530 nm and an OD of 0.22. The template curve set to these values was used as the M pigment component for estimating L:M cone proportions in all normals.

All the deuteranopes included in this study met two criteria: They had a single cone photopigment gene on the X chromosome, and the sequence of that gene corresponded to that found in one of the color-normal subjects whom we tested (see Table 1). Template curve fits to the deuteranopes provided a set of L-cone spectra to be used as the L components in estimating cone proportions in normal subjects.

B. Measurement Reliability

The accuracy of the estimates of the cone ratios for the normal subjects ultimately depends on the accuracy of the deuteranope data from which the L-cone spectral curves are derived. To assess this accuracy we tested several of the deuteranopes repeatedly. Example results from one deuteranope are shown in Fig. 4A. Spectral sensitivities for subject 130 were determined five times over a 2-month test period. The average λ_{max} obtained from these measurements was 552.55 nm (Fig. 4A, inset). The largest deviation from the average value on any single day was 0.33 nm. For the different measurements the average absolute deviation from the mean was 0.15 nm. Repeated measurements of other deuteranopes gave similar results. These results suggest that a single measurement of spectral sensitivity from one deuteranope is sufficient to yield an estimate of his λ_{max} that will be within a few tenths of a nanometer (usually <0.2 nm) of what would be obtained if additional measurements were made on the same person.

In this study, estimates of L:M cone proportions in normal subjects are corrected based on the gene sequence of the L pigment. In this method it is assumed that the gene sequence specifies a pigment with precisely the same spectrum when it is found in different subjects. To begin to assess the extent that this assumption is true we examined the results for different deuteranopes who had

the identical L-cone pigment gene sequence. One of the sequences was found in four deuteranopes. The results for those four individuals are shown in Fig. 4B. The average λ_{max} determined for this group was 559.11 nm. Each individual measurement can be compared with the average of the four (Fig. 4B, inset). The average absolute deviation of the individual measurements from the mean for the four subjects was 0.54 nm. This suggests that one individual can usually represent an L pigment sequence type with an error in λ_{max} of only ~0.5 nm. It is noteworthy that measuring photopigments produced by the same sequence in different individuals produces only a slightly larger absolute error than measuring the same person repeatedly. We presume that part of the reason that variation across subjects with the same sequences is so small is that the effects of variations in photopigment optical density that might occur across subjects and the effects of variations in macular pigment are both reduced in these experiments that employ a large stimulus field.

One of the major criticisms of psychophysical flicker photometry as a method for estimating L:M ratios is that large differences in the L:M ratio produce relatively small changes in spectral sensitivity; thus, even small measurement errors can lead to large errors in estimates of cone ratio. ERG flicker photometry has inherently low measurement error, and when complete spectral sensitivity functions are measured, small errors at individual points tend to offset one another such that an error at any one point has little effect on the final estimate of L:M ratio. To determine the reliability of the ERG estimates of L:M ratio we took measurements on two male subjects repeatedly over a period of 3 months. The results are shown in Figs. 4C and 4D. Five spectral-sensitivity functions were obtained for each observer, and an estimate of the percent of L cones {[L/(L + M)] × 100} was calculated from each function (Figs. 4C and 4D, insets). For each observer the individual estimates of %L, the relative contributions of L and M cones, can be compared with the mean for the five

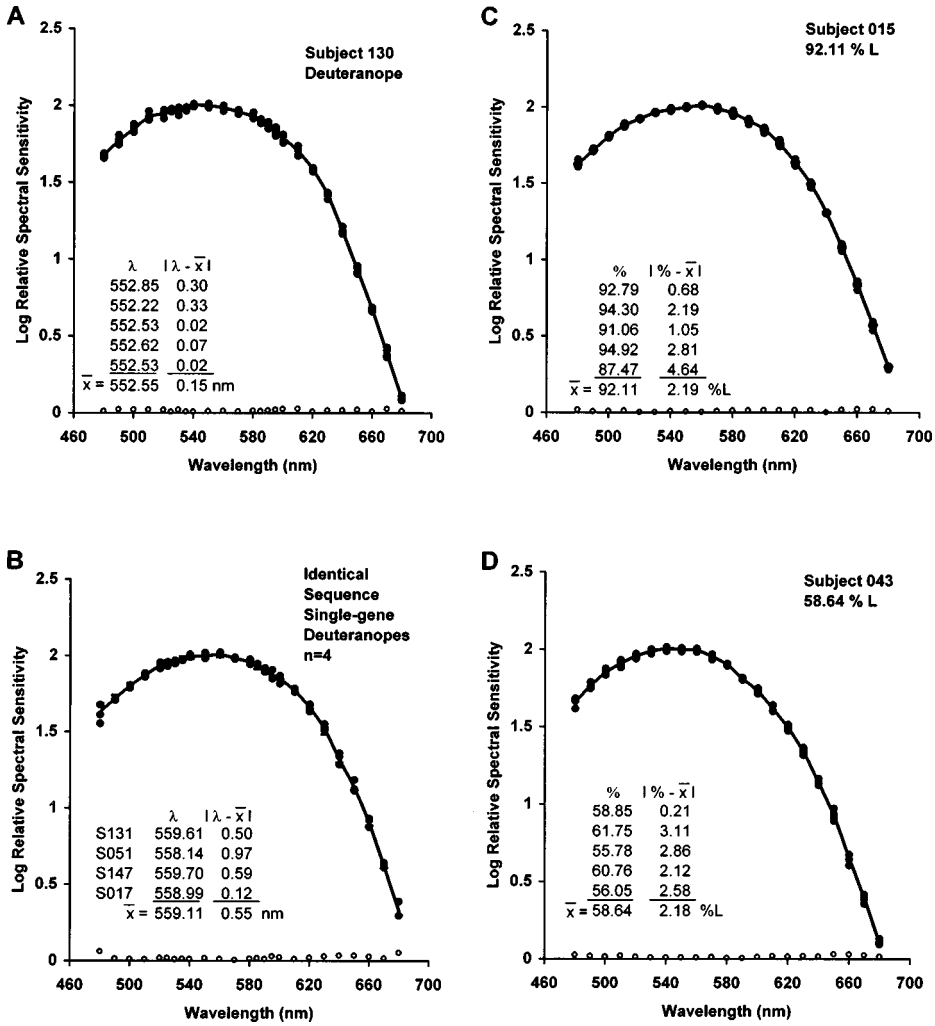

Fig. 4. Reliability of ERG spectral sensitivity functions. Spectral sensitivity values are shown as filled circles; standard deviations from the trials are shown as open circles. The solid curves represent the averages. A, Five independent measurements of spectral sensitivity on a single deuteranope (subjects 015) over a 2-month test period. Each spectral sensitivity function was fitted to a template curve from our expression (with OD fixed at 0.35), and λ_{max} was recorded. B, Spectral sensitivity functions for four single gene deuteranopes with identical L pigment sequences. The test for each subject was run once, and his spectral sensitivity function was fitted to the template curve. The resultant λ_{max} values were recorded. C, D, Each panel plots five repeated measures of spectral sensitivity for one color-normal subject. %L was calculated as the weighted sum of the subject's individualized L and the standard M fundamental. (L, λ_{max} = 559.09, OD = 0.35 and λ_{max} = 559.11, OD = 0.35, respectively; M, λ_{max} = 530, OD = 0.22.)

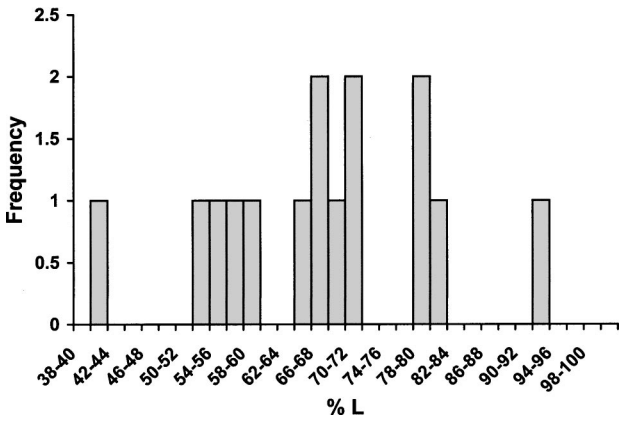

Fig. 5. Distribution of L-cone relative to M-cone contributions to ERG spectral sensitivity determined for 15 men with normal color vision. The relative L:M contributions are represented in terms of %L in linear combination with M required to best fit each subject's flicker-photometric ERG spectral sensitivity function. An individualized L pigment spectral sensitivity curve was derived from each subject's L pigment gene sequence.

measurements. In the ten cases, the individual estimates were never more than 5% different from the mean ($N = 5$ for each subject), and the average absolute difference of individual estimates from the mean was 2.2%. Thus an individual estimate of L:M ratio, taken from one ERG spectral sensitivity function, will have an error, on average, of only ~2% compared with the value that would be obtained from repeated measurements on the same subject.

C. L:M Cone Ratios of Normal Subjects

We determined the L:M cone ratios for each of 15 normal male subjects by finding the weighted sum of L- and M-cone fundamentals required for the best fit of the spectral sensitivity data. The results are summarized in Table 1 and in Fig. 5 in terms of %L. Each male subject was assigned an individualized λ_{max} for his L cone, and the template expression, shifted to that peak value, was used as his L fundamental. Each λ_{max} value was assigned based on the sequences of the L pigment genes for that subject. Exons 2–4 of the pigment genes encode eleven-polymorphic amino sites: three sites in exon 2, five in exon 3, and three in exon 4. The identities of the amino acid sites are given in Table 1 by their single-letter amino acid codes. There are no known amino acid polymorphisms in exons 1 and 6. Amino acids in exon 5 are responsible for the large spectral difference between L- and M-cone classes[15,16,34]; however, none of the amino acids in exon 5 that influence the spectrum varies within the subtypes of normal L pigments.[35,36] Thus the amino acid variations in L pigment sequences that could control the λ_{max} of normal subjects are completely specified by the amino acid letter codes given in Table 1. Male subjects with normal color vision can have more than one gene that encodes an L-type pigment,[17] and the additional L pigments can be expressed at significant levels in the retina.[11,35] However, only one L sequence was detected in each of the subjects in this study. The results from this study would suggest a lower frequency of men with multiple L genes than would have been expected from an earlier study designed to estimate the numbers and ratios of cone pigment genes.[17] The present study would identify a man as having more than one L gene only if the sequences differed. It is possible that some of the subjects whose results are reported here could have multiple L genes with the same sequence.

The λ_{max} specified by each L-cone pigment gene sequence was determined from the spectral sensitivities of single-gene deuteranopes with the same deduced L pigment sequence. Five of the normal subjects had L pigment sequences that did not exactly match any deuteranope in our sample. In those cases there were only one or two amino acid differences from the deuteranope sequences. Thus we were able to make a reasonable estimate of the specified λ_{max} for those few subjects by extrapolation. For two of the subjects, the amino acid differences observed were not found to influence the spectrum.[16,30] However, the other three men had polymorphisms that produced a significant shift in the spectrum.[16,30] The photopigment template expression that we used to fit the deuteranope data was set to specify a fixed pigment OD of 0.35. The same OD was maintained for the L fundamental when we fitted the data from the normal men.

The issue of OD deserves some discussion. In a preliminary analysis, the data from dichromats were fitted with the photopigment template, and both λ_{max} and OD were allowed to vary. The average OD that best fitted the deuteranope data was higher than that for protanopes. Thus the fixed OD values used here in fitting the normal spectral sensitivity curves reflect a difference in effective OD values between the M and the L cones. A higher OD for L than for M cones was suggested earlier.[37,38] In the present case, however, the estimate of an OD difference is based solely on subtle differences in the spectral shapes of the dichromat curves, which are influenced by assumptions about the shape invariance of the template curve and by assumptions used in correcting for preretinal absorption. Thus, at least from this study, evidence for an OD difference between L and M cones should be viewed with caution. A second issue is that there is evidence that the effective OD of the cones can vary in color normal observers[37] and that sequence variation in the cone pigment genes can contribute to OD differences.[39] Our method relies on having the template provide a good description of the dichromat curves. We chose fixed OD's and allowed λ_{max} to vary to account for spectral differences among the deuteranopes. Allowing both λ_{max} and OD to vary when we fitted the dichromats did not give significantly different estimates of L:M ratio in color-normal individuals (normals). A third issue concerns the possibility that the OD values for dichromats are different from those of normals. Berendschot *et al.* have reported a small but significant difference between dichromats and color normals in OD as assessed by optical reflectance spectra.[40] The reflectance data are affected by both the average OD per cone and the packing density of the cones, whereas the ERG spectral curves are affected only by a change in the average OD per cone. Thus it is possible that the difference suggested from reflection densitometry is not relevant to the ERG measurements. Also, inasmuch as there is evidence that OD differences arise from differences in the cone pigment

genes,[39] it is possible that the reported differences in OD between dichromats and normals reflects differences in the distribution of sequence variants in the dichromat and normal populations. If this were true, the differences between normals and dichromats would be largely accounted for by the practice of matching the sequences of the normals to the respective dichromats, as was done here. Barring both of those possibilities, if there were systematic differences in OD between normals and dichromats they would result in small systematic errors in the estimated cone ratios reported here.

To determine the effect of using the individualized L-cone spectra, we analyzed each subject in two ways, with his individual L-cone spectrum and with a standard L (Table 1). For the standard L we chose the highest λ_{max} value from our sample. We were forced to use a high value for λ_{max}, as choosing a fixed L even as low as the mean resulted in %L values of greater than 100% for some subjects. Using the individualized L fundamentals had a substantial effect on the estimates. For nearly half of the men the estimated %L changed by nearly 10% when the individualized L fundamental was used. The largest discrepancy was for subject 057. A %L of 62% was obtained for him with the standard L, but a value of 80% L was obtained with the correction for his individualized L fundamental. This corresponds to a difference in estimated cone ratio of 4:1 L to M cones, compared with 1.6:1 calculated with the standard L. Of course, the exact pattern of discrepancies depends on the choice of the standard L, but this exercise does give an indication of the number and magnitude of discrepancies that come from using a fixed L fundamental.

We used a fixed standard M fundamental because there is less variation in M pigment spectra in the population[15,16] and because the effects of variations in λ_{max} of the M pigment on estimates of cone ratio are dramatically smaller than for L.[13] For example, for subject 015 (Fig. 4C), a 6-nm change in the M fundamental produced a change in %L of 1%; a 1-nm shift in the L fundamental produced a change of 5.39 %L. This result might be expected purely because the subject had an extremely high contribution from his L cones. However, examination of a subject with much lower %L (subject 023, 40.13 %L) showed a similar effect. A 1-nm shift in the L fundamental changed the derived %L by 2.89%. It takes almost a 4-nm shift of the M fundamental to produce exactly the same change.

We estimate that λ_{max} values taken from the dichromats are usually accurate to ~0.5 nm, as shown in Fig. 4B (inset), but how does a 0.5-nm variation in L pigment λ_{max} affect the derived %L in the color-normal subjects? Each of the 15 subjects was analyzed at his appropriate L fundamental and then again with an L fundamental that differed by 0.5 nm, both above and below the original fun-

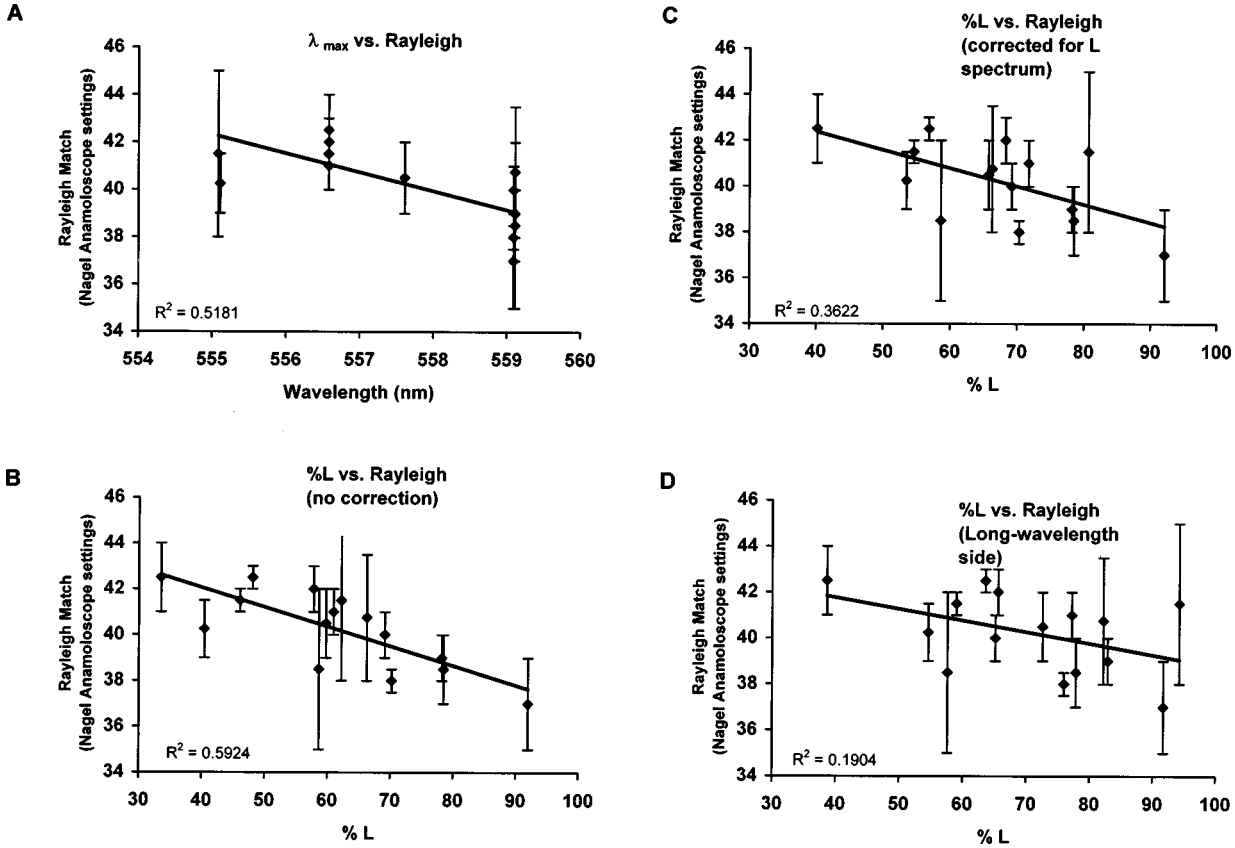

Fig. 6. L-cone pigment λ_{max} and estimated %L-cone contributions compared with Nagel anomaloscope Rayleigh match settings for 15 men with normal color vision. A, λ_{max} of the L cone determined from L pigment gene sequences versus anomaloscope setting. B, %L-cone contribution calculated with a fixed standard L-cone fundamental versus anomaloscope setting. C, Same as B, except that the %L contribution was corrected by use of individualized L-cone fundamentals for each subject. D, Same as C, except that %L values were calculated by use of only the spectral sensitivity points at wavelengths that were ≥550 nm.

damental. On average, a 0.5-nm change in the L fundamental produces an absolute difference of 2.3 %L.

To empirically evaluate the effectiveness of the procedure of using individualized L fundamentals in removing error from the estimated cone ratios we compared the individual cone ratio estimates with the subjects' Rayleigh matches on the Nagel anomaloscope. The logic is this: The Rayleigh matches are linearly related to the spectral sensitivities of the cones but are theoretically completely insensitive to variations in cone ratio. Conversely, our correction procedure has been designed to make the measurement of cone ratios insensitive to variations in the spectral sensitivities of the cones. We assume that the cone ratio and the individual spectral sensitivities of the cones are unrelated (although we cannot rule out the possibility that they could be related, e.g., through genetic linkage). Thus, if our procedure were completely effective at removing errors in the cone ratio estimates that come from variations in λ_{max}, there should be no correlation between estimates of %L and the Rayleigh match. Figure 6 shows the results of the comparisons. First, we confirmed that there was a high correlation between the Rayleigh match and the predicted λ_{max} of the L pigment (Fig. 6A). The correlation is high ($r^2 = 0.6$, $p = 0.0006$) but far from perfect, presumably because of experimental error in the Rayleigh match measurement and the influence of other factors, such as variations in preretinal absorption[41] and variation in λ_{max} of the M pigments, on the Rayleigh match. Figure 6B shows that when the estimates of %L are not corrected for individual differences in λ_{max} of the L pigment (i.e., the fixed standard L is used), there is a very high correlation with the Rayleigh match ($r^2 = 0.6$, $p = 0.0008$). This is presumably caused principally by the large errors in the %L estimates introduced by the λ_{max} variation. When the same comparison is made after the correction for λ_{max} of the L pigment, the correlation is markedly reduced ($r^2 = 0.4$, $p = 0.02$). Thus the procedure is highly effective at removing error. However, some correlation persists, and it is just statistically significant ($p = 0.02$). The residual correlation may still be the result of variations in preretinal absorption that would be expected to have a small effect on both the Rayleigh match and the spectral luminosity. To evaluate this possibility we made new estimates of %L for each subject, using his individualized L fundamental but fitting his spectral sensitivity curve only at wavelengths longer than 550 nm. This should theoretically remove much of the effect of preretinal absorption on the %L estimates. The result is shown in Fig. 6D; the correlation is reduced to a value that is not significantly different from zero ($r^2 = 0.19$, $p = 0.10$). This result indicates that there is a small residual error that comes from variations in preretinal absorption but that our method effectively removes errors that come from variations in the spectral sensitivities of the cone pigments and raises the issue of whether it is better to use the estimates of %L that come from the full spectral luminosity function or those from fitting only the long-wavelength side of the function. On inspection, the two methods (full function and long-wavelength side only) do not differ much in the range of %L values obtained or in the mean %L for the group (the mean is 67% L full function or 70% L long-wavelength function). Using the long-wavelength side rather than the full function makes for only subtle differences in the estimates for a few subjects. There are probably trade-offs. Using the full function may introduce small errors from preretinal absorption, but using only the long-wavelength side, and thus basing the estimate on fewer points, may slightly increase the error of measurement.

The intersubject variation in spectral luminosity is strikingly large, as one can see from Fig. 7, which shows the results for the two subjects who represent the extremes in our sample of 15 men. Also plotted in the figure are the L fundamentals (solid curves) that we used to analyze each subject. The spectral sensitivity function for subject 015 shown in Fig. 7A nearly matches his L fundamental, indicating a very small contribution of M cones

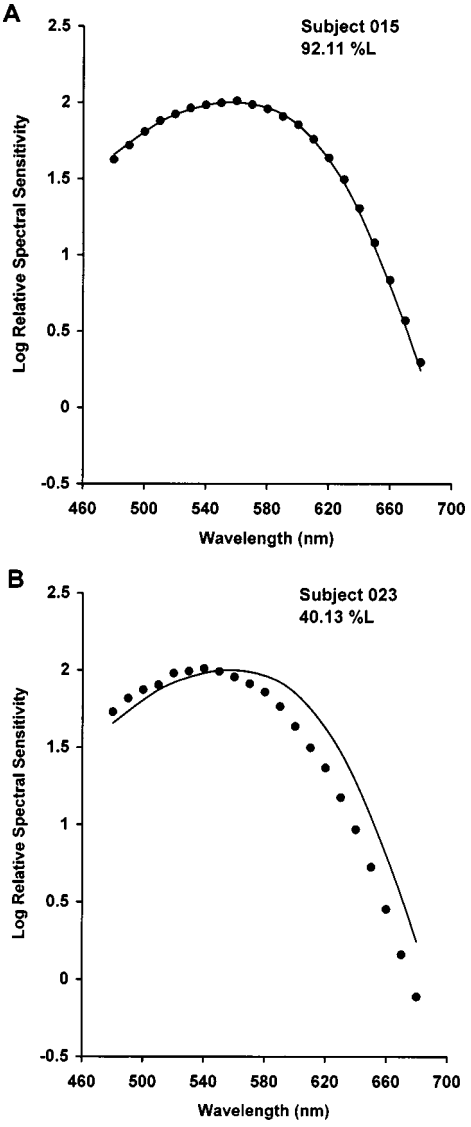

Fig. 7. Individual variations in %L observed in the spectral sensitivity function. The L-cone template curve that corresponds to that pigment is shown by the solid curve in A and in B. The two subjects (who represent the extremes observed in these experiments) have dramatically different spectral sensitivity functions (filled circles). The differences in spectral sensitivity presumably represent large differences in L:M cone contributions to the ERG signal.

to his sensitivity function. At the other extreme, the spectral sensitivity curve of subject 023 shown in Fig. 7B falls far below his L fundamental in the long wavelengths. This indicates a very robust contribution of M cones to his sensitivity.

4. SUMMARY

A major source of error in estimates of cone proportions from spectral luminosity functions is the known variation in λ_{max} of the normal photopigments. We have corrected for those errors, using individualized L-cone spectra sensitivity curves deduced from photopigment gene sequences from each male subject. The uncorrected L:M ratios estimated from spectral sensitivities were found to be highly correlated with Rayleigh match settings on a standard Nagel anomaloscope. This result is evidence that the L:M ratio estimates from spectral luminosity functions with a standard L pigment are substantially contaminated by variations in the cone pigments. We were able to reduce the correlation to chance levels by correcting for variation in the L pigment λ_{max} and additionally by excluding sensitivity values in the short wavelengths. The Rayleigh match is sensitive to any factor that influences the spectral sensitivities of the cones, including variations in λ_{max} of the L and M cones and their photopigment optical densities. The fact that the procedures were effective in nullifying the correlation between L:M ratio estimates and color matches confirms that the L pigment λ_{max} is the major photopigment spectral-sensitivity-related factor that influences estimates of L:M ratio from spectral sensitivity functions. The results indicate that our procedure corrects for that factor effectively.

Previously, a large range of individual variability had been reported for L:M ratios obtained from photometry. Here we demonstrate only a very small measurement error for the ERG procedure and present evidence indicating that we have removed much of the error that comes from variation in the photopigment spectral sensitivities. However, there was still a very large range of individual differences among our 15 subjects. Estimated L:M ratios for individuals ranged from 0.6:1 to 12:1 L:M (with an average of 2:1 L:M. It seems certain that these numbers represent real differences among individuals in the relative contributions of the L and M cones. Brainard *et al.*[42] have shown that ERG flicker photometry with corrected L-cone fundamentals accurately predicts the two widely different cone ratios (as measured by direct imaging[1]) of two male subjects. However, it is still possible that, in some subjects, differences in cone ratio are compounded with real variability in other factors that affect the relative contributions of L and M cones to the ERG. There may be normal variations in the relative strength of the contribution of the individual cone classes to the ERG signals, e.g., perhaps in some people each L cone produces a larger ERG signal than each M cone. If this were true, even corrected estimates of the relative numbers of L:M ratio from flicker photometry could overestimate the range of variation. We thus must be cautious when interpreting results obtained with this method in terms the relative numbers of L and M cones. Nonetheless, the ERG flicker photometry method with corrections made for L-cone λ_{max} is a promising technique for efficiently obtaining reliable estimates of L:M cone ratios in larger samples of normal subjects.

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