

Electroretinogram measurements of cone spectral sensitivity in dichromatic monkeys

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The corneal electroretinogram (ERG) was used to investigate the spectral sensitivities of cones in 12 dichromatic squirrel monkeys (*Saimiri sciureus*) whose color-vision capacities were established in behavioral tests. Three different varieties of dichromacy were represented among these animals. A flicker-photometric procedure was used in which the ERG response to a rapidly flickering monochromatic test light was compared with the response elicited by a similarly flickering reference light. The spectral-sensitivity functions obtained by the use of this technique are similar to previous estimates of cone spectral sensitivity in dichromatic squirrel monkeys derived from direct microspectrophotometric measurements.

INTRODUCTION

The corneal electroretinogram (ERG) provides a noninvasive, convenient index of retinal activity. It is thus not surprising that it has often been employed to attempt measurements of the spectral mechanisms in the retina (early research in this area was reviewed by Riggs and Wooten¹ and by Armington²). A formidable problem inherent in this approach is that responses from the rods and all the cone classes can contribute to the recorded signals, making the separation of component contributions difficult. It has long been known that rod contributions to the ERG can be minimized by utilizing rapidly flickering lights as stimuli.³ This procedure, however, often also results in small signals and in unfavorable signal-to-noise ratios. To ease this difficulty, the fundamental, stimulus-locked component of the flicker ERG has been enhanced relative to the noise through the use of a vector voltmeter⁴ or with active narrow-bandpass filtering.⁵ The second aspect of this problem, that of separating the contributions from multiple cone classes, is also of long standing. The usual solution for ERG studies of spectral mechanisms, as for analogous psychophysical studies, has been to employ concurrent chromatic adaptation as a tool for rendering one or more of the constituent cone classes less effective. In recent adaptations of this approach, it has proved possible to use the ERG to measure directly the field sensitivities of the spectral mechanisms.^{5,6} Another obstacle to making ERG measurements of spectral sensitivity is that any spontaneous fluctuations in ERG sensitivity occurring during the test session make the application of a constant response criterion difficult. One solution is to use a flicker-photometric procedure to measure cone spectral sensitivity. In this case, the intensity of a flickering monochromatic test light is adjusted so that it produces an ERG response that matches the response elicited by a similarly flickering reference light.⁷⁻⁹ The advantage of this procedure is that any spontaneous changes that occur in ERG amplitude during the experiment will be equally reflected in the responses to both test and reference lights and so will only minimally affect measured spectral sensitivity.

All these recently developed techniques have made it easier to obtain ERG measurements of cone mechanisms. A re-

maining issue, however, is that it has been difficult to provide an independent validation of the results. This is because these procedures have usually been employed to study retinas in which either the spectral properties of the cone mechanisms are not accurately known from independent measurements and/or retinas in which multiple, spectrally overlapping cone classes exist; in this case, investigators must make assumptions about the combination of ERG signals from different cone classes. In our attempts to develop a technique to measure accurately the spectral properties of cones by using the ERG, measurements have been made on the squirrel monkey (*Saimiri sciureus*), a subject that allows both of these problems to be obviated.

Over the past several years, it has been established that there are striking within-species variations in color vision among squirrel monkeys. Behavioral tests show that there are both dichromatic and trichromatic individuals in this species.¹⁰ Further subtypes can be differentiated within each of these broad groups. For instance, there are three distinct dichromatic phenotypes. In concurrent experiments, the absorbance characteristics of individual photoreceptors have been measured by microspectrophotometry in squirrel monkeys whose color vision had been previously established by behavioral tests.¹¹⁻¹³ These measurements indicate that the variations in color vision in this species result from variations in the spectral properties of the cones present in individual animals. Thus the retinas of dichromatic squirrel monkeys contain a short-wavelength-sensitive photopigment ($\lambda_{\max} = 433$ nm) and, depending on the individual, a second, single cone class absorbing maximally at 536, 551, or 563 nm.

In the experiments reported here, we developed an ERG flicker-photometric procedure involving the use of rapidly flickering stimuli and narrow-bandpass filtering to examine the spectral sensitivity of the cone mechanisms in each of the three types of dichromatic squirrel monkeys.

METHODS

Subjects

Twelve adult squirrel monkeys (ten male and two female) were tested. The color vision of each of these animals had

been established in behavioral tests of the type described in detail elsewhere.¹⁰ All these monkeys were judged to have dichromatic color vision on the basis of more than one of the following test outcomes: (1) Their wavelength-discrimination functions were U shaped with a minimum located near 500 nm; (2) they had neutral points located in the spectral range between 489 and 505 nm; (3) they failed to discriminate either monochromatic red or monochromatic green light from equiluminant yellow light and to discriminate any mixture of red and green lights from yellow. All these criteria have been classically employed to differentiate the dichromatic from the trichromatic observer.¹⁴

Apparatus

A three-channel optical system was used. The test light came from a Bausch & Lomb high-intensity grating monochromator (half-energy passband, 10 nm). The reference light was produced by a tungsten halide lamp (50 W, 12 V). A third beam, which could be used to provide accessory adaptation lights, originated from a similar tungsten halide lamp. All lights were underrun at 11 V from a regulated DC power supply. The intensity of the test light was controlled by the use of a circular neutral-density wedge. The chromatic content and the intensity of the lights in both reference and adaptation beams were varied by using neutral-density step filters and interference filters (Ditric, half-energy passband, 10 nm). High-speed electromagnetic shutters (Vincent Associates Uniblitz) located in each of the three beams were used to control the presentation of the three lights. The shutters were driven from a programmable digital timer. The three beams were optically superimposed and then focused with a final lens so that they illuminated a circular 53° patch of the retina in Maxwellian view. Light calibrations were made with a P-I-N 10-DF silicon photodiode (United Detector Technology).

ERG's were recorded with a Burian-Allen bipolar contact-lens electrode. The ground electrode was placed against the inside of the cheek. A flicker-photometric procedure was employed whereby ERG responses to flickering monochromatic test flashes of variable intensity were compared with the responses elicited by a similarly flickering, fixed reference light. The sequence of these two lights is illustrated in Fig. 1. It consisted of an alternating train of flashes from the test and the reference lights separated by a dark interval. A complete stimulus cycle thus consisted of test flash, no light, reference flash, and no light. The number of such cycles per second is here referred to as the stimulus frequency. Note that the flicker rate is twice the stimulus frequency. When accessory adaptation was used, this entire sequence was superimposed upon a continuously present adaptation light.

Figure 1 also illustrates the method used to process ERG responses. The ERG elicited by high-frequency flicker contains a fundamental frequency equal to the stimulus frequency. This stimulus-locked component was greatly enhanced relative to the others by passing the differentially amplified signal through an active narrow-bandpass filter. The filter consisted of two two-pole stages (for each stage $Q = 5$). The half-voltage bandpass of the filter was 0.2 times the center frequency. The center frequency of this filter was set equal to the frequency at which both test and reference lights were individually modulated. When the intensities of test and reference lights were greatly disparate, the filter

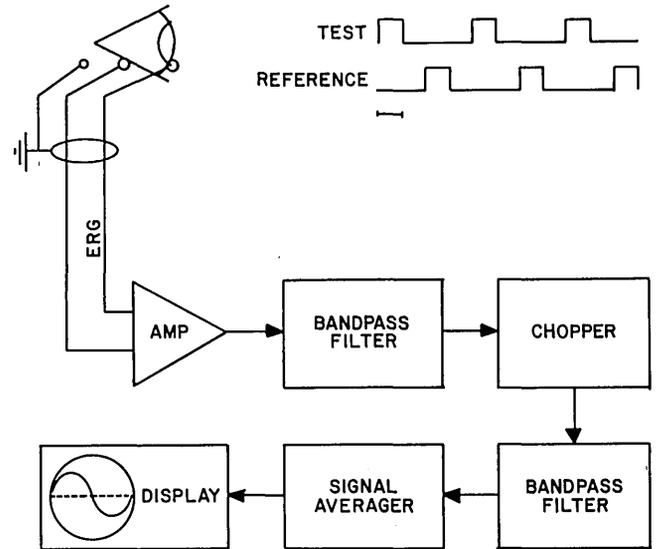


Fig. 1. Schematic representations of the stimulus sequence and the recording system used to make ERG measurements of cone spectral sensitivity using a flicker-photometric procedure. The sequence of test and reference lights is illustrated in the upper-right-hand corner. Both lights were flickered at 25 Hz. The test light, reference light, and interstimulus durations were all equal. Calibration bar: 10 msec. The block diagram shows the major components of the recording system. The characteristics and functions of each block are described in the text.

output was nearly sinusoidal. A reversal of the relative intensities of the two lights caused the sinusoidal output from the filter to be phase shifted by 180°. At an intermediate intensity ratio, the amplitude of the signal was minimized, and the phase was intermediate. To improve the cancellation of the signal at the point where the test and reference lights are equally effective, the output of the bandpass filter was chopped (Fig. 1) so as to invert each response during the period from onset of the test light to onset of the reference light (see Fig. 1). This signal was then filtered with a second narrow-bandpass filter, which was identical in character with the first but had its center frequency set equal to the flicker frequency (i.e., twice the frequency at which the test light and the reference light were individually modulated). As the signal produced by the test light was inverted before entering this filter, the filter effectively subtracted it from the signal produced by the reference light. The filtered output was averaged with an Ortec 4623 signal averager. The signal-averager output could be read directly from an oscilloscopic display and written out on an XY plotter.

Procedure

Monkeys were anesthetized by an initial intramuscular injection of 15 mg of ketamine hydrochloride plus 0.15 mg of acepromazine maleate and by subsequent intraperitoneal injection of sodium pentobarbital (10 mg/kg). Atropine sulfate was used to inhibit salivation and respiratory-tract secretions. The pupil of the test eye was dilated by topical application of atropine sulfate (0.04%) and Neosynephrine (phenylephrine HCl).

The animals were placed in a modified stereotaxic instrument. A specially designed head holder was used to restrain the animal painlessly and position the head for recording.

The holder consisted of four large neoprene pads, two for each side of the head. One set was shaped so as to support the cantilevered portion of the occipital pole of the monkey; the other pair rested on top of the head. These pads could be adjusted to accommodate individual variations in head size. Normal body temperature was maintained through the use of a circulating-hot-water heating pad.

As noted above, spectral sensitivity was measured using a flicker-photometric procedure in which the intensity of a flickering monochromatic light was adjusted until the ERG it produced best nulled the response produced by a flickering reference light. In these experiments, the standard flicker rate employed was 50 Hz, with the durations of the test, reference, and no-light intervals all equal. On occasion, higher flicker rates were employed. The reference light was achromatic and produced a corneal irradiance of $32.8 \mu\text{W}/\text{cm}^2$. In some experiments an accessory short-wavelength-adaptation light was used (wavelength, 440 nm; corneal irradiance, $115 \mu\text{W}/\text{cm}^2$). All measurements were made with the room lights on. The overhead lights produced a corneal irradiance of $25 \mu\text{W}/\text{cm}^2$.

To measure sensitivity at a given test wavelength, the density wedge was set to an arbitrary position, and a train of 100 stimulus cycles was presented. The responses to the last 60 of these were averaged. The phase and the amplitude of the resultant response were used to indicate the change in the test-light intensity required to better null the reference signal. This procedure was repeated until the best null position was determined, a process usually requiring four to seven iterations. Sensitivity was measured at 10-nm steps, typically from 450 to 660 nm.

The sensitivity values so determined were first corrected for lens absorbance in this species by using measurements made in earlier experiments in this laboratory (unpublished). These corrected values were then compared with wavelength-dependent visual pigment nomograms.¹⁵ The nomograms were calculated from the polynomial expressions given by Dawis.¹⁶ A computer was used to determine the spectral positioning (to the nearest nanometer) of the relevant nomogram that gave the best fit to the corrected spectral-sensitivity data. To do this, the difference between each sensitivity measurement and the nomogram was computed for each peak value of the nomogram. The computer then selected the nomogram that gave the least mean difference squared. That value was used as a measure of goodness of fit.

RESULTS

It was a relatively easy task to measure spectral sensitivity in squirrel monkeys using the ERG flicker-photometric procedure. Figure 2 illustrates the technique. Shown are superimposed XY plotter records obtained in a test involving a comparison of the effects of a 640-nm test light and the reference light. Five different intensity settings of the test light are shown. In one trace (labeled 0.00 in Fig. 2), the test light was adjusted to be equal in effectiveness to the reference light; in the other cases, the test light was offset so as to be either more or less intense than the value required at the null setting. It can be seen that small intensity shifts led to large, easily discerned changes in the recorded signals. As is seen in Fig. 2, in most cases changes in the intensity of the test light of the order of 0.02 to 0.04 log unit could be discriminated reliably

in the ERG output. The settings were also reliable: In re-checking sensitivity values throughout the experimental session, it was only infrequently necessary to revise any of the determined values. Finally, the procedure was also quite efficient since, usually, only 45 min to 1 h was required to determine an entire spectral-sensitivity function and to re-check it thoroughly.

The spectral-sensitivity functions obtained from this sample of dichromatic monkeys fell into three groups. These groups are shown separately in Figs. 3–5. In each instance, the solid line represents the sensitivity curve for the best-fitted visual pigment nomogram with the spectral location of the peak of each curve indicated on the function. So separated, the spectral-sensitivity curves for two animals peak at 535 and 538 nm (Fig. 3), five animals had peak sensitivity between 548 and 550 nm (Fig. 4), and the remaining five animals had sensitivity peaks from 559 to 561 nm (Fig. 5). Without exception, the relevant nomograms provided excellent fits to the full range of sensitivity values. The measurement of goodness of fit for the 12 animals ranged from 6×10^{-4} to 3.9×10^{-3} log unit. The only indication of any systematic deviations in the data from the nomograms is that, in some of the animals, the shortest test wavelengths yielded sensitivity values lower than those predicted by the nomograms. This deviation is small. It might be explained as resulting from some variable degree of filtering by the macular pigment, which is said to be present in the squirrel monkey.^{17–19} No measurements of the absorbance properties of that pigment are available yet to our knowledge.

The extremely close fits between the sensitivity values obtained for each animal and a single visual pigment nomogram argues strongly that this ERG procedure in fact isolates signals from single cone mechanisms. Further evidence for that conclusion was obtained by examining the effects of chromatic adaptation on spectral sensitivity. Responses produced by a long-wavelength reference light (630 nm, $90 \mu\text{W}/\text{cm}^2$) were balanced against the responses produced by a 540-nm test light. The intensity of the 540-nm light required to achieve the balance was determined under conditions of (1) no accessory adaptation, (2) concurrent long-wavelength adaptation (630 nm, $86 \mu\text{W}/\text{cm}^2$), and (3) concurrent middle-wavelength adaptation (540 nm, $28 \mu\text{W}/\text{cm}^2$). The intensities of the two chromatic adaptation lights were determined in preliminary experiments to be sufficient to elevate the threshold for a homochromatic test light flickering at 50 Hz by about 1 log unit. The results of the chromatic adaptation were clear cut: The balance equations determined

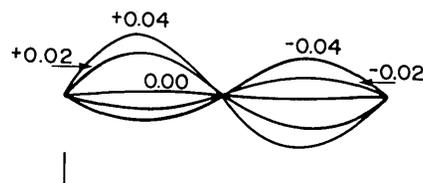


Fig. 2. Superimposed XY-plotter records illustrating the flicker-photometric procedure. Each of the five traces represents the ERG response produced from a comparison of a 640-nm test light with an achromatic reference light. The trace labeled 0.00 shows the response obtained when the intensity of the test light was adjusted so as to be equally effective to the reference light. The other traces represent the responses to systematic mismatches of the two lights (mismatch values given in log units). Calibration bars, $1 \mu\text{V}$ and 10 msec.

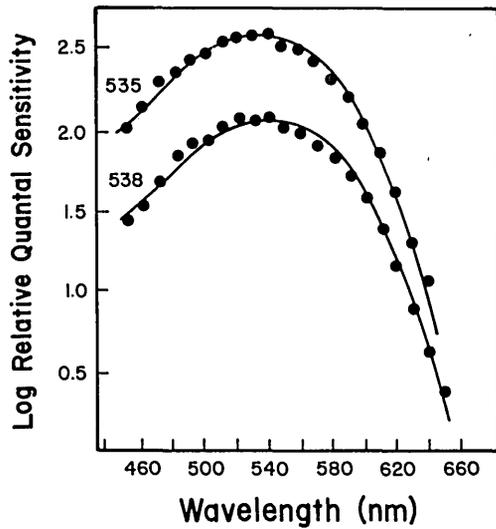


Fig. 3. Flicker-photometric spectral-sensitivity functions obtained from two squirrel monkeys. These animals represent the first of three types of dichromatic color vision seen in this species. The filled circles show sensitivity values obtained by equating the effectiveness of monochromatic test lights and an achromatic reference light. These values have been corrected for absorbance by the squirrel-monkey lens. The solid lines are the best-fitting visual pigment nomograms, the spectral peak of which is shown on each function. The two curves have been arbitrarily positioned on the sensitivity axis.

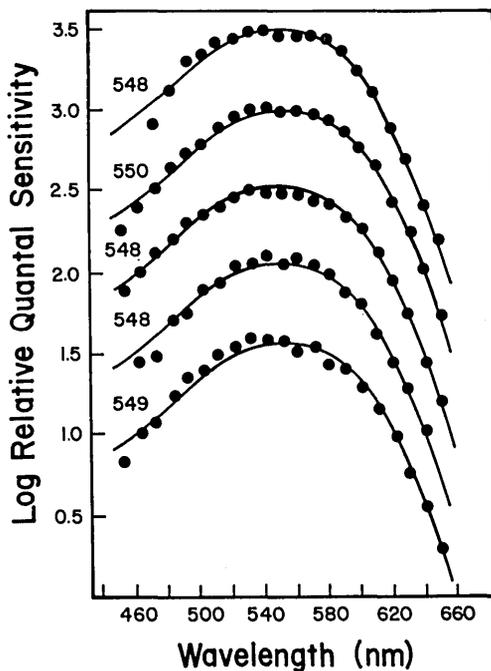


Fig. 4. Flicker-photometric spectral-sensitivity functions obtained from five squirrel monkeys. These animals have the second variety of dichromatic color vision seen in this species. Details are the same as those given for Fig. 3.

under the three conditions showed no significant differences. The actual equation values never differed by more than 0.03 log unit, and, when they did differ, even by this amount, they were as frequently as not in a direction opposite what would be expected if a typical chromatic adaptation effect were occurring (e.g., less green light was required to balance the red in the presence of a green adapting light). For comparison, we tested a number of trichromatic squirrel monkeys in ex-

actly the same way. Each of these animals showed systematic threshold changes under the two conditions of chromatic adaptation.

DISCUSSION

The retinas of dichromatic squirrel monkeys contain three classes of receptors: rods, short-wavelength cones, and cones absorbing maximally in the middle to long wavelengths.¹¹⁻¹³ The results shown in Figs. 3-5 indicate that, under the stimulus conditions employed in this experiment, neither the rods nor the short-wavelength cones provide any obvious contributions to the ERG spectral-sensitivity data. Comparisons of these electrophysiological results with both behavioral results and direct pigment measurements make it additionally clear that this technique provides a valid indication of the spectral sensitivities of the middle- to long-wavelength cone mechanisms found in these animals.

Eleven of the twelve monkeys had served as subjects in behavioral experiments involving the measurement of increment-threshold sensitivity for two monochromatic test lights, 540 and 640 nm (for details of this experiment, see Ref. 20). For the dichromatic subject it would be expected that relative sensitivity to these two test lights would be completely determined by the operation of the (single) cone pigment absorbing through this spectral range. If so, the behavioral sensitivity to the 540- and 640-nm test lights should, in each case, be accounted for by the cone mechanisms whose sensitivities appear in Figs. 3-5. Figure 6 indicates that this expectation is correct. In Fig. 6, in the form of a scatterplot, the sensitivity values obtained from each animal in the behavioral experiment are shown plotted against the sensitivity values determined from the ERG of the same animal. The two indices are significantly correlated ($r = 0.85$, $df = 9$, $p < 0.01$). Further, the slope of the best-fit line (1.07) and its intercept

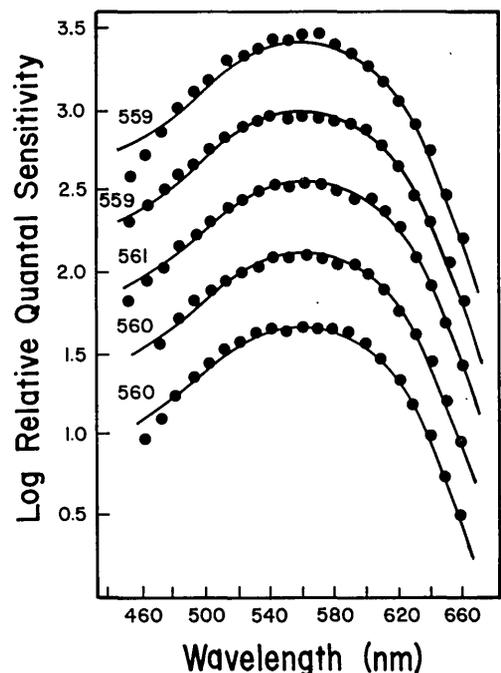


Fig. 5. Flicker-photometric spectral-sensitivity functions obtained from five squirrel monkeys. These animals have the third variety of dichromatic color vision seen in this species. Details are the same as those given for Fig. 3.

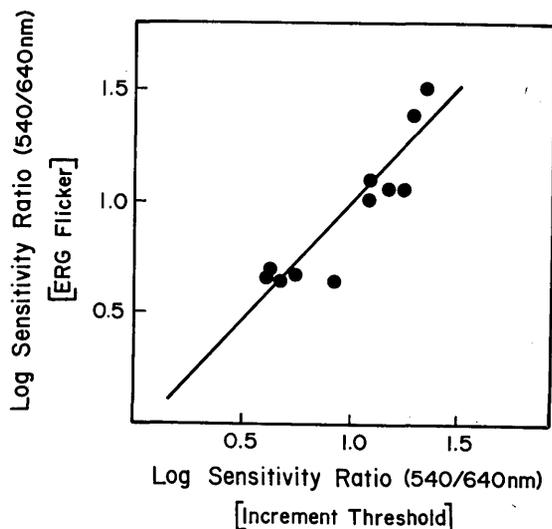


Fig. 6. Relationship between the 540/640-nm sensitivity ratios obtained from ERG flicker photometry and from behavioral measurements. Each point corresponds to an individual animal. The line represents a least-squares fit to the data. The two sets of data are significantly correlated ($r = 0.85$; $df = 9$; $p < 0.01$).

value (-0.08) are quite close to what would be expected if there were an exact correspondence between the behavioral and the electrophysiological data. Thus the variation in increment-threshold sensitivity among individual dichromatic squirrel monkeys can be accounted for by the variations in sensitivity of the cone mechanisms found in individual retinas.

The separation of the cone spectral sensitivities into the three groups of Figs. 3–5 is also paralleled by results from a color-vision test. Each of these subjects was tested to determine if it had a spectral neutral point. All did, and the locations of these neutral points differed for the three types of dichromats. Thus, for the animals whose sensitivity curves are shown in Fig. 3, the average neutral-point location was 490.4 nm ($N = 2$), for the animals of Fig. 4, the average location was 492.1 nm ($N = 5$), and the animals whose ERG data are shown in Fig. 5 had an average neutral-point location of 497.9 nm ($N = 5$). Note that, although the range in neutral-point locations for these three dichromatic subtypes is not great in absolute terms, it is similar to results obtained from human dichromats. Human protanopes and deuteranopes give neutral-point locations that are on average about 6 to 7 nm different.^{21,22} That difference is indistinguishable from the difference in neutral-point locations for the two classes of squirrel monkeys whose dichromacies are most similar to human protanopia and deuteranopia, i.e., the animals whose results are given in Figs. 3 and 5, respectively. These ERG data thus correlate well with behavioral measurements of both visual sensitivity and color vision.

The cone sensitivity curves obtained from ERG flicker photometry are also remarkably similar to the earlier direct measurements of cone pigments in squirrel-monkey retinas. Microspectrophotometry measurements made in known dichromatic squirrel monkeys yielded three different classes of cones: those having an average absorbance peak at 536.6 nm (results from 189 cones), those having a mean absorbance peak at 551.0 nm (63 cones), and those having a peak at 563.1 nm (56 cones).¹³ Without any further corrections or assumptions, these pigment peaks can be seen to be essentially identical

with the average sensitivity peaks for the three groups of subjects tested here: mean values of 536.5, 548.6, and 559.8 nm, respectively. These values are well within one standard deviation of the estimates given elsewhere for the peaks of the squirrel-monkey cone pigments.¹³

In summary, the present experiments indicate that the ERG flicker-photometric procedure provides a sensitive, reliable, and efficient method for obtaining spectral-sensitivity functions from cone mechanisms. Utilization of known dichromatic subjects shows that these measurements are also a valid reflection of the actual cone pigments present in the retina. This technique thus provides a promising tool for investigating cone spectral sensitivities in other subjects, including those intriguing cases in which the effects of two or more spectrally overlapping mechanisms need to be sorted out.

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