Electroretinogram flicker photometry and its applications

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Received May 9, 1995; revised manuscript received July 31, 1995; accepted August 31, 1995

The electroretinogram (ERG) has been a traditional tool for the measurement and the analysis of spectral sensitivity. With the appropriate choices of stimulus and measurement conditions, the ERG permits a noninvasive examination of photopigment complement and provides the means for studying the combination of spectral signals at various locations throughout the retina. There are a number of practical problems associated with making spectral measurements with the ERG. One approach to minimizing these problems is to exploit the advantages of a flicker-photometric procedure. We summarize a method used to conduct ERG flicker photometry and illustrate a range of problems to which this technique can be successfully applied. © 1996 Optical Society of America

1. INTRODUCTION

Over the long history of its employment, the electroretinogram (ERG) has provided one of the most commonly used electrophysiological approaches to the measurement of spectral sensitivity and its concomitants.[1,2] But, as everyone who utilizes the ERG for this purpose learns, there are a number of practical drawbacks. Among these, one of the most serious is that any thorough spectral measurements must be accomplished across an often-extended recording session. During this period, for any of a myriad of reasons (for example, shifts in the position of the eye or the electrode or changes in the metabolic state of the retina), the amplitude of the ERG component of interest may change. If so, any spectral sensitivity measurement dependent on a comparison of amplitudes or latencies across the duration of the recording session, as most are, may be seriously compromised. One common solution to this problem is to test repetitively a single spectral point and then allow the responses to that stimulus to serve as a reference value to which the responses to all other spectral stimuli can be subsequently normalized. This is, at best, an inefficient solution to the problem, as it requires many extra measurements. Spectral sensitivity measurements based on amplitude or latency criteria also require that measurements be made at a number of different intensity settings at each test wavelength to allow for the construction of response/intensity functions. This too makes for an extended test session with its attendant problems.

One possible means to alleviate these problems is to employ an adaptation of a standard psychophysical technique, flicker photometry. In this case the intensity of a flickering test stimulus is adjusted until it produces an ERG comparable with that produced by a similarly flickering reference light. The principal advantage of making spectral measurements in this fashion is that any changes that occur in the ERG over a test session will likely be equally reflected in the responses to both test and reference lights, and so the changes should have minimal influence on measured spectral sensitivity. Another advantage is that only a single measurement need be made at each test wavelength, thus minimizing the duration of the test session. Approximately a decade ago we were faced with the problem of having to make a large number of accurate spectral measurements in monkeys, and as a result we eventually adopted an ERG flicker-photometric technique.[3] Although others have utilized somewhat similar flicker-photometric procedures with the ERG,[4–8] we have perhaps accumulated more experience with this approach to the ERG in a wider variety of contexts than others. The purposes of this paper are to summarize the details for carrying out ERG flicker photometry as it has evolved in our hands, to point out some of the advantages of this technique, and to document some of the purposes to which it has been and may profitably be directed.

2. METHODS

A. Recording Techniques

ERG's are differentially recorded. As noted above, ERG flicker photometry is relatively immune to contaminating influences from fluctuations in ERG amplitude that might result from changes in electrode position or conductivity over the course of a recording session or similar variations from one session to the next. Consequently a variety of different electrode configurations have been used with

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little differences in success. One virtue of the procedure, then, is that choice of electrode can be dictated more by matters of availability and convenience rather than for any differences in recording quality. We have used Burian–Allen bipolar contact lens electrodes, silver-ring electrodes, and DTL fiber electrodes[9] with good results.

B. Stimuli

Most of the ERG flicker-photometric experiments have used a three-channel Maxwellian optical system to produce stimuli.[3] Two of the beams in this system provide the test and the reference lights, respectively. The third channel can be used to provide a source of steady adaptation. Lights from the three channels are superimposed to illuminate a circular portion of the retina subtending 57 deg. High-speed electromagnetic shutters control the presentation of the test and the reference lights. The intensity of the test light is controlled by a circular neutral-density wedge. The frequency of the stimulus, the intensity of the reference light, and the retinal adaptation state may be varied depending on the goal of the particular experiment. We illustrate the uses of some of these variations below. Application of this procedure is not limited to this particular means for stimulus generation. For example, ERG flicker photometry has been used successfully with stimuli generated by a video display.

C. Electroretinogram Signal Processing

We designed the signal-processing system to try to achieve a single goal, namely, to extract a signal that can be used to determine precisely the test-light intensity that will produce a response that matches in amplitude the response to a standard reference light. Thus the ideal signal-processing system for ERG flicker photometry would output a signal having the following characteristics: (1) When the test and the reference lights are identically effective, the processed ERG signal should be a perfectly flat null. (2) The response should be very sensitive to small changes in the intensity of the test light relative to the reference light. This feature is especially desirable in the vicinity of the null point. (3) The signal that results when the test light is more effective than the reference light should be the exact inverse of the signal that results when the reference light is more effective. Thus the sign of the response will indicate the direction of intensity change (i.e., increment or decrement) required for production of a null. The signal-processing scheme illustrated in Fig. 1 was developed to have, as nearly as possible, these characteristics.

The development of this processing scheme reflects a considerable amount of trial and error. For example, the top two rows of Fig. 1 illustrates that both the test and the reference lights are modulated with a 25% duty cycle. Thus, in the combined stimulus, the test and the reference light presentations are separated by a dark interval. This is different from traditional flicker photometry, in which the test and the reference lights are exactly substituted, with no intervening dark interval. In early explorations we found that the introduction of the dark interval, when combined with the subsequent processing steps outlined here, often resulted in much larger amplitude changes for small intensity changes near the null point and that this greatly enhanced the sensitivity of the technique. The reasons for the effect have not been systematically studied. However, as we illustrate below, the use of this particular stimulus sequencing is not a necessary condition for the successful use of the procedure; indeed, the stimuli for ERG flicker photometry can be made identical to those for behavioral flicker photometry, i.e., having a 50% duty cycle. Other steps in the processing sequence were similarly chosen because of perceived improvements in performance. These optimizations were done primarily in the context of recording from a variety of diurnal mammals (principally human and nonhuman primates and ground squirrels).

The ERG is first differentially amplified in two stages. The first amplifier is positioned near the recording electrode, and the second is within the signal-processing sys-

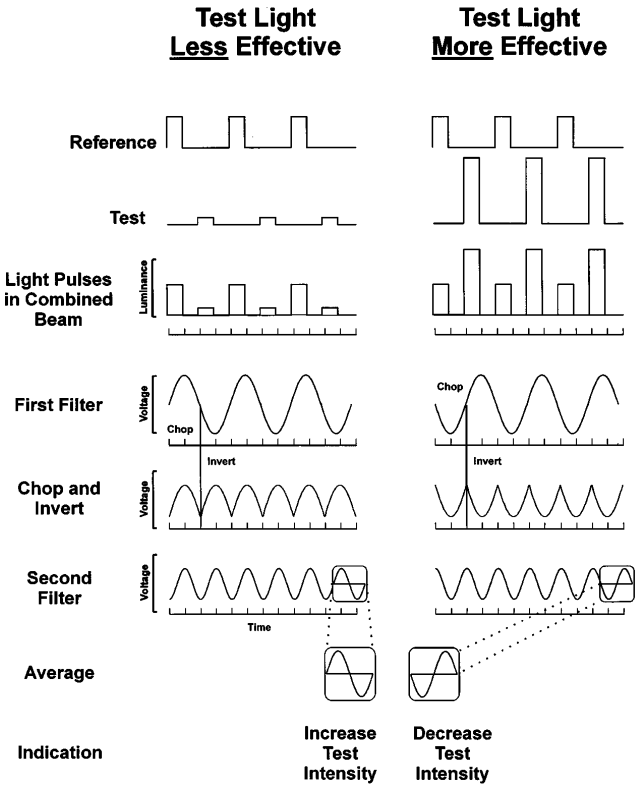

Fig. 1. Signal processing for ERG flicker photometry is schematized for two conditions. In the left-hand column the test light is less effective in producing an ERG signal than is the reference light; in the right-hand column the reverse holds. The time courses and the relative intensities of the test and the reference lights are illustrated individually and are then superimposed (as they are in the actual stimulus; rows 1–3). The next four rows illustrate the sequence of steps in signal processing. The signal is first passed through a narrow-bandpass filter. The signal is then chopped, and the output is inverted during each on-period of the test light and its subsequent off-period. The output is again filtered through a second narrow-bandpass filter. This filter is set to pass a frequency exactly twice that of the first filter. The waveforms illustrated correspond to the sine waves that are passed at the center frequency of each bandpass filter. The final processing stage averages over a period that corresponds to one cycle at the center frequency of the second filter. The final outputs for the two conditions are 180 deg out of phase. As indicated, the phase and the amplitude of the output are used to direct further changes in the test-light intensity.

tem. The signal-processing system is currently implemented as a custom-made, single plug-in board in an IBM-compatible personal computer. The amplified signal is processed through the four stages that are illustrated as rows 4–7 in Fig. 1. First, the signal is passed through an active narrow-bandpass filter (the half-voltage bandpass of this filter is 0.2 times the center frequency). The filter has a center frequency set equal to the individual modulation frequencies of the test and the reference lights. We note that if the output of this filter is directly averaged without further processing, the result is a system that performs reasonably well as a flicker photometer. However, the addition of the next two states (Fig. 1) appears to improve performance by giving better noise rejection, flatter nulls, and responses on each side of the null that are more antiphasic.

In the third stage (Fig 1, row 5) the signal is chopped, and the output during the test light and its dark afterperiod are inverted relative to that during the reference light presentation and its dark afterperiod. The signal is then filtered with a second active, narrow-bandpass filter. The bandpass of the second filter is the same as that of the first, but its center frequency is set to be exactly twice the center frequency of the first filter. The final output is averaged over an interval equal to the period of the sine wave passed by the second filter. To ensure that any transients in the response that occur at the onset of the stimulus pulses will not be included in the average, the initiation of signal averaging is delayed by a preset number of stimulus cycles (typically a minimum of 10). The phase of the final sine wave can be adjusted for each experiment. One can accomplish this by adjusting the phase of the stimulus relative to that of the chopping and averaging. The resulting ERG is an extremely noise-free, single-cycle, sinusoidal waveform. The sine wave reverses phase when the relative intensities of the test and the reference lights reverse. With iterative adjustment of the test light intensity, a null of minimum signal and intermediate phase can be found. This is taken as the point where test and reference lights are equally effective.

3. RESULTS AND DISCUSSION

A. Sensitivity

As in the analogous behavioral paradigm, the goal of ERG flicker photometry is to adjust test and reference lights to be equally effective. In this case the equation is completed when the intensity of the test light has been set such that the test and the reference lights produce equivalent ERG signals. Inasmuch as the intensity of the test light is continuously adjustable, these equations can typically be set with considerable precision. Based on our early experience with this procedure it was suggested that deviations from equation values in the amounts of 0.02–0.04 log unit could rather reliably be discerned.[3] On the basis of much more extensive experience, those values now seem to provide a rather good indication of the sensitivity of this technique. That point is illustrated in Fig. 2, which shows the average amplitudes of the ERG signal recorded in one experiment for a number of wedge-density settings in the vicinity of the photometric equation. We recorded the mean amplitudes at eight steps (taken at intervals of 0.05 log unit) that bracketed the equation value. Those shown by filled symbols to the left in the figure are for cases in which the test light was more effective than the reference light, whereas those indicated by open symbols to the right indicate the reverse. Subsequently, five photometric equation settings were also made. These equations settings were made blindly (i.e., without looking at the wedge values) from starting positions on the test beam wedge that were randomly determined. The total range of these five is given by the spacing between the two vertical lines at the center of the figure. Note that the changes in the wedge setting required for producing an ERG having an average amplitude of only $\sim 0.5\ \mu$V (produced by an offset from the equation value amounting to ~ 0.03 log unit) are well outside the total range of the photometric settings. Naturally the sensitivity of the procedure varies somewhat from experiment to experiment, but it has proved sufficiently consistent that, if on two repetitions the derived equation values differ by 0.05 log unit or more, that equation is repeated a third time under the assumption that the variability for the first two settings is atypical for ERG flicker photometry. On occasion we made ERG flicker-photometric measurements on human subjects who were then asked to set traditional flicker-photometric equations for the exact same set of stimuli (50% duty cycle in each case). The consistent result of these side-by-side comparisons is that the variability of the equations made with ERG flicker photometry are considerably smaller (perhaps only approximately one third as large) than the variability characteristic of settings made with behavioral flicker photometry.

A second advantage provided by the flicker-photometric ERG technique associated with its high sensitivity and the use of stringent signal filtering is that it allows one to

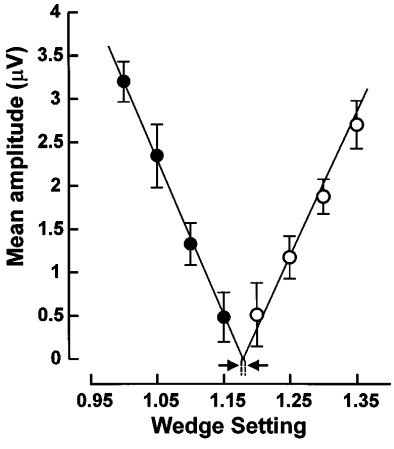

Fig. 2. Demonstration of the sensitivity of ERG flicker photometry. ERG amplitudes were measured for a number of wedge density settings in the vicinity of the position required for an equation to be attained. The latter is indicated by the two vertical lines at the bottom (see text). Each plotted value represents the mean obtained for 10 separate averages (error bars are ± 1 standard deviation). The filled symbols at the left are for cases in which the test light was relatively too bright; open symbols to the right are for settings at which the test light was relatively too dim. The recordings were those of cone signals from the eye of a Siberian hamster (*Phodopus sungorus*). Stimulus: 500 nm, 12.5 Hz.

work quite effectively under conditions that tend to yield small ERG signals. A number of species on which we have employed this technique have restricted populations of cones in their retinas, yet it has proved rather easy to record their signals reliably. For example, only ~0.1% of all the receptors in the rat retina contain UV-sensitive photopigment.[10] Despite this restricted representation, the signals that these receptors generate have proved to be more than sufficient to permit an examination of spectral sensitivity in the UV wavelengths.[11]

B. Reliability

Spectral sensitivity measurements made with ERG flicker photometry also turn out to have quite high reliability. On a number of occasions, repeated measurements were made on the same subjects over an extended period of time. An example is illustrated in Fig. 3, which summarizes spectral sensitivity data obtained from a human protanope in two separate test sessions. In this case the test dates were separated by 49 months. At the top of the figure is the average spectral sensitivity function obtained from the two determinations. Shown at the bottom are the wavelength-by-wavelength differences in the photometric equations from the two separate experiments. As can be seen , these differences are quite small (the mean difference in the two tests was 0.031 log unit) and unsystematic across wavelengths. A similar indication that the technique yields highly reliable results comes from an experiment involving measurement of spectral sensitivity in rats. In this case repeated measurements were made of a scotopic spectral sensitivity function. Each such measurement resulted in a best-fit spectral sensitivity function. The average total range of the peak sensitivity (λ_{MAX}) values for 10 separate determinations in 8 animals made over the duration of a 6-month experiment was 4.3 nm (standard deviation, 1.04). Clearly, ERG flicker photometry can provide very highly reliable measurements.

Experience suggests that ERG flicker photometry provides a technique of considerable versatility. Below we illustrate a variety of problems to which its application has proved useful. Many of these involve the examination of fundamental problems of spectral sensitivity and color vision.

C. Estimates of Middle- and Long-Wavelength Photopigment Spectra

As is the case for its psychophysical ancestor, ERG flicker photometry provides a standard method for assessing spectral sensitivity. In general, access to cone signals is achieved by use of higher flicker rates and brighter lights. Spectral sensitivity has been measured with this technique from a wide variety of different types of eye with many different end points in mind. The exact conditions required for effectively isolating cone signals depend on the species under study. For human subjects studied under conditions of light adaptation, stimulus frequencies of 20–25 Hz or higher are sufficient.[12] A frequent goal has been to assess the photopigment complement of the eye. In many cases it has proved possible to compare the ERG measurements with those obtained from other techniques and thus to establish the validity of the procedure. For instance, many New World monkeys show significant cone-pigment polymorphisms with four different classes of cone pigment represented in varying combinations among the individuals of a species, thus yielding both dichromatic and trichromatic phenotypes.[13] The spectra for these pigments have been inferred from spectral sensitivity measurements made with ERG flicker photometry in dichromatic animals.[14] Figure 4 shows the mean spectral sensitivity data obtained from 17 dichromatic monkeys, all of whom have the same long-wavelength-sensitive (L) cone pigment. The best-fit curve drawn

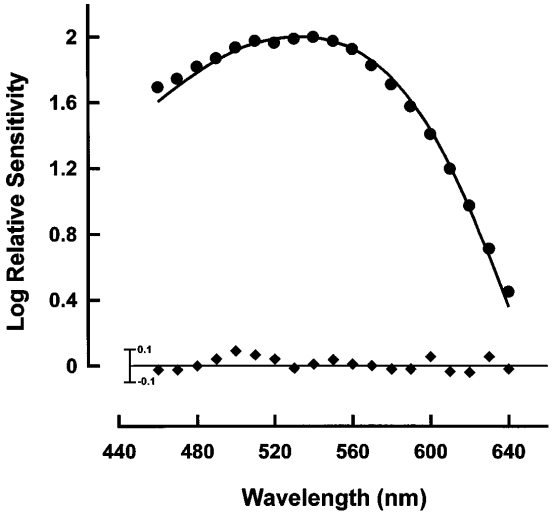

Fig. 3. ERG spectral sensitivity function recorded from a human protanope. The filled circles are mean values obtained from two test sessions that were separated by a period of ~4 years. The absolute difference in sensitivity for the two sessions at each test wavelength is plotted by the filled diamonds. The continuous curve is an absorption spectrum for a standard photopigment having a peak value of 530 nm. Stimulus: 31.25 Hz.

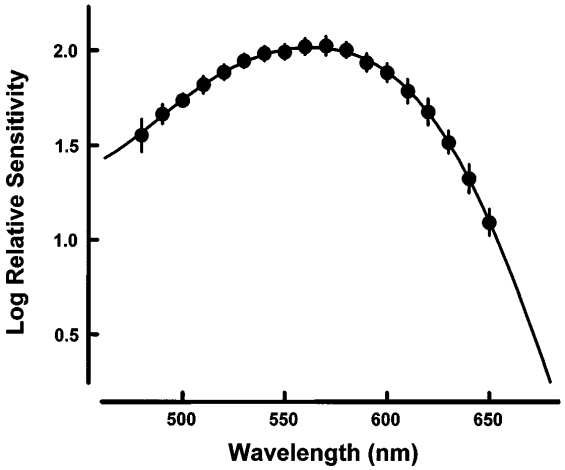

Fig. 4. Comparison of spectral sensitivity assessed with ERG flicker photometry and photopigment spectra. The filled circles are the mean sensitivity values for 17 dichromatic squirrel monkeys (*Saimiri sciureus*), each of which had the same type of L/M cone photopigment. Before the data were combined, the spectral sensitivity function for each animal was adjusted on the sensitivity axis so that each had the same average sensitivity. The vertical bars enclose ±2 standard deviations. The continuous curve is that for the best-fitting visual pigment absorption curve (λ_{MAX} = 560 nm). Stimulus: 31.25 Hz.

through the data points represents the absorption spectrum for a standard photopigment.[15] Here, as in other cases in which the recording is arranged to reflect the operation of a single spectral mechanism, the spectral sensitivity data are well accounted for by the assumption that they index directly the contribution of a single type of cone.

Direct photopigment measurements have been made with microspectrophotometry on the same animals, and thus it is possible to assess the validity of the ERG measurements.[16–18] The λ_{MAX} values obtained with the two approaches agree well, e.g., the L-cone photopigment of the squirrel monkey has a mean λ_{MAX} value of 563 nm from direct microspectrophotometry measurements, whereas the comparable value for ERG measurements (Fig. 4) is 560.6 (standard deviation, 1.5) nm. These values are not significantly different, and thus it is possible rather accurately to infer photopigment complements from ERG measurements. This is a substantial advantage because it allows such information to be noninvasively gathered from large numbers of subjects when those goals are desirable.[19]

How good is ERG flicker photometry as a technique for making subtle discriminations between individuals with differing spectral sensitivities? One example that speaks to this point comes from measurements of the pigment spectra in human deuteranopes. It has been established that two polymorphic versions of the L-cone opsin gene can yield two spectrally discrete versions of the L-cone pigment.[20,21] ERG spectra were obtained from four deuteranopic subjects later discovered to possess alternative forms of the L-cone opsin gene.[22] These spectra are shown in Fig. 5. Note that the spectra obtained from the two types are clearly discriminated in these measurements with an associated λ_{MAX} shift of ~5 nm. This suggests that ERG flicker photometry is quite efficient as a means of accurately and rapidly assessing small differences in photopigment complement.

D. Assessment of Short-Wavelength-Sensitive Cone Spectra

Signals from short-wavelength-sensitive (S) cones are more difficult to detect in gross-potential recordings than signals originating from other cone classes.[23,24] Flicker photometry provides a means of doing so, and earlier we showed that S-cone contributions can easily be discerned in recordings made from both nonhuman, e.g., Ref. 25, and human[26] eyes. The procedure in each case involves an optimization of the stimulus conditions for recording S-cone signals. This includes the use of concurrent chromatic adaptation to suppress sensitivity of middle-wavelength-sensitive- (M-) and L-cone signals and the use of a short-wavelength reference light in conjunction with a slower pulse frequency. All these features maximize S-cone contribution. Figure 6 illustrates the results from such an experiment. In this case we recorded spectral sensitivity from a human deuteranope by using a 12.5-Hz stimulus and concurrent, intense yellow adaptation. Under these conditions a substantial contribution from the S cone is revealed. The continuous curve in Fig. 6 represents the summative contributions to the ERG signal from the two cone types of the deuteranope. In a number of cases the spectral sensitivities of the S-cone spectra thus recorded correspond reasonably to spectral measurements obtained from more direct recordings, for example, from microspectrophotometry or single photoreceptor electrophysiology. Changes in the responses from S cones are a frequent concomitant of diseases affecting the human retina.[27–30] ERG flicker photometry provides a convenient means to index both S-cone presence and relative contribution.

E. Assessment of Rod Spectra

Under many of the standard recording paradigms signals from rods dominate the ERG.[1] Indeed, one motivation for perfecting ERG flicker photometry was to have a technique that would make it easily possible to record cone signals and avoid rod contributions. Nevertheless, there are many occasions in which access to rod signals is

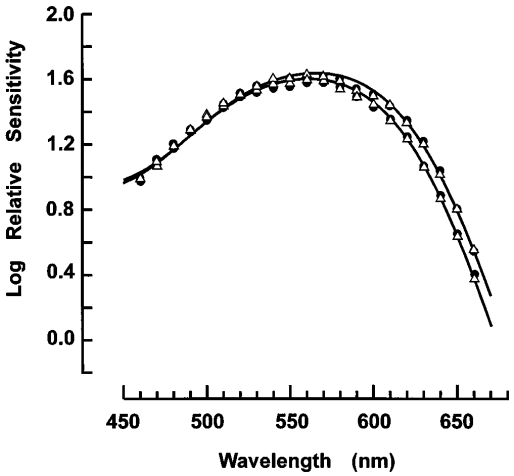

Fig. 5. Spectral sensitivity curves obtained with ERG flicker photometry from four deuteranopic subjects (the open triangles and the filled circles represent sensitivity of individual subjects). The L-cone pigments for the pair whose data are shifted slightly to the longer wavelengths differ from those of the other pair by an amino acid substitution at position 180 in their L-cone opsins. The continuous curves are photopigment absorption spectra having respective peaks of 558 and 563 nm.

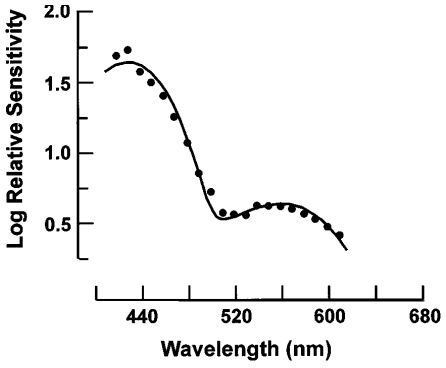

Fig. 6. S-cone signals recorded by ERG flicker photometry. The filled symbols show spectral sensitivity measured from a human deuteranope. The conditions were arranged to maximize the contribution of S-cone signals to the ERG (see text for description). The continuous curve is that for the best-fitting summative combination of two different photopigment absorption curves (λ_{MAX} values of 430 and 558 nm, respectively). Stimulus: 12.5 Hz.

necessary. This is easily accomplished with ERG flicker photometry. To do so, the stimulus rate is greatly reduced (e.g., to 2–4 Hz), the reference light is made much dimmer, and recording is carried out under conditions of dark adaptation. The several advantages of ERG flicker photometry remain intact under these test conditions. One of the principal advantages of flicker photometry noted above is that spectra may be recorded much more rapidly than with traditional techniques. This feature is particularly attractive in studies of rod signals by diurnal investigators who often find it unappealing to spend long periods of time in the dark. We have successfully recorded rod spectra from various primates, carnivores, ungulates, and rodents; see, e.g., Ref. 31. In many cases it has been possible to validate the results by comparing the ERG spectra with other measurements of rod pigments.

F. Signals from Multiple Cone Classes and Their Analysis

The cases above involve measurements made from dichromatic eyes for which one can constrain the ERG spectral measurements by adjusting conditions of chromatic adaptation and flicker rate to reflect principally the operation of only a single class of cone. Of course, in many cases of interest (e.g., most human eyes) there may be considerable spectral overlap among the photopigments present. In fact, it is frequently important to determine whether that is the case. ERG flicker photometry provides a tool for making such an assessment. One way to do this is to adapt a standard test for photopigment univariance. For example, to discriminate between dichromatic and trichromatic primates (human or nonhuman) we made an ERG flicker-photometric equation between a middle- and a long-wavelength light. The equations were made under conditions of achromatic adaptation and then, alternatively, as the eye was concurrently adapted to either a middle or a long-wavelength light. A consistent change in the equation value obtained under the two conditions of chromatic adaptation indicates a failure of univariance and the presence of more than one class of photopigment over the wavelengths spanned by the equation components. This procedure has been routinely employed to discriminate dichromats from trichromats. Further, the magnitude of the shift in the equation is correlated with the spectral separation of the underlying pigments. For instance, trichromatic squirrel monkeys whose L and M pigments are spectrally separated by ~ 25 nm show consistently larger adaptation effects than do those whose pigments are peak separated by an amount of half that size.[13] And in tests of human dichromats and trichromats the results from such an ERG test have consistently corresponded to conclusions drawn from traditional psychophysical measurements (plate tests, color matching, etc.).[32] As a diagnostic tool for sorting among the various varieties of trichromacy and dichromacy, ERG flicker photometry has proved very accurate.

The spectral sensitivity functions obtained from trichromats traditionally have provided opportunities both for drawing inferences about spectral mechanisms and for understanding their interactions. Here we illustrate two ways in which spectra obtained with ERG flicker photometry have contributed to these goals.

Measurements made with classical flicker photometry reveal large individual differences in spectral sensitivity among people with normal color vision.[33,34] These differences in spectral sensitivity have often been argued to reflect individual differences in the ratio of M- to L-cone populations in the human retina.[35] One specific hypothesis is that these differences in spectral sensitivity might be related to the highly variable ratios of M- and L-cone pigment opsin genes now know to characterize normal human trichromats.[36] To test this idea we recently measured ERG flicker-photometric spectral sensitivity functions for 10 young male subjects having normal color vision. The ERG spectral sensitivity curve of each subject could be accounted for by a theoretical curve derived by adding together the spectral sensitivities of individual M and L cones. The spectra of the M and L cones for each subject were inferred from an examination of the sequences of their M- and L-cone opsin genes. We used an iterative computer program to vary the relative weighting of these pigment sensitivities to find the linear sum of the two that best fit each of the spectral sensitivity curves. Across the 10 subjects the scaled weights of the cone spectra that best fit the spectral sensitivities varied in M to L ratio over the range from 1.6:1 to 1:7.3 (average ratio, 1:1.8). This average M:L ratio is similar to previous estimates; the range of variation accords with some, but not all, of the earlier studies.[35] The ratios of M- to L-photopigment genes had been previously determined for the same subjects,[36] and we found that higher proportions of M-pigment genes were significantly correlated (Spearman $r = 0.80$; $P = 0.007$) with larger M-cone contributions to spectral sensitivity. These results suggest that the ratio of M- to L-pigment genes plays a major role in controlling the ratio of the respective cone populations. Experiments like these require precise and reliable spectral measurements from substantial samples of naïve subjects. One of the strengths of ERG flicker photometry is that it requires no subject training and thus makes a much more efficient experiment involving large samples of naïve subjects.

The stimulus conditions employed for recording the ERG in the example just described tap signals that apparently contain purely summative combinations of signals originating from M and L cones. However, there is also abundant evidence to show that under certain test conditions various aspects of ERG signals can be best explained as indexing other than summative combinations of cone signals.[7,37–39] These combinations have frequently been modeled as representing a reflection of L/M cone opponency. Such features of the ERG are of interest because they provide an indication of the early stages of color processing, one that is obtainable through noninvasive recording. By appropriate choice of stimulus parameters, evidence for nonsummative combinations of L- and M-cone signals can easily be seen with ERG flicker photometry.[40] One such parameter is the duty cycle of the stimulus. Figure 7 shows two ERG spectral sensitivity functions obtained in a single session from a normal human trichromat. In both cases the stimulus rate was 20 Hz, but in one instance (filled triangles) a 25% duty cycle was employed, whereas in the other a 50% duty cycle was used (filled circles). The spectrum obtained with the shorter duty cycle is reasonably accounted for

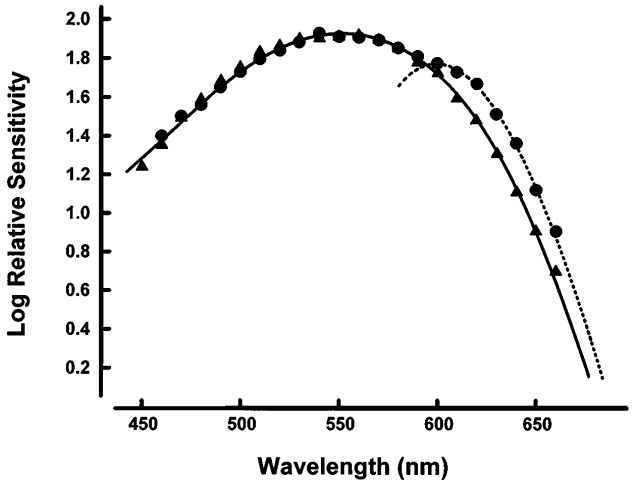

Fig. 7. Effects of duty cycle on spectral sensitivity measured with ERG flicker photometry. Two spectral sensitivity functions were recorded for a normal human trichromat under identical stimulus conditions (20-Hz pulse rate) except for the stimulus duty cycle, which was either 25% (triangles) or 50% (circles). The curve for the 25% duty-cycle condition was best fitted with a summative combination of L- and M-cone fundamentals (λ_{MAX} of 561 and 530 nm, respectively). The curve obtained with the 50% duty cycle is well fitted by summation for test wavelengths shorter than ~600 nm; beyond that point the function is well accounted for by subtractive combination (dashed curve) of the same two cone fundamentals.

by a weighted summation of signals from mechanisms having the spectral sensitivity of M and L cones. With the longer duty cycle, however, this procedure does not work. Rather, one can better account for spectral sensitivity recorded under these conditions by assuming that the ERG indexes both summative and subtractive combinations of the L- and M-cone signals. The continuous best-fit line drawn through the spectral sensitivity points reflects that fact. ERG flicker photometry thus provides a tool that may be used to examine interactions that occur between spectral mechanisms in the outer retina.

G. Extensions of the Electroretinogram Flicker-Photometric Technique

All the examples of the uses of ERG flicker photometry to this point involve stimuli traditionally employed to examine spectral mechanisms with the ERG. This technique, however, has considerable potential for use in other situations. Brief mention of a few examples follows. In one case ERG flicker photometry was used to see whether a striking regional anisotropy of different cone classes in the retina of the mouse that had been established anatomically could be shown to have functional consequences.[41] To accomplish this, ERG's were recorded in response to regional stimulation of the retina. In each region a flicker-photometric equation was made between two spectral stimuli that were selected to be, respectively, maximally effective for the two cone classes found in the mouse retina. These regional equations were compared with similar equations made with diffuse retinal illumination. From this set of equations, indication of the variation in contribution from the two classes of cone for various retinal regions was derived. The regional ERG variation showed good qualitative agreement with a map derived from direct cone counts. This result suggests that ERG flicker photometry might be usefully employed in cases in which it is important to sample regional activity from the retina.

ERG flicker photometry has been used in other situations involving other less-traditional ERG stimuli. For instance, it has proved possible to use the flicker-photometric paradigm in record ERG's by use of cone-isolating stimuli.[42] A color monitor is used to generate such stimuli. A number of different problems may be addressed. For example, the effectiveness of L-cone-isolating stimuli may be balanced against those M- or S-cone-isolating stimuli, against the effectiveness of pure-luminance modulation, or against any combinations of these. This should allow one to examine directly the details of interactions among cone signals in the ERG and the influence of a variety of important stimulus parameters, e.g., pulse frequency and adaptation state. We have also verified that the pattern electroretinogram may be examined in the same fashion. In each case, the advantages of flicker photometry can be exploited to increase sensitivity and reliability and to decrease the time required for a useful data set to be produced.

4. SUMMARY

ERG flicker photometry is a direct adaptation to the recording situation of a standard psychophysical technique. For many purposes in which a gross retinal potential can be exploited to provide useful information, particularly those that require measurements of spectral sensitivity, this technique seems superior to traditional ERG approaches that require measurements of ERG amplitudes or latencies. In more than a decade of using this technique we have encountered few situations in which ERG flicker photometry cannot be profitably applied. It seems likely to us that the potential range of application of ERG flicker photometry is considerably broader than its present use.

ACKNOWLEDGMENTS

We thank Jack Calderone, Michael Crognale, Jess Deegan II, and John Fenwick for many contributions to the development and successful application of ERG flicker photometry. This research was supported by National Eye Institute grants EY02052 and EY09303.

REFERENCES

1. J. C. Armington, *The Electroretinogram* (Academic, New York, 1974).
2. D. van Norren, "Electroretinographic aspects of colour vision deficiencies," in *Inherited and Acquired Colour Vision Deficiencies,* D. H. Foster, ed. (Macmillan, London, 1991), pp. 56–63.
3. J. Neitz and G. H. Jacobs, "Electroretinogram measurements of cone spectral sensitivity in dichromatic monkeys," J. Opt. Soc. Am. A **1,** 1175–1180 (1984).
4. C. R. Cavonius, "Color sensitive response in the human flicker-ERG," Doc. Ophthalmol. **18,** 101–113 (1964).
5. J. F. W. Nuboer, W. M. van Nuys, and J. F. Wortel, "Cone systems in the rabbit retina revealed by ERG null detection," J. Comp. Physiol. **151,** 347–352 (1983).
6. E. Brenner, J. P. Spaan, J. F. Wortel, and J. F. W. Nuboer, "Early color deprivation in the pigeon," Behav. Brain Res. **8,** 343–350 (1983).

7. Y. Chang, S. A. Burns, and M. R. Kreitz, "Red–green flicker photometry and nonlinearities in the flicker electroretinogram," J. Opt. Soc. Am. A **10,** 1413–1422 (1993).
8. R. M. Shapley and S. Brodie, "Responses of human ERG to rapid color exchange: implications for M/L cone ratio," Invest. Ophthalmol. Vis. Sci. **34,** 911 (1993).
9. W. W. Dawson, G. L. Trick, and C. A. Litzkow, "Improved electrode for electroretinography," Invest. Ophthalmol. Vis. Sci. **19,** 988–991 (1979).
10. A. Szel, T. Diamantstein, and P. Rohlich, "Identification of blue-sensitive cones in the mammalian retina by anti-visual pigment antibody," J. Comp. Neurol. **273,** 593–602 (1988).
11. J. F. Deegan II and G. H. Jacobs, "On the identity of the cone types of the rat retina," Exp. Eye Res. **56,** 375–377 (1993).
12. F. A. Abraham, M. Alpern, and D. B. Kirk, "Electroretinograms evoked by sinusoidal excitation of human cones," J. Physiol. (London) **363,** 135–150 (1985).
13. G. H. Jacobs, "Color-vision polymorphisms in New World monkeys: implications for the evolution of primate trichromacy," in *New World Primates: Ecology, Evolution and Behavior,* W. G. Kinzey, ed. (de Gruyter, New York, 1996).
14. G. H. Jacobs and J. Neitz, "Inheritance of color vision in a New World monkey (*Saimiri sciureus*)," Proc. Natl. Acad. Sci. (USA) **84,** 2545–2549 (1987).
15. S. M. Dawis, "Polynomial expressions of pigment nomograms," Vision Res. **21,** 1427–1430 (1981).
16. J. D. Mollon, J. K. Bowmaker, and G. H. Jacobs, "Variations of colour vision in a New World primate can be explained by polymorphism of retinal photopigments," Proc. R. Soc. London Ser. B **222,** 373–399 (1984).
17. J. K. Bowmaker, G. H. Jacobs, D. H. Spiegelhalter, and J. D. Mollon, "Two types of trichromatic squirrel monkey share a pigment in the red–green spectral region," Vision Res. **25,** 1937–1946 (1985).
18. J. K. Bowmaker, G. H. Jacobs, and J. D. Mollon, "Polymorphism of photopigments in the squirrel monkey: a sixth phenotype," Proc. R. Soc. London Ser. B **231,** 383–390 (1987).
19. G. H. Jacobs, J. Neitz, and M. Neitz, "Genetic basis of polymorphism in the color vision of platyrrhine monkeys," Vision Res. **33,** 269–274 (1993).
20. S. L. Merbs and J. Nathans, "Role of hydroxyl-bearing amino acids in differentially tuning the absorption spectra of the human red and green cone pigments," Photochem. Photobiol. **58,** 706–710 (1993).
21. A. B. Asenjo, J. Rim, and D. D. Oprian, "Molecular determinants of human red/green color discrimination," Neuron **12,** 1131–1138 (1994).
22. M. Neitz, J. Neitz, and G. H. Jacobs, "Genetic basis of photopigment variations in human dichromats," Vision Res. **35,** 2095–2103 (1995).
23. M. Sawusch, J. Pokorny, and V. C. Smith, "Clinical electroretinography for short wavelength sensitive cones," Invest. Ophthalmol. Vis. Sci. **28,** 966–974 (1987).
24. P. Gouras, C. J. MacKay, and S. Yamamoto, "The human S-cone electroretinogram and its variation among subjects with and without L- and M-cone function." Invest. Ophthalmol. Vis. Sci. **34,** 2437–2442 (1993).
25. G. H. Jacobs, J. Neitz, and M. Crognale, "Spectral sensitivity of ground squirrel cones measured with ERG flicker photometry," J. Comp. Physiol. A **156,** 503–509 (1985).
26. M. Crognale, G. H. Jacobs, and J. Neitz, "Flicker photometric ERG measurements of short wavelength sensitive cones," Doc. Ophthalmol. Proc. Ser. **10,** 341–346 (1991).
27. V. C. Greenstein, D. C. Hood, and R. E. Carr, "A comparison of S cone pathway sensitivity loss in patients with diabetes and retinitis pigmentosa," in *Colour Vision Deficiencies IX,* B. Drum and G. Verriest, eds. (Kluwer, Dordrecht, The Netherlands, 1989), pp. 233–241.
28. H. Krastel and J. D. Moreland, "Colour vision deficiencies in ophthalmic disease," in *Inherited and Acquired Colour Vision Deficiencies,* D. H. Foster, ed. (Macmillan, London, 1991), pp. 115–172.
29. W. H. Swanson, H. Fellman, J. R. Lyon, and R. J. Starita, "S-cone contrast sensitivity in glaucoma as a function of mean luminance," in *Colour Vision Deficiencies XII,* B. Drum, ed. (Kluwer, Dordrecht, The Netherlands, 1994), pp. 63–71.
30. M. A. Crognale, E. Switkes, J. Rabin, M. E. Schneck, G. Haegerstrom-Portnoy, and A. J. Adams, "Objective assessment of short wavelength sensitive (SWS) mechanisms with the spatiochromatic VEP: X-linked achromatopsia and transient tritanopia," in *Colour Vision Deficiencies XIII,* B. Drum, ed. (Kluwer, Dordrecht, The Netherlands, 1994), pp. 407–413.
31. G. H. Jacobs, J. F. Deegan II, M. A. Crognale, and J. A. Fenwick, "Photopigments of dogs and foxes and their implications for canid vision," Vis. Neurosci. **10,** 173–180 (1993).
32. G. H. Jacobs and J. Neitz, "ERG flicker photometric evaluation of spectral sensitivity in protanopes and protanomalous trichromats," in *Colour Vision Deficiencies XI*, B. Drum, ed. (Kluwer, Dordrecht, The Netherlands, 1993), pp. 25–31.
33. C. M. Cicerone and J. M. Nerger, "The relative numbers of long-wavelength-sensitive to middle-wavelength-sensitive cones in the human fovea centralis," Vision Res. **29,** 115–128 (1989).
34. M. F. Wesner, J. Pokorny, V. C. Smith, and S. K. Shevell, "Foveal cone detection statistics in color normals and dichromats," Vision Res. **31,** 1021–1037 (1991).
35. G. H. Jacobs and J. Neitz, "Electrophysiological estimates of individual variation in the L/M cone ratio," in *Colour Vision Deficiencies XI,* B. Drum, ed. (Kluwer, Dordrecht, The Netherlands, 1993), pp. 107–112.
36. M. Neitz and J. Neitz, "Numbers and ratios of visual pigments genes for normal red–green color vision," Science **267,** 1013–1016 (1995).
37. W. J. Donovan and W. S. Baron, "Identification of the R-G-cone difference signal in the corneal electroretinogram of the primate," J. Opt. Soc. Am. **72,** 1014–1020 (1982).
38. S. L. Mills and H. G. Sperling, "Red/green opponency in the rhesus macaque ERG spectral sensitivity is reduced by bicuculline," Vis. Neurosci. **5,** 217–221 (1990).
39. W. Spileers, F. Reis-Falcao, C. Hogg, and G. B. Arden, "Evidence from human electroretinogram A and off responses that color processing occurs in the cones," Invest. Ophthalmol. Vis. Sci. **34,** 2079–2091 (1993).
40. G. H. Jacobs and J. B. Calderone, "Contributions from cone mechanisms to the flicker ERG," Invest. Ophthalmol. Vis. Sci. **35,** 2045 (1994).
41. J. B. Calderone and G. H. Jacobs, "Regional variations in the relative sensitivity to UV light in the mouse retina," Vis. Neurosci. **12,** 463–468 (1995).
42. D. H. Brainard, J. B. Calderone, and G. H. Jacobs, "Contrast flicker ERG responses to cone-isolating stimuli," Soc. Neurosci. Abstr. **21,** 1644 (1995).