

# Rod Electrophoretograms Elicited by Silent Substitution Stimuli from the Light-Adapted Human Eye

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**Purpose:** To demonstrate that silent substitution stimuli can be used to generate electroretinograms (ERGs) that effectively isolate rod photoreceptor function in humans without the need for dark adaptation, and that this approach constitutes a viable alternative to current clinical standard testing protocols.

**Methods:** Rod-isolating and non-isolating sinusoidal flicker stimuli were generated on a 4 primary light-emitting diode (LED) Ganzfeld stimulator to elicit ERGs from participants with normal and compromised rod function who had not undergone dark-adaptation. Responses were subjected to Fourier analysis, and the amplitude and phase of the fundamental were used to examine temporal frequency and retinal illuminance response characteristics.

**Results:** Electroretinograms elicited by rod-isolating silent substitution stimuli exhibit low-pass temporal frequency response characteristics with an upper response limit of 30 Hz. Responses are optimal between 5 and 8 Hz and between 10 and 100 photopic trolands (Td). There is a significant correlation between the response amplitudes obtained with the silent substitution method and current standard clinical protocols. Analysis of signal-to-noise ratios reveals significant differences between subjects with normal and compromised rod function.

**Conclusions:** Silent substitution provides an effective method for the isolation of human rod photoreceptor function in subjects with normal as well as compromised rod function when stimuli are used within appropriate parameter ranges.

**Translational Relevance:** This method of generating rod-mediated ERGs can be achieved without time-consuming periods of dark adaptation, provides improved isolation of rod- from cone-based activity, and will lead to the development of faster clinical electrophysiologic testing protocols with improved selectivity.

## Introduction

The flash electroretinogram (ERG) is an electrical response elicited from the retina in response to stimulation by light. The ERG is generated by contributions from many different retinal cells types, but with appropriate manipulation of the temporal, chromatic, and luminance characteristics of the stimulus, as well as the subject's adaptational state, it is possible to selectively stimulate and assess the

functional characteristics of discrete populations of retinal neurons.<sup>1,2</sup> In particular, the isolation of rod photoreceptor activity has long been considered important from a clinical perspective as many congenital and acquired visual disorders can differentially affect rod relative to cone function. The ability to elicit ERGs that selectively reflect the activity of rods has had a key role in the diagnosis and monitoring of conditions, such as retinitis pigmentosa, congenital stationary night blindness (CSNB), and vitamin A deficiency.<sup>3-7</sup> In age-related

macular degeneration (ARMD), some of the earliest pathological and functional changes occur in rod-mediated vision in geographically localized regions of the retina.<sup>8</sup> In addition, it has been shown that normal younger individuals who carry a high genetic risk of ARMD developing in later life exhibit subtle changes in rod-mediated mesopic vision.<sup>9</sup> Thus, there are compelling clinical reasons for methods that selectively assess rod function in humans.

The most frequently employed method of isolating rod function has centered on the use of stimuli of low light intensity after rod sensitivity has been maximized by a 20- to 30-minute period of dark adaptation.<sup>10</sup> An alternative, but less frequently used, means of isolating ERGs from rods involves the method of silent substitution,<sup>11,12</sup> which is based on the principle of univariance.<sup>13</sup> The isolation of rod photoreceptor activity requires alternation between two stimuli which contain mixtures of wavelengths at different intensities. The alternation elicits no overall change in excitation in the L-, M-, and S-cone classes, but does elicit a change in rod excitation. The basic rule is that the isolation of 1 of  $n$  classes of photoreceptor requires a minimum of  $n$  primaries tuned to different wavelengths. Theoretically, any desired combination of photoreceptor excitation modulation can be achieved without changing the state of adaptation, a major advantage of this approach. With the increased commercial availability of light-emitting diode (LED) Ganzfeld stimulators containing at least 4 primaries, researchers now have the prospect of more precise control of ERG stimuli. This improved precision, coupled with our knowledge of cone and rod spectral characteristics, enables better control of photoreceptor excitation.<sup>14</sup> Stimuli based on the silent substitution method already have been applied in previous ERG studies of rod function.<sup>15–19</sup> However, despite the obvious advantages afforded by silent substitution, there is a clear need to demonstrate that the ERGs elicited by such rod-isolating stimuli do, in fact, selectively reflect rod function and are free from intrusions from cone photoreceptors, which normally predominate at higher mesopic and photopic light levels in the light-adapted human retina.<sup>20,21</sup>

The aim of this study was to demonstrate that stimuli generated using the silent substitution method enable the functional assessment of rods without the confounding effects of cone intrusion. Specifically, we examined ERGs obtained using rod-isolating stimuli in terms of temporal frequency and luminance characteristics. Rod vision has a lower temporal

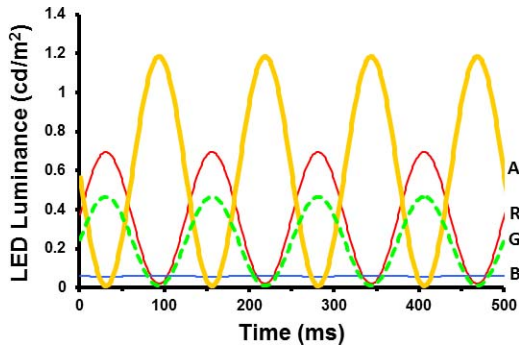
resolution limit than that mediated by cones.<sup>22–28</sup> At high scotopic levels of illumination rod temporal resolution can reach up to 28 Hz.<sup>26–28</sup> Cones, by comparison, can support a temporal resolution limit in excess of 60 Hz<sup>28</sup> and previous work has demonstrated that the cone flicker ERG can be recorded at frequencies up to 100 Hz in visually normal subjects.<sup>29</sup> Therefore, we wanted to exploit this difference to test the selectivity of our rod-isolating stimuli. We also assessed retinal illuminance response characteristics. Rod ERGs typically are measured using low intensity stimuli at scotopic levels of illumination.<sup>30–34</sup> This allows the study of rod responses free from the cone intrusions, which become increasingly more predominant as the stimuli increase to mesopic and photopic light levels.<sup>20,21</sup> However, in the light-adapted eye, stimuli of higher intensity must be used and this will require caution as we have to ensure rod selectivity is maintained and that cone intrusions are minimized. Measuring the ERG response as a function of retinal illuminance will help us to gauge the extent of such intrusions.

By examination of the temporal and retinal illuminance response characteristics we assessed the suitability of ERGs generated by silent substitution for the assessment of rod function in humans. In doing so, we attempted to define stimulus conditions for which rod responses can be optimized and identify parameter ranges beyond which the effects of cone intrusion can be demonstrated. Overall, this approach will lead to the development of better clinical testing protocols for the acquisition of rod-mediated ERGs for which there will be improved selectivity and reduced clinical testing times. To demonstrate this and assess the wider clinical applicability of our light-adapted silent substitution protocol, a second aim of this study was to compare the results to existing International Society for Clinical Electrophysiology of Vision (ISCEV) standard protocols in patient groups with normal and compromised rod function.

## Methods

### Stimuli

Sinusoidal, full-field flicker stimuli with temporal frequencies ranging between 5 and 100 Hz were presented using a ColorDome (Diagnosis LLC, Lowell, MA) four primary Ganzfeld stimulator with blue (460 nm), green (514 nm), amber (592 nm), and red (632 nm) LEDs. The spectral characteristics, chromaticities, and luminances of each class of LED



**Figure 1.** The luminance profiles and relative phases of the *blue* (B), *green* (G), *amber* (A) and *red* (R) LEDs that are required to generate a 63 Td, 8 Hz rod-isolating silent substitution stimulus. The modulation of rod excitation for the resultant stimulus = 0.25.

were measured and calibrated using a PR650 spectrophotometer (Photo Research, Inc., Chatsworth, CA). To obtain silent substitution stimuli photoreceptor excitations were calculated by multiplying the emission spectra of the LEDs with cone fundamentals and the  $V'_\lambda$   $10^\circ$  function.<sup>35,36</sup> (see Appendix 1). Wavelength and intensity combinations produced no changes in net excitation in three of the four photoreceptor populations; thus, these were triple silent substitution stimuli.<sup>11,12,37,38</sup> Figure 1 shows how the luminance outputs of the four LEDs vary as a function of time to produce a silent substitution rod-isolating stimulus (8 Hz, 63 Td). Contrast was defined as the Michelson contrast of rod excitation and was set at 0.25 for all stimuli. Retinal illuminance varied between 1 and 12,000 photopic trolands (Td). We have used photopic as opposed to scotopic Tds throughout the study, since it would be arbitrary to change units when going from high to low stimulus intensities and also would confuse the examination of ERGs across mesopic-photopic illumination transitions. For the stimulus set used in this study, conversion from photopic to scotopic Tds is achieved by multiplying by a factor of 2.489.<sup>36</sup>

We also used L-cone isolating stimuli ( $C = 0.25$ ) to compare ERGs mediated by the two photoreceptor populations. In addition, we also generated non-selective, non-isolating stimuli that elicited simultaneous excitation of rods and cones. These stimuli were generated by the same method described in Appendix 1, except that L- and M-cone modulations ( $C = 0.3$ ) were added to the standard rod-isolating stimulus. Another non-selective “white” stimulus also was used and this was generated by modulating all of the LEDs in phase, the resultant stimulus produced the same excitation (0.25) across all four photoreceptors.

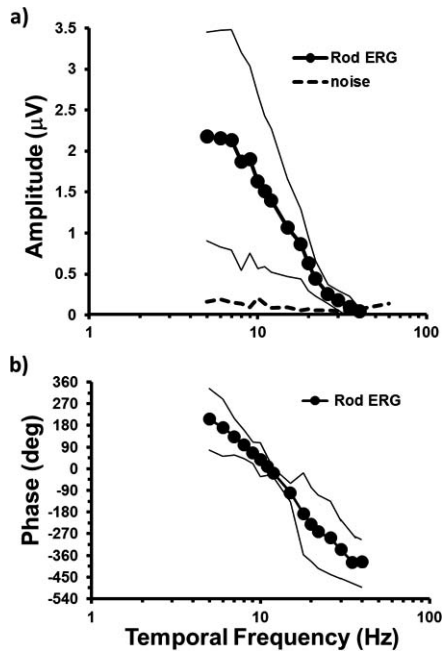
## ERG Recording

Electroretinograms were recorded from the right eye using a silver/nylon corneal fiber electrode (Department of Physics and Clinical Engineering, Royal Liverpool University Hospital, UK) referenced to a 9-mm Ag/AgCl electrode (Biosense Medical, Chelmsford, UK) on the outer canthus; a similar electrode was affixed to the forehead to serve as ground. Impedance was maintained below 5 k $\Omega$ . Signals were recorded using the Espion E<sup>2</sup> system (Diagnosys LLC) which amplified and filtered (bandwidth = 1 to 300 Hz) the ERGs and digitized them at a rate of 1000 Hz. Retinal responses to the flicker stimuli were acquired over 4-second epochs with subsequent offline analysis being performed on an average of a minimum of 8 of these epochs. Participants viewed the stimuli monocularly and fixation was maintained on a central point which subtended approximately  $0.5^\circ$ . Participants underwent pupillary dilation (1% tropicamide); the mean (dilated) pupil diameter across the 7 subjects was 8 mm (SD = 1.78), and this value was used in the computation of retinal illuminance. Before each recording session they sat in the testing room, which had an illumination level of 500 lux, for 5 minutes.

## Data Analysis

Following acquisition, the averaged traces were subjected to a two-stage offline analysis involving, firstly, resampling of the traces and then, secondly, subjecting these resampled traces to Fourier analysis. The first stage was necessary because the Espion system samples at 1000 Hz producing 4000 points over the recording epoch. To perform a Fast Fourier Transform (FFT)  $2^n$  data points (where  $n =$  integer value) are required, so a method of interpolation was used to resample the averaged traces to give 4096 data points. The resampled traces then were imported into Signal software (version 2.16; Cambridge Electronic Design, Cambridge, UK) and subjected to a FFT. This analysis provided a measure of the amplitude and phase of the response at the stimulation frequency (i.e., the fundamental, F) as well as higher harmonics (2F). The phase values generated by the FFT can provide values that cycle in multiples of  $360^\circ$ . To “unwrap” these phase values we either added or subtracted multiples of  $2\pi$  radians ( $360^\circ$ ) to minimize the phase differences measured between the adjacent sampled temporal frequencies.<sup>38</sup> Noise (N) was defined as the mean amplitude (A) of the response at the stimulus frequency minus 1 Hz and





**Figure 2.** Electretinogram (ERG) amplitude (a) and phase (b) of the fundamental component as function of temporal frequency obtained for a 63 Td rod-isolating silent substitution stimulus ( $C = 0.25$ ). The data shown are the group ( $n = 5$ ) averaged results and the *thin solid lines* represent  $\pm 1$  SD from the mean. The *thick dashed line* (a) plots the measure of noise (see Methods).

plus 1 Hz:

$$N = \frac{A_{(F-1\text{Hz})} + A_{(F+1\text{Hz})}}{2} \quad (1)$$

A response was considered significant if the measured ERG amplitude was a least 2.82 times greater than the computed noise amplitude for that frequency.<sup>39</sup>

## Participants

In the first part of this study where the objectives were to characterize the response properties of the rod-mediated ERGs and optimize stimulus parameters, a total of 7 color normal trichromats (3 males; mean age, 28 years; age range, 35 years) were used. Color vision in all subjects was assessed using the City University Colour Vision Test (second edition), the Farnsworth Munsell 100 Hue test, and the HMC Anomaloscope (Oculus, Wetzlar, Germany). In the second part of the study we wanted to assess the wider clinical application of our techniques. As well as measuring responses to our 8 Hz 63 Td rod-isolating stimulus, we also measured rod- and cone (30 Hz) ERGs using standard ISCEV clinical protocols<sup>10</sup> in a cohort of 28 subjects (17 females; mean age, 36.8

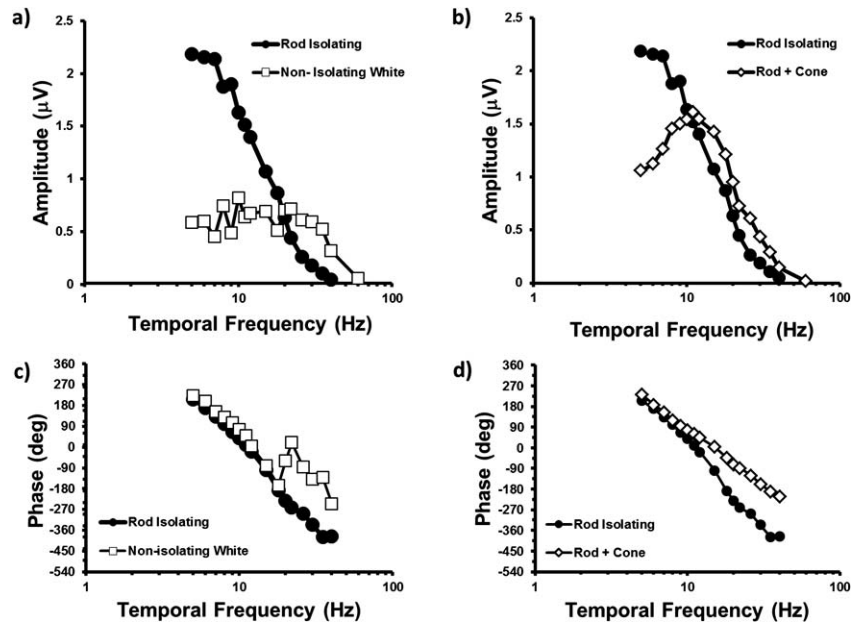
years; age range, 52 years), 18 of whom had normal rod function and 10 of whom had compromised rod function (6 diagnosed with rod or rod/cone dystrophy, 2 with CSNB type 1, 2 with CSNB type 2). All participants gave informed consent before the commencement of the experiments, which were conducted in accordance with the Declaration of Helsinki and were approved by the University of Bradford Ethics Committee.

## Results

### Temporal Frequency Response Characteristics

Figure 2 shows the variation in ERG amplitude and phase as a function of temporal frequency for a 63 Td rod-isolating stimulus. The data shown are the group (vector) averaged responses ( $n = 5$ ) and were obtained without dark adaptation. These results are similar to previous studies.<sup>29</sup> The data describe a low-pass temporal function and, consistent with psychophysically obtained estimates of the temporal resolution limit of rods,<sup>26</sup> the ERG amplitude relative to noise falls below significance between 26 and 30 Hz.

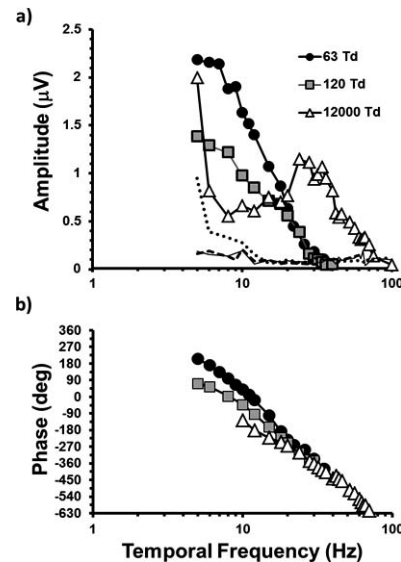
We compared the temporal frequency response functions obtained using silent substitution rod-isolating stimuli with those using non-isolating stimuli. Figure 3 shows the amplitude (Fig. 3a) and phase (Fig. 3c) of the ERG response fundamental obtained using a dim (63 Td) white light stimulus. Compared to those generated by the rod-isolating stimuli (also shown in Fig. 3) the temporal response functions elicited by the non-isolating stimuli are very different. In terms of amplitude, ERGs elicited by the dim white stimulus are reduced at low temporal frequencies ( $< 15$  Hz) while at frequencies normally considered beyond the range of rod photoreceptors ( $> 30$  Hz) responses still are obtainable well above noise levels. The phase plots show that beyond 18 Hz there is a discontinuity in response which is likely to reflect contributions from other (presumably cone-based) mechanisms at higher temporal frequencies. Figures 3b and 3d show the temporal response functions obtained from rod-isolating stimuli to which we have intentionally added L-cone modulation ( $C = 0.30$ ), via manipulation of the luminance outputs and relative phases of the four LEDs. The addition of cone modulation to the erstwhile rod-isolating stimulus again has characteristic effects on the amplitude and phase responses as a function of temporal frequency. In terms of amplitude, the



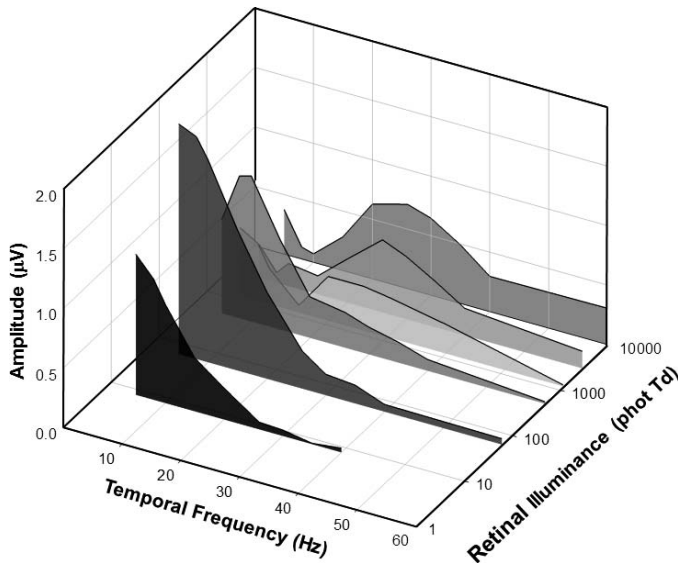
**Figure 3.** Left: Comparison between the variations in ERG (fundamental) amplitude (a) and phase (c) as function of temporal frequency obtained for a 63 Td rod-isolating (filled circles) and a 63 Td non-isolating white stimulus (empty squares). Right: Comparison between the variations in ERG (fundamental) amplitude (b) and phase (c) as function of temporal frequency obtained for a 63Td rod-isolating (filled circles) and a 63 Td stimulus which modulates rods (0.25) and L-cones (0.30; empty diamonds).

addition of cone modulation generates a more band-pass temporal response function, compared to the low-pass function obtained using a purely rod-isolating stimuli, with response amplitude being markedly reduced at low temporal frequencies. Similar reductions for combined rod/cone stimuli have been noted previously<sup>19</sup> and have been attributed to destructive interference between signals emanating from the different photoreceptor populations. The addition of cone modulation leads to a gradual phase advance of the response relative to that of the isolated rod response at higher temporal frequencies.

To eliminate intrusions from cones, rod ERGs typically have been elicited using low intensity scotopic stimuli.<sup>32</sup> To what extent does the use of silent substitution stimuli free the experimenter from this constraint? Figure 4 shows the amplitude and phase variation of the ERG fundamental as a function of temporal frequency obtained using rod-isolating stimuli with retinal illuminances extending well into the photopic range. Increasing the retinal illuminance of the stimulus up to 120 Td decreases the response amplitude at low temporal frequencies but the limit of temporal response is similar to that obtained at 63 Td as signal amplitude relative to noise falls below significance between 26 and 30 Hz. When stimulus illuminance is increased to high photopic



**Figure 4.** ERG temporal response functions for (a) amplitude and (b) phase of the fundamental obtained using rod-isolating stimuli at retinal illuminances equal to 63, 120, and 12,000 Td. The data represent the group vector average ( $n = 5$ ). The thick dashed line, solid thin line, and dotted line represent the noise for 63, 120, and 12,000 Td conditions, respectively, and phase is plotted only for temporal frequencies where signal was  $2.82\times$  greater than noise.

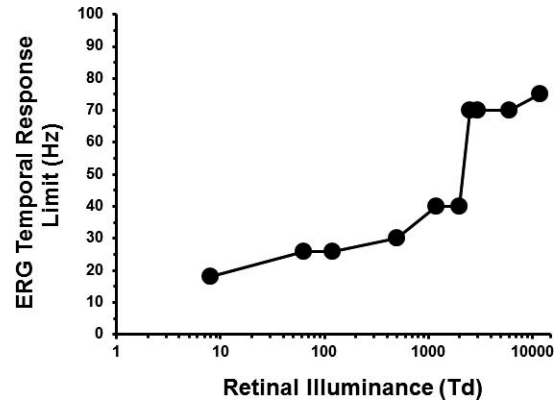


**Figure 5.** ERG (fundamental) temporal response functions obtained using rod-isolating stimuli at retinal illuminances: 8, 63, 500, 1200, 3000, and 10,000 photopic Td. The data represent vector averaged group ( $n = 4$ ) responses.

illumination levels (12,000 Td) the temporal response function of the ERG takes on a very different form and becomes more band-pass in appearance, peaking at approximately 30 Hz. In addition the temporal response limit extends to higher frequencies. The function in effect becomes more like the cone temporal response function<sup>29,30</sup> and clearly indicates a loss of rod selectivity in the ERG at these light levels.

To examine more closely the stimulus intensity range over which the transition from rod- to cone-like temporal frequency response characteristic occurred, we measured a series of temporal response curves in four subjects. These were sampled less frequently in the temporal domain compared to the previous experiment, but used a larger range of retinal illuminances (8, 63, 500, 1200, 3000, and 10,000 photopic Td). The results are shown as a three-dimensional plot in [Figure 5](#). The temporal functions generated by the stimuli of lower (8, 63, and 500 Td) and higher (1200, 3000, and 10,000 Td) illuminance are qualitatively very different; the former are low-pass in nature contrasting with the latter, which have more band-pass shape where responses still can be obtained for frequencies greater than 30 Hz.

In [Figure 6](#), a measure of the temporal response limit of the ERG is plotted as a function of stimulus illuminance. This value was computed as the temporal frequency at which signal amplitude fell below the



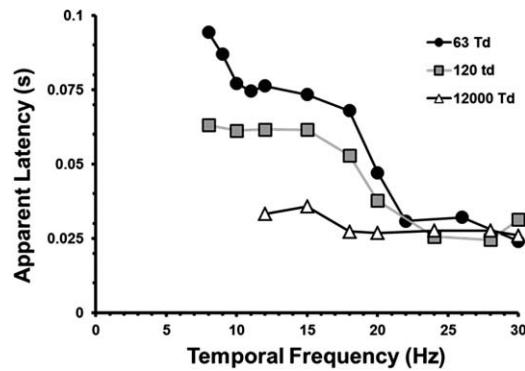
**Figure 6.** The temporal response limit of the ERG elicited by a rod-isolating stimulus as a function of retinal illuminance (see text for calculation).

criterion for significance (i.e.,  $2.82 \times$  noise amplitude). These temporal response functions were obtained for a single subject using rod-isolating stimuli ranging from 8 up to 12,000 Td and the ERG temporal response limit was calculated for each condition. The resultant plot shows that, at high illuminance levels ( $>2000$  Td), the temporal response limit of the ERG is in excess of 60 Hz—a level that is incompatible with rod function but is more in keeping with the properties of cone photoreceptors.<sup>33,34</sup> Below 1000 Td the temporal response limit falls to 20 to 30 Hz, a value that is consistent with psychophysical measures of rod temporal properties obtained at higher scotopic illumination levels.<sup>26–28</sup>

The phase data plotted in [Figure 4b](#) for the 63, 120, and 12,000 Td stimuli are useful as they can provide information about the temporal characteristics of the neuronal mechanisms that underpin the generation of the response. Specifically, the slope of the function provides a measure of what is known as apparent latency and provides a measure of response delay.<sup>40,41</sup> Apparent latency ( $\tau$ ) is given by:

$$\tau = -\frac{1}{360} \times \left( \frac{\Delta\phi}{\Delta ft} \right) \quad (2)$$

where  $\phi$  is response phase and  $ft$  the temporal frequency of the stimulation. In [Figure 7](#), the apparent latency is plotted as a function of temporal frequency for the 63, 1200, and 12,000 Td “rod-isolating” stimuli. Each data point is calculated from the slope of a linear regression line fitted to 5 adjacent data points on the phase versus linear temporal frequency function. Constant values of apparent latency imply that physiologic mechanisms that underpin the generation of the response have similar

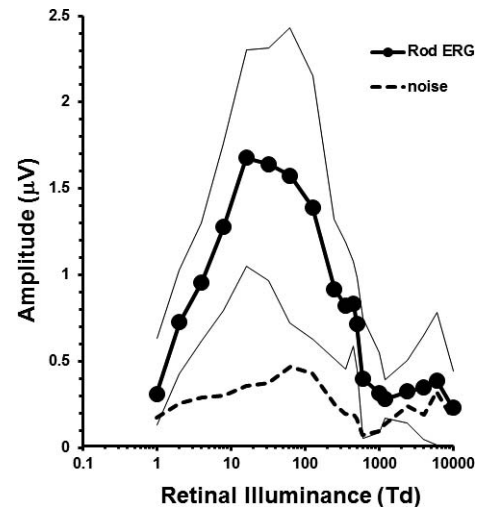


**Figure 7.** Apparent latency plotted as a function of temporal frequency for ERGs elicited by rod-isolating stimuli of retinal illuminance equal to 63, 1200, and 12,000 Td.

temporal response properties.<sup>29</sup> In this respect, the function derived from the 12,000 Td phase data is the simplest in that a relatively constant value for apparent latency is returned across the temporal frequency range tested. This suggests that a common mechanism with a short response delay (i.e., fast temporal response characteristics) underpins the ERG response to this stimulus. In view of the temporal response functions shown above for such high intensity stimuli, it would seem likely that cone-based mechanisms are the most likely generators of these responses. For the 120 and 63 Td stimuli, the apparent latency functions have two regions where a constant value is returned; the first is between 22 and 30 Hz where apparent latency values converge on a value similar to ERGs elicited by the 12,000 Td stimuli, implying that they are generated by mechanisms with common temporal response properties. A second constant region, indicating a different temporal mechanism, is found between 10 and 15 Hz. Between the two lies a transitional region (15–22 Hz) where the function has a non-zero slope. For the 63 Td stimuli, there also is an additional transitional region below 8 Hz which points to the possible existence of a third even slower mechanism (i.e., with increased apparent latency) that operates across low stimulus intensities and low temporal frequencies. The existence of multiple mechanisms with different temporal properties that contribute to the generation of rod-mediated ERG is consistent with previous studies.<sup>26,28,31,42–44</sup>

### Retinal Illuminance Response Characteristics

**Figure 8** shows the group averaged data where ERG amplitude measured with an 8 Hz rod-isolating stimulus is plotted as a function of retinal illumi-



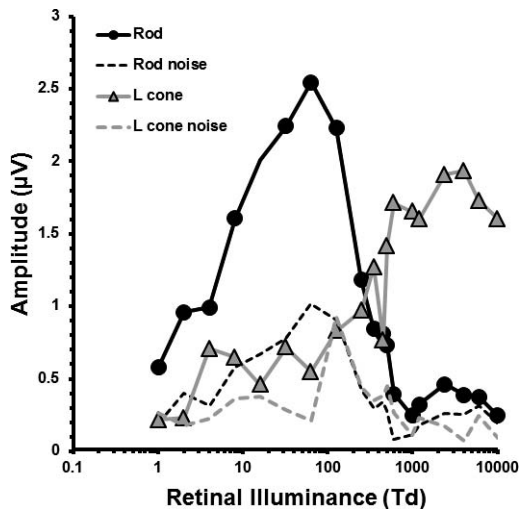
**Figure 8.** ERG (fundamental) response amplitude for an 8 Hz sinusoidal rod-isolating flicker stimulus plotted as a function of retinal illuminance (photopic Td). The *dashed line* plots the measure of noise as a function of stimulus illuminance. The data shown (*thick solid line filled circles*) are the group ( $n = 5$ ) averaged data and the *thin solid lines* represent  $\pm 1$  SD from the mean.

nance. Response amplitude increases as a function of illuminance reaching a peak between 10 and 100 Td where the response is significantly greater than noise. Response amplitude then falls towards noise levels at 1000 to 2000 Td; there may be a small increase for higher illuminances but this rarely exceeds our criterion for significance ( $>2.82 \times$  noise).

By way of comparison, **Figure 9** shows ERG amplitude as a function of retinal illuminance for an 8 Hz L-cone isolating stimulus of the same temporal frequency and with a cone contrast of 0.25. The data plotted were obtained from a subset ( $n = 2$ ) of the main experimental group and demonstrate that the cone-mediated response behaves very differently from that of the rod ERG. Unlike the rod-mediated ERG the L-cone response exhibits an increase in response amplitude with increasing retinal illuminance showing no sign of the peak response between 10 and 100 Td. Another key point is that, in the region where the rod response reaches its maximum ( $\sim 30$  Td) the cone response barely rises above noise levels.

In addition to 8 Hz stimulation, we also examined ERG amplitude as a function of retinal illuminance (less densely sampled) at other stimulation frequencies. **Figure 10** shows the averaged data from 4 subjects for rod-isolated ERGs elicited by stimulation frequencies of 5, 10, 15, and 30 Hz. For all but the highest stimulation frequency the responses are similar to the 8 Hz data in that the responses all



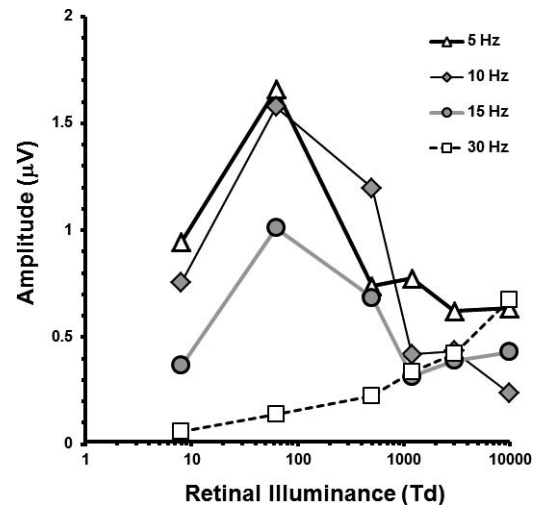


**Figure 9.** ERG (fundamental) response amplitude for 8 Hz sinusoidal rod (black circles) and L-cone (gray triangles) isolating flicker stimuli plotted as a function of retinal illuminance (photopic Td). The dashed lines plot the measure of noise as a function of stimulus illuminance. The data shown are averaged data from a subset of 2 subjects.

reach maximum amplitude at approximately 100 Td, then decrease with increasing illuminance. The data obtained from the 30 Hz stimulus follow a different response pattern; below 1000 Td it is barely recordable above noise levels but exhibits a steady increase in amplitude with increasing retinal illuminance, similar to the L-cone isolated response shown in Figure 9.

### Comparison with Current Clinical Protocols

To assess the wider clinical applications of the silent substitution stimuli, we recorded ERGs in a group of normal participants ( $n = 18$ ) using our 8 Hz rod-isolating stimulus and compared them to ERGs obtained from the same cohort using current ISCEV standard protocols<sup>10</sup> for isolating rod- (dark-adapted;  $0.01 \text{ cd/s/m}^2$ ) as well as cone (light-adapted  $3.0 \text{ cd/s/m}^2$  flicker [30 Hz])–mediated ERGs. Figure 11a plots the amplitude of the ERGs obtained using the 8 Hz silent substitution stimulus against those obtained for the ISCEV dark-adapted 0.01 protocol. There is a significant positive correlation between these two measures of rod function ( $r = 0.626$ ,  $n = 18$ ,  $P < 0.005$ ,  $R^2 = 0.39$ ). Figure 11b plots the correlation between the 8 Hz rod-isolating stimulus and the ISCEV light-adapted  $3.0 \text{ cd/s/m}^2$  flicker response. These two measures would not be expected to exhibit a significant correlation as they purportedly separately assay rod and cone function, respectively. Our results



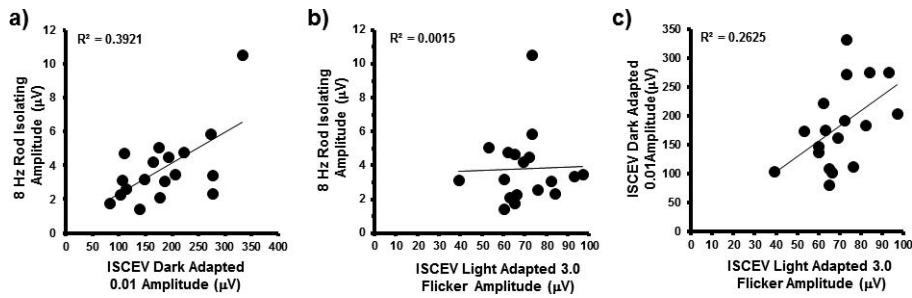
**Figure 10.** ERG (fundamental) response amplitude sinusoidal rod-isolating flicker stimuli plotted as a function of retinal illuminance (photopic Td) for stimuli of temporal frequency = 5, 10, 15, and 30 Hz. The data represent the group average from  $n = 4$  subjects.

demonstrated that this, indeed, is the case ( $r = 0.38$ ,  $n = 18$ ,  $P = 0.881$ ,  $R^2 = 0.0015$ ). However, when a similar analysis is performed for the data collected with the ISCEV dark-adapted 0.01 and light-adapted 3.0 flicker protocols a significant positive correlation is found ( $r = 0.512$ ,  $n = 18$ ,  $P = 0.03$ ,  $R^2 = 0.26$ ).

So far, we have considered the use of the silent substitution protocol in light-adapted participants with normal rod function. An important question is whether this new approach can be applied usefully in a clinical population with compromised rod function who may exhibit additional complications, such as reduced visual acuity or nystagmus, for example. To address this issue we recorded ERGs from a cohort of 10 individuals, all of whom have compromised or severely reduced rod function due to rod/cone dystrophy ( $n = 6$ ) or CSNB (type 1 [ $n = 2$ ], type 2 [ $n = 2$ ]). Figure 12a shows this group, now added to the normals previously shown in Figure 11a, where response amplitude for the 8 Hz rod-isolating is plotted against the ISCEV dark-adapted 0.01 protocol. Analysis, now including this patient group, demonstrates a stronger correlation ( $r = 0.785$ ,  $n = 28$ ,  $P < 0.001$ ,  $R^2 = 0.62$ ) between both measures of rod function.

Compared to the ISCEV clinical standard for generating rod ERGs, the absolute amplitude values are lower for our method. However, what is arguably more important than the absolute amplitude in this context is the signal-to-noise ratio (SNR). In Figure





**Figure 11.** Correlation between ERG response amplitudes elicited by different testing protocols: (a) compares the amplitudes of the 8 Hz silent substitution rod-isolating (63 photopic Td) ERGs with those obtained for the ISCEV dark adapted 0.01 protocol, (b) compares the 8 Hz silent substitution with the ISCEV light adapted 3.0 flicker (30 Hz) response, and (c) compares the ISCEV dark-adapted and light-adapted responses. The data were collected from 18 normal participants.

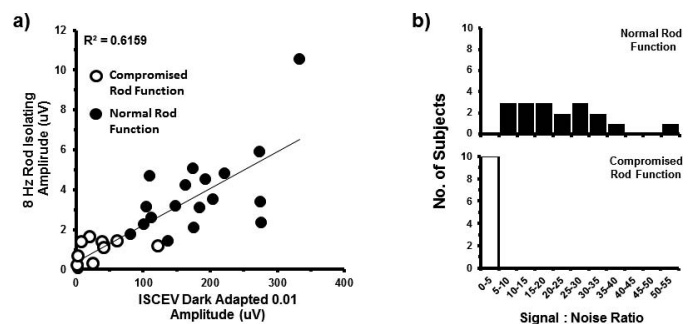
12b the distributions of SNR are plotted for the normal and compromised rod function groups. Comparison of these distributions (Mann-Whitney  $U$  test) shows that the SNR ratio in the normal group is statistically significantly higher than in the group with abnormal rod function ( $U = 0.0$ ,  $P < 0.001$ ) and demonstrates that the rod-isolating silent substitution protocol has the potential to differentiate between subjects with normal and compromised rod function.

## Discussion

In this study, we have demonstrated that, using silent substitution stimuli, it is possible to elicit ERGs with response characteristics that are consistent with known properties of rod-mediated vision. Rod ERGs are optimal for stimuli of temporal frequencies between 5 and 8 Hz and retinal illuminances between 10 and 100 photopic Td. Importantly, isolation of rod function can be achieved without prior dark adaptation and without the need for stimuli restricted to low scotopic light intensities. The low-pass, low resolution ( $<30$  Hz) ERG temporal frequency response functions generated by rod-isolating stimuli constitute a key piece of evidence supporting the fact that silent substitution stimuli provide a selective assay of rod-mediated visual function.<sup>30</sup> Importantly, our measures of the temporal response limit of the rod ERG are consistent with psychophysical measures of rod function obtained at high scotopic light levels.<sup>26,27</sup> While this functional selectivity for rods is maintained for ERG responses elicited by silent substitution stimuli at mesopic and low photopic intensity levels, it is absent at levels of retinal illumination greater than 1000 Td. Above this level ERG temporal frequency response curves take on a more band-pass form and responses can be elicited by stimuli of frequencies in

excess of 60 Hz. Such properties are incompatible with rod function. They are more consistent with their mediation by cone photoreceptors<sup>28,30</sup> and indicate that ERGs elicited beyond this parameter range are no longer rod-selective.

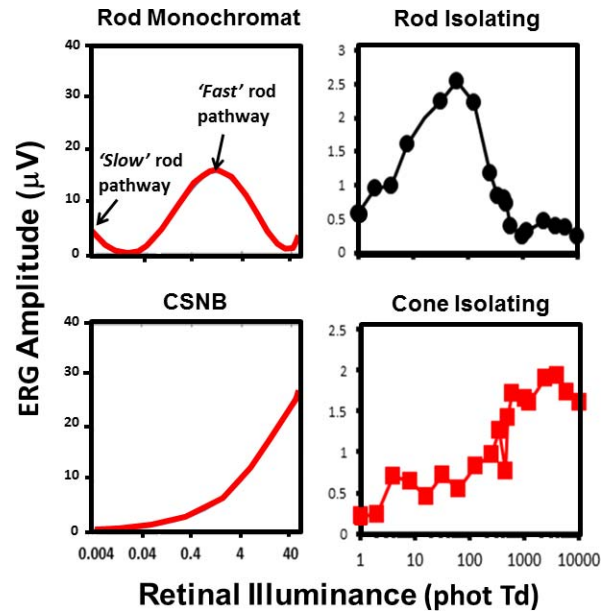
A number of studies have demonstrated the existence of separate pathways for the transmission of temporal information by rods (see prior review<sup>44</sup>). The existence of these pathways has been revealed by changes in the temporal resolution of the rod system with increasing stimulus intensity as well as phase-dependent interactions observed in psychophysical and electrophysiologic experiments.<sup>20,26–29,42–45</sup> These multiple processing pathways are based on the fact that rod signals have at least two, but probably more,<sup>46</sup> routes via which they can pass from outer to inner retina.<sup>48</sup> One route is via rod bipolar cells to AII amacrine cells.<sup>48–50</sup> This forms the so-called “slow” rod pathway, which operates over scotopic levels of



**Figure 12.** (a) Correlation between ERGs obtained with a standard ISCEV dark adapted 0.01 stimulus and those obtained with an 8Hz sinusoidal rod-isolating silent substitution stimulus. Recordings were made from 18 subjects with normal rod function (filled circles) and 10 subjects with compromised rod function. (b) Distribution of the signal-to-noise ratio calculated for the 8 Hz rod-isolating ERG data are shown for the normal (upper panel) and compromised (lower panel) rod function groups.

illumination. A “fast” rod pathway, which operates at higher intensity levels, is thought to be mediated anatomically by gap junctions that allow the passage of rod signals directly to cones and then via cone bipolar cells to ganglion cells.<sup>49,51–53</sup> A key question is whether ERGs elicited by silent substitution stimuli show evidence of similar temporal mechanisms. Examination of the apparent latency data (Fig. 7) would indicate that this is, indeed, the case. The plots of apparent latency versus temporal frequency exhibit distinct lobes for low intensity rod-isolating stimuli, indicating the existence of multiple generators of the ERG response with different temporal characteristics. Furthermore, an important transitional region between one mechanism and the other occurs between 15 and 20 Hz. This is consistent with psychophysical studies where the measurements of critical fusion frequency versus intensity also show this to be a key region in the transfer from slow to fast rod pathways.<sup>44,54</sup> Previous studies that have used silent substitution to generate rod-isolating stimuli have found that rod function can be assessed over a wider range of stimulus intensities than that which might be expected using non-isolating flash stimuli.<sup>17,18</sup> However, with the use of more intense stimuli comes the need for reassurance that, despite the employment of intensities that extend well beyond the scotopic range, rod selectively is maintained and is free of confounding contributions from cones. Hence, the emphasis in this study has been on defining parameter boundaries within which we can be confident about the selective stimulation of rod function. Our data showed that rod-isolating silent substitution stimuli generate ERGs that rise to a maximum amplitude between 10 and 100 Td, then decrease with increasing stimulus intensity. This “band-pass”-shaped function is similar to rod ERG amplitude versus intensity functions obtained in previous studies that have used either low intensity (scotopic) stimuli<sup>32,33</sup> or rod-isolating silent substitution stimuli<sup>18</sup> in dark-adapted participants. For comparison, in Figure 13 we have replotted rod ERG amplitude versus intensity functions obtained by Bijveld et al. Using a 15 Hz flickering stimulus, they measured ERGs in patients with either absent or reduced cone function (rod monochromats) or defective rod pathways (CSNB).<sup>33,34</sup> Alongside these data, we show 8 Hz amplitude versus intensity functions obtained in this study for rod- and L-cone isolating stimuli.

There are clear qualitative similarities between the different data sets. Importantly, the band-pass amplitude versus intensity function for the ERG



**Figure 13.** *Left column:* ERG amplitude as a function of stimulus intensity recorded from rod monochromats (*upper left panel*) and CSNB patients (*lower left panel*). These data are replotted from the study of Bijveld et al.<sup>33</sup> and were generated using 15 Hz flickering white light stimuli of low scotopic intensity. *Right hand column:* ERGs recorded from normal trichromats in this study which were elicited using an 8 Hz flickering rod-isolating stimulus (*upper right panel*) and an 8 Hz L-cone isolating stimulus (*lower right panel*).

obtained using rod-isolating silent substitution stimuli can be directly linked to rod activity on the basis that a similarly shaped response function is evident in ERG recordings from rod monochromats.<sup>17,33,34</sup> Rod ERGs obtained within this optimal intensity region are purported to reflect the activity of the fast rod pathway.<sup>5,33,34</sup> At higher illuminance levels our data showed a reduction in rod ERG amplitude where responses fall to a minimum at approximately 1000 Td. This minimum at higher intensities has been attributed to destructive interference between rod and cone signals.<sup>33,34,42–44</sup> The data from CSNB patients (who have dysfunctional rod signaling pathways) and normal trichromats using L-cone isolating ERG responses showed that cone responses behave in a different manner, clearly increasing in amplitude with increasing stimulus intensity. At higher intensities the temporal frequency response functions of the silent substitution rod ERGs become more “cone-like” in terms of their properties – that is, they take on a more temporally band-pass form and support high (> 60 Hz) temporal response limits. This increase in cone activation to ostensibly rod-isolating stimuli may arise from a number of possible sources. Firstly, the anatomy of the rod signaling pathway itself provides

multiple points of contact between the rod and cone systems. Studies have demonstrated a high degree of complexity in the extent to which rod signals can gain access to cone signaling pathways via direct photoreceptor coupling as well as via multiple connective pathways that are found in the inner retinal layers.<sup>46,50</sup> The increases observed in the ERG amplitude at the high stimulus intensities could, in theory, be mediated by any of these pathways. Secondly, increased cone contributions at high stimulus intensities could be the result of small departures from complete rod isolation by our stimuli. Such departures from isolation could arise as a result of inter-individual variations in photoreceptor fundamentals and as well as differences in preretinal absorption characteristics.<sup>14</sup> The responses elicited at high stimulus intensities ( $>1000$  Td), therefore, are not rod selective and are contaminated by intrusions from cone activation that potentially may be derived from a number of separate physical as well as physiologic sources.

The reduction in rod ERG amplitude for stimuli above 100 Td is interesting because it coincides with illumination levels over which rod saturation begins.<sup>20,55,56</sup> Earlier studies have referred to this as “rod insensitivity” at higher intensities.<sup>18</sup> But, what is the mechanism for this reduction? Previously, observed decreases in flicker ERG amplitude with increasing stimulus intensity have been modelled accurately on the basis of destructive interference and cancellation between rod and cone signals that are delayed with respect to each other.<sup>42–44</sup> Such interactions have been demonstrated clearly when ERGs have been elicited using non-isolating luminance flicker stimuli.<sup>31</sup> However, with the use of silent substitution stimuli to isolate rod function, the extent of cone modulation should be minimal. Thus, the potential for interference between rod and cone signals is likely to be reduced for silent substitution stimuli. Furthermore, the decrease in rod ERG for stimuli  $>10$  to 100 Td is observed at all the temporal frequencies that we have tested (see Fig. 8). Stimulus frequencies at and around 7.5 Hz are important for revealing interactions between rods and cones because, at this frequency, the delay between rod and cone signals (66 ms) produces a  $180^\circ$  difference in phase between the rod and cone-mediated responses, leading to almost complete cancellation between the two signals.<sup>42–44</sup> However, at higher and lower temporal frequencies there should be constructive interference between the two signals which should augment the ERG signal. Figure 8 shows that, below

30 Hz, decreases in rod ERG amplitude occur regardless of the stimulation frequency suggesting that another mechanism must be responsible for this rod insensitivity at high illuminance levels. One possibility is that rod polarization remains essentially constant during the stimulus, therefore, generating no response to the silent substitution. Another is that the decrease in rod ERG amplitude is the result of a generalized suppression of rod activity that occurs abruptly with increasing illumination. Such a mechanism has been described in the mouse retina where a retinal circuit has been described, which mediates rapid switching from rod to cone-mediated vision at illumination levels where cone bipolars become activated.<sup>57</sup> We speculate that a similar suppression of rod function also may exist in the human retina and that the decreases in rod ERG amplitude that occur at high light intensities, regardless of the temporal frequency, may constitute an electrophysiologic correlate of this suppression in humans.

The assessment of rod-mediated visual function is becoming increasingly clinically relevant with the growing realization that some of the earliest pathological and functional changes that occur in ARMD are found in rod photoreceptors.<sup>8</sup> In addition, rod function also may constitute an important biomarker in the identification of individuals who carry a high genetic risk of ARMD developing in later life.<sup>9</sup> Thus, growing clinical demands are driving the need for the development and improvement of methods that selectively assess rod function in humans. Our data demonstrated that it is possible to elicit ERGs with response characteristics that are consistent with known properties of rod-mediated vision using silent substitution stimuli. Furthermore, we have delineated parameter ranges over which these responses can be optimized. From a clinical perspective, our approach offers potential advantages over current standard methods of assessing rod function. Importantly, the use of silent substitution stimuli provides an opportunity for the assessment of human rod function without the need for subjects having to undergo time-consuming periods of dark adaptation, offering the prospect of more time-efficient testing protocols. A second advantage is that the adaptation state is constant throughout the test session. Specifically, rods are maximally sensitive immediately following dark adaptation and begin to lose sensitivity following repeated stimulation. This can result in much larger responses at the beginning of the test session compared to the end. Excluding dark adaptation largely obviates this problem. Another advantage of



silent substitution stimuli is that they appear to provide better isolation of rod activity and, unlike current standard clinical protocols, do not exhibit a significant correlation with cone responses. The data presented here provide an important translational link between basic and clinical and demonstrate that silent substitution stimuli can efficiently and effectively isolate rod function in humans, providing an alternative approach to current standard clinical protocols.

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## Appendix 1

To generate silent substitution stimuli the spectral characteristics of the 4 LEDs were obtained using a PR650 spectrophotometer. These spectra then were multiplied by each of the four photoreceptor fundamentals,<sup>34,35</sup> integrating across the visible spectrum of wavelengths (see Equation A1). Equation A1 shows the calculation of the excitation of the rod photoreceptors by the red LED:

$$E_{r,R}(t) = F_R \times L_R(t) \times \sum_{\lambda} I_R(\lambda) A_r(\lambda) \quad (A1)$$

where  $E_{r,R}$  is the excitation of the rod by the red LED, changing as a function of time  $t$ .  $F_R$  is a conversion factor for the red LED relating to photometric measurements,  $L_R$  is the luminance of the red LED,  $I_R(\lambda)$  is the emission spectrum of the red LED and  $A_r(\lambda)$  is the  $V'_\lambda$  10° function.<sup>35</sup> When the above calculation is carried out for all four photoreceptors for each of the individual LEDs the resultant is a 4 × 4

matrix,  $\mathbf{A}$  (see Equation 2).

$$\mathbf{A} = \begin{bmatrix} E_{l,R}(t) & E_{m,R}(t) & E_{s,R}(t) & E_{r,R}(t) \\ E_{l,G}(t) & E_{m,G}(t) & E_{s,G}(t) & E_{r,G}(t) \\ E_{l,B}(t) & E_{m,B}(t) & E_{s,B}(t) & E_{r,B}(t) \\ E_{l,A}(t) & E_{m,A}(t) & E_{s,A}(t) & E_{r,A}(t) \end{bmatrix} \quad (A2)$$

The subscripts  $R, G, B, A$  represent the four LEDs (red, green, blue, and amber) and  $l, m, s, r$  the L-, M-, S-cone, and rod photoreceptors, respectively. To calculate maximal and minimal excitation and then cone or rod Michelson contrast from LED Michelson contrast a further step is required. With sine-wave stimuli, the maximal or minimal excitations coincide with the time-point  $t$  where LED luminance is maximal or minimal. Thus, from matrix  $\mathbf{A}$  maximal and minimal excitations are calculated for each photoreceptor. From that we can calculate the photoreceptor contrast from LED contrast (RCd, GCd, BCd, and ACd, and so forth) which is matrix  $\mathbf{B}$ .

As an example, let us say we wish to generate a rod-isolating stimulus that produces a 0.25 modulation of rod excitation. Our desired photoreceptor contrast setting (PC<sub>d</sub>) would be 0% for L cone, 0% for M cone, 0% for S cone, and 25% for rods:

$$PC_d = \begin{bmatrix} L \\ M \\ S \\ r \end{bmatrix} = \mathbf{B} \times \begin{bmatrix} RCd \\ GCd \\ BCd \\ ACd \end{bmatrix} \quad PC_d = \begin{bmatrix} 0 \\ 0 \\ 0 \\ 0.25 \end{bmatrix} \quad (A3)$$

We then can multiply our desired photoreceptor contrast with the inverse of  $\mathbf{B}$  ( $\mathbf{B}^{-1}$ ), to obtain the desired LED contrast (LC<sub>d</sub>) required to achieve our level of photoreceptor isolation.

$$LC_d = PC_d \times \mathbf{B}^{-1} \quad (A4)$$