1. The long duration flash ERG protocol.

2. Scope and applications:

This guideline describes an extended full-field clinical electroretinography (ERG) protocol for the recording of the long-duration flash ERG (On-Off ERG). The basic technique is already widely used as an aid to diagnosis of various retinal disorders and may be recorded on most modern commercially available equipment, usually as an addition to the ISCEV-standard ERG protocol [1].

The standard light-adapted (LA) 3.0 ERG b-wave is evoked with flashes of a short duration (<5 ms) on a rod-saturating background, and largely reflects overlapping contributions from the On- and Off- bipolar cell pathways. Separation of the function of the On- and Off- pathways requires long-duration stimuli (e.g. 150-200 ms) in the presence of a rod-saturating background. The long-duration ERG has two major components, the On- response and the Off- response. The On- response occurs after the stimulus onset, and consists of two prominent waves, the negative polarity a-wave and the positive b-wave. The Off- response, or d-wave, is a positive polarity component in response to stimulus offset [2–4]. The sources of On- and Off- responses were elucidated by experimental pharmacological studies in non-human primates, whose ERGs are very similar to those in humans. These studies showed that the a-wave of the On- response originates from cone photoreceptors, with a significant contribution from Off- (hyperpolarizing) bipolar cells [5]. The b-wave of the On- response reflects the function of the On- (depolarizing) bipolar cells, although its amplitude and shape may be influenced by Off- bipolar and horizontal cells [6]. The d-wave is a complex response; the initial rapid phase originates from Off- bipolar cell activity, but cone photoreceptors contribute to the later slow phase and On- bipolar cells act in an opposite direction [7, 8].

Common diagnoses that may benefit from additional On-Off ERG testing include retinal dystrophies and retinal disorders that cause dysfunction post-phototransduction or at a post-receptoral level. The On-Off ERG allows evaluation of the relative or selective involvement of On- and Off- pathways, not fully enabled by the standard LA 3.0 ERG responses [2-4, 9-15]. Common forms of congenital stationary night blindness (CSNB) are good illustrative examples. In complete CSNB there is generalised On- bipolar cell dysfunction and the waveform shows an electronegative On- response but a preserved Off-response. In contrast, incomplete CSNB is associated with abnormalities affecting both the On- and Off- responses [2]. Other retinal disorders associated with selective On- pathway dysfunction include melanoma associated retinopathy, early cases of phosphomannomutase deficiency (PMM2-CDG) [12], and some forms of autoimmune retinopathy [15]. Long duration ERGs may also be useful in X-linked retinoschisis, Batten disease, Duchenne muscular dystrophy, spinocerebellar degeneration, quinine toxicity and other disorders [16, 17].

3. Identification:

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4. Patient population for whom the protocol is targeted:

Patients of all ages able to tolerate Ganzfeld stimulation, referred for investigation of possible retinal dysfunction, especially those with decreased light-adapted and/or dark-adapted ERG b-wave and relatively preserved a-wave (electronegative ERG or low b:a ratio), suggesting dysfunction post-phototransduction or at the level of the inner retina.

5. Technical issues:

The long-duration ERG protocol will follow the specifications of the current ISCEV standard ERG [1]. Additional considerations include the following:

a) Stimulus duration. The duration of the light stimulus should be long enough to separate the On- and Off- responses. Most studies used durations of between 150-200 ms (Table 1), based on the largest d-wave amplitude attained, although the increase in amplitude for flash durations greater than 75 ms was not statistically confirmed [18]. Further increase of the d-wave amplitude was observed with flashes up to 900 ms duration [19], but responses to such long durations take longer to record. Patient comfort is a consideration when selecting the light duration, also to minimize possible eye closure and blink artefacts.

b) Stimulus wavelength. The flash and background wavelength for the ISCEV-standard ERG are defined as visibly white, with CIE co-ordinates near x = 0.31, y = 0.32. Both white flashes and chromatic (blue and green) flashes have been used to elicit long-duration ERGs of similar waveform [18]. Some laboratories use orange stimuli in the presence of green background, for more selective stimulation of L-and M-cone system with simultaneous suppression of the rods and S-cones [17, 20]. These stimuli are effective at eliciting On- and Off- responses and have been shown to be informative in numerous studies (Table 1). Longer wavelength stimuli (red) may decrease the d-wave and change the shape of the b-wave [18, 19] and should be avoided.

c) Stimulus strength and background luminance. Brighter backgrounds require stronger stimuli to elicit detectable responses [18]. If stimuli are too weak responses are small. If stimuli are too strong the b-wave becomes broader and peak time variable and difficult to determine [18], while the d-wave becomes either decreased or dominated by a component of longer peak time, (the basis of the photopic hill phenomenon) [21]. Strong stimuli and backgrounds may also be poorly tolerated by some patients.

6. Calibration:

The protocol is technically similar to that for the ISCEV standard ERG, and the calibration and frequency of calibration should follow the latest ISCEV standard [1]. The strength of the

stimulus and background luminance should be specified in photopic candelas per meter squared (phot cd.m^{-2}).

7. Protocol Specifications:

Patient preparation follows that for the current ISCEV standard ERG [1]. It is suggested that for routine applications the long-duration ERG is added to the ISCEV-standard protocol after the other LA ERGs. The following additional specifications are suggested:

a) Stimulus duration. It is suggested to use durations of 150ms or 200ms, to allow clear separation of On and Off responses, efficient signal averaging and for consistency with the majority of published clinical studies to date (Table 1).

b) Stimulus wavelength. A white stimulus on a white background (as used in majority of published studies, Table 1) or a chromatic light on a chromatic background (e.g. to saturate S-cones) may be used providing longer wavelength (red) stimuli are avoided. Orange (620 nm) stimuli in the presence of green (560 nm) background may allow more selective stimulation of L-and M-cone systems with simultaneous suppression of the rods and S-cones.

c) Stimulus strength and background luminance. It is suggested that stimulus luminance for white stimuli is 250 cd.m^{-2} or within the range phot 150-350 cd.m^{-2} with a background luminance of 30 cd.m^{-2}.

d) Inter-stimulus time (rate). A maximum rate of 0.5 per s (≤2.0 Hz) conforms to the current ISCEV Standard for the LA 3.0 ERG.

8. Response evaluation:

The long-duration flash ERG response consists of three prominent waves, the a-, and b-waves as part of the On- response and the Off- response complex, mainly the d-wave (Figure 1). A negative a-wave appears in response to the onset of the stimulus, followed by the positive-going On-response b-wave. The d-wave of the Off- response is the positive peak, which appears after the termination of the stimulus. The amplitude of the a-wave is measured from the baseline to the first negative trough. The amplitude of the b-wave is measured from the trough of the a-wave to the peak of the b-wave. The amplitude of the d-wave is measured from the time point of stimulus offset to the peak of the d-wave. The peak time of the a- and b-waves is measured from the beginning of the stimulus flash to the peak of each of the waves. The peak time of the d-wave is measured from the stimulus offset to the peak of the d-wave.

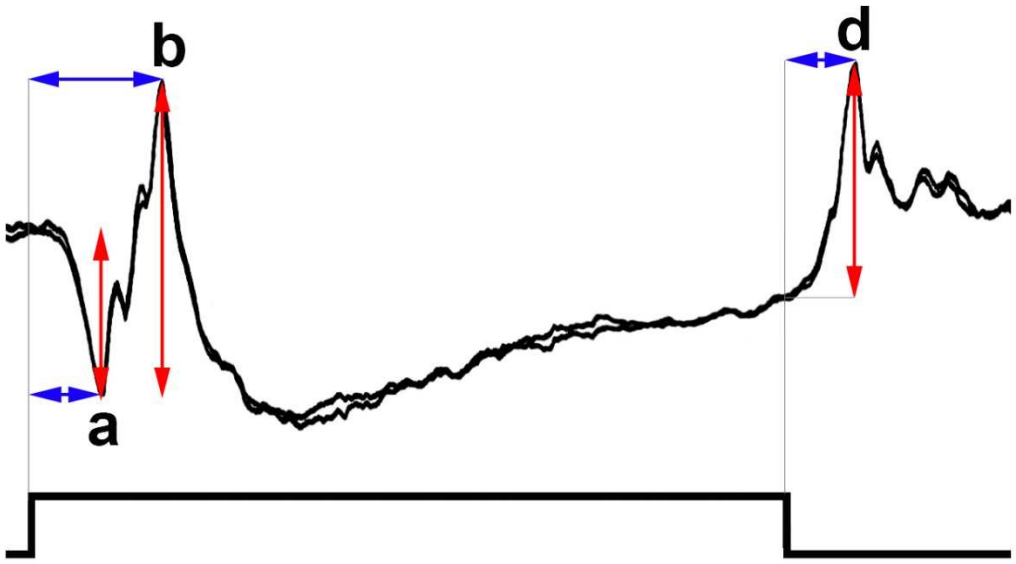

Figure 1: Diagram of the three main components of the long-duration flash ERG and their measurement. Stimulus duration is indicated at the bottom of the figure; red arrows indicate amplitude measurement, blue arrows indicate peak time measurement.

The amplitude and peak time values should be evaluated according to the reference values, which should be established in each laboratory for its own equipment, recording protocols and patient population.

9. Reporting:

This protocol is intended to be used for routine applications as an extension to the standard ERG protocol, and reporting should follow the latest ISCEV Standard for ERG [1]. Additionally the spectral characteristics of the stimulus and background should be acknowledged if different from the standard LA ERG (e.g. peak wavelength and bandwidth). The duration (in ms if different from 150 or 200ms) and luminance (in phot cd.m^{-2}) of stimulus and background should be stated. Unless already embedded within the ISCEV standard ERG protocol pupil size and duration of light adaption should be stated. The amplitude of the a-, b- and d-waves and respective time to peaks may be reported along with age-appropriate laboratory reference normative data. It is acknowledged that in diagnostic studies involving ISCEV standard ERGs it may be sufficiently informative to describe the relative involvement of a-, b-, and d- waves qualitatively.

10. Experimental procedures excluded from this guideline:

ERG recordings with long-duration flashes can be affected by technical issues, and blink and squint artefacts may disturb the recordings. Some authors suggest that this may be alleviated by using stimuli with sawtooth luminance profiles. The recordings are typically performed at photopic luminances. Instead of a flash upon a background, these stimuli are modulated around a mean. The stimulus strength is quantified by Michelson contrast (C): $C= (L_{max}+L_{min})/ (L_{max}-L_{min})$ in which L_{max} and L_{min} are the maximal and minimal luminance in the stimulus. Since this stimulus is given repetitively with a frequency between 2 and 8 Hz, blink artefacts do not play a large role. Higher frequencies are not recommended because the responses to subsequent stimuli may merge. On- and Off-responses are obtained separately

by using rapid-ON and rapid-OFF stimuli. They have been used in a variety of disorders [22-27] and may have benefits, but have not been widely available on commercial systems.

On- and Off- responses might not be the same for all stimulus types. It has been reported that On- and Off- responses originating in the L-cones have the same morphology as those obtained with luminance stimuli. In contrast, M-cone driven On-responses resemble Off-responses with L-cone isolating and luminance stimuli and vice versa, suggesting that cone opponent processes may be involved [28-30].

Beside the sawtooth stimulation, increment and decrement stimulation is also one of alternative ways for eliciting the d-wave without a major impact of blinking artefacts [31].

An alternative method extracts On- and Off- responses from the LA 3.0 ERG through the quantification of wavelet coefficients by discrete wavelet transform (DWT) analyses [32]. This approach suggests the activity of the retinal On- pathway to be related to a 20Hz component of the photopic ERG, while the Off- pathway activity is reflected by a 40Hz component. This finding was based on the fact that 20Hz and 40Hz components of the photopic b-wave are selectively attenuated in case of imbalanced dysfunction of the On- and Off- pathways in some diseases [32], confirmed with the DWT of photopic long-duration flash ERGs [33].

Since long-duration flash ERG is potentially valuable ERG method in animal studies, the researchers should be aware that positive On- and Off- responses, as those in humans, can only be recorded in some non-human primates, while On- and Off- responses with electronegative waveform are presented in rodents including mice and rats [34].

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Part B. Justification for the protocol details and description of the consultation process

A literature review was performed with Medline search engine to find publications that reported the long-duration or On-Off ERG using following keywords: electroretinogram or full-field ERG or long duration flash, b-wave or ON-response, d-wave or OFF-response. Out of 110 matches, animal studies were excluded, as well as studies using multifocal, focal and sawtooth type of stimulation, as well as studies focusing on the function of rod or ganglion cell systems. Studies from the year 1986 to 2016 were reviewed and those with specified stimulus parameters are summarized in Table 1.

Table 1: Recording parameters, used in some of the previous studies.

Ref.	Stimulus strength	Background luminance	Stimulus duration	Stimulus and background wavelength
[4]	185 cd/m^2	43 cd/m^2	150 ms	white flash on white background
[3]	3.7 log cd/m^2 (5011 cd/m^2)	2.1 log cd/m^2 (125 cd/m^2)	5-100 ms	white flash on white background
[9, 53]	3 cd s/m^2 (15 cd/m^2)	10 cd/m^2	200 and 250 ms	red and green flash on white background
[10]	750 cd/m^2	42 cd/m^2	500 ms	white flash on white background
[11]	3 cd s/m^2 (12 cd/m^2)	10 cd/m^2	256 ms	630 nm flash on white background
[12]	200 cd/m^2	43 cd/m^2	90, 120ms	white 6500K
[13]	3 log cd s/m^2 (5000 cd/m^2)	2 log cd/m^2 (100 cd/m^2)	200 ms	white flash on white background
[14]	398 cd/m^2	48 cd/m^2	200 ms	white flash on white background
[21]	0.6-3.5 log cd/m^2 (4-3162 cd/m^2)	40 cd/m^2	250 ms	white flash on white background
[18, 56]	0.4 - 2.1 log cd s/m^2 (12.5-629 cd/m^2)	20-50 cd/m^2	5-200 ms	white, 460, 508 and 667 nm flash on white background
[15, 17, 20, 38, 41, 52]	560 cd/m^2	160 cd/m^2	150-200 ms	620 nm flash on 530 nm background
[35]	440 cd/m^2	160 cd/m^2	200 ms	612 nm flash on 530 nm background
[36]	133 cd/m^2	43 cd/m^2	120 ms	white 6500K
[37]	1.7 log cd s/m^2 (250 cd/m^2)	40 cd/m^2	200 ms	white flash (6500 K) on white background
[39]	1 cd s/m^2 (4 cd/m^2)	30 cd/m^2	250 ms	white 6500K
[40]	200 cd/m^2	42 cd/m^2	150 ms	white flash on white background
[42]	4 log ph td (200 cd/m^2)*	3.3 log ph td (40 cd/m^2)*	150 ms	white flash on white background
[43]	200 cd/m^2	30 cd/m^2	100 ms	white flash on white background
[44]	225 cd/m^2	30 cd/m^2	188 ms	white flash on white background
[45-48]	300 cd/m^2	40 cd/m^2	150 ms	white flash on white

				background
[49]	650 cd/m^2	160 cd/m^2	120, 200 ms	orange flash on green background
[50]	200 cd/m^2	42 cd/m^2	150 ms	white flash on white background
[51]	1700 cd/m^2	28 cd/m^2	125 ms	white flash on white background
[54]	360 cd/m^2	40 cd/m^2	100 ms	white flash on white background
[55, 57]	1120 cd/m^2	30 cd/m^2	200 ms	white flash on white background
[58]	40,60,80 cd/m^2	20 cd/m^2	240 ms	white flash on white background
[59]	2.5 log cd/m^2	40 cd/m^2	150 ms	white flash on white background

434 * calculated for 8 mm pupil diameter