Severe Loss of Tritan Color Discrimination in RPE65-Associated Leber Congenital Amaurosis

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PURPOSE. RPE65-associated Leber congenital amaurosis (RPE65-LCA) is a progressive severe retinal dystrophy with early profound dysfunction of rod photoreceptors followed by progressive cone photoreceptor degeneration. We aim to provide detailed information about how cone dysfunction affects color discrimination.

METHODS. Seven adults (aged 16–21) with RPE65-LCA underwent monocular color discrimination assessment using the Trivector and Ellipse versions of three computerized tests: Cambridge Colour Test (CCT), low vision version of the Cambridge Colour Test (IvvCCT), and the Universal Colour Discrimination Test (UCDT). For comparison, subjects were also tested using the American Optical Hardy Rand Rittler (AO-HRR) plates. Each assessment was repeated three times.

RESULTS. The Trivector version of the tests demonstrated that color discrimination along the tritan axis was undetachable in four subjects, and severely reduced in three subjects. These findings were confirmed by the Ellipse version of the tests. Color discrimination along the protan and deutan axes was evident but reduced in six of seven subjects. Four of seven subjects were unable to read any of the HRR plates.

CONCLUSIONS. The computerized color vision tests adopted in this study provide detailed information about color discrimination in adult RPE65-LCA patients. The condition is associated with severe impairment of color discrimination, particularly along the tritan axis indicating early involvement of S-cones, with additional protan and deutan loss to a lesser extent. This psychophysical assessment strategy is likely to be valuable in measuring the impact of therapeutic intervention on cone function.

Keywords: color vision, retina, Leber congenital amaurosis, LCA, LCA2, clinical trials, endpoints
up of discrete discs that vary in size and luminance. This ensures that the object is only identifiable from its background by its chromatic difference and not from a difference in perceived luminance. Computerized color vision testing is less frequently used in clinical practice and more so in research settings. Computerized tests such as the Colour Assessment and Diagnosis test (CAD), the low-vision version of the Cambridge Colour Test (CCT), the Cambridge Colour Test (IvvCCT) and the Universal Colour Discrimination Test (UCDT) offer multiple advantages. Firstly, through using methods described below, they allow greater quantitative characterization of color discrimination. Secondly, by incorporating the chromatophotometer, computerized color tests change the chromatic difference between the stimulus and the background in response to patient performance, enabling more precise measurement of color discrimination. Moreover, they are able to randomize the presentation of stimuli to counteract any learning effect.

The CCT was the first popular computerized test to measure color discrimination. The CCT has been shown to be suitable only for patients with a visual acuity lower, and thereby better, than 0.78 LogMAR. Identifying the limitation of poor visual acuity in assessing color discrimination, a modified version of the CCT, the IvvCCT, was developed. Subsequently, the UCDT was established and shown to accurately measure color discrimination in both adults and children, even with a visual acuity higher, and thereby worse, than 1.00 logMAR.

There are two versions of each computerized test: the Trivector version and the Ellipse version. Both versions measure the amount of saturation required to discriminate a color target from a series of gray distractors. The Trivector version is a screening version because it allows rapid testing of color discrimination along three vectors; namely the protan, deutan, and tritan confusion axes. Figure 1A shows the saturation thresholds obtained from an ideal observer with normal color discrimination, with a saturation threshold of 5.43, 2.44, and 7.29 in the protan, deutan, and tritan confusion axes, respectively. The Ellipse version, shown in Figure 1B, assesses color discrimination along more than three confusion axes, which allows an Ellipse to be determined.

In this study, we aim to provide detailed information about how cone dysfunction in RPE65-LCA affects color vision by using the American Optical Hardy Rand Rittler (AO-HRR) plate test and three computerized color discrimination tests: the CCT, IvvCCT, and UCDT. The AO-HRR plate test was used for comparison, as such plate tests are commonly used in a clinical setting, while, the computerized tests were chosen as they have been shown to be effective in testing subjects with inherited or acquired color vision defects, including subjects with low vision.

METHODS

Subjects

Seven subjects (aged 16–21) were each molecularly confirmed as having two likely disease-causing sequence variants in RPE65 following targeted next generation sequencing of the coding regions of 176 retina-associated genes from genomic DNA extracted from peripheral blood leukocytes (performed at The Manchester Centre for Genomic Medicine, Manchester, UK). Parental blood was used to confirm the variants to be in trans by cosegregation analysis with Sanger direct sequencing, where possible. The L/M-opsin genes were not screened. The study protocol adhered to the tenets of the Declaration of Helsinki and was approved by the Moorfields Eye Hospital Ethics Committee. Informed consent was obtained from all subjects prior to entering the study.

Assessments of Color Discrimination

All patients were assessed monocularly, with their current spectacle correction where used, using the AO-HRR chart first and then with the three computerized tests in a randomized fashion. Furthermore, the first eye tested was also randomized and all patients were instructed using standardized text instructions, specific for each individual assessment. The
advantages, and disadvantages of the four color vision assessments used in this study.

The Display++ resolution is 1920 × 1080 pixels and its frame rate is 120 Hz. It provides 16-bit RGB color resolution. A five-key RB-540 Cedrus response box (Cedrus, San Pedro, CA, USA) was used to collect observers’ responses. The tests were controlled by the Metropolis system (Cambridge Research Systems Ltd., Rochester, Kent, UK).

AO-HRR plates were presented under a Daylight Illuminator (Richmond Products, Inc., Albuquerque, NM, USA) and scored using the accompanying worksheet. Best-corrected visual acuity (BCVA) was assessed using a Lighthouse (Long Island City, NY, USA) or Precision Vision Lightbox (Woodstock, IL, USA) and a stand, illuminated with 2 cool daylight 20-W fluorescent tubes (each with a color temperature of 6500 K and a color rendering index of 75).

Stimuli

Low-Vision Version of the Cambridge Colour Test (ivvCCT). In this assessment, four discs are presented on a 2- cd/m² neutral background in a diamond-shaped array. Each subtends 4° at the test distance (1.5 m) and was separated by 2.5° from the adjacent discs. On each presentation, one of the discs differs in chromaticity from the remaining three (which remain of neutral hue) and the patient was asked to identify which of the four discs is different in color.22

Cambridge Colour Test (CCT). The CCT involves the presentation of a 5° Landolt C-like ring in four orientations, against a background that differs in chromaticity only. Based on the principles of Stilling,19 the target and background are made up of many discrete discs, each with its own contour, and the luminance of the individual discs are randomized. These two maneuvers allow discrimination of the target from the background using chromaticity alone. The observer was instructed to report the position of the 1° gap in the C-like ring presented.21

Universal Colour Discrimination Test (UCDT). The UCDT is a two alternative forced-choice assessment involves the presentation of circles of random luminance. A subset of circles delineates a 8.58° stimulus that varies in saturation and hue only, on the left or right hand side of the screen, with the patient being asked to distinguish the laterality.25

Assessment of Best-Corrected Visual Acuity

Each patient underwent a subjective refraction and subsequent assessment of BCVA. The number of Early Treatment Diabetic Retinopathy Study (ETDRS) letters read was scored. This was performed three times, on different days, and the number of ETDRS letters read was averaged and converted into a logMAR equivalent.

Statistical Analysis

Statistical analyses were performed using the Stata statistical software package (StataCorp, College Station, TX, USA). For Trivector assessment of the three computerized tests, a one-way repeated measures ANOVA was used to investigate any statistically significant difference in the mean saturation threshold between the three (protan, deutan, and tritan) confusion axes. In cases where a statistically significant difference was found, a post hoc pairwise comparison with Bonferroni correction was used to compare the mean saturation thresholds between the (1) protan and deutan, (2) protan and tritan, and (3) deutan and tritan axes, respectively. To minimize the clustering effect of using data from both eyes, only results from the right eye of all subjects were analyzed.
The same statistical analysis was performed on data from the left eye and provided comparable results.

**Results**

Table 3 shows the demographics, RPE65 variants, and average BCVA for all seven subjects.

**AO-HRR Test**

Only three subjects (subjects number 4, 5, and 6) were able to read at least one full plate. The total number of plates read by these three subjects ranged from one to six. Of note, the plates read were distributed equally throughout the deutan, protan, tritan, and tetartan plates. A normal trichromat should be able to identify all 14 diagnostic plates.

**Low-Vision Version of the Cambridge Colour Test (IvvCCT) Trivector Test**

Figure 2 shows the mean saturation threshold and standard error of each eye, of each patient in the protan, deutan, and tritan axes, as calculated by the Trivector assessment of the lvvCCT. As described above, a normal trichromat has a saturation threshold of less than 10 (shown by the gray shaded area in each row in Fig. 2). The maximum saturation threshold the monitor is able to present is 110 in each axis (as shown by the dotted line in each row in Fig. 2).

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**Table 2.** Example Stimulus, Advantages, and Disadvantages of the Four Color Vision Assessments Used in This Study

<table>
<thead>
<tr>
<th>Test</th>
<th>Example Stimulus</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hardy Rand Rittler Test</td>
<td><img src="image" alt="Hardy Rand Rittler Test" /></td>
<td>All cone classes. Quick (&lt;5 min).</td>
<td>Need &quot;good&quot; acuity. Not sensitive to small changes. Plates can fade over time.</td>
</tr>
<tr>
<td>CCT</td>
<td><img src="image" alt="CCT" /></td>
<td>All cone classes. Sensitive to change. Stimuli are randomized to counteract memorization.</td>
<td>Need &quot;good&quot; acuity, due to 1&quot; gap in C stimulus. Takes longer (~10 min).</td>
</tr>
<tr>
<td>IvvCCT</td>
<td><img src="image" alt="IvvCCT" /></td>
<td>All cone classes. Sensitive to change. Stimuli are randomized to counteract memorization. Appropriate for low vision.</td>
<td>Takes longer (~10 min).</td>
</tr>
<tr>
<td>UCDT</td>
<td><img src="image" alt="UCDT" /></td>
<td>All cone classes. Sensitive to change. Stimuli are randomized to counteract memorization. Appropriate for low vision. Appropriate for small visual field.</td>
<td>Takes longer (~10 min).</td>
</tr>
</tbody>
</table>

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The same statistical analysis was performed on data from the left eye and provided comparable results.

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**Table 3.** Cohort Demographics Including Sex, Age, RPE65 Variants, and Best-Corrected Visual Acuities (logMAR)

<table>
<thead>
<tr>
<th>Subject Number</th>
<th>Sex</th>
<th>Age</th>
<th>RPE65 Variant 1</th>
<th>RPE65 Nucleotide 1</th>
<th>RPE65 Variant 2</th>
<th>RPE65 Nucleotide 2</th>
<th>Right Eye BCVA</th>
<th>Left Eye BCVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Female</td>
<td>21</td>
<td>c.118G&gt;A</td>
<td>p.Gly40Ser</td>
<td>c.955G&gt;A</td>
<td>p.Glu319Lys</td>
<td>0.3</td>
<td>0.4</td>
</tr>
<tr>
<td>2</td>
<td>Female</td>
<td>20</td>
<td>c.989G&gt;A</td>
<td>p.Cys330Tyr</td>
<td>c.1443_1445delAGA</td>
<td>p.Glu481del</td>
<td>0.7</td>
<td>0.7</td>
</tr>
<tr>
<td>3</td>
<td>Male</td>
<td>20</td>
<td>c.1451G&gt;A</td>
<td>p.Gly484Asp</td>
<td>c.1451G&gt;A</td>
<td>p.Gly484Asp</td>
<td>0.5</td>
<td>0.6</td>
</tr>
<tr>
<td>4</td>
<td>Female</td>
<td>20</td>
<td>c.11+5G&gt;A</td>
<td>Splice region</td>
<td>c.1341_1342dupCT</td>
<td>p.Cys+488SerfeTecri</td>
<td>0.8</td>
<td>0.7</td>
</tr>
<tr>
<td>5</td>
<td>Female</td>
<td>19</td>
<td>c.1078C&gt;A</td>
<td>p.Pro363Thr</td>
<td>c.1078C&gt;A</td>
<td>p.Pro363Thr</td>
<td>0.6</td>
<td>0.6</td>
</tr>
<tr>
<td>6</td>
<td>Male</td>
<td>18</td>
<td>c.370C&gt;T</td>
<td>p.Arg124Ter</td>
<td>c.952T&gt;A</td>
<td>p.Tyr318Asn</td>
<td>0.9</td>
<td>0.5</td>
</tr>
<tr>
<td>7</td>
<td>Male</td>
<td>16</td>
<td>c.11+5G&gt;A</td>
<td>Splice region</td>
<td>c.245G&gt;A</td>
<td>p.Arg821lys</td>
<td>0.7</td>
<td>0.6</td>
</tr>
</tbody>
</table>

* Sequence variants preceded with an asterisk are noted to be novel.
Four participants (subjects 1, 2, 3, and 7) were unable to see the stimulus in the tritan axis despite presentation of a maximally saturated stimulus (Fig. 2). Conversely three participants (subjects 4, 5, and 6) were able to identify the tritan stimulus below the maximal threshold, suggesting that these participants had some color discrimination in the tritan axis. Given that these saturation threshold values are close to the maximal level, their tritan discrimination can be described as poor. Interestingly, when reviewing the same in the protan and deutan axes, all participants, except subject 7, exhibited varying degrees of color discrimination in the protan and deutan axes.

**Figure 2.** Low vision version CCT Trivector test results. Shown are the mean (symbols) and one standard error (vertical bars) of the mean saturation along the tritan (blue), deutan (green), and protan (red) axes, for each eye of each of the seven tested subjects. The dotted line in each row represents the maximum saturation threshold the monitor is able to present (110). The gray shaded area in each row corresponds to the normal trichromatic range (0–10).

**Figure 3.** CCT Trivector test results. Shown are the mean (symbols) and one standard error (vertical bars) of the mean saturation along the tritan, deutan, and protan axes, for each eye of each of the seven tested subjects. The dotted line in each row represents the maximum saturation threshold the monitor is able to present (110). The gray shaded area in each row corresponds to the normal trichromatic range (0–10).
There was a statistically significant difference ($F_{2,12} = 11.42, P = 0.0017$) in mean saturation threshold between the three confusion axes, suggesting a difference in color discrimination between the three confusion axes. A Bonferroni post hoc test identified subjects to have a significantly better mean saturation threshold in the protan axis (39.44 ± 9.15, $P = 0.003$) and the deutan axis (36.02 ± 9.15, $P = 0.006$) as compared with the tritan axis. This suggests significantly worse color discrimination in the tritan axis as compared with the protan axis and the deutan axis.

**Cambridge Colour Test (CCT): Trivector Test**

Figure 3 illustrates the mean saturation threshold and standard error of each eye, of each patient in the protan, deutan, and tritan axes as calculated by the Trivector assessment of the CCT. Subjects 2, 3, 6, and 7 showed no evidence of color discrimination, using the CCT trivector test, as indicated by the maximum possible saturation thresholds. Subjects 1 and 5 showed evidence of color discrimination along the deutan and protan confusion axes. Subject 4 appeared to retain color discrimination along all three confusion axes, with a markedly variable performance in the deutan axis of the right eye (as indicated by the respective error bars).

No statistically significant difference in the mean saturation threshold and standard error of each eye, of each patient in the protan, deutan, and tritan axes as calculated by the Trivector assessment of the CCT. Subjects 2, 3, 6, and 7 showed no evidence of color discrimination, using the CCT trivector test, as indicated by the maximum possible saturation thresholds. Subjects 1 and 5 showed evidence of color discrimination along the deutan and protan confusion axes. Subject 4 appeared to retain color discrimination along all three confusion axes, with a markedly variable performance in the deutan axis of the right eye (as indicated by the respective error bars).

**Universal Colour Discrimination Test (UCDT): Trivector Test**

Figure 4 shows the mean saturation threshold and standard error of each eye, of each patient in the protan, deutan, and tritan axes as calculated by the Trivector assessment of the UCDT.

Subject 4 exhibited discrimination in all three confusion axes in both eyes, but as shown by the error bars (Fig. 4), this patient’s responses were very variable in four of the six axes tested over the two eyes. Of the remaining six subjects, only subject six demonstrated residual tritan discrimination (left eye), which was both variable and at a level close to the maximum threshold the monitor is able to present. Furthermore, of these six subjects, two (subjects 3 and 5) demonstrated discrimination in the protan and deutan axes.

In keeping with the CCT Trivector results, statistical analysis did not show a statistically significant difference in the mean saturation threshold between the three axes ($F_{2,12} = 0.63, P = 0.551$).

**CCT, LvvCCT, and UCDT Ellipse Test**

The subset of 10 saturation thresholds identified using the Ellipse test were fitted with a ‘best-fit’ Ellipse using a least-square procedure. This allows color discrimination to be described using three parameters; the orientation of the Ellipse, the axial ratio (the ratio between the major and minor axes), and the area within the Ellipse. Each parameter allows quantification of a different aspect of color discrimination. The orientation of the Ellipse provides information regarding the axes in which the patient lacks discrimination and can suggest loss of tritan, protan, or deutan discrimination if it correlates with these confusion axes: the greater the axial ratio, the more selective the loss of color discrimination in the relevant confusion axis. The area within the Ellipse can provide a quantification of the discrimination ability of the patient: the smaller the area the better the color discrimination ability. Color discrimination Ellipses of a normal trichromat have an axial ratio and area within the Ellipse typically less than 2 and 340 (using a 10³ unit multiplication in keeping with previously published and accepted methods), respectively.

Figure 5 shows the mean and standard error of the areas of the best-fit color discrimination Ellipse for each test. Furthermore, the dashed line represents the maximum threshold the...
monitor is able to present. Figure 5 confirms that color discrimination can be measured, with differing variability in six of seven patients; however, subject 7 demonstrated very poor color discrimination with all three tests. The LvvCCT had a lower average Ellipse area in five of the remaining six tested subjects than the other two tests, suggesting that these patients had better color discrimination when assessed with the LvvCCT, as compared with either the CCT or UCDT; the

![Figure 5](image_url)

**Figure 5.** Color discrimination Ellipse areas. Shown are the mean (symbols) and one standard error (vertical bars) of the area of the Ellipse for the LvvCCT, CCT, and UCDT, for each eye of each of the seven tested subjects. The dotted line represents the maximum saturation threshold the monitor is able to present. A normal trichromatic region is from 0 to 340.

![Figure 6](image_url)

**Figure 6.** Color discrimination Ellipse axis orientation. Shown are the mean (symbols) and one standard error (vertical bars) of the axes of the Ellipse for the LvvCCT, CCT, and UCDT, for each eye of each of the seven tested subjects. The corresponding deutan (green), tritan (blue), and protan (red) confusion axes are also shown.
notable exception to this being the performance of the right eye in subject 4.

Figure 6 shows the mean and standard error of the axes of the best-fit color discrimination Ellipse for each test. The protan, deutan, and tritan axes are also highlighted. As shown, the axes of the majority of the Ellipse assessments fall closest to the tritan axis, in keeping with these subjects having poor color discrimination in the tritan axis.

**Discussion**

In this study, we investigated in detail how cone dysfunction affects color vision in a cohort of seven young adults with RPE65-associated LCA. We have identified a severe loss of tritan color discrimination. The lvvCCT Trivector assessment identified significantly worse color discrimination in the tritan axis compared with the protan and deutan axes (Fig. 2). This was further supported by poorer discrimination in the tritan axis, for the majority of patients, as compared with the protan and deutan axes in the CCT and UCDT Trivector assessments, respectively (Figs. 3, 4).

Subjects performed worse overall in the CCT, including four subjects not seeing the stimulus at all. This may be due to their low level of visual acuity preventing them from being able to discern the 1º gap in the C-stimulus. Furthermore, we observed that with the UCDT Trivector assessment, three of seven subjects were also unable to see the stimulus across the three tested axes. All three computerized color vision assessments require subjects to accomplish a visual search to compare the stimulus and the background. We suggest that subjects find the lvvCCT easier than the other two tests, as the stimulus comprises of large (4º) uniform discs, which may be easier to search across. This may explain why the CCT and UCDT are less sensitive in identifying a difference in performance in the tritan, deutan, and protan axes. Interestingly, subject 4, who had the worst level of visual acuity performed variably with the CCT and UCDT. However, when assessed with the lvvCCT, performed more consistently and in keeping with the rest of the cohort.

The Trivector findings, identifying severe loss of tritan color discrimination, are further supported by the axes of the respective Ellipse assessments for the lvvCCT, CCT, and UCDT (Fig. 6).

As tritan color discrimination correlates to S-cone function, we suggest that S-cone function is lost earlier than L- and M-cone function in RPE65-LCA. There are several, not necessarily mutually exclusive, hypotheses that may account for this observation. Firstly, the ‘scarcity hypothesis’ suggests that if a fixed number of cones are lost, the effect will be greatest on S-cones due to their paucity and hence the overrepresentation of the S-cone signal. Secondly, it has been suggested that the S-cone pathway is more vulnerable than that of the L- and M-cones. Finally, it has also been suggested, in mouse models, that L- and M-cones are only partially reliant on the RPE for visual pigment recycling, whereas S-cone reliance on RPE-derived visual pigment has not been explored. It is therefore possible that S-cones may be more reliant on RPE derived visual pigment than L- and M-cones, and hence affected earlier and more severely in the progressive cone dysfunction seen in RPE65-LCA. Further support to our findings in this study can be found from studies in dog and mouse models of RPE65-LCA, where S-cone loss is seen prior to L- and M-cone loss.

This study demonstrates that color vision can be quantified in most RPE65-LCA subjects. Through using appropriate assessments of color vision, we have identified that these subjects have a severe loss of tritan color discrimination, prior to loss of protan or deutan discrimination. This suggests that S-cone function is lost earlier in the natural history of RPE65-LCA compared with L- and M-cones. Gene therapy for RPE65-LCA has been shown to be safe with varying levels of efficacy and durability noted in both phase I/II and III studies. Subsequently, multiple new clinical trials are currently underway to investigate potential benefits of gene therapy.

Therefore, in-depth assessments of visual function are becoming more critical. Furthermore, knowledge and sensitive assessments of color discrimination are valuable tools in the measurement of the impact of intervention on cone function, both to accurately describe change and to inform patient experience.

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