Cone opsin gene variants in color blindness and other vision disorders

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Abstract

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The long wavelength sensitive (L) and middle wavelength sensitive (M) photopigments are encoded in a tandem array on the X chromosome. Due to their close proximity and high sequence homology, this locus is prone to unequal homologous recombination resulting in intermixing of the L and M opsin genes. This thesis explores the enormous variability in both L and M gene sequence and in total opsin gene copy number that results from this process. In a sample of 1022 normal trichromatic males, there were 89 unique L sequences and 31 unique M sequences. A majority of opsin gene arrays contained extra M opsin genes (55%) and a small number (5%) contained extra L opsin genes. Array structure was found to vary by race: African Americans were more likely to have extra L genes (10%); Caucasians to have extra M genes (64%); and Asians to have arrays without extra genes (54%). A second study of 335 women unselected for color vision phenotype was used to explore the frequency of extra cone types in the retina. Between 42% and 60% had opsin gene arrays indicative of having four cone types and between 2.4% and 11% had arrays indicative of five. The ability of the visual system to make use of these extra cone types to create a new chromatic channel is exceedingly rare though there may be a possibility of training women who have a fourth cone type to use it by creating

artificial stimuli that specifically activate the extra cone in isolation of the other three.

In a second project, knowledge of the gene mutations and rearrangements that lead to color vision deficiencies underpinned the creation of a genetic assay to find and classify color defects. That assay was tested on 1872 human subjects and its potential for use in clinical diagnosis was evaluated. Our results indicate that information about the underlying pathology of color blindness derived from genetic analyses can be extremely valuable in accurate diagnosis, and that a test incorporating gene analyses supplemented with behavioral testing approaches the ideal color vision test.

While most opsin variants lead to normally functioning photopigments, certain variants that encode "toxic" sequences in exon three lead to serious eye diseases with symptoms that include cone dystrophy, diminished ERGs, color blindness, and pathologically high myopia. A virally-mediated gene therapy treatment aimed at curing these diseases was designed using the modified AAV2 capsid 7M8 that has enhanced tropism toward photoreceptors and an optimized expression cassette to drive high expression in cone cells. Initial work in a mouse model shows that this construct is capable of driving high and highly specific expression in cones following minimally invasive intravitreal injections. Further examination of the construct in mice using immunohistochemistry, spectral ERG, and behavioral testing will determine whether this is a viable approach to curing these diseases of the cones in human patients.

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Chapter One

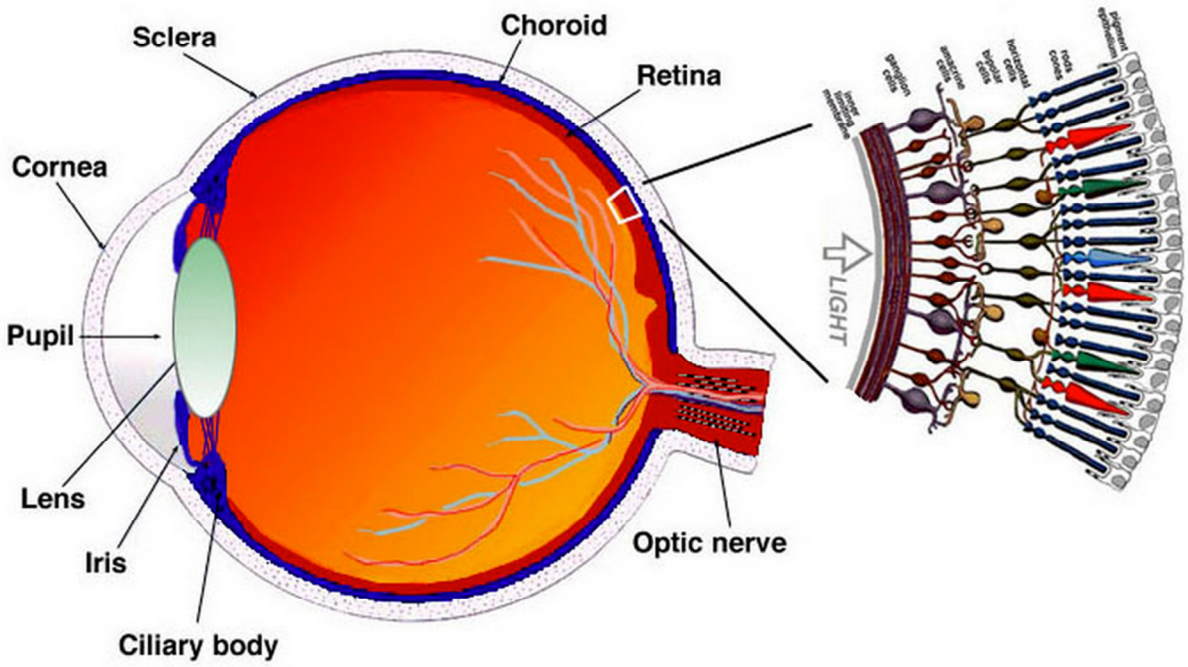
General Introduction

The motivation for this work was to explore the genetic variability of the cone photopigments, elements crucial to our visual experience. These molecules are responsible for the very first steps in seeing - the absorption of light reflecting off of objects in the world around us. The cone photopigments give us our most detailed images of the world and color those images with the splendor of a million hues. I studied the variability of the genes encoding the cone photopigments in people with normal color vision, and investigated the potential for extra dimensions of color vision in women. Using knowledge of the gene mutations and rearrangements that lead to color vision deficiencies, I created a genetic assay to find and classify color defects and tested that assay on over 1800 people. Finally, I designed virally-mediated gene therapy treatments with the aim of curing blinding diseases caused by known toxic gene variants. This work would have been impossible without the enormous body of knowledge gained by my predecessors in this field. The remainder of this chapter will discuss some of that work, as it pertains to the research described in this dissertation.

Photoreceptors

Human visual perception begins in the retina with the activity of two classes of light-sensitive photoreceptor cells: rods and cones (Figure 1.1). Rod photoreceptors are extremely sensitive, able to reliably give rise to the perception of light from the absorption of as few as five photons (Hecht et al., 1941). However, because all rods have the same spectral sensitivity, rod vision is achromatic. Spectral sensitivity can be thought of as the probability that a photon of a

Figure 1.1 – Diagram of the basic structure of the eye, rods, and cones



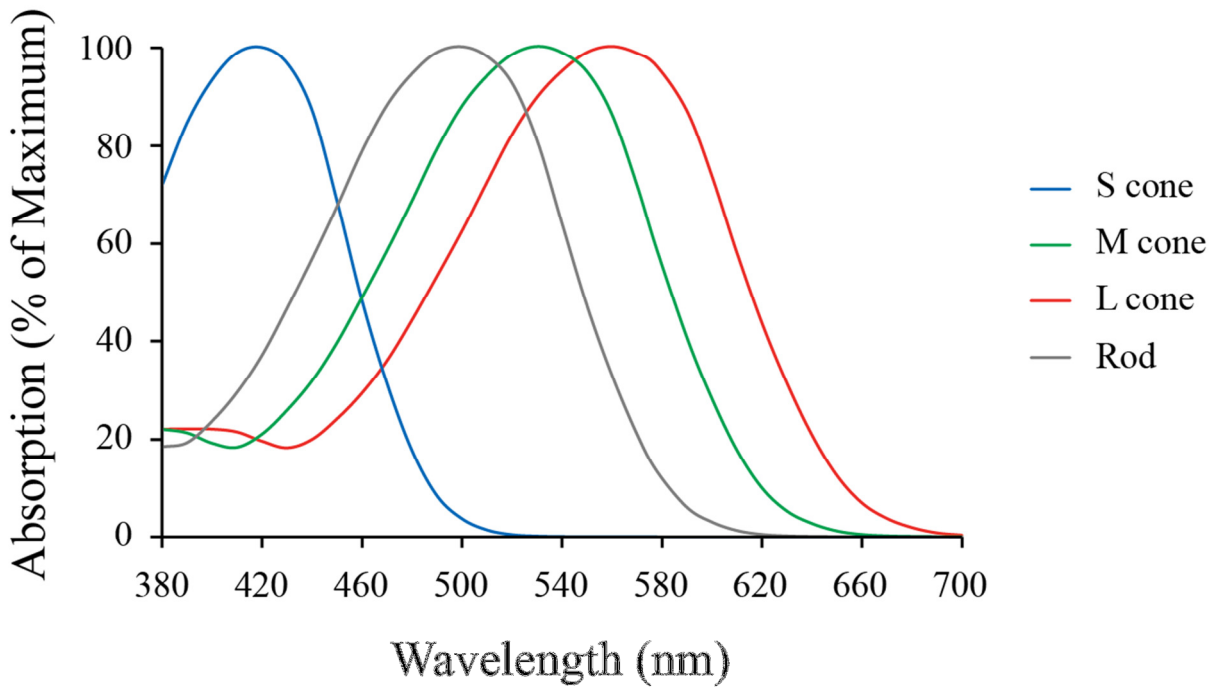
This is a drawing of a cross section through the human eye and a diagram of the layers of the retina. The retina (in red) spans the back of the eye and the photoreceptor layer is at the back of the retina, meaning light must traverse through many cellular layers before reaching light sensitive tissue. Visual signals exit the eye and travel through the brain via the optic nerve.

Image courtesy of Helga Kolb at http://webvision.med.utah.edu

given wavelength will be absorbed. Once absorbed, information about the wavelength of that photon is lost because all absorption events have the same result: isomerization of 11-cis retinal to its all-trans form. Thus, a dim light of a preferred wavelength will evoke the same response as a bright light of non-preferred wavelength. Color vision is the ability to resolve the wavelength of light independent of its intensity. This determination requires comparison of the responses of light detectors with different spectral sensitivities. Our color vision and our high acuity vision both derive from cone photoreceptors. Normal human color vision is trichromatic, served by three types of cone photoreceptors that differ in their spectral sensitivity.

The light-sensitive elements of the photoreceptors, photopigments, are composed of a membrane protein bound to a chromophore. All mammalian photoreceptors use the same chromophore, the molecule 11-cis retinal, which changes conformation to become all-trans retinal upon absorption of a photon (Wald, 1967, 1968). The protein components of the photopigments, called opsins, are membrane-bound proteins with seven transmembrane helices belonging to the superfamily of G-protein coupled receptors. Each type of photoreceptor expresses a different opsin protein. The amino acid sequence of the opsin alters the absorption properties of the chromophore it binds to and thereby gives each photoreceptor its unique spectral sensitivity. All visual pigments have absorption spectra of the same shape (a broad curve with a single peak that falls off gradually to either side) but differ in their wavelength of maximal sensitivity. All human rods share the same photopigment, rhodopsin, that has an absorption spectrum that peaks around 498 nm (Figure 1.2). Humans have three different cone types each expressing a unique photopigment. They are known colloquially as blue cones, green cones, and red cones, referring roughly to the part of the visible spectrum they preferentially absorb. More precisely, they are called short-wavelength sensitive (S) cones, middle-wavelength

Figure 1.2 – Spectral sensitivity of photoreceptors



The spectral sensitivity of the three human cone photoreceptors (S, M, and L) and the rod photoreceptors are plotted across the visible spectrum. The probability of absorbing a photon of any given wavelength is normalized to that of the wavelength of peak sensitivity. That peak is about 417 nm for S cones, 530 nm for M cones, 559 for L cones, and 498 nm for rods. The plotted data is derived from the Neitz spectral sensitivity curves found at http://www.neitzvision.com/content/research.html.

sensitive (M) cones, and long-wavelength sensitive (L) cones, and those are the names that will be used in this dissertation. The absorption spectra of the human S, M, and L cones peak around 417 nm, 530 nm, and 559 nm respectively.

Cone opsin genes

A great deal is understood about the genetic underpinnings of human color vision. Pioneering work reported in a series of three papers from Nathans et al in 1986 determined the chromosomal location and nucleotide sequences of the genes encoding the three cone opsin proteins (Nathans et al., 1986a; Nathans et al., 1986b). The OPN1SW gene, which encodes the S cone opsin protein, is autosomal with a locus at 7q32. OPN1SW has five exons (regions that code proteins) spanning 17 kb. From work sequencing OPN1SW in 282 people with normal color vision (Gunther et al., 2006) and several studies of people with color vision deficiencies (see next section), it was determined that there is likely a single S cone opsin gene sequence that results in normal S cone photopigment function and thus normal color vision.

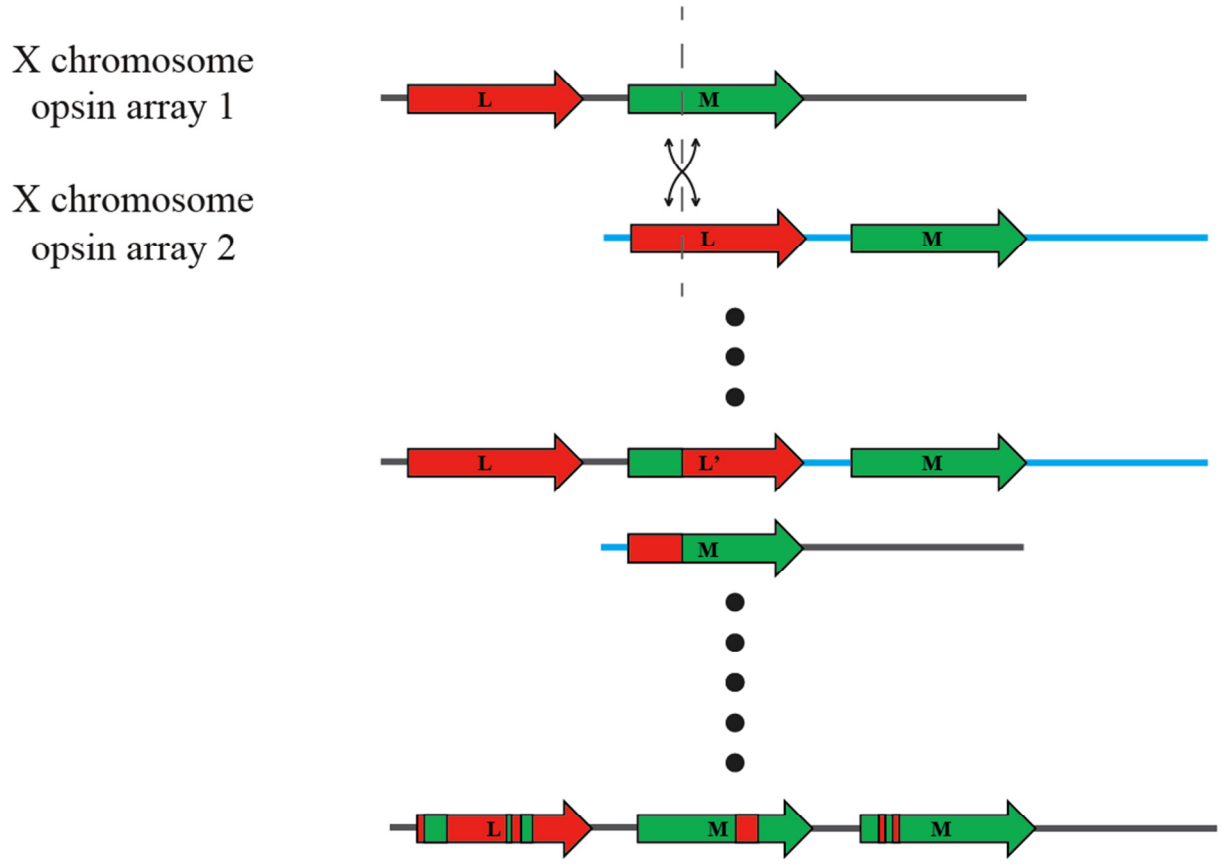
The genetics of the L and M cone opsins are more complicated. The genes OPN1LW and OPN1MW, which encode the L and M cone opsin photopigments respectively, are arranged in a tandem array on the long arm of the X-chromosome at Xq28 (Nathans et al., 1986b). The two genes are highly similar, containing six exons each and sharing about 98% nucleotide identity. The M cone opsin gene likely arose from duplication of the L cone opsin gene approximately 30 to 40 million years ago.

The X chromosome opsin array has two interesting features: variability in opsin gene copy number(Nathans et al., 1986b; Neitz and Neitz, 1995) and a large number of opsin gene variants that all encode normal photopigments(Neitz et al., 1993; Winderickx et al., 1992). This

means that in humans, rather than a single L cone photopigment and a single M cone photopigment as with the S cone, there exists a family of each. The variability in sequence arises from misalignment of the L and M cone opsin genes during meiosis due their close proximity and extremely high sequence homology. As shown in Figure 1.3, the ancestral L and M cone opsin gene array presumably contained an L opsin gene followed by one M opsin gene. During meiosis, the L opsin gene of one X chromosome paired with the M opsin gene of the other and crossed over, resulting in two abnormal arrays. One contains a single opsin gene, a configuration which would result in a red-green color vision deficiency. The other chromosome has three opsin genes: a normal L and a normal M with a hybrid pigment between them. Over generations, this intermixing continued, eventually giving rise to the large variability seen in the modern human X chromosome cone opsin gene array.

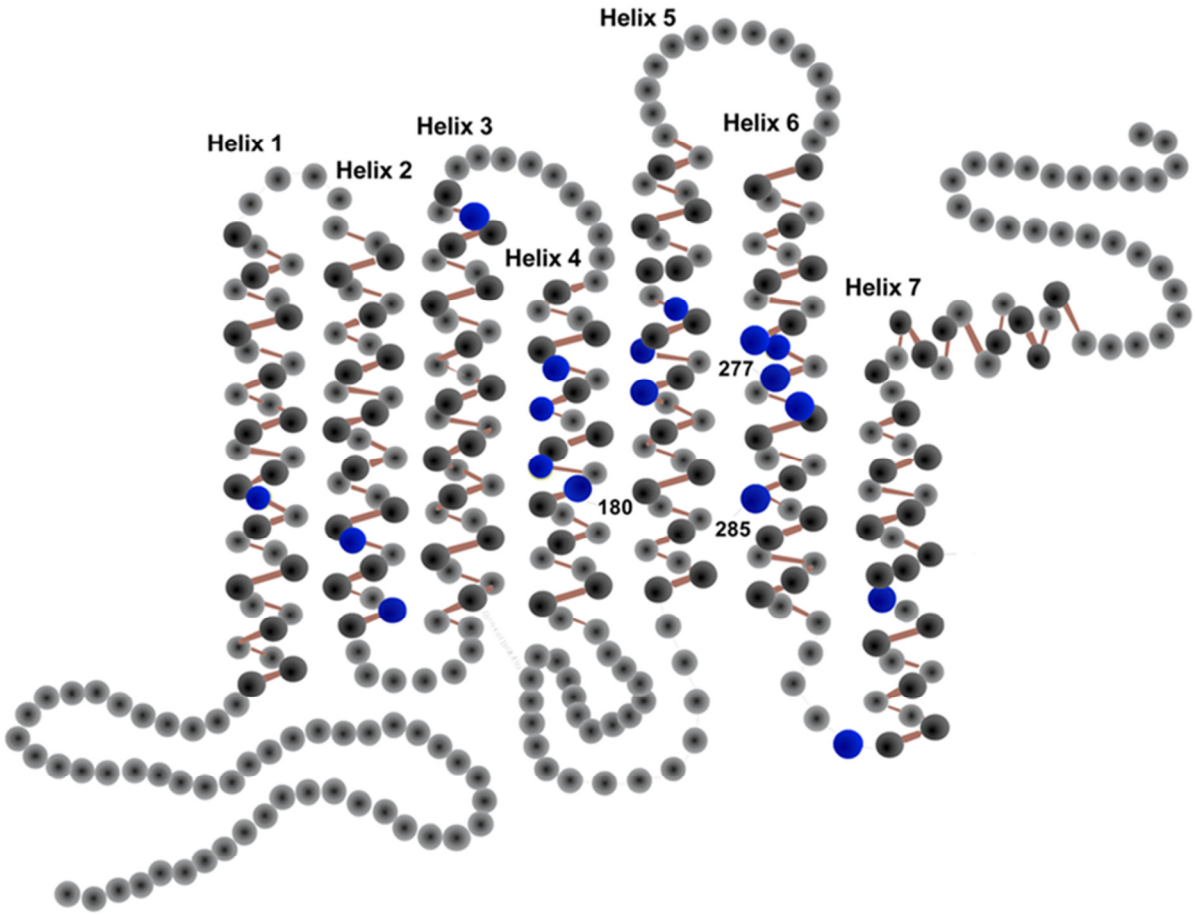
The ancestral L and ancestral M opsins likely differed at 18 amino acid positions. Thus, modern opsin variants have a binary amino acid choice at each of those locations, one drawn from the ancestral L and one drawn from the ancestral M (Figure 1.4). We term these variants "exchange mutants" to distinguish them from spontaneous mutations resulting from errors during DNA replication. The amino acids at positions 277 and 285 produce a spectral shift of about 20 nm in the photopigment, and thus define the difference *between* an L and an M class pigment (Asenjo et al., 1994; Neitz et al., 1991). The amino acid acids at positions 116, 180, and 230/233 produce smaller spectral shifts in the photopigments of 2-4 nm and thus define the differences in spectral sensitivity of photopigments *within* a class (Asenjo et al., 1994; Merbs and Nathans, 1992a; Neitz and Neitz, 2011).

Figure 1.3 – Unequal homologous crossover leading to opsin array variation



During meiosis, the process of cell division to produce gametes (sperm or eggs cells), homologous chromosomes engage in crossing over, where equivalent regions of DNA are swapped as a mechanism to enhance genetic diversity in the population. Because of the high degree of similarity and the proximity of the L and M opsin genes, the X chromosome is prone to misalignment at this locus. When crossovers occur at this point, the result is intermixing of the L and M opsin genes. Over generations, this intermixing produced the enormous variability seen today in opsin gene sequence and copy number. Many of these opsin gene arrays confer color vision defects.

Figure 1.4 – L/M opsin ribbon diagram



This ribbon diagram of the L/M opsin protein shows the seven helical regions that are embedded in the membrane. The residues highlighted in blue are the dimorphic positions where each photopigment has a choice of amino acids drawn from the ancestral L or M photopigment. The identity of amino acids in blue positions 277 and 285 determines whether the protein is of the L or M class.

Color Vision Defects

Blue-yellow color vision deficiencies, termed tritan defects or tritanopia, are rare, affecting about one in 10,000 people. They result from loss of function or viability of S cones. As with rhodopsin, almost any missense mutation in the S cone opsin gene appears to cause disease. Currently six known mutations of OPN1SW have been linked to S cone dysfunction and result in tritanopia (Baraas et al., 2007; Baraas et al., 2012; Gunther et al., 2006; Weitz et al., 1992a; Weitz et al., 1992b). The color vision loss in these individuals appears progressive (Baraas et al., 2007); normal trichromatic color vision in childhood gradually succumbs to tritan defects as S cones slowly die. The mutations are autosomal dominant, meaning only one copy of the mutation-containing variant is sufficient to cause disease. The mechanism of this dysfunction is currently unknown.

Inherited red-green color vision defects are congenital and come in two forms, deutan and protan. Deutan defects result from loss of functional M cones and protan defects from loss of functional L cones. The phenotypic difference between the two is in the color confusion line they produce, meaning which exact hues of red and green are indistinguishable from grey. By far the most common cause of red-green defects is gene rearrangements caused by unequal homologous recombination during meiosis. As described in the previous section, some X chromosome opsin gene arrays contain only a single gene. These arrays will result in dichromacy, the most severe form of red-green defect in which only two of the normal three classes of cones are present in the retina. The single gene array in Figure 1.3 contains only an L opsin gene and a male with this X chromosome will have only L and S cones. All cones that would have expressed M opsin will instead express L opsin so that the total number of cones is unchanged, meaning visual acuity is unaffected. Dichromacy is indicated by the suffix “opia” so

that male would have deuteranopia and be called a deuteranope. A male with multiple L opsin genes that all encode L photopigments with identical spectral sensitivity would have the exact same deuteranopic phenotype. Similarly, a person with an X chromosome containing only a single M opsin gene or multiple M opsin genes encoding spectrally identical photopigments is called a protanope and the defect is called protanopia.

A milder form of red-green color blindness results from an opsin gene array that contains two genes that encode photopigments of the same class with different spectral sensitivity. People with such genotypes are called anomalous trichromats because their retinas still have three cone types but two are of the same class – either S, L, and L' or S, M, and M'. These cone types lead to deuteranomalous and protanomalous deficiencies respectively. Anomalous trichromats have some degree of red-green sensitivity but with higher thresholds than normal trichromats. The greater the spectral distance between their longer-wavelength photopigments, the better their red-green sensitivity.

It is important to note that regardless of how many cone opsin genes are present in the array, usually only those occupying the first two positions are expressed in a cone photoreceptor (Hayashi et al., 1999). Thus, the array shown in Figure 1.3 with three cone opsin genes would lead to a red-green defect, as the first two genes both belong to the L class of photopigments. Since the first gene is the ancestral L photopigment and the second is slightly green-shifted due to the presence of some portion of the ancestral M gene, the individual would be deuteranomalous.

Like tritan defects, spontaneous mutations that interfere with the expression or function of L or M opsin will cause color vision deficiencies. One common missense mutation in Caucasians results in the replacement of cysteine with arginine in position 203, which causes

complete degeneration of the cones that express the mutant gene (Carroll et al., 2009). Another mutation common in Asian populations is a substitution of the nucleotide cytidine for adenosine in position -71 in the promoter region of the M opsin gene. This mutation appears to cause decreased expression of M opsin in the cones or decreased numbers of M cones (Ueyama et al., 2015a). Around a dozen missense mutations and insertion/deletion mutations in L or M opsin genes have been linked to red-green color vision defects.

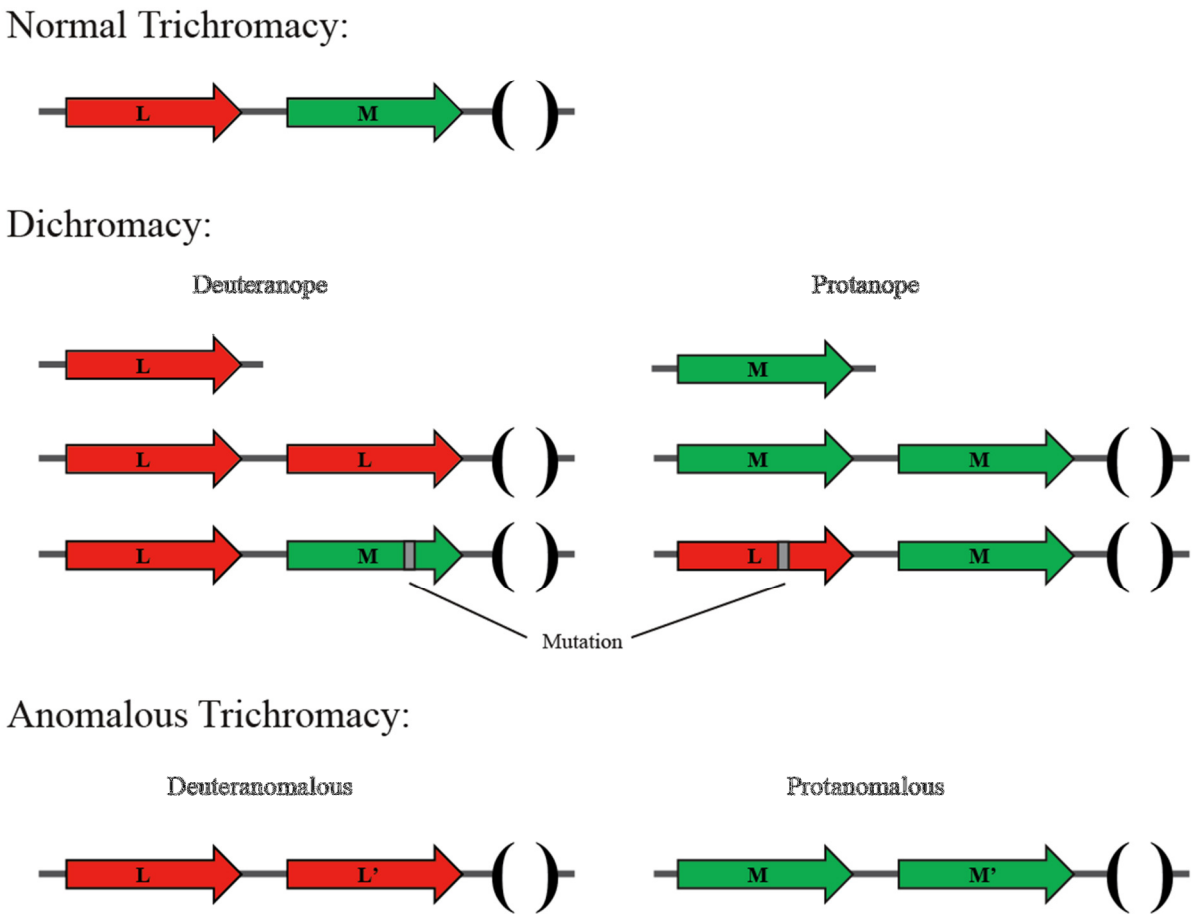
Finally, there are 'toxic' variants of the opsin genes with a particular grouping of exchange mutations that have been shown to cause opsin dysfunction. At dimorphic positions 153, 171, 174, 178 and 180 in exon 3, the genes specify the amino acid sequences MIAVA, LIAVA, LIVVA, or LVAVA. They can be found in either L or M genes with identical effect. Each of the five dimorphisms is benign on its own, but the combination produces disastrous consequences for cone function or viability.

Red green color blindness is far more common in males than females, as females have two X chromosomes and thus two opportunities for functional copies of both the L and M opsin genes. Overall, rates of red-green defects in Caucasian men are about 1% protanopia, 1% deuteranopia, 1% protanomaly, and 5% deuteranomaly (Waaler, 1927). About 1 in 250 women has a red-green defect and about 1 in 7 is a carrier of a defect. Opsin gene array conformations that confer various color vision phenotypes are illustrated in Figure 1.5.

Mouse cones

For the research described in the final chapter of this thesis, I make use of a mouse model to investigate the possibility of using virally-mediated gene therapy treatments to cure human cone diseases that are caused by the toxic cone opsin variants described above. To understand

Figure 1.5 – Opsin gene arrays conferring normal and defective color vision



Normal color vision requires that a functional copy of both the L and the M opsin gene occupy the first two position in the array. Additional opsin genes may exist downstream of those two, as shown by the open brackets. Dichromatic color vision comes in two types – deutan and protan – and can be caused by loss of either the L or M gene, the presence of identical genes in the first two array positions, or mutations that interfere with the expression or function of the encoded opsin protein. Anomalous trichromacy occurs when the first two array positions are occupied by genes of the same class that have different spectral peaks.

this work, it is necessary to appreciate the ways in which mouse cones differ from human cones.

In the mouse, there are two cone photopigments, one that is sensitive to ultraviolet light with spectral peak of around 360 nm and one similar in sensitivity to the human M cone with a spectral peak of around 510 nm (Jacobs et al., 1991). An important difference in the mouse retina is that while human cones only express one photopigment gene in each cell, mouse cones can dual express both of their photopigments genes in the same cone cell (Applebury et al., 2000). There exists a gradient of opsin expression across the mouse retina, such that cones in the ventral retina contain primarily S opsin and cones in the dorsal retina contain primarily M opsin. In order to isolate the toxic opsin variant, the UV opsin gene was knocked out of the mice used in this study.

Chapter Two

Variation of Opsin Genotypes Among People with Normal Color Vision

Introduction

The genes OPN1LW and OPN1MW, which encode the human long wavelength sensitive (L) and middle wavelength sensitive (M) cone photopigments respectively, are arranged in a tandem array on the X chromosome at Xq28. As a consequence of their close proximity and high (98%) nucleotide identity, the L and M opsin genes are prone to unequal homologous recombination during meiosis, resulting in intermixing between the two genes. Modern human opsin arrays thereby vary both in gene sequence and in gene copy number. At those positions where the ancestral L and M opsin genes differed, modern opsin variants have a dimorphic nucleotide choice with one option drawn from the ancestral L opsin and one from the M. The nucleotide differences in exon 5 encode a 20 nm spectral shift, defining the difference *between* L and M class photopigments. Other nucleotide dimorphisms in exons 2, 3, and 4 shift the spectral sensitivity by 2-4 nm and thus create differences *within* the L or M class.

Normal color vision is served by expression of at least one functional M and one functional L class photopigment. As only the first two genes in the array are generally expressed (Hayashi et al., 1999), displacement of either gene from an expressed position by a gene of the other class will cause red-green defects. If the two expressed positions are occupied by spectrally different genes of the same class, the result will be a milder form of red-green defect called anomalous trichromacy. If they are occupied by identical genes of the same class, or if only one functional gene is present, the result is dichromacy, a complete loss of the red-green color system. A typical array conferring normal color vision consists of a single L opsin gene

followed by at least one M opsin gene, though in rare cases additional L opsin genes are present downstream of the expressed positions.

In this work we use a large pool of 1022 male subjects with normal color vision to explore array composition and opsin haplotype distribution in the normal population. We also use a pool of female subjects unselected for color vision to investigate the potential for extra dimensions of color vision in humans. Because women have four expressed opsin positions across their two X chromosomes, they have the potential to have up to five different cone types due to random X inactivation in each cone (four longer wavelength absorbing cones and S cones). In our sample of 335 women, we investigated the frequency of trichromacy, tetrachromacy, and pentachromacy in the population.

Methods

Subject recruitment

Experiments involving human subjects were conducted in accordance with the principles embodied in the declaration of Helsinki, and were approved by Institutional Review Boards at the University of Washington.

1022 male subjects participated in this research. The ages ranged from 18 to 82 with a mean of 36. The racial composition of the subject pool was: 463 (45%) Caucasian, 247 (24%) African-American, 161 (16%) Asian, 28 (3%) Hispanic, 16 (2%) Alaska Native/Native American, 107 (10%) other/mixed/not reported. All were determined to have normal color vision by assessment with the AO-HRR and the Lanthony D-15. A DNA sample was collected from blood or saliva for cone opsin gene analysis.

A second subject pool that provided DNA samples was comprised of 335 women. 201

women were participants in a study of glaucoma and high myopia. They were unselected for color vision phenotype though there may be a relationship between opsin haplotype and glaucoma. Behavioral color vision data and age and racial information is not available for these subjects. The remaining 134 subjects were recruited for color vision studies or for myopia studies which had passing the 4th edition HRR test as a criteria. Frequencies of genotypes did not vary between the group unselected for color vision and those that were tested and so both groups were included in the final analysis.

Analysis of X-chromosome opsin array composition

Analysis of the number and class of opsin genes on the X chromosome was performed using a technique described in detail in the following chapter. Briefly, using the MassArray genotyping platform (Sequenom), one can determine the proportion of genes in a sample that have a particular single-nucleotide polymorphism (SNP) variant. To determine the number of opsin genes in the array, the measured SNP is in the promoter region and differs between the first gene in the array and all downstream opsin genes. The inverse of this proportion is thus the number of genes in the array per X chromosome. To determine how many of those opsin genes are of the L and M classes, a second SNP that encodes residue 309 is used. L photopigments generally encode a tyrosine at that positon and M photopigments generally encode a phenylalanine.

Gene sequencing

Genetic analysis was carried out as described in detail in Chapter 3. Briefly, for each sample, the L and M opsin genes were separately and specifically amplified in a long-range PCR

spanning from intron 1 to the middle of exon 5. Opsin-type specificity was conferred using 3' primers that bind to the site of characteristic polymorphisms in exon 5 that differ between L and M opsin genes. The products of those reactions were used as the template for a second round of PCR that amplified individual exons. In this way, we derived PCR products for exon 2, exon 3, and exon 4 from both L opsin genes and M opsin genes.

For some subjects, the first gene or the last gene in the photopigment gene array was specifically amplified and individual exons were sequenced. This was accomplished by using PCR primers outside of the repeat unit of the array, the 5' primer for the first gene and the 3' primer for the last gene. Finally, for some subjects, exon 5 was non-specifically amplified from all opsin genes, from the first gene PCR product, or from the last gene PCR product and sequenced. Direct sequencing was performed using the Applied BioSystems BigDye Terminator v3.1 cycle sequencing kit and analyzed on an ABI 3500 Genetic Analyzer.

Identification of the last gene in the array as encoding an L or M pigment was performed for selected subjects by amplifying exon 5 from the last gene PCR product. The sequence encoding residue 277 differs between L and M opsin genes. The L opsin variant creates an RsaI restriction site, thus digestion with RsaI followed by gel electrophoresis will yield two bands for L opsin genes and a single band for M opsin genes.

Estimate of Photopigment Spectral Peak

To estimate the photopigment spectral peak, we use averages of several estimates of amino acid contribution to spectral tuning (Asenjo et al., 1994; Neitz and Neitz, 2011). We estimated the longest L photopigment to have a λmax of 559 nm and the spectral tuning to be shifted 2.5 nm shorter for a tyrosine at 116, 3.5 nm shorter for an alanine at 180, and 4 nm

shorter for a threonine at 230. We estimated the shortest M photopigment to have a λmax of 530 nm and the spectral tuning to be shifted 3 nm longer for either a serine at 180 or an isoleucine at 230.

Results

Array Composition of Males

Plotted in Figure 2.1 are the results of the analysis of the male subjects' X chromosome opsin gene arrays. The abscissa is the proportion of "first genes" and the ordinate is the proportion of genes in the array belonging to the L class of opsins. Subjects cluster into groups based on their array composition. Those at [0.5, 0.5] have two total genes of which half belong to the L class, indicating an LM array. Those with extra M genes are also on the unity line with decreasing distance from the origin as the number of extra M genes increases. Those with extra L opsin genes are above the unity line, as they have a higher proportion of L genes for a given array length. For example, the subject at [0.33, 0.35] has a three-gene array containing one L while the subject at [0.33, 0.72] has a three-gene array containing two Ls.

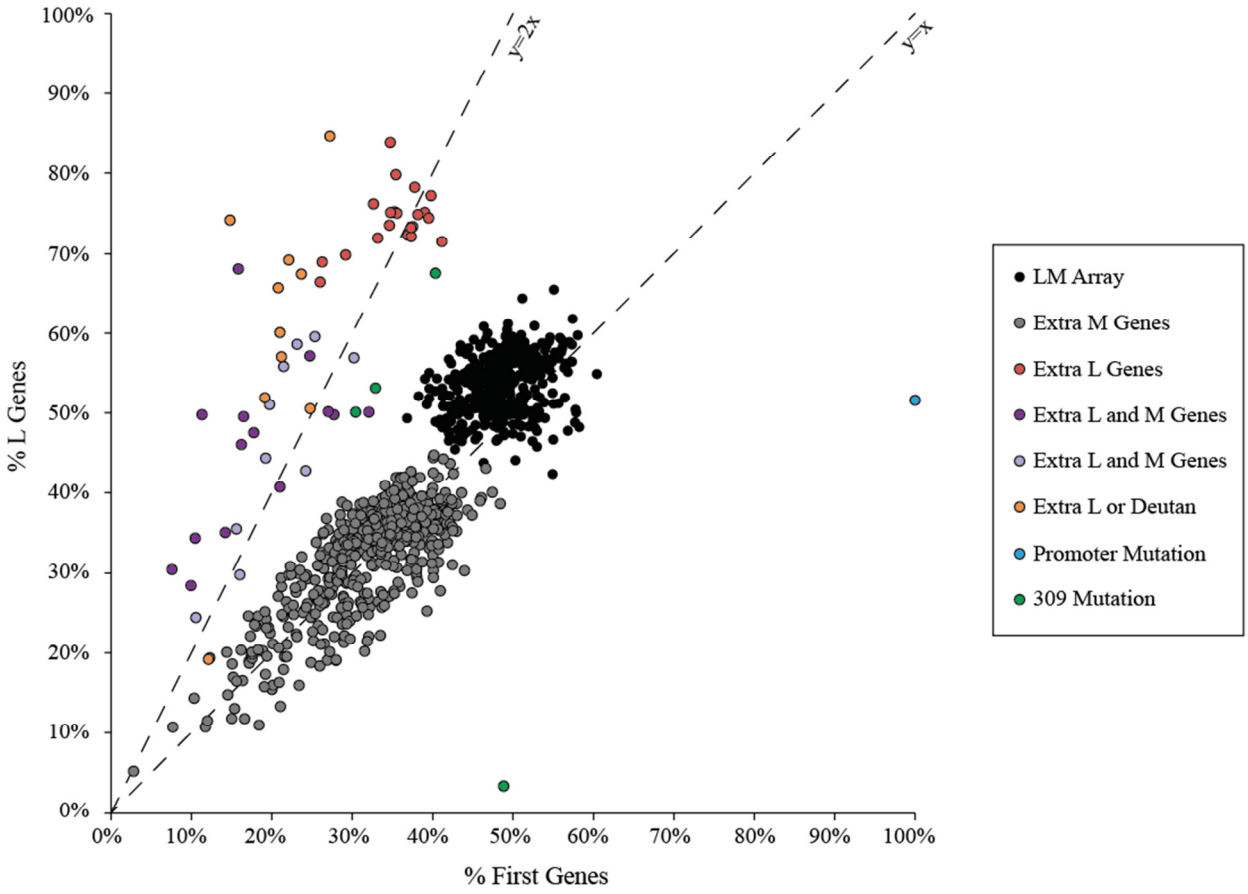
Generally, only the first two opsin genes in the array are expressed in cone cells. For subjects with extra L opsin genes, additional analysis was conducted on the last gene in the array to confirm that the extra L opsin gene was not in an expressed position; otherwise the subject would be a deutan with mild enough defects to pass our screening tests. Subjects who were confirmed to have the extra L gene in the unexpressed last position (e.g. an LML array) are included in the present study. Subjects who were confirmed to have the extra L gene in the expressed second array position (e.g. an LLM array) were excluded, as they are necessarily deutans.

With longer arrays, it becomes increasingly difficult to determine which gene is in the expressed second position. Consider a subject with two L genes and two M genes. The class of the first and last genes can be determined by specific PCR reactions. If both are L, then the array is necessarily LMML and the subject would be categorized as "Extra M and L." However, if the last gene is an M, there are two possible arrays: the deutan LLMM and the trichromatic LMLM. In this situation, we classified the subject as having normal color vision if the two L genes were spectrally identical, since a deutan with two spectrally identical L cones would be a dichromat and very likely to have failed the color vision tests. If the two L genes were spectrally distinct, we cannot disambiguate an array conferring normal color vision from one conferring very mild deutan defects. Such subjects were classified as "Extra L genes, possibly expressed."

Finally, we identified several subjects who had mutations in the SNPs used by our assay of array composition. Gene sequencing revealed that one subject at [1, 0.5] had an LM array but with the "first gene" promoter variant in both of his genes instead of one "first gene" and one "downstream" variant. Four other subjects had analogous mutations in the SNP encoding position 309 that we use to distinguish L from M genes in this assay. Three had M genes that encoded the tyrosine normally found in L photopigments and one had an L gene that encoded the phenylalanine normally found in M photopigments.

We find that deviations from the presumably ancestral opsin gene array containing a single L and single M opsin gene are extremely common among men with normal color vision. Over half the men in our sample had extra unexpressed M opsin genes and about 1 in 20 men had extra unexpressed L genes. In about 1% of our sample, we could not definitively determine whether the subject had normal color vision with extra opsin genes of both classes or whether the subject was in fact a mild deutan able to pass standard color vision tests.

Figure 2.1 MassArray data for 1022 males with normal color vision.



The data plotted in Figure 2.1 is derived from MassArray test, which uses SNPs that vary between the first gene in the array and downstream genes and that vary between L and M genes in order to characterize the L and M opsin gene array. The x axis is the proportion of the total opsin genes that have the "first gene" promoter allele. The y axis is the proportion of the total opsin genes that are of the L opsin class, as measured by a SNP in codon 309. Dotted lines indicate where arrays with different L opsin gene numbers are expected to fall, with the slope of the line indicating L gene number.

We found significant differences in array structure across racial groups. Subjects who identified as Asian, Hispanic, or Native American/Native Alaskan had lower opsin copy number than Caucasians or African Americans. Caucasians more commonly had extra M genes, with fully two thirds of the sample having at least one extra M gene. A striking 10% of African American men had extra L opsin genes, twice as often as Caucasians and seventeen times as often as Asian men. The differences between the Asian, African American, and Caucasian groups were highly significant (Fisher's exact test: Caucasian/African American $p=.0211$, Asian/African American $p<.0001$, Asian/Caucasian $p<.0001$). Trends toward shorter opsin gene arrays in Hispanic and Native populations are intriguing but we cannot draw conclusions due to the small size of our samples of these populations. These results are summarized in Table 2.1.

Array Composition of Females

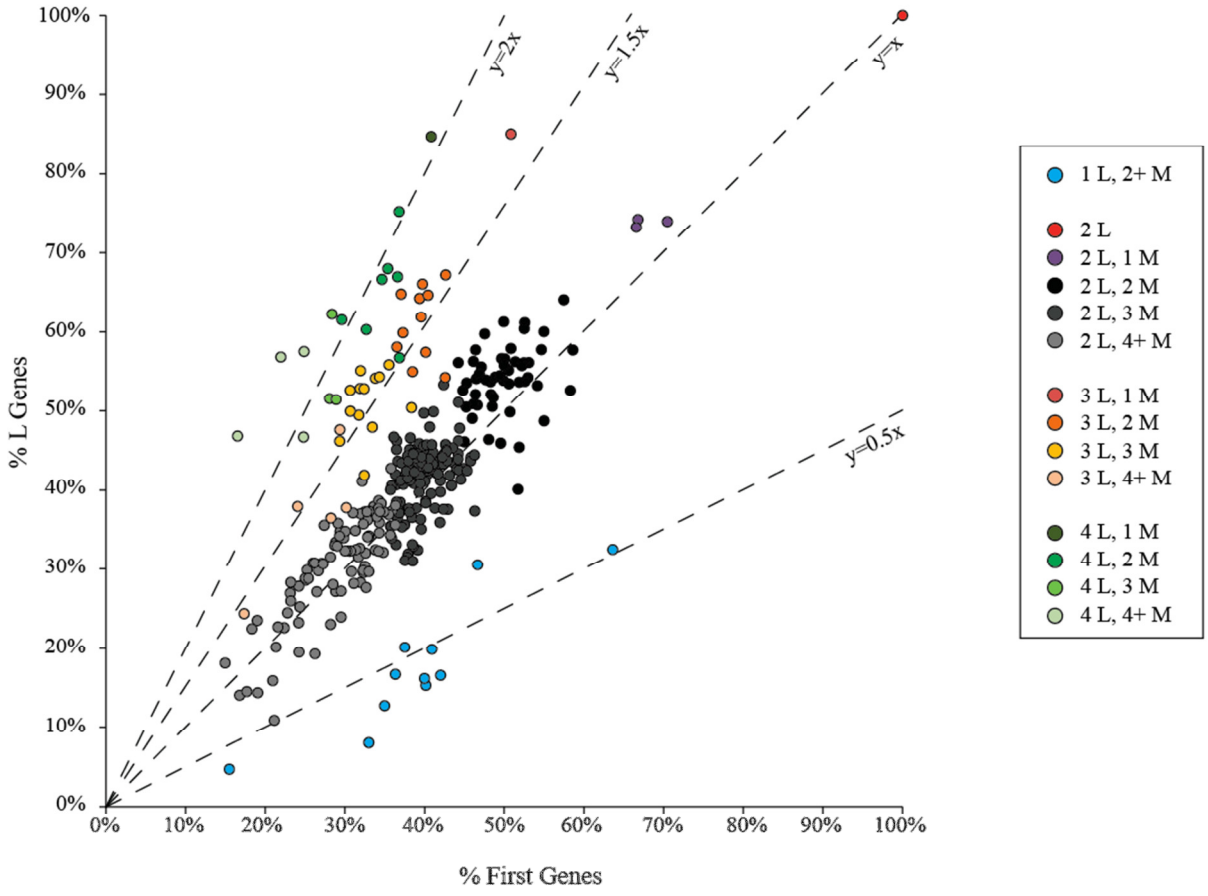
Determining the opsin array structure of women is complicated by the presence of two X chromosomes. Using the MassArray SNP assay, we can determine the number and type of opsin genes across both arrays (Figure 2.2). For women, the inverse of the abscissa multiplied by two is the total number of genes. For any line drawn through the origin, the slope is the average number of L genes per X chromosome. Protan carriers fall along the $y=0.5x$ line as they have half an L gene per X chromosome. Women with two L genes fall on the $y=x$ line, women with three L gene on $y=1.5x$, women with four L genes on $y=2x$, and so on. With information about the number of L and M genes, we can use specific sequencing of L genes, M genes, genes in the first array position, and genes in the last array position to deduce the genes present in the first two positions of the two arrays for most women (271 out of 335). For the rest, we can narrow their arrays down to two to three possibilities.

Table 2.1 Summary of opsin array types

Array Compostion	All Subjects		Caucasian		African Amer		Asian		Hispanic		Native	
"Ancestral" LM Array	388	38.0%	145	31.3%	83	33.6%	87	54.0%	16	57.1%	12	75.0%
Extra M genes	573	56.1%	295	63.7%	136	55.1%	70	43.5%	12	42.9%	4	25.0%
Extra L genes	21	2.1%	10	2.2%	9	3.6%	0	0.0%	0	0.0%	0	0.0%
Extra M and L genes	25	2.4%	11	2.4%	11	4.5%	1	0.6%	0	0.0%	0	0.0%
Extra L genes, maybe expressed	10	1.0%	2	0.4%	6	2.4%	0	0.0%	0	0.0%	0	0.0%
309 mutation	4	0.5%	0	0.0%	2	0.8%	2	1.9%	0	0.0%	0	0.0%
Promoter mutation	1		0		0		1		0		0	
Total	**1022**		**463**		**247**		**161**		**28**		**16**	

Data from the MassArray assay and gene sequencing are combined to deduce opsin gene array configurations. The data are given for the total pool of 1022 men and then are subdivided by racial background where possible (some subjects did not report their race). For ten subjects with extra L genes, it was impossible to determine if that gene was in an expressed position, and such people might be very mild deutans with red-green sensitivity high enough to pass our screening tests.

Figure 2.2 MassArray data for 335 females



The data plotted in Figure 2.2 is derived from MassArray test, which uses SNPs that vary between the first gene in the array and downstream genes and that vary between L and M genes in order to characterize the L and M opsin gene array. The x axis is the proportion of the total opsin genes that have the "first gene" promoter allele. The y axis is the proportion of the total opsin genes that are of the L opsin class, as measured by a SNP in codon 309. Dotted lines indicate where arrays with various L opsin gene numbers are expected to fall, with the slope of the line indicating average L gene number across both X chromosomes.

Table 2.2 Summary of cone types found in 335 female subjects.

# Cone Types	Physiological Phenotype	Number of Subjects	Frequency
2	Deuteranomalous	1	0.3%
3	Trichromatic; protan carrier	6	1.8%
	Trichromatic	119	35.5%
3-4	Trichromatic or tetrachromatic (M'); protan carrier	5	1.5%
	Trichromatic or tetrachromatic (M')	23	6.9%
	Trichromatic or tetrachromatic (L')	3	0.9%
4	Tetrachromatic (L' or M')	1	0.3%
	Tetrachromatic (L')	137	40.9%
	Tetrachromatic (M')	3	0.9%
4-5	Tetrachromatic (L') or pentachromatic (L' and M')	28	8.4%
	Tetrachromatic (L') or pentachromatic (L' and L'')	1	0.3%
5	Pentachromatic (L' and M')	6	1.8%
	Pentachromatic (L' and L'')	2	0.6%

Using a combination of MassArray data and gene sequencing of L opsins, M opsins, first genes, and last genes, we deduced the number of cone types present in the retinas of 335 female subjects. Cone types are defined by their estimated spectral peak. For cases where it was impossible to be certain of which opsin genes were in expressed positions, a range of possible numbers of cone type was given.

The results of our analysis are summarized in Table 2.2. One woman had two arrays with only a single L opsin gene; as they encoded spectrally different pigments, she is deuteranomalous. 125 women were trichromatic with opsins encoding a single type of L and a single type of M photopigment; of those, 119 women were normal trichromats and additional 6 were protan carriers. 141 women were tetrachromatic with either two L photopigments (137), were protan carriers. 141 women were tetrachromatic with either two L photopigments (137), two M photopigments (3), or either two L or two M photopigments (1). 8 women were pentachromatic, 6 of whom had two distinct L photopigments and two distinct M photopigments and 2 of whom had three distinct L photopigments and one M photopigment. An additional 31 women could be trichromats or tetrachromats and 29 could be tetrachromats or pentachromats. Overall, 0.3% of the sample were anomalous trichromats, 37.3% to 47.6% were normal trichromats, 42.1% to 60.0% were tetrachromatic, and 2.4% to 11.0% were pentachromatic.

Haplotype and spectral peak distribution

We used our sample of male subjects to investigate haplotype diversity in the population of "normal" L and M opsin genes both because we could confirm their color vision phenotype with behavioral testing and because determining exact sequences for females is often impossible. For subjects with multiple spectrally distinct L genes, the first gene sequence was used. For subjects with multiple spectrally distinct M genes, the last gene sequence was used to deduce the expressed M gene sequence, when possible. For 178 subjects, we could not definitively determine the sequence of the expressed M gene. This occurred for three reasons. First, the amplified region of the last gene PCR (30kb) is close to the limit of the ability of long-range PCR enzymes and we were unable to amplify it for some subjects. Second, for subjects with a

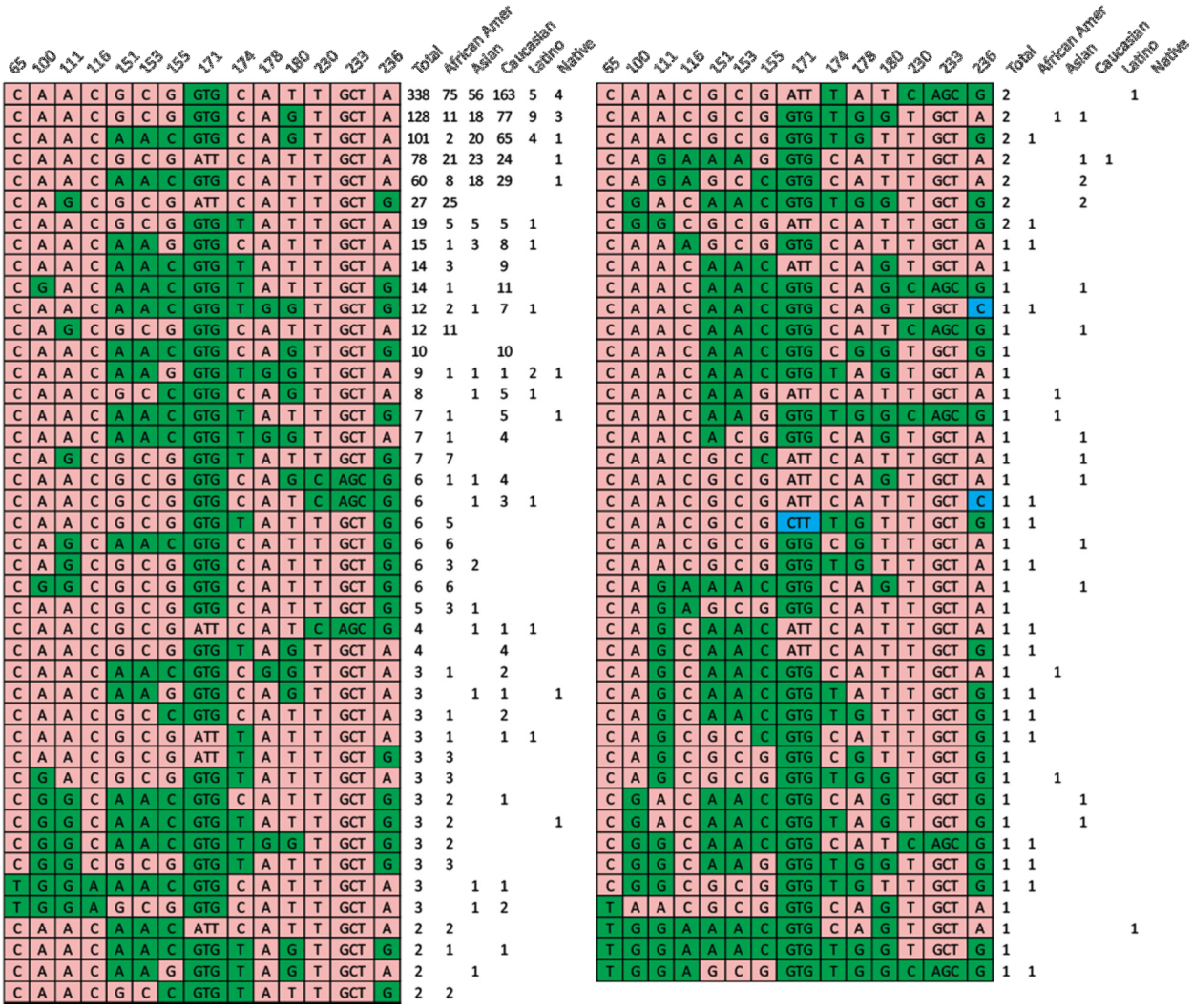
large number of M genes, we may be unable to detect opsin variants with exchange mutations. A minority species that comprises less than about 15-20% of a sample is below the detection threshold for our gene sequencing (unpublished data), so in a large array an allele found only in one gene may produce a signal too low to be detected. Third, for subjects with more than two M opsins with different spectral peaks, the peak of the expressed M may be undeterminable. Consider a subject with one L and three M genes in which one M gene has 180Ser and two have 180Ala. If we do not find the 180Ser photopigment in the last array position, then there is no way to determine whether it is in the gene in expressed second array position or in the unexpressed third position.

Overall, we found sequences for 1022 expressed L genes and 844 expressed M genes. Figure 2.3 shows haplotypes for 1008 expressed L opsins and 839 expressed M opsins (14 L genes and 5 M genes had other point mutations outside of the dimorphic positions). As has been reported previously, the L opsin genes were more variable then the M genes, and exon 3 was the most variable region of both genes.

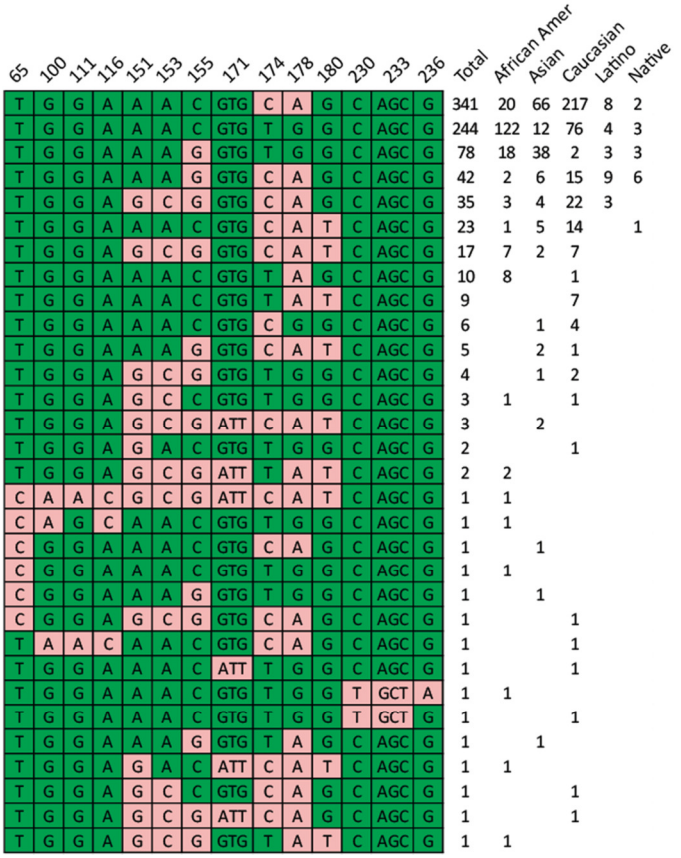
Next, we used this gene sequence information to estimate the spectral peaks of the L and M cones. This data is plotted in Figures 2.4A and 2.4B respectively. We find that the M genes are quite homogenous: 93% of the men had unshifted 530 nm M photopigments, 7% had either 180Ser or 230Met alleles that shifted the pigment to 533nm, and none had the combination of both mutations to generate the maximum shift to 536 nm. The L photopigments were far more variable; only 66% of subjects had unshifted 559 nm L peaks. The most common spectral tuning shift in L opsin variants was due to the replacement of serine with alanine at position 180, which occurred in almost a third of the sample. Several subjects had multiple amino acid changes affecting tuning, including one subject with a maximally shifted 549 nm L photopigment.

Figure 2.3 Haplotypes at dimorphic positions in exons 2, 3, and 4 in expressed opsin genes

A



B



65	100	111	116	151	153	155	171	174	178	180	230	233	236	Total	African Amer	Asian	Caucasian	Latino	Native
T	G	G	A	A	A	C	GTG	C	A	G	C	AGC	G	341	20	66	217	8	2
T	G	G	A	A	A	C	GTG	T	G	G	C	AGC	G	244	122	12	76	4	3
T	G	G	A	A	A	G	GTG	T	G	G	C	AGC	G	78	18	38	2	3	3
T	G	G	A	A	A	G	GTG	C	A	G	C	AGC	G	42	2	6	15	9	6
T	G	G	A	G	C	G	GTG	C	A	G	C	AGC	G	35	3	4	22	3	
T	G	G	A	A	A	C	GTG	C	A	T	C	AGC	G	23	1	5	14		1
T	G	G	A	G	C	G	GTG	C	A	T	C	AGC	G	17	7	2	7		
T	G	G	A	A	A	C	GTG	T	A	G	C	AGC	G	10	8		1		
T	G	G	A	A	A	C	GTG	T	A	T	C	AGC	G	9			7		
T	G	G	A	A	A	C	GTG	C	G	G	C	AGC	G	6		1	4		
T	G	G	A	A	A	G	GTG	C	A	T	C	AGC	G	5		2	1		
T	G	G	A	G	C	G	GTG	T	G	G	C	AGC	G	4		1	2		
T	G	G	A	G	C	C	GTG	T	G	G	C	AGC	G	3	1		1		
T	G	G	A	G	C	G	ATT	C	A	T	C	AGC	G	3		2			
T	G	G	A	G	A	C	GTG	T	G	G	C	AGC	G	2			1		
T	G	G	A	G	C	G	ATT	T	A	T	C	AGC	G	2	2				
C	A	A	C	G	C	G	ATT	C	A	T	C	AGC	G	1	1				
C	A	G	C	A	A	C	GTG	T	G	G	C	AGC	G	1	1				
C	G	G	A	A	A	C	GTG	C	A	G	C	AGC	G	1		1			
C	G	G	A	A	A	C	GTG	T	G	G	C	AGC	G	1	1				
C	G	G	A	A	A	G	GTG	T	G	G	C	AGC	G	1		1			
C	G	G	A	G	C	G	GTG	C	A	G	C	AGC	G	1			1		
T	A	A	C	A	A	C	GTG	C	A	G	C	AGC	G	1			1		
T	G	G	A	A	A	C	ATT	T	G	G	C	AGC	G	1			1		
T	G	G	A	A	A	C	GTG	T	G	G	T	GCT	A	1	1				
T	G	G	A	A	A	C	GTG	T	G	G	T	GCT	G	1			1		
T	G	G	A	A	A	G	GTG	T	A	G	C	AGC	G	1		1			
T	G	G	A	G	A	C	ATT	C	A	T	C	AGC	G	1	1				
T	G	G	A	G	C	C	GTG	C	A	G	C	AGC	G	1			1		
T	G	G	A	G	C	G	ATT	C	A	G	C	AGC	G	1			1		
T	G	G	A	G	C	G	GTG	T	A	T	C	AGC	G	1	1				

All haplotypes found in both L (A) and M (B) opsin genes in expressed array positions are listed here. The codons containing dimorphic nucleotides are given in the top row. Alleles from the ancestral L are colored in red, those from the ancestral M in green, and new mutations in blue. The frequency that each haplotype was found in the full subject pool and in each racial subgroup are given at the right of the haplotype.

Table 2.3 L and M cone spectral peaks

L peak	Number of Subjects	M peak	Number of Subjects
549 nm	1	530 nm	872
551.5 nm	8	533 nm	63
553 nm	3	536 nm	0
555 nm	15	**Total**	**935**
555.5 nm	308		
556.5 nm	13	**Indeterminate**	**87**
559 nm	674		
Total	**1022**		

Estimates of the spectral peaks of the L and M cones in normal males are derived from gene sequences of the opsins in the first two opsin array positions. It was impossible to determine the M cone peak definively some some subjets. This data is plotted in Figure 2.4.

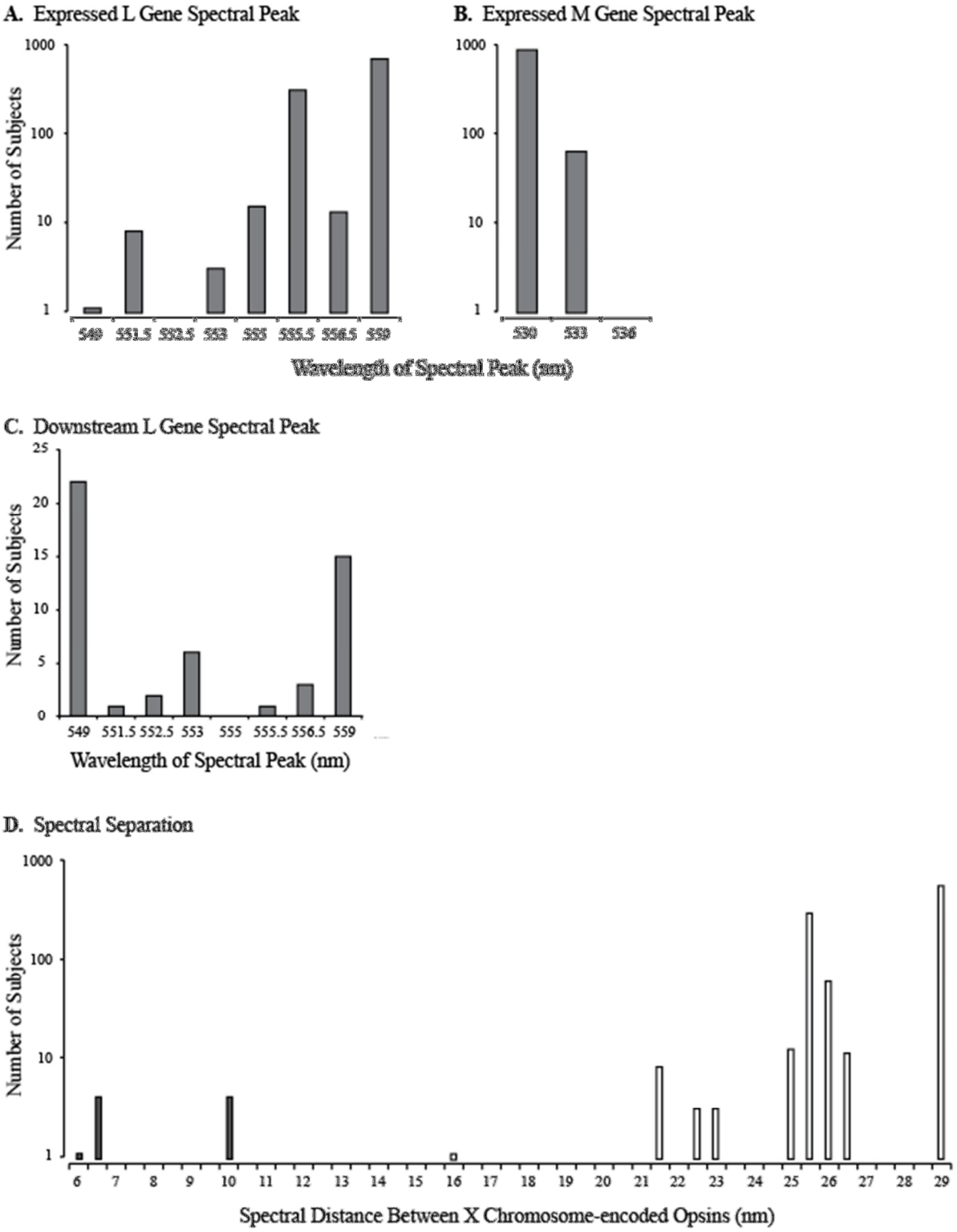
In addition to the expressed cone photopigments, we also estimated the spectral peaks for downstream, unexpressed L opsin genes (Fig 2.4C). These genes are far more green-shifted in their absorbance than expressed L genes. We also found that a significantly smaller proportion of M genes have a λmax of 533 nm among unexpressed genes at the end of the opsin array than in expressed M genes (20 out of 548 for last M genes compared to 63 out of 935 for expressed M genes; Fisher's exact test, p=0.014). Together, this indicates that alleles that derived from the ancestral L photopigment tend to be found in more upstream genes and those derived from the ancestral M photopigment tend be found in more downstream genes. Since red-shifted M genes are more likely to be upstream, many of the 23 potential M opsin tetrachromats from Table 2.2 are probably real M tetrachromats.

Figure 2.4D plots the spectral distance between the two expressed cone photopigments for each individual subject. Only 41% of our sample had the maximal 29 nm spectral separation that was presumably present in the ancestral L and M photopigments. The majority of subjects had less well separated photopigments. Strikingly, one subject had two photopigments only separated by 16 nm, closer to a mild deutan's 10 nm separation than to the canonical normal 29 nm separation. Despite his close photopigments, this subject made no errors on the AO-HRR and only minor inversions on the D15. Due to his decreased sensitivity to red light, we would have expected a slight protan shift in his Rayleigh match, but his match range was 36-40, consistent with normal color vision.

Frequency of Mutations

Table 2.4 summarizes all mutations found in exons 2, 3, and 4 for both L and M genes aside from previously known dimorphic sites in both the male and female subject pools. We found 12

Figure 2.4 Estimated spectral peaks of cones and spectral distance between cones in males with normal color vision



In A and B are plotted the distribution of spectral peaks of L and M cones respectively. These estimates are derived from gene sequences of the opsins in the first two opsin array positions. C shows the spectral peaks of L genes in unexpressed positions in the opsin gene array. D shows the spectral distance between L and M cones for each subject. Note that the data is plotted on a log scale in A, B, and D to allow visualization of less frequent spectral peaks and spectral distances.

previously unreported polymorphisms, 7 of which were in expressed genes. The expressed polymorphisms evidently do not affect opsin function, as all subjects with those mutations had normal acuity and color vision. Consistent with that observation, all mutations are either silent or lead to conserved amino acid changes. Unsurprisingly, the mutations that lead to non-conserved amino acid changes (Cys203Arg and Ser212Leu) are in unexpressed genes; we hypothesize that Thr103Ala is likely not in an expressed gene although we could not confirm that due to the large array length of those subjects.

Interestingly, the most common polymorphism, a T to C in codon 161, was found exclusively in African-American subjects. Four had the variant in their L gene, four in both L and M, and one in an unexpressed M gene. This polymorphism was also observed by (Verrelli and Tishkoff, 2004) in a study of the L opsin genes including 163 African males though they do not report its frequency. Only one out of 161 Asian subjects had a point mutation, the silent polymorphism in codon 76.

Discussion

In this work we report on opsin gene array composition and on linked haplotypes across dimorphic exons 2, 3, and 4 in both L and M opsin genes for 1022 color normal male subjects. We used this data to investigate the variability of cone opsin genotypes that can confer normal trichromatic color vision. We find that array composition varies across racial groups: the frequency of multiple L opsin genes was 0.6%, 5.0%, and 10.5%, and the frequency of multiple M opsin genes was 45.3%, 66.7%, and 62.8% in Asians, Caucasians, and African Americans respectively. Our data expands on previous work from several past studies that investigated the opsin gene arrays of people with normal color vision. Early work from Drummond-Borg

Table 2.4 Opsin gene mutations

# Subj		
	In Expressed Gene	
2	Ala076Val	both nonpolar
2	Ala96silent	silent
2	Val104Ile	both nonpolar
8	Asn161silent	silent
2	171Leu	all nonpolar
1	His197silent	silent
3	236Leu	all nonpolar
1	Ala248Val	both nonpolar
	Unsure if in Expressed Gene	
1	Ala076silent	silent
4	Thr103Ala	polar to nonpolar
3	Cys203Arg	polar to basic
2	Val207Met	both nonpolar
1	Met224Val	both nonpolar
	Not in Expressed Gene	
1	Val068Ile	both nonpolar
1	Asn161silent	silent
7	Cys203Arg	polar to basic
1	Ser212Leu	polar to nonpolar

This table lists all mutations found both groups of subjects. Mutations are group by whether they were found in genes in expressed array positions or not. To the right of all mutations is the class of the standard and substituted amino acid. All mutations in expressed genes were in subjects whose color vision was tested and was determined to be normal, indicating these mutations do not affect opsin function.

(Drummond-Borg et al., 1989) and Jorgenson (Jørgensen et al., 1990) on array composition used similar populations: 102 African American, 101 Japanese, and 134 Caucasian males. However, as they have no information about gene order and did not perform color vision tests, they cannot distinguish trichromatic subjects with unexpressed L genes from deutan subjects. Rather, they rely (circularly) on what was known about the molecular genetic characteristics of the normal opsin array to determine which subjects had normal color vision and then used those subjects to study the molecular genetics of subjects with normal color vision. Deeb et al (Deeb et al., 1992) tested their subjects by anomaloscopy and found that out of 52 color normal Caucasian males, 2 (3.8%) had multiple L genes. Neitz and Neitz (Neitz et al., 1995) studied 27 color normal males using PCR amplification followed by quantitation of restriction fragment length polymorphism and reported that 12 (44.4%) had multiple L genes. Hayashi (Hayashi et al., 2001) and Ueyama (Ueyama et al., 2001) studied 121 color normal Japanese males, finding that 57% of subjects had multiple M genes and that <1% had multiple L genes. Our results are in broad agreement with the findings of Deeb, Hayashi, and Ueyama.

A novel aspect of this study was an analysis of the haplotypes of the expressed L and M photopigment genes of normal subjects and an estimate of the spectral peak of the L and M cones derived from that gene sequence information. Our data shows that normal color vision can be served by photopigments far closer together in spectral sensitivity than the 29 nm of the ancestral L and M photopigments. The majority of subjects had shifted photopigment spectral peaks so that the spectral distance between longer wavelength cones was 21-25 nm. In one subject, cones whose spectral sensitivity was separated by only 16 nm allowed normal color vision as measured by the anomaloscope, the AO-HRR, and the D15.

We found an enormous number of distinct haplotypes in our sample. This huge

variability may explain some of the genetic risk for other eye diseases involving the cone opsin genes besides color blindness. Certain "toxic" opsin variants that encode the amino acid sequence LVAVA in dimorphic exon 3 positions have been linked to disorders leading to extremely high grade myopia (McClements et al., 2013); the many "normal" opsin variants may explain some of genetic contribution to common "school grade" myopia.

Across all 1357 subjects of both sexes, we found 13 new polymorphisms in opsin genes, seven of which do not seem to interfere with normal opsin function. A number of non-conserved mutations were found in unexpressed genes. These downstream genes may harbor deleterious mutations that have no phenotypic consequence but that can cross back into an expressed position during a recombination event.

Using our pool of female subjects, we investigated the frequency of women possessing more than three cone types. Strikingly, we found that over half of women had at least one additional cone type. While the presence of fourth and even fifth cone types in the retinas of women are very common, this does not imply widespread tetrachromatic and pentachromatic color vision in the human population. Most women had extra photopigments with spectral separations of only 2-4 nanometers so the extra chromatic information gained may not be enough to support a new dimension of color vision. The highest potential for behavioral tetrachromacy would be found in women with cones as far separated as possible, which would be an M with a λmax of 530 nm, an L with a λmax of 559 nm, and a second L with a maximally green-shifted λmax of 549 nm. This configuration was found in only between one and nine of our 335 subjects.

Even well separated photopigments are not sufficient for tetrachromatic color vision: Jordan and colleagues (Jordan et al., 2010) studied at least three such women and found only

one, cDA29, whose behavior was consistent with a third color dimension. They propose that factors such as cone ratio, cone spatial distribution, and photopigment optical density may explain the difference in behavior between women with identical photopigment types. Another possibility is that different visual experience has allowed cDA29's visual system to make use of her fourth cone type while Jordan's other subjects could not. Because no biochemical or morphological difference has ever be observed between L and M cones aside from the cone opsin itself, the visual system must learn to differentiate cone types through their pattern of responses to light. A focus of computational research has been modelling how the visual system learns to identify cone class (L, M, or S) based on their response properties. Two studies have addressed the ability of the visual system to distinguish a fourth cone type (Benson et al., 2014; Wachtler et al., 2007) and found that responses of different cones of the same class are so highly correlated that it may be impossible to differentiate them based solely on their response properties. It's likely that for the vast majority of physiologically tetrachromatic and pentachromatic women, information from all cones of the same class (L or M) are combined, effectively widening the spectral sensitivity of that class. This may explain why carriers of color vision defects, who are likely to have more than three cones types, perform worse on color vision tests (Baraas, 2008; Crone, 1959; Feig and Ropers, 1978; Jordan and Mollon, 1993). However, because these studies used natural images as their stimuli, that indicates only that there is not enough differential activation of the fourth cone in the natural world. It is possible that if people who had four well separated cone types were trained on stimuli that were specifically designed to produce different responses from their two types of L cones, they may be able to learn to identify the shifted L as a separate chromatic channel.

Chapter Three

Genetic Testing as a New Standard for Clinical Diagnosis of Color Vision Deficiencies

Introduction

Color vision deficiency encompasses a broad range of disorders with a similarly large spectrum of consequences for those affected. An ideal color vision test should accomplish three aims: separate people with normal color vision from those with a color vision deficiency, classify the type of defect, and quantify its severity. Common clinical practice uses color vision tests like the Ishihara or Farnsworth D15, which attempt to deduce the cause of color vision deficiency indirectly from behavior. However, such tests often fail in at least one of the above objectives. The standard for research on color vision defects for more than twenty years has been analysis of the cone opsin genes to identify deleterious mutations and gene rearrangements, a more direct method of diagnosis that determines the underlying pathophysiology of the disorder. The purpose of this study is to evaluate the potential of analysis of the photopigment genes as a clinical tool for diagnosing color vision deficiency.

A great deal about the genetic underpinnings of human color vison is understood. The genes *OPN1LW* and *OPN1MW* encode the long wavelength sensitive (L) and middle wavelength sensitive (M) cone opsin respectively. They are arranged in a tandem array on the long arm of the X-chromosome at Xq28 and the arrays vary in the number of genes (Nathans et al., 1986b). As shown in Figure 1.3, the ancestral L and M opsin array presumably contained one L opsin gene followed by one M opsin gene. Gene rearrangements associated with common color vision deficiencies occur during meiosis; the L opsin gene of one X chromosome can pair with the M opsin gene of the other and an “unequal” cross-over occur, resulting in two new arrays, both of

which confer color vision deficiency in males. One contains a single opsin gene, a configuration that results in dichromacy. The other array has three opsin genes: an ancestral L, an ancestral M and, between them, an extra gene produced by the unequal crossover that encodes an L pigment that may or may not be green-shifted depending on the location of the crossover. This array confers a color vision defect in a males because only the first two genes in the array are usually expressed, and thus, the M opsin gene has been displaced to a non-expressed position (Hayashi et al., 1999). Over generations, this intermixing has continued, and because of relaxed selection against colorblindness in modern humans it has given rise to the large variability seen in the X chromosome cone opsin gene array (Asenjo et al., 1994; Merbs and Nathans, 1992b; Neitz and Neitz, 2011).

The amino acids in positions 277 and 285 together produce a spectral shift of about 20 nm in the photopigment, and thus define the difference *between* genes specifying an L versus an M class pigment (Asenjo et al., 1994; Neitz et al., 1991). The amino acids at positions 116, 180, and 230/233 each shift the photopigment spectra by 2-4 nm and define the differences in spectral sensitivity of photopigments *within* a class (Asenjo et al., 1994; Merbs and Nathans, 1992b; Neitz and Neitz, 2011). These differences determine the severity of the color vision defect in males in which the first two genes in the array specify a pigment of the same class (Neitz et al., 1996). If the encoded pigments do not differ at any sites involved in spectral tuning or at sites that affect optical density (Neitz et al., 1999), the array confers dichromacy. If the encoded pigments differ in peak sensitivity, the array confers anomalous trichromacy, and the severity of the defect is determined by the magnitude of the spectral difference.

The genetics of the S cone opsin are simpler. The *OPN1SW* gene is autosomal with a locus at 7q32 and lacks the variability of the L and M opsin genes. As with rhodopsin, almost

any missense mutation is expected to cause disease. Currently six known mutations of the gene have been linked to S cone dysfunction and result in tritanopia (Baraas et al., 2007; Baraas et al., 2012; Gunther et al., 2006; Weitz et al., 1992a; Weitz et al., 1992b). The color vision loss in these individuals appears progressive (Baraas et al., 2007); normal trichromatic color vision in childhood gradually succumbs to tritan defects as S cones slowly die.

In this work, we used knowledge of the cone opsin genes to examine the molecular substrates for color vision in 1,872 subjects and to evaluate the potential for use of such genetic techniques for clinical diagnosis of color vision deficiencies. Our results indicate that information about the underlying pathology derived from genetic analyses can be extremely valuable to patients in avoiding problems associated with undiagnosed or misdiagnosed colorblindness.

Subjects and Methods

Subject recruitment

We recruited 1,074 subjects for behavioral and genetic color vision testing. Subjects were assessed using the fourth edition HRR pseudoisochromatic plate test and the Lanthony D-15 arrangement test. Those whose performance indicated the presence of a color vision deficiency were given additional color vision tests including the second edition Dvorine pseudoisochromatic plate test and the Nagel anomaloscope. DNA was extracted from blood or saliva from each subject for cone opsin gene analysis.

DNA samples from 798 participants in the Age Related Eye Disease Study (AREDS) were purchased from Coriell. DNA samples were from approximately equal numbers of individuals with age-related macular degeneration and matched controls. These patients were not

selected for color vision and thus are assumed to be representative of the population as a whole in terms of the frequency of inherited color vision defects. The cone opsin genes of these samples were amplified and sequenced. No behavioral color vision assessment was possible.

Gene sequencing

The sequencing technique was adapted from (Oda et al., 2000). For each sample, the L and M photopigment genes were separately and specifically amplified using primer pairs 1 and 2 listed in Table 3.1. Specificity for L or M genes was conferred by the reverse primers because they hybridize to sequences within exon 5 unique to L or M genes. The PCR products obtained with primer pairs 1 and 2 were used in another round of PCR to amplify exons 2, 3, and 4 using primer pairs 4, 5, and 6 (Table 3.1). For a subset of subjects, exons 1, 5, and 6 were amplified non-specifically from all genes in the array using primer pairs 3, 7, and 8 (Table 3.1). Primer pairs 3 through 8 amplify individual exons including about 50 base pairs of flanking introns. Primer pair 9 specifically amplifies the first gene in the array, primer pair 10 specifically amplifies downstream genes (all genes after the first), and primer pair 11 amplifies part of the final gene in the array. PCR thermal cycling parameters for each primer pair are given in legend of Table 3.1.

DNA segments smaller than 10 kilobase pairs (kb) were amplified either with the AmpliTaq Gold PCR kit or the XL-PCR kit in conjunction with AmpliWax Gems until the latter product was discontinued. The Invitrogen Platinum Taq kit was used for the remainder of the samples. DNA segments larger than 10 kb were amplified with Takara LA Taq kit (Clonetech). The final reaction volume of each PCR was 50 ul with primer concentrations of 200 nM. Concentrations of all other reaction components were those recommended by the manufacturers.

Table 3.1 Sequencing Primers and Conditions

PCR #	Region Amplified	Sequence	Primer Binding Position	Length (base pairs)
1	intron 1 to exon 5, M genes only	GTCTCTGGCTTGAGGGACAG	intron 1, 180 bp upstream of exon 2	5912
		AAGCAGAATGCCAGGACCATC	M gene exon 5, codon 279	
2	intron 1 to exon 5, L genes only	GTCTCTGGCTTGAGGGACAG	intron 1, 180 bp upstream of exon 2	5911
		GCAGTACGCAAAGATCATCACC	L gene exon 5, codon 278	
3	L/M exon 1	AGTCCCAGGCCCAATTAAGAGAT	155 bp upstream of ATG start codon	301
		CAGCCACCCAGCCTCCAC	intron 1, 35 bp downstream of exon 1	
4	L/M exon 2	GGTGGGATCAGCACTGGTAT	74bp upstream of exon 2	420
		GCAGGGTGAATGAGTGGTTT	49 bp downstream of exon 2	
5	L/M exon 3	TGTCGTTTTTTCCACCTCAGTCC	intron 2, 136 bp upstream of exon 3	351
		CAGAGTCTGACCCTGCCCACT	intron 3, 46 bp downstream of exon 3	
6	L/M exon 4	TGGCTGCCGGCCCTTC	intron 3, 23bp upstream of exon 4	251
		TTGAGGGCAGAGCAGCTTAGG	intron 4, 62bp downstream of exon 4	
7	L/M exon 5	TCCAACCCCCGACTCACTATCC	intron 4, 35bp upstream of exon 5	314
		ACGGTATTTTGAGTGGGATCTGCT	intron 5, 39 bp downstream of exon 5	
8	L/M exon 6	ACCCTTCCCTGCTCTGCTCAA	intron 5, 42bp upstream of exon 6	201
		GGAGAGGTGGCCAAAGCCC	intron 6, 51bp downstream of exon 6	
9	First gene in the L/M array	CCTGGGCTTTCAAGAGAACCACATG	459 bp upstream of ATG start codon	12912
		CACCTAAGCCTTCTGCTAAGGGCCA	202 bp downstream of exon 5	
10	Nonspecific down-stream L/M genes	ATACCCTGCAAGTGGGAATCTA	736 bp upstream of ATG start codon	11747
		ACGGTATTTTGAGTGGGATCTGCT	intron 5, 39 bp downstream of exon 5	
11	Last gene in the L/M array, intron 4 to end	CCACGCCCAGTCATCAATCAAATC	intron 4, 331bp upstream of exon 5	27792
		GAATGTGCTCGCCCTGTGTCTGAA	25kb downstream of exon 6	

For each primer pair, the forward primer is listed first and the reverse primer is underneath. All primer sequences are 5' to 3'. For the L or M specific PCRs, cycling conditions were: (1x) 94C for 3 minutes; (30x) 94C for 30 seconds, 61C for 30 seconds, 68C for 6 minutes; (1x) 68C for 20 minutes. For exon-amplifying PCRs, cycling conditions were: (1x) 94C for 3 minutes; (30x) 94C for 30 seconds, 61C for 30 seconds, 68C for 30 seconds; (1x) 68C for 3 minutes. For the first gene and downstream gene PCRs, cycling conditions were: (1x) 94C for 3 minutes; (30x) 94C for 10 seconds, 56C for 30 seconds, 68C for 11 minutes plus 20 seconds per cycle starting in cycle 11; (1x) 68C for 20 minutes. For the last gene PCR, cycling conditions were: (1x) 94C for 3 minutes; (30x) 98C for 10 seconds, 68C for 20 minutes plus 20 seconds per cycle starting in cycle 16; (1x) 68C for 20 minutes.

The PCR products obtained with primer pairs 3 through 8 were sequenced with the same primers using BigDye Terminator v3.1 cycle sequencing (Applied BioSystem). Reactions were analyzed on an ABI 3500 Genetic Analyzer.

The spectral class of the pigment encoded by the last gene in array was identified by selectively amplifying the last gene using primer pair 11 with a reverse primer that lies outside the repeat unit of the array, then amplifying exon 5 from the last gene using primer pair 8, and finally subjecting the PCR product to Rsa I restriction enzyme digestion. The Rsa I site is created when codon 277 specifies tyrosine and the third position of codon 276 is a G residue. These are both present in L class pigment genes and absent in M class pigment genes; thus, digestion of PCR-amplified exon 5 with Rsa I followed by gel electrophoresis will yield two bands for L pigments and a single band for M pigments (Nathans et al., 1986a).

Estimates of Photopigment Spectral Peak

To estimate the photopigment spectral peak, the identities of the amino acids at positions 116, 180, and 230 were determined. We estimate the longest L photopigment to have maximal sensitivity to 559 nm light and the spectral peak to be shifted 2.5 nm shorter when tyrosine is present at amino acid position 116, 3.5 nm shorter when alanine is present at amino acid position 180, and 4 nm shorter when threonine is present at position 230. We estimate the shortest M photopigment to have maximal sensitivity to 530 nm light and the spectral peak to be shifted 3 nm longer when serine is present at amino acid position 180 or when isoleucine is at amino acid position 230 (Neitz and Neitz, 2011).

Analysis of X-chromosome opsin array composition

We conducted a novel high throughput genetic color vision assay using the MassArray

system (Sequenom), which allows genotyping of multiple single nucleotide polymorphisms (SNPs) simultaneously using a primer extension process followed by mass spectrometry. An initial round of PCR amplifies from genomic DNA a short length of DNA surrounding each SNP. This is followed by single-base extensions of a primer that anneals directly adjacent to the SNP such that the extended primer carries the dideoxynucleotide that is the complement of the SNP. The nucleotides used for this extension are mass modified such that all four possible extension products are distinguishable by MALDI-TOF mass spectrometry.

A first version of the MassArray color vision assay was comprised of five SNPs that include: three sites that control spectral tuning differences within L and M genes (in codons 116, 180, and 230), one site that distinguishes L and M opsin (in codon 309), and a final site in the promoter region (+1) that differs between the first gene in the array and all downstream genes. Codon 309 was chosen to distinguish L from M opsin genes because it is closely linked with positions 277 and 285 and, unlike those important spectral tuning sites, is not surrounded by silent polymorphisms that make a SNP difficult to assay in the MassArray format. The assay reports opsin gene copy number, the proportion of L genes, and an estimate of spectral sensitivity differences between opsins encoded by the genes present. A second iteration of the test added sites of known mutations in both L/M cone opsin genes as well as the S cone opsin gene. These mutations are C203R for L/M opsin and L56P, G79R, T190I, S214P, P264S, and R283Q for S opsin. All PCR and extension primers are listed in Table 3.2.

For samples with MassArray results indicating the presence of extra L opsin genes, a long range PCR of the final gene in the array was performed to determine if the extra L opsin gene had displaced the M opsin gene from an expressed position.

Table 3.2 MassArray Primers

SNP Location	Purpose	PCR Primer Sequences	Extension Primer Sequence
L/M opsin nucleotide +1	Characterize L/M opsin array	ACGTTGGATGTTTTAAGGTGAAGAGGCCCG ACGTTGGATGTGGCTATGGAAAGCCCTGTC	GGGGTGGCAGCCGGCCCTGG
L/M opsin codon 309		ACGTTGGATGCTTCCACCCTTTGATGGCTG ACGTTGGATGACCTGCCGGTTCATAAAGAC	CCTGCCCTGCCGGCCT
L/M opsin codon 116	Spectral tuning sites	ACGTTGGATGGTCATCGCCAGCACTATCAG ACGTTGGATGCTCCAGGACACACATAGGGT	CCCAGCACGAAGTAGCCA
L/M opsin codon 180		ACGTTGGATGTGAGATTTGATGCCAAGCTG ACGTTGGATGCTCCAACCAAAGATGGGCG	GGGACTGTCCACACAGCAG
L/M opsin codon 230		ACGTTGGATGATTGTCCTCATGGTCACCTG ACGTTGGATGCACACTTGGAGGTAGCAGAG	TCACCTGCTGCATCA
L/M opsin codon 203	Known deleterious mutations	ACGTTGGATGTTGGTTGGAGCAGGTACTGG ACGTTGGATGATCATGTAAGACTGCACCCC	CACCACGGCCTGAAGACTTCA
S opsin codon 56		ACGTTGGATGCTTCCTTATAGGGTTCCCAC ACGTTGGATGGCTGCCGCAACTTTTTGTAG	ACTCAATGCCATGGTGC
S opsin codon 79		ACGTTGGATGAGCCCCTCAACTACATTCTG ACGTTGGATGTTACAGCTGGCGACGAAGAC	GAAGAGGAGGAAGCCTC
S opsin codon 190		ACGTTGGATGGTTTGGTCCTTTGCAGGTTC ACGTTGGATGAGGACTCGCTGCGGTATTTG	GGCCCTGACTGGTACA
S opsin codon 214		ACGTTGGATGAGTCCTATACGTGGTTCCTC ACGTTGGATGTCAGCAGCTGAGTGTAGGAG	TCTGCTTCATTGTGCCTCTC
S opsin codon 264		ACGTTGGATGGTGATGGTAGGATCCTTCTG ACGTTGGATGCCATGGTTACGGTTGTTGAC	CGAAGGCCGCGTAGG
S opsin codon 283		ACGTTGGATGTCAACAACCGTAACCATGGG ACGTTGGATGGTAGATGCAAGCACTCTTGG	GAACCATGGGCTGGACTTAC

For each PCR primer pair, the forward primer is listed first and the reverse primer is underneath. All primer sequences are 5' to 3'. PCR conditions are the standard recommended condition given by Sequenom for use with the MassArray system.

Severity of deficiency in deuteranomalous trichromats

For a subset of subjects, a highly-specialized two-alternative forced-choice procedure on the Nagel anomaloscope was used to measure the severity of color deficiencies. We argue that this procedure provides a valid measure of severity that can be used as a standard of comparison for evaluating clinical color vision tests and estimates of severity from genetics. While the anomaloscope is used as a clinical instrument, the procedure used here is too demanding and too time-consuming (usually requiring more than thirty minutes to complete) for clinical use. For each ratio of the 547 nm and 658 nm mixture lights set by the experimenter, the subject was asked to adjust the brightness of the 589 nm light to make the closest possible match. When this match was achieved, the subject was asked to describe the mixture light as either too red or too green compared to the 589 nm light. This procedure was repeated until two 547 nm/ 658 nm ratios were found: one that the subject always described as red and one as green across 10 trials. Ten measurements were taken at intermediate ratios between the too-red and too-green ratios to generate a psychometric function describing the ability of the subject to discriminate mixed red and green lights from monochromatic yellow.

The data for each subject was fitted with a cumulative Gaussian weighted three times more heavily on the tails, the points that were always too red or too green. An estimate of the match range was calculated from the fitted curve; it was defined to be the range of values for which we are $\leq$ 95% confident that the 547 nm /658 nm mixture light was indistinguishable from the 589 nm standard light. Larger matching ranges are indicative of poorer red-green color vision.

Color sensitivity of dichromats

The trivector Cambridge Color Test was used with coordinates designed to test neutral points from dominant wavelengths 492 to 508 in 2 nm increments. The 498 nm, 500 nm, and 502 nm lines were tested three times and the other six were each tested twice. For each subject, the thresholds were averaged for each wavelength and then normalized by dividing by the subject's maximum threshold to determine the axis of lowest sensitivity.

Experiments involving human subjects were conducted in accordance with the principles embodied in the declaration of Helsinki, and were approved by Institutional Review Boards at the University of Washington.

Results

Detection and categorization of color vision defects

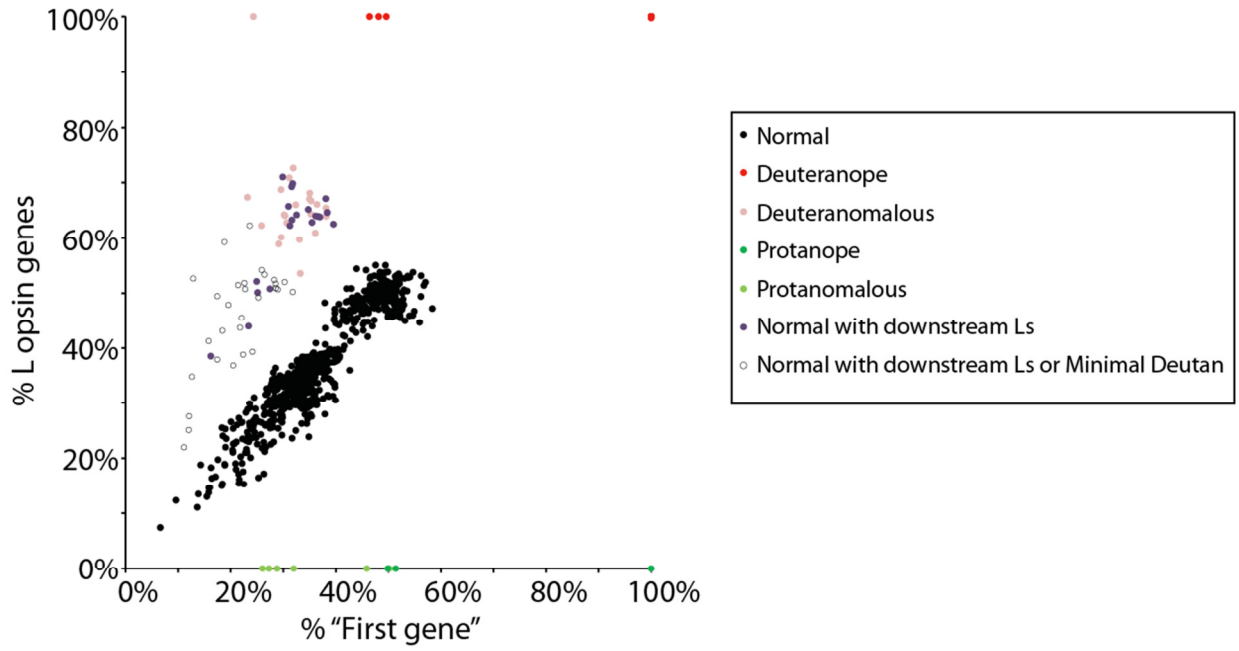
We analyzed the composition of the opsin gene array using the MassArray assay for two subject pools: a group of male subjects unselected for color vision status who participated in the AREDS study and a group of subjects for whom we performed standard color vision tests in our laboratory. The results for both subject pools are shown in Figures 3.1 and 3.2, where the percentage of genes in the array that are in the first position (the inverse of this proportion is opsin gene copy number) is compared to the percentage of the total genes that encode an L opsin. Subjects cluster into groups based on the characteristics of their arrays, and this provides a first pass diagnosis of the best possible color vision each subject can have.

Males with normal color vision typically have one L gene followed by one or more M genes (Nathans et al., 1986b), and they fall along the unity line below about [60%, 60%] in the MassArray assay. Rarely, males with dichromatic color vision (protanopic or deuteranopic) will

also fall in this area because they have inactivating mutations in one of the first two genes in the array that are not detected by the MassArray assay (Hayashi et al., 1999; Neitz et al., 2004). Subjects who lack L genes fall along the X-axis and all of these have, at best, protan color vision deficiencies, with single-gene protanopes clustering at [100%, 0%]. Subjects who lack M genes fall along the y=100% line and all of these can have no better than deutan color vision deficiencies, with single-gene deuteranopes clustering at [100%, 0%]. Individuals whose results plot above the unity line possess at least one extra L opsin gene and may either be color normal or have a deutan-type color vision deficiency, depending on whether the first two genes in the array encode an L and an M pigment or whether both encode an L pigment. Female carriers of red green color vision deficiency are also distinguishable with a high degree of reliability from both color anomalous individuals and non-carrier normals: carriers of protan defects tend to have a paucity of L opsin genes compared to normal and thus should fall between the normal (one L gene) line and the X-axis, while carriers of deutan defects tend to have extra L genes compared to normal and thus should fall between the normal line and the deutan range.

Data from the pool of male AREDS subjects is shown in Figure 3.1. Of 798 men, 708 fell along the unity line and are predicted to have normal color vision. Eight males were identified as single-gene deuteranopes based on clustering at [100%,100%] and two multigene deuteranopes were identified based on clustering along the y=100% line along with absence of differences in SNPs in codons that influence spectral tuning. One person whose results fell along the y=100% line was determined to be (at best) deuteranomalous with multiple L genes that differed in spectral tuning. Nine males were identified as protan: 1 single-gene protanope at [100%, 0%], 2 multigene protanopes with no differences in their M genes at SNPs that influence spectral tuning, and 3 protan-type individuals were identified whose M genes did encode spectral

Figure 3.1 MassArray results for the AREDs subject pool.



The classification of 798 males is based solely on genetic analysis was accomplished using the first version of the MassArray test, which used five SNPs to characterize the L and M opsin gene array. The x axis is the proportion of the total opsin genes that have the "first gene" promoter allele. The y axis is the proportion of the total opsin genes that are of the L opsin class, as measured by a SNP in codon 309.

differences. The other 3 protan males had M genes that differed only in exon 2, which encodes amino acid differences that may affect optical density and give rise to protanomalous behavior in the anomaloscope test (Neitz et al., 1999). The final 70 of the 798 males fell above the unity line but below the y=100% line, indicative of arrays with at least two L opsin genes and at least one M opsin gene. These individuals may either be color normal or deutan, and distinguishing the two requires identification of the last gene in the array to deduce the order of the genes. For 20 of these subjects, there was a single extra L gene and it was at the 3' end of the array; they are predicted to have normal color vision. For 21 of the subjects, the extra L gene was deduced to be in the second position; their arrays had only 1 M gene and it was found in the last position. Their L genes were different at spectral tuning sites so they are predicted to be, at best, deuteranomalous. The remaining 29 subjects had too many genes to determine whether the first and second genes both encoded L pigments and their diagnosis remains ambiguous.

We also performed some direct gene sequencing for these subjects. There were two previously unreported missense mutations in expressed L opsin genes, L232V and L55V, and one silent mutation, GTC>GTT in codon 97. Additionally, one subject was homozygous and four were heterozygous for the deleterious C203R mutation in M genes. One of the heterozygous subjects had two M genes and the C203R mutation was found in the gene in the last gene position, meaning the expressed M did not have the mutation. The other three subjects had more than two M genes and so it could not be determined if the C203R mutation was in an expressed gene. Therefore, between one and seven subjects in this pool would be dichromatic depending on the position of the gene encoding C203R and on the effects of the L232V and L55V mutations (which could affect protein folding or function) and the synonymous mutation (which could affect splicing). The L232V substitution is predicted by Mutation Taster (Schwarz et al., 2014) to

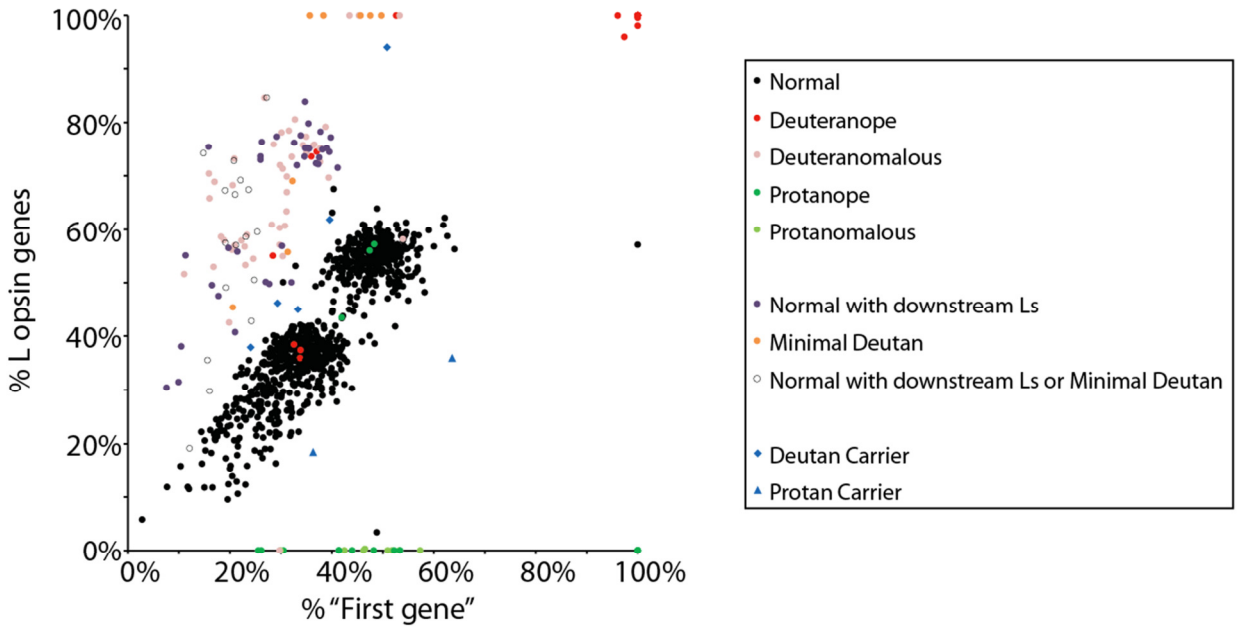
be disease-causing while the L55V is not, and none of the three are predicted by Human Splicing Finder (Desmet et al., 2009) to affect splicing.

In the second subject pool, we used MassArray data, gene order, gene sequencing, and behavioral tests to evaluate color vision status. The MassArray data for these subjects is shown in Figure 3.2. All subjects are summarized in Table 3.3. Our subject pool included 31 females, 29 of whom passed the HRR and D15, one of whom made protan errors on those tests, and one of whom made deutan errors. The MassArray test classified all 29 women who were color normal in behavioral assessments as having genes required for normal color vision. Additionally the genetic test identified 4 of the women as deutan carriers and 2 as protan carriers based on established characteristics of opsin genes in carriers (Kainz et al., 1997; Oda et al., 2000). The protan female was found to have five spectrally identical M opsin genes in her array. The deuteranomalous female was found to have an extra L opsin on one chromosome, displacing the M opsin gene from an expressed position, and a missense mutation (V63M) in the expressed M gene of the other chromosome, leaving her with three L opsins and a presumably non-functional M opsin. The V63M substitution is predicted by Mutation Taster to be disease-causing.

Among the male subjects (n=1043), the MassArray identified 22 as having arrays comprised of only a single L gene ([100%, 0%]), making them deuteranopes; another 5 had arrays comprised of only a single M gene ([100%, 100%]), making them protanopes. All of these obligate dichromats failed the behavioral tests.

The MassArray identified 9 males who had L opsin genes with distinct spectral tuning and no M opsin gene, making them, at best, deuteranomalous. However, only 4 made deutan errors on the behavioral tests; the others, who we call “minimal deutans”, made no errors at all on either test. Examination of their L opsin sequences revealed that these subjects all had

Figure 3.2 MassArray results for the behaviorally-tested subject pool.



The classification of 1043 males and 31 is based on both genetic and behavioral diagnosis. Some subjects were tested using the first version of the MassArray test and some using the second; results from the first version were normalized to allow plotting on the same graph. The x axis is the proportion of the total opsin genes that have the “first gene” promoter allele. The y axis is the proportion of the total opsin genes that are of the L opsin class, as measured by a SNP in codon 309.

L photopigments with spectral separations of 6.5 to 10 nm. Three of these 5 agreed to return for testing with the Nagel anomaloscope, which confirmed that they were very mild deutans.

There were 18 males who had no L opsin gene and multiple M opsin genes. Five of them had arrays comprised solely of multiple identical M genes, making them obligate protanopes. Seven subjects had M opsin genes with differences only in exon 2, which does not affect spectral tuning but may create optical density differences; another 5 had M opsins with differences in both spectral tuning sites and in exon 2, making them, at best, protanomalous. The eighteenth protan male had three M opsin genes, but direct sequencing showed that one of them encoded a tyrosine at 309 (normally found in L pigments) making him appear normal in the MassArray assay. Seventeen of these 18 protans made protan errors on the color vision tests, but one (who had differences in spectral tuning and optical density controlling sites) was misdiagnosed by the HRR and D15 as a deutan.

887 of the behaviorally tested male subjects were found to have one L gene followed by a variable number of M opsin genes, a configuration normally associated with normal color vision. In 7 of those subjects, direct sequencing revealed the presence of mutations in the opsin genes that lead to red-green color vision defects. One had a Cys203Arg mutation in his M gene, one had a Cys203Opal mutation in his M gene, one had a single base insertion in his M gene introducing a frame shift in exon 3, one had a gene rearrangement where his L was displaced to the third position leaving two spectrally identical M genes in expressed positons, and three had toxic opsin LIAVA or LVAVA variants (Carroll et al., 2012). These seven subjects all failed the behavioral color vision tests.

Sequencing found that 5 of the 888 subjects with normal arrays had mutations in the SNPs the MassArray assays uses to characterize the array. One of those subjects was outside all

diagnosis clusters at [100%, 50%] and appeared to have an impossible array – 50% of his genes were of the L class, but he seemed to have only a single gene in his array. Sequencing of the promoter region revealed that he had in all genes the "first gene version" of the SNP which the MassArray assay uses to determine gene number (i.e. a G at nucleotide +1) but was heterozygous at other positions, meaning he had an LM array sufficient for normal color vision. The other four subjects had mutations in the codon 309 SNP that the MassArray uses to distinguish L and M opsin genes. One had an LM array in which the L had a phenylalanine at 309 (typically associated with M genes), making him appear protan to the MassArray. Similarly, three subjects who appeared to have deutan LLM arrays actually had normal LMM arrays because one M opsin gene encoded a tyrosine at 309, normally found in L pigments. All of these five subjects passed the color vision tests.

Direct sequencing of the opsin genes for the other 875 of the 888 subjects with normal numbers of L and M genes showed no deleterious mutations, together indicating the genetic basis for normal color vision. 869 of these 875 subjects passed both the HRR and D15 color vision tests. The remaining 6 showed mild defects in protan, deutan, or, in two cases, tritan stimuli on behavioral color vision tests. No genetic cause for color vision deficiency was ultimately found for these subjects.

The final group of behaviorally-tested subjects were 102 men found to have at least two L opsin genes and at least one M opsin gene. In 26 of those subjects, the extra L opsin gene was found to be in the last position of the array, not displacing the M gene. These arrays (LML or LMML) are consistent with normal color vision, and, indeed, 25 of these men passed the behavioral color vision tests. The last man had an LMML array but made mild errors on the HRR and D15, had a Rayleigh match range of 44-59, and was of East Asian descent. We

sequenced his promoter region and found he was heterozygous for an A-71C mutation, a common cause of red-green defects in Asian populations (Ueyama et al., 2015b). In another 28 men, two L opsin genes were found to be in expressed positions by deduction from last gene sequencing (LLM or LLLM arrays). L opsin sequences indicated that there was no spectral difference between the L opsins for 2 of these 28, making them multigene deuteranopes, and there was a spectral difference for the other 26, making them, at best, deuteranomalous. Twenty-two of the 26 deuteranomals were classified as mild or medium deutans by the HRR and D15. However, two were minimal deutans who made no errors on either test. The last two deuteranomals had a 2.5 nm spectral separation but behaved more poorly on color vision tests than we would predict from having two pigments with that separation (see next section). One had very poor acuity and amblyopia, which likely led him to perform worse on the behavioral tests than his color vision defect would have allowed; the other was not tested for visual acuity and reported no other eye disorders.

The remaining 48 of the 102 subjects with extra L opsin genes had long arrays containing 4 or more opsin genes. While we can specifically analyze the first gene and the last gene in the array, we cannot access the second gene. For longer arrays, we cannot always deduce the identity of the second expressed gene. Of the subjects with extra L opsin genes and a long array, 11 had spectrally identical L genes. If their extra L was in an expressed position, these men would be deuteranopes, otherwise, they would have normal color vision. All of them passed the HRR and D15, consistent with normal color vision. Another 19 failed the behavioral tests so we assume the extra L gene is in the second position. The final 18 subjects with long arrays and extra L genes passed the HRR and D15 but had a spectral separation between their Ls of at least 6.5 nm; these men may be normal with the extra L genes downstream of the expressed positions

Table 3.3 Summary of Behaviorally Tested Subjects

31	Total Female Subjects			
23	Female normal trichromat			
4	Female deutan carrier			
2	Female protan carrier			
1	Female deuteranomal			
1	Female protanope			
1043	Total Male Subjects			
22	Male with single L gene - deuteranope			
5	Male with single M gene - protanope			
9	Male with multiple L genes and no M genes			
	4	Deuteranomal - fail HRR/D15		
	5	Minimal deutan - pass HRR/D15		
18	Male with multiple M genes and no L genes			
	5	Multi-gene protanope		
	7	Protan – heterozygous at optical density sites		
	5	Protan - heterozygous at tuning and optical density sites		
	1	Protan - 309 mutation (appears normal to MassArray)		
887	Male with one L gene and at least one M gene			
	869	Normal trichromat		
	7	Dichromat with deleterious mutations		
	5	Normal trichromat with MassArray SNP mutations		
	6	Ambiguous - mild defects on HRR/D15, no genetic cause		
102	Male with multiple L genes and at least one M gene			
	1	LMML array with A-71C mutation		
	25	Normal trichromat with sole extra L gene in unexpressed position		
	28	Deutan with sole M gene in unexpressed position		
		2	Multi-gene deuteranope	
		26	Deuteranomal	
			2	Minimal deutan - pass HRR/D15
			22	HRR mild or medium
			2	HRR strong
	48	Array too long to deduce identity of expressed genes		
		11	Probably normal trichromat - spectrally identical Ls, pass HRR/D15	
		19	Deuteranomal - spectrally different Ls, fail HRR/D15	
		18	Ambiguous - spectrally different Ls, pass HRR/D15	
			3	Normal Rayleigh match - normal trichromat with extra L gene in unexpressed position
			1	Shifted Rayleigh match - minimal deutan who passes HRR/D15
			14	No anomaloscopy data

Sub groups within a larger group are denoted by indentations. Subjects with normal color vision are listed in plain text, those with inherited color vision deficiencies in bold, and those that are ambiguous in italics. The diagnoses are made based on a combination of genetic and behavioral data for each subject.

or be minimal deutans able to pass the behavioral tests. Four of these agreed to return for testing with the Nagel anomaloscope, which revealed that three were normal trichromats and one was a minimal deutan.

Estimates of severity of the deficit in anomalous trichromats

The SNPs at amino acid positions 116, 180, and 230/233 each encode a shift of 2-4 nm in spectral sensitivity. By determining the identity of these amino acids, one can estimate the spectral separation between two opsins of the same class and thus predict the severity of the deficiency in anomalous trichromats. It is possible to determine the spectral peaks of the expressed opsins for almost all subjects using direct sequencing of relevant exons specifically amplified from first genes, last genes, L genes, and M genes. The MassArray also provides an estimate of the spectral separation of photopigments by identifying the proportion of genes with each allele present at codons 116, 180, and 230. However, it does not give information about which SNPs are present specifically in L versus M genes or about the SNPs associated with genes in a particular order in the array. When using only information from the MassArray we estimated spectral sensitivities under the assumption that ancestral L opsin amino acids would tend to be in genes more upstream in the array and ancestral M opsin amino acids would be in more downstream genes. Results using this assumption agreed for 19 of 22 subjects diagnosed as deuteranomalous with estimates obtained from gene sequencing.

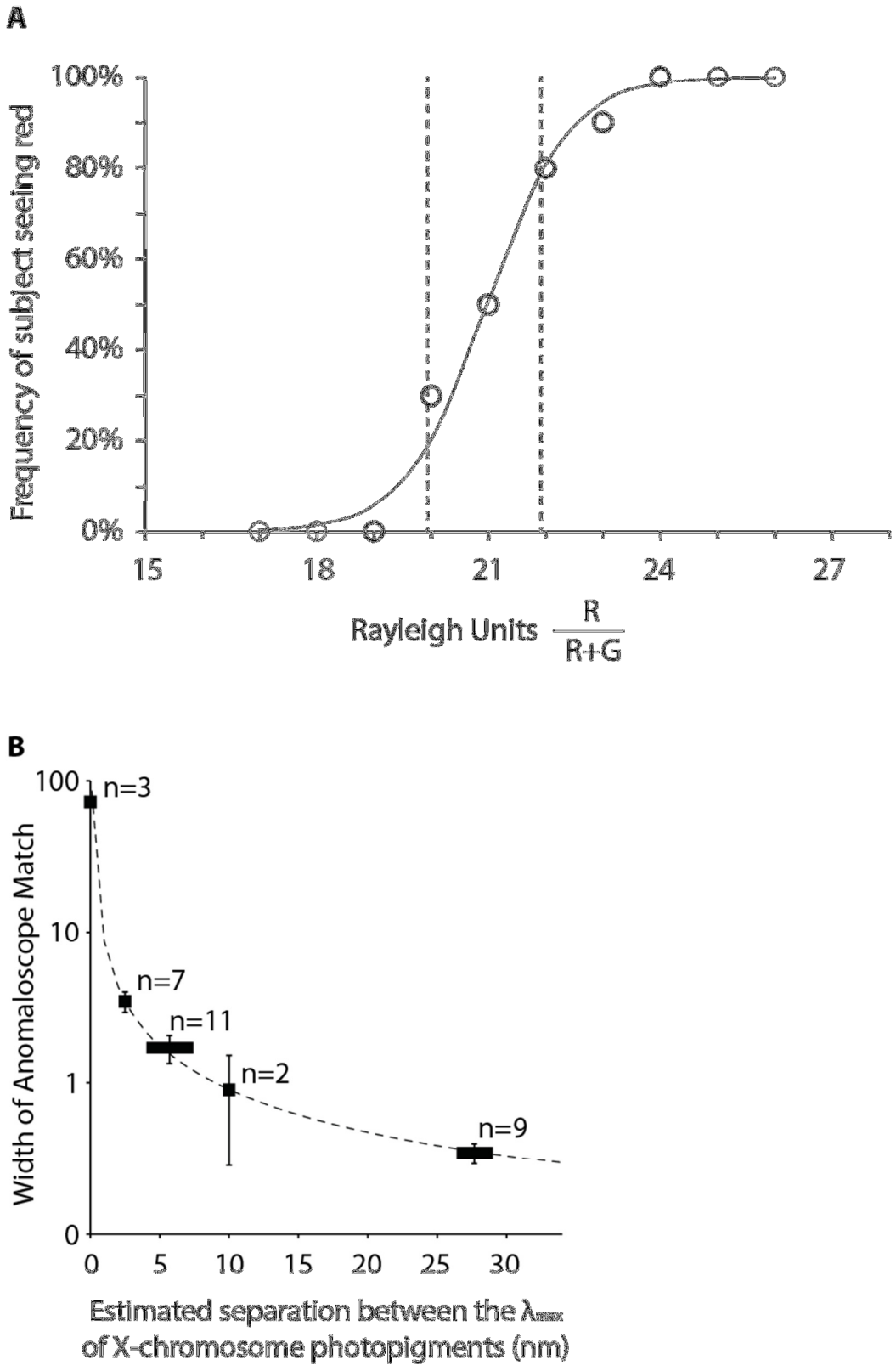
To test the accuracy of genetic estimates of color sensitivity from spectral separation, the severity of the deficiency in 20 deutan subjects and 9 normal controls was assessed by a forced choice procedure on the Nagel anomaloscope (see methods) and compared to estimates of spectral separation derived from gene sequence information.

Figure 3.3A shows example results from a deuteranomalous male with L pigments separated by 2.5 nm. His midpoint is about 21 and his match range is 2 Nagel units. Data from subjects with similar spectral separations were averaged to generate Figure 3.3B to show the relation between spectral separations predicted from genetics and defect severity assessed by the Nagel anomaloscope. The genetic estimates agree well with the anomaloscope assessment. Subjects with a single X chromosome opsin gene or no separation between multiple opsin genes, perform far worse than all anomalous trichromats and cannot even distinguish the pure red or green lights from the yellow light. We measured the spectra of the lights in the Nagel anomaloscope with a Konica Minolta CS-2000 spectroradiometer and found that the average Nagel range of 0.345 units for our normal subjects equates to a cone contrast of only 0.174%. With such impressive sensitivity, even the tenfold higher thresholds of moderate anomalous trichromats still represent a considerable color discrimination ability compared to that of a dichromat.

Color Discrimination Thresholds in Dichromats

As described earlier, rather than a single L and a single M photopigment, there exists a family of each, and members of each family can differ in their spectral sensitivity. The peak absorbance of L pigments can range from 549 nm to 559 nm while the peak absorbance of M pigments can range from 530 nm to 536 nm. This means that not all dichromats will have the same color confusion lines. This variability presents a problem for discriminating dichromats from anomalous trichromats using cone contrast type tests such as the HRR. Because color decrease with distance from that axis, stimuli designed to produce confusions along a given color axis will fail to correctly diagnosis people with confusion lines that differ from the standard

Figure 3.3 Results of forced choice Rayleigh match procedure.

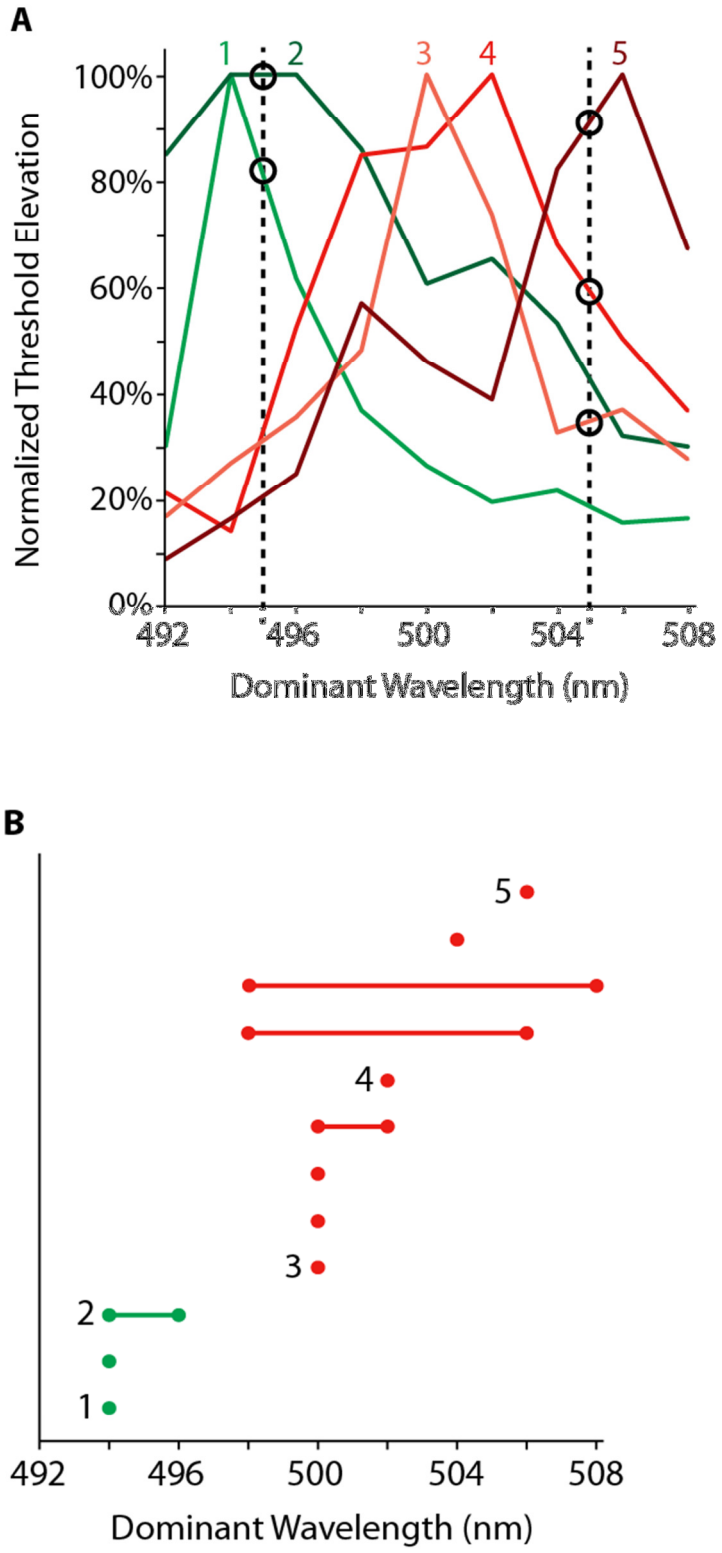


A) Example results from a deuteranomalous male with 2.5 nm separating the spectral peaks of his L opsin photopigments. B) An estimate of the spectral separation between X chromosome photopigments was derived from gene sequence information for deuteranopic, deuteranomalous, and normal trichromatic subjects (deuteranopes were given a separation of 0 nm). This estimate was compared to the width of the Rayleigh match range obtained from the forced choice protocol.

discrimination thresholds of red-green color defectives peak at a single confusion line and chosen for a particular cone contrast type test. To evaluate this, we measured color discrimination thresholds for twelve dichromats - three protanopes and nine deuteranopes using the Cambridge Color Test (Figure 3.4) which allows measurement of thresholds along a variety of hypothetical confusion lines. All subjects had only a single X chromosome cone opsin gene or two genes with the same amino acids at spectral tuning sites, and were categorized as protanopes or deuteranopes based on the identity of those genes. Deuteranopes varied greatly in their color discrimination for different confusion lines, with some displaying their worst performance along a line through a dominant wavelength of 506 nm and others of 500 nm. For a stimulus designed to test a confusion line for the deuteranope whose threshold peaks at 500 nm, the deuteranope who peaks at 506 nm would have a threshold around 50% below his maximum and thus would be indistinguishable from an anomalous trichromat whose confusion line is at 500 nm.

Testing along a single standard confusion line for protan, deutan and tritan defects is predicted to produce errors in determining the severity of color vision defects and, indeed, they were found to occur quite frequently. Using the 4th edition HRR plate test we assessed the color vision of 22 subjects with only a single opsin gene on the X chromosome, multiple genes with the same sequence, or genes with inactivating mutations. Even though such subjects are necessarily dichromats, 7 (32%) were categorized as “medium” by the HRR plate test.

Figure 3.4. Finding dichromat confusion lines using the Cambridge Color Test.



The thresholds of five sample dichromats across a range of confusion lines are shown in (A). All thresholds are normalized to the neutral point, the wavelength where the subject performed worst. Two protanopes are shown in green and three deuteranopes in red. The two vertical dotted lines show the standard neutral points for protans and deutans with circles to show how well the individual subjects perform at that point. The neutral points are shown for all twelve dichromats in (B). Dots represent individuals who had a single worst performance and bars represent individuals who performed equally badly on multiple wavelengths. The five individuals from (A) are numbered and shown again in (B).

Discussion

Separating normal color vision from congenital red-green deficiency using an assay of the number and ratio of L and M opsin genes

Red-green color vision deficiency is by far the most common single locus disorder in humans. Unlike many other common genetic polymorphisms that have been driven to high frequency by selective pressure, the high frequency of red-green color vison defects is the result of "mutation pressure" from the extremely high frequency of rearrangements at this locus. Relaxation of selection against colorblindness in humans in the presence of the high mutation rate accounts for its high frequency (Neitz & Neitz 2011).

Taking advantage of the fact that almost all common color vision defects are the result of gene rearrangements, we developed an efficient and practical MassArray assay designed to separate people with normal color vision from those with different forms of red-green colorblindness by determining the number and ratio of L and M genes. The assay also included a quantitation of the spectral tuning sites allowing multigene protanopes and deuteranopes to be differentiated from individuals with the genetic potential to be anomalous trichromats and allowing us to predict their relative severity. In addition, the MassArray assay included a probe for a C203R mutation that interrupts photopigment function and occurs at a relatively high frequency in the population.

The sample of subjects that we studied from the Seattle area (discussed below) who underwent color vision testing included individuals who volunteered because they knew (or suspected) that they might have color vision defects so it cannot be considered to represent a random sample from the population. Thus, a sample of 798 male AREDS subjects, who were unselected for color vision phenotype, shown in Figure 3.1, were run to give an estimate of how

results for the assay might be distributed for the general population, and to evaluate the sensitivity and selectivity of this assay as a clinical test for congenital red-green color vision deficiency.

Some large studies have employed behavioral color vision testing methods that were designed to have sensitivity for detecting congenital red-green color vision deficiencies that is very close to 100%. For example, Waaler (Waaler, 1927) screened his subjects using the Ishihara, but knowing that some mild deuteranomalous individuals can pass the Ishihara making zero errors, he used a criteria of selecting subjects who made errors or even showed difficulty on the Ishihara for additional testing using the anomaloscope. Subjects chosen for anomaloscope testing were classified into 5 categories based on the results with that test: normal, protanope, deuteranope, protanomalous, or deuteranomalous. Most clinics do not have an anomaloscope or personal trained to use one so this is not a practical clinical testing method; however, results from this approach do represent a gold standard for sensitivity in detecting red-green color vision defects. Waaler found that 8.01% of males had red-green color vision deficiency in which 0.88% were protanopes, 1.03% were deuteranopes, 1.04% were protanomalous, and 5.06% were deuteranomalous.

For the AREDS sample, we diagnosed people based solely on the MassArray plus one extra step of assaying the last gene for 71 subjects (9%) who had extra L genes and 4 with a C203R mutation. Opsin gene arrays conferring color vision defects were found in 42 subjects (5.3%) including obligate single-gene and multigene-dichromats, obligate protans with no L genes and 21 people with an extra L gene that was deduced to be in the second position, making their best possible color vision deuteranomalous. In 724 subjects (90.7%), no gene rearrangements were detected that would cause a red-green color vision defect, thus, they were

classified by this test as having normal red-green color vision. However, from sequencing of exons 2, 3, 4 of the L and M genes of all subjects, three previously unreported point mutations in expressed L opsin genes - L55V, L232V, and a silent substitution in codon 97- were discovered. If any of these changes cause the loss of L-cone function, these people could be protanopes that were not detected by the MassArray assay (up to 0.3% of the population). There may also have been deleterious mutations in exons 1, 5 and 6 or the regulatory regions which were not sequenced but, except for the A-71C (discussed below) that occurs at high frequency in some Asian populations, people with such mutations are expected to be exceedingly rare.

Finally, in 29 subjects with extra L genes and in 3 that were heterozygous for the C203R mutation there were too many genes to determine whether the first and second genes encoded functional L and M pigments or not, so all 32 (4.0% of the sample) were categorized as "deutan suspects." To compare with prior studies, we can estimate how many of these men had color vision defects by assuming a similar proportion of deutans as found in men with shorter arrays. For men in this sample with arrays that could be resolved by last gene analysis, about 50% (21/41) had two L genes in expressed positions and are thus deutans. If a similar percentage of longer arrays with multiple L genes are deutan then there were 0.5% protanopes, 1.3% deuteranopes, 0.8% protanomals, and 4.6% deuteranomals in the AREDS sample. These rates are very similar to the previously published population estimates (Koliopoulos et al., 1976; Waaler, 1927) and thus, a purely genetic population survey is consistent with the classic results using the anomaloscope as a gold standard.

Sensitivity and Specificity of the MassArray Assay

The MassArray test separates subjects into three categories (normal red-green color

vision, red-green color vision defects, and deutan suspects) instead of two, as needed for conventional measures of sensitivity and specificity. Nonetheless, the following statements can be made. The assay missed two new point missense mutations; if these disrupt cone function then only 61 out of an estimated 63 red-green color vison defects were detected giving a sensitivity of 97% of people with red-green color vision defects in the AREDS sample correctly identified as either having a color vision deficiency or being a deutan suspect. All point mutations that cause color vision defects are expected to interrupt photopigment function and be associated with dichromacy rather than anomalous trichromacy. Earlier, we examined the genes of 128 red-green dichromats and, retrospectively, we can say that 6 (4.7%) had mutations that would not be caught by the MassArray assay (Neitz et al, 1994). Estimating that 25% of all males with red-green color vision deficiencies are dichromats provides an estimate of 1.4% of all color defects not detected by the assay giving a sensitivity of 98.6% for the purely genetic MassArray test.

Since everyone identified as having a color vision deficiency had a gene defect that positively prevents either the normal L or normal M pigment from being produced the MassArray assay is expected to have 100% specificity. However, in comparing genetics with behavioral tests of color vision in our Seattle sample (discussed below) out of 942 men and women who passed color vision tests five of them had mutations in SNPs used by the MassArray to determine the number and ratio of L and M genes and they would have been misidentified as red-green color blind. This gives the MassArray assay genetic test a specificity of 99.5%.

As an alternative genetic testing method, sequencing the entirety of the L and M genes and the promoter region for all subjects would give close to 100% sensitivity with the technology presently available; however, it would be impractical for use as part of a routine clinical

assessment for red-green color vision deficiency. The great virtue of the MassArray test is that it is quick and high throughput. As a diagnostic tool, its separation of deutan defects from protan is perfect, and its ability to identify a subset of people as obligate dichromats is very valuable since separating dichromats from anomalous trichromats is one of the most difficult distinctions for behavioral color vision tests. The two weaknesses of the MassArray test are that it can miss people with the most severe form of red-green defect and that diagnosis is ambiguous for the 4% identified as "deutan suspects." Nonetheless, as a much more direct measure of the underlying pathophysiology, the genetic test described here has many advantages over conventional clinical color vision testing methods and the following discussion serves to compare the genetic test with conventional methods used clinically to diagnose color vision deficiencies.

Current methods of diagnosing color vision defects

The widely used Ishihara pseudoisochromatic plate test has high sensitivity for discriminating individuals with some type of inherited red-green deficiency from normal individuals. However, whereas the MassArray assay, with an estimated sensitivity of 98.6%, will fail to detect missense mutations that cause dichromacy, the Ishihara instead can fail to detect the mildest forms of inherited red-green color vision deficiency. Using the anomaloscope as a standard, around 98% of individuals with a red-green deficiency have been identified with the Ishihara when used with a failure criterion of 3 or 4 errors on the 38 plate version (Birch, 1997, 2010). Everyone who passes the Ishihara either has normal color vision or they are one of those relatively rare individuals with a minimal deuteranomalous red-green color vision deficiency. Thus, the Ishihara can be useful in eliminating people from occupations that require perfect red-green color vision. Even cheating by memorizing the plates can be foiled by randomizing the

order in which the plates are presented.

One of several downfalls of the Ishihara as a stand-alone clinical diagnostic tool, however, is its poor specificity. For example, even in a sample composed primarily of college students Miyahara (Miyahara, 2008) found that 9% of individuals who test as normal trichromats on the anomaloscope made at least three errors. Performance issues are the main reason for failing normal trichromats. Numerical confusions, likely due to the curvy script of the numbers, were made by a striking 75% of school children with normal color vision, with 13.1% making four or more errors (Cosstick et al., 2005). The Ishihara also cannot reliably distinguish protans from deutans (Birch, 1997), nor can it be used to determine the severity of color vision loss by either the identity or number of plates missed (Rodriguez-Carmona et al., 2012). Finally, the Ishihara plates do not include tritan stimuli and thus are unable to diagnosis blue-yellow color vision deficiencies. For patients (or their parents) who see a clinician seeking an accurate color vision diagnosis so they can minimize the impact of color vision deficiency, the Ishihara is an inadequate instrument.

A number of tests have been designed to provide information about the type and severity of color vision defects where the Ishihara fails. One of the best examples is the Farnsworth D-15 arrangement test which is meant to dichotomize the population into those with normal color vision or mild anomalous trichromacy and those with moderate to severe anomalous trichromacy and dichromacy (Farnsworth, 1947). Unfortunately, when compared to the anomaloscope, the D15 has an error rate of 1.5-6% in correctly failing dichromats depending on the failure criterion used. It performs even less well with anomalous trichromats, with over 13% of slight or minimal anomalous trichromats failing and 24% of severe anomalous trichromats passing (Birch, 2008a, b). A positive attribute of the D-15 is that it pits blue-yellow color vision against red-green and

thus people with losses specific to one system make systematic errors that characterize protan, deutan and tritan defects and distinguish them from diffuse color vision defects. However, people with less severe defects can fail if they attend unduly to the wrong color vision dimension and people with severe defects can pass by paying attention to subtle cues in color and luminance.

The Hardy-Rand-Rittler (HRR) pseudoisochromatic plate test is designed to address all three goals of an ideal color vision test. By having figures with colors that lie on either protan or deutan confusion lines, it aims to distinguish the type of red-green defect. It also includes figures colored along tritan and (now defunct) tetartan confusion lines to find blue-yellow defects. The color of the figures increases in saturation with each plate to provide an estimate of the severity of the deficiency. Depending on the criteria used, the sensitivity of the 2002 4th edition HRR is at least 97.5% (Bailey et al., 2004; Cole et al., 2006). However, Cole et al. found that only 86% of red-green color defective individuals were correctly classified as protan versus deutan. In addition, as illustrated in Figure 3.4, failures to accurately determine defect severity are inherent to all "cone contrast tests" that rely on a single set of confusion lines being appropriate for all observers. Even dichromats of the same class (protan or deutan) will have different confusion axes depending on the spectral sensitivity of their longer wavelength photopigment and on differences in preretinal filtering by the lens and macular pigment. Thus, tests like the HRR will mischaracterize the severity of the defect for dichromats whose confusion axes are different from the tested one. Indeed, both results from our laboratory (described here) and from Cole agree that around a third of dichromats are classified as "medium" by the HRR.

Often considered to be the gold standard, even the Nagel anomaloscope, in many practitioners' hands, is prone to errors in distinguishing dichromats from anomalous trichromats.

This became evident in the very first paper describing the association between genetics and color vision in which a person diagnosed as an extreme anomalous trichromat by anomaloscope was found to have a single opsin gene on the X-chromosome and was therefore an obligate deuteranope (Nathans et al., 1986a). Traditionally, the difference between a dichromat and an anomalous trichromat has been defined by performance on the anomaloscope. However, dichromats do not experience the red and green colors experienced by a normal trichromat. During an anomaloscope exam, a dichromat may randomly say, sometimes very insistently, that a particular red-green mixture is redder or greener than the monochromatic standard and it if happens to be in the right direction for a normal trichromat, the examiner will record a correct discrimination leading to misdiagnosis of a dichromat as an anomalous trichromat. Such mistakes can be avoided using the forced choice procedure described here where subjects are shown the same red-green mixtures repeatedly, however, the procedure is so demanding for the subject and so time-consuming that it is not practical in a clinical setting.

No behavioral color vision test that is practical for use in the clinic exists that can dependably identify protans, deutans, tritans, and normal trichromats and provide reliable information about the severity of color vision defects. Pigment-based behavioral tests are also vulnerable to changes in lighting conditions and viewing distance, degradation of testing materials, and performance of subjects. Decreased visual acuity can result in poorer performance in behavioral color vision testing (McCulley et al., 2006). Additionally, female carriers have been reported to have poorer color discrimination than normal trichromats, resulting in higher failure rates on behavioral color vision tests and increased difficulty distinguishing color deficient people from carriers (Baraas, 2008; Crone, 1959; Feig and Ropers, 1978; Jordan and Mollon, 1993).

Finally, there are inherited color vision deficiencies that do not lend themselves very well to conventional tests of color vision. A mutation common in Asian populations, an A–71C substitution in the M promoter region, has been linked to color vision defects (Ueyama et al., 2015a). Most of the affected individuals have one normal L and one normal M gene and they express both photopigments. Thus, they are not protan or deutan according to the way the terms are classically defined and they do not fall easily into these categories in color vision tests. Many individuals with this promoter mutation have Rayleigh match midpoints in the normal range but the matching range may be enlarged and they can make confusing errors on standard plate and arrangement tests. Including a probe to test for this SNP in the genetic screen would provide a simple solution to identifying this cause of color vision deficiency.

Genetic testing for severity of anomalous trichromats

The same problem, discussed above, with testing dichromats using a cone contrast test that employs a single fixed set of dichromatic confusion lines applies to anomalous trichromats. If the subject's individual confusion line is different than the standard used in the test, it will underestimate severity. Thus the accuracy of the genetically-deduced severity from spectral separation shown in Figure 3.3B suggests that the genetic test is a better indicator than the HRR or other "cone contrast" type behavioral tests not just of anomalous trichromacy versus dichromacy but also of severity among anomalous trichromats.

The ability to discriminate colors in the middle-to-long wavelengths is determined by the spectral proximity of photopigments encoded on the X-chromosome (Kainz et al., 1996). Here, as shown in Figure 3.3, we have examined the relationship between the separation between photopigments and red-green color vision thresholds with a high degree of precision using a

forced choice anomaloscope task. Two aspects of this relationship are striking. First, a reduction in spectral separation from the normal ~30 nm to only 10 nm results in a very modest elevation in thresholds of only 2-3 times normal. Second, even deuteranomalous individuals with very small spectral separations of about 2.5 nm experience color vision threshold elevations that are only about 10 times higher than normal, which is dramatically better than dichromats that are more than 100 times or more worse than normal. Thus, dichromats have color vision that is about 10 times worse than the common more severe anomalous trichromats. This has important implications for the real life capabilities of dichromats versus anomalous trichromats.

Anomalous trichromats will have difficulty distinguishing colors that are close together in the spectrum, for example, orange from red-orange and they will have trouble distinguishing very pastel colors, but will have little trouble distinguishing saturated primary colors if they are more than ten times normal threshold such as those used as signals in the transportation industry and many educational materials in primary school. This highlights the great significance in clinical color vision testing of separating dichromats from anomalous trichromats. As introduced above, the Ishihara and HRR cannot reliably distinguish the two categories. The ability to correctly identify dichromats is a very strong reason to incorporate genetic testing into the standard diagnosis protocol for clinical color vision testing.

Interestingly, as introduced above, mild deuteranomalous individuals can have red-green color vision thresholds that are only 2-3 times higher than normal (Figure 3.3). The color discriminations for the most difficult plates in the HRR and the Ishihara are also about 3-4 times above normal threshold. This means that the screening plates of the Ishihara and HRR are very near threshold for deuteranomalous individuals with the best discrimination and it predicts that depending on the circumstances, a fraction of deuteranomalous individuals with the largest

spectral separations will pass color vision screening tests. That was true of the subjects we tested. Eight out of 55 individuals diagnosed as deuteranomalous trichromats from the MassArray assay followed by last gene analysis made no errors at all on the HRR showing that genetic screening is more sensitive than the HRR in detecting mild defects.

Combining genetic and behavioral approaches

Given the extremely high frequency of color vision defects, the fact that they usually appear in children for whom neither parent is affected, and the damage that can result from being undiagnosed or misdiagnosed, we suggest that everyone should have an accurate diagnosis of color vision deficiency prior to entering preschool. Colors are used extensively in the early school years in teaching subjects such as reading and math, and colorblind children, particularly dichromats, can be seriously disadvantaged if they are not accurately diagnosed and appropriate accommodations are not made. The administration of a genetic test can be done at a very early age, even at birth, and from the discussion above, it may correctly diagnose an estimated 98.6% of everyone tested, as either normal, color defective or a deutan suspect. Subsequently, a simple color vision screening test could be used to separate the normals from deutans among the "deutan suspect" group from the genetic test and identify anyone who is a dichromat as the result of a missense mutation prior to preschool.

Starting with the invention of the ophthalmoscope, the fields of ophthalmology and optometry have been continually revolutionized by the development of new methods that provide more direct information about underlying pathophysiology than can be obtained through vision assessment alone. Moreover, in the clinic, vision assessment and more direct measures of pathology almost always complement each other. For example, in the case of patients with age

related macular degeneration (AMD), OCT measurements and visual acuity are used together in decision making about treatments.

Analogously, information from behavioral color vision testing complements information gained from the practical genetic test described here. People with missense mutations that are not picked up in the MassArray assay will be dichromats. Such people would appear normal in the genetic screen but be diagnosed as having a moderate to severe red-green defect on a behavioral test, as was the case for 7 out of 887 male subjects in our behaviorally-tested group. The combination of a normal appearing gene array coupled with obvious failure on a behavioral color vision test could be used as a criteria indicating the need for sequencing the L and M genes and their regulatory regions in such individuals, which would both detect and correctly identify the type of all red-green dichromats. Sequencing the L and M genes of people who fail a behavioral screening test would also identify toxic sequence combinations such as "LIAVA" and "LVAVA" that result in splicing errors (Ueyama et al., 2012), and it would identify the small percentage of colorblind people who appear normal in the MassArray assay because of mutations in the SNPs used by the assay to determine array configurations.

The combination of a color vision screening test with the MassArray assay also provides a method of separating people with an inherited color vision defect from those with reduced color vision because of some other cause, which is impossible with color vision screening alone. It is important to separate color vision defects in the absence of L, M, or S opsin gene mutations from inherited color vision deficiencies because their detection can indicate more serious vision disorders, including damage to the retina, optic nerve or visual areas of the brain (Katz, 1995; Pearlman et al., 1979), solvent and heavy metal exposure (Gobba and Cavalleri, 2003), reactions to certain medicines (Koliopoulos and Palimeris, 1972; Perdriel and Manent, 1982), alcohol

toxicity (Smith and Layden, 1971), and the presence of other serious diseases like multiple sclerosis (Shaygannejad et al., 2012), diabetes (Shoji et al., 2011), and age-related macular degeneration (O'Neill-Biba et al., 2010).

In our behaviorally tested subjects, we found six people who showed mild defects in protan, deutan, or tritan stimuli on behavioral color vision tests. No cause for these results was found in the L and M pigment gene array or in the S-opsin gene. One subject had poor acuity and had undergone eight eye surgeries to correct his amblyopia; his failure on the behavioral color vision tests can be attributed to other eye problems and not an inherited color vision defect. A second man in this group also had amblyopia; another suffered from night blindness with nystagmus. The other three men may have made errors due to presence of an acquired color vision deficiency or to inattention or performance issues during the behavioral testing.

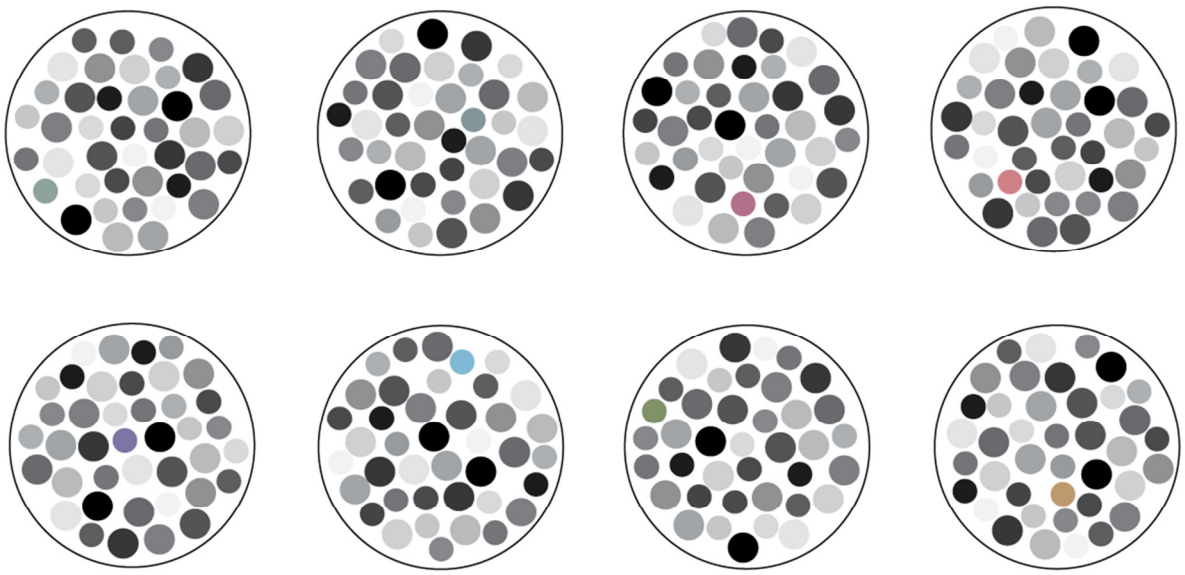
Diagnosis of tritan (or blue-yellow) color vision deficiencies also lends itself to the combined approach of genetics and behavioral screening. Tritan defects can be inherited as a result of mutations in the S-opsin genes but they often result from other kinds of damage to the S-cone pathways. The MassArray assay includes SNPs associated with tritanopia, all of which are mutations in S opsin: L56P, G79R, T190I, S214P, P264S, and R283Q. When used in conjunction with a tritan screening test, people who are found to have a mutation and/or make errors on the behavioral screening test will fall into three categories. 1) Those with both an identified mutation from the MassArray and who make errors on a behavioral tritan screening test would be diagnosed as having an inherited tritan color vision defect. 2) Subjects who have one of the mutations from the MassArray test who pass the tritan screening test would be diagnosed as "future tritans." Tritanopia is a progressive autosomal dominant disorder that is, in many ways, analogous to retinitis pigmentosa (Baraas et al., 2007). The age of onset is variable

but presumably everyone with one of the identified disease-causing alleles will ultimately develop a tritan color vision defect. 3) People who make errors on the screening test but do not have one of the mutations identified by the MassArray should have their S-opsin genes sequenced. If a novel mutation is identified, the person is diagnosed as having an inherited tritan color vision deficiency. Alternatively, if no mutation is found, they are diagnosed as having a blue-yellow color anomaly that is not inherited.

The ideal protocol for clinical color vision diagnosis combines a behavioral color vision screening test with the genetic test described here. It is most efficient to use a simple screening test focused on separating normals from deutans among the "deutan suspects" and identifying subjects with rare mutations that require further genetic analysis including complete sequencing of the opsin genes. With this in mind, we developed the disposable minimalist "paper and pencil" behavioral color vision screening test shown in Figure 3.5. This is designed to detect both blue-yellow and red-green color vision deficiencies and it can be part of a color vision testing kit which could also include materials for collecting buccal swabs or saliva samples. Such a "kit" represents a complete color vision diagnosis solution that can be administered in the clinic in just a few minutes—about 2 minutes to administer the paper and pencil test and just a few more minutes to collect a buccal swab or saliva sample. It can also be used as pediatric test with no lower age limit and the screening test could be administered separately prior to kindergarten entry.

With a failure criteria of a single missed dot, the minimalist test shown in Figure 3.5 was 89% sensitive when used to examine 55 subjects with red-green deficiencies. It had a specificity of 99% specific (n=260 color normal subjects). As introduced above minimal deutans are a challenge for all screening tests. While the minimalist test did correctly fail two of five minimal

Figure 3.5. Sample behavioral color vision test to accompany genetic assay.



A simple color vision test that can be administered quickly and with no experimenter training. Each group of dots contains one colored dot whose chromaticity falls along a protan, deutan, or tritan confusion line. The subject must identify the colored dot in each group and a single mistake constitutes failure.

deutans who passed the HRR and D15, there were still minimal deutans and a few mild deutans who passed, accounting for the 89% sensitivity. However, as summarized in Table 3.3, a fraction of deuteranomalous subjects have only L genes and no M genes, and well over half of subjects with multiple L genes have only three genes total so deutans among them can be separated from normal with the last gene assay. Thus, the combination of the minimalist screen with the MassArray test is estimated to be greater than 96% sensitive missing only mild deutans. However, the small fraction of people who pass the screening test but have multiple L genes could be referred for additional screening to insure that everyone with a color vision deficiency is identified.

Summary

Overall, the MassArray test described here is capable of directly identifying the underlying pathobiology of color vision deficiencies and offers many important advantages over traditional behavioral color vision tests. When combined with a simplified behavioral screening test, the MassArray opsin gene assay provides a powerful tool that can accomplish all the aims of an ideal color vision test. It is the best way, clinically, to separate protans from deutans and dichromats from anomalous trichromats, and it performs well in stratifying different levels of severity among anomalous trichromats. It can distinguish inherited color vision deficiencies from those that arise from other causes. An additional benefit of genetic testing is the ability to distinguish female carriers of color vision deficiencies from normal trichromatic women, a feat impossible for behavioral tests. The test can even predict future color vision loss in younger people with known S cone mutations. Moreover, as gene therapies for color blindness become available, knowledge of the causal mutations leading to color defects will identify subjects who

are good candidates and determine which type of therapy they would require.

Chapter Four

Virally-Mediated Gene Therapy for Toxic Cone Opsin Variants

Introduction

The L and M cone photopigments are highly variable due to frequent recombination between the two genes that encode them, OPN1LW and OPN1MW. Most of the resulting intermixed sequences function normally and a large variety of different opsin genes are found in people with normal color vision, as detailed in chapter two. However, some rare combinations of the exon 3 polymorphic sites have recently been linked to vision disorders with symptoms including colorblindness, high-grade myopia, and cone dystrophy (McClements et al., 2013). These "toxic" haplotypes involve amino acid residues 153, 171, 174, 178, and 180, and are by convention named according to the specific amino acids present at those locations. So far, four toxic opsin variants have been discovered in humans: LVAVA, LIAVA, LIVVA, and MIAVA (Carroll et al., 2012; Carroll et al., 2004; Gardner et al., 2014; McClements et al., 2013; Mizrahi-Meissonnier et al., 2010; Neitz and Neitz, 2011; Neitz et al., 2004).

Ueyama and colleagues (Ueyama et al., 2012) discovered that the disease mechanism of these variants is at least partly due to an error in gene splicing. In LIAVA-encoding genes, exon 3 loses definition as an exon and is excluded from the final messenger RNA. The consequent joining of exon 2 to exon 4 leads to a frame shift and a premature stop at codon 199 and presumably leads to nonsense-mediated decay of the transcript. LVAVA, MVAVA, and MIAVA-encoding sequences also showed the same exon 3 splicing error but a portion of the full length message remains. Indeed, humans with LVAVA opsins who have been tested by electroretinography (ERG) show residual function of LVAVA-containing cones.

To better understand the etiology of these diseases, our laboratory created knockin/knockout mouse lines in which the mouse S opsin was knocked out and the endogenous M opsin was replaced by an engineered opsin containing either a toxic LVAVA exon 3 haplotype or a functional control LIAIS exon 3 haplotype (Greenwald, 2013). These engineered opsins contained only intron 1 so that all exons after exon 2 were "pre-spliced" meaning that disease caused by exon 3 splice defects should not affect these mice. Yet, compared to controls, LVAVA mice showed significantly lower light responses by ERG and had abnormally foreshortened cone outer segments, recapitulating functional and physiological defects observed in human patients. Still, even though LVAVA cones are structurally and functionally defective, it appears that they remain alive and weakly responsive to light. This finding that LVAVA cones remain viable, albeit dysfunctional, suggests that it may be possible to rescue vision using gene therapy. The current work explored this possibility using recombinant AAV technology to deliver functional opsin genes to dysfunction mouse LVAVA-containing M cones.

Materials and methods

Animals

All experiments using animals conformed to the principles regarding the care and use of animals adopted by the American Physiological Society and the Society for Neuroscience and were approved by the Animal Care and Use Committee at the University of Washington.

Two lines of genetically modified mice were created as described previously (Greenwald, 2013). Briefly, in both C57BL6 lines the mouse UV sensitive opsin gene OPN1SW was knocked out through targeted deletion of exons 2, 3, and 4 which introduces a stop codon in the beginning of exon 5. Secondly, a segment of the endogenous mouse OPN1MW gene starting from codon

66 in exon 2 was replaced with a corresponding segment of human L opsin cDNA, and codons 58, 62, and 65 were altered to encode the same amino acids as the corresponding codons in human Opn1lw. The opsin encoded by the final modified locus retained the amino acids specified by mouse exon 1, but the amino acids specified by exon 2 through 6 corresponded to those encoded by a human L opsin. In one mouse line, OPN1LW-LIAIS / Opn1sw−/−, the haplotype of exon 3 specified an amino acid sequence of L153, I171, A174, I178, S180. In the other line, OPN1LW-LVAVA / Opn1sw−/−, the haplotype of exon 3 specified an amino acid sequence of L153, V171, A174, VI178, A180. In both lines, exon 2 encoded T65, I111, S116 and exon 4 encoded I230, A233, and M236 at dimorphic positions. To summarize, one mouse line (OPN1LW-LIAIS / Opn1sw−/−, hereafter called LIAIS) has a single functional opsin gene that encodes a photopigment that has been associated with normal cone function and that has a λmax of 559 nm. The other mouse line (OPN1LW-LVAVA / Opn1sw−/−, hereafter called LVAVA) has a single functional opsin gene that encodes a photopigment that has been associated with aberrant cone function and has a λmax of 555.5 nm.

Viral Vectors

Two different recombinant adeno-associated virus (rAAV) constructs were used in the experiments reported here. Both have a modified AAV capsid called 7M8 created by Dalkara et al (Dalkara et al., 2013) that was reported to display enhanced tropism toward photoreceptors. Both also share a novel L/M cone specific promoter that contains an optimized version of human L opsin promoter PR2.1(Komáromy et al., 2008; Mancuso et al., 2009), a mini intron, and an SV40 polyadenylation signal. The construct rAAV.7M8.GFP contains a gene encoding green fluorescent protein and the construct rAAV.7M8.hOPN1SW contains a gene encoding human S

opsin that has been codon optimized for expression in mouse. The sequences of all vectors were verified by direct sequencing. Viruses were packaged and purified following the triple transfection protocol in HEK293T cells described in (Flannery and Visel, 2013). Final viral titers determined by quantitative PCR are as follows: rAAV.7M8.GFP $2.2x10^{12}$ vg/mL, rAAV.7M8.hOPN1SW prep 1 $6.4x10^{11}$ vg/mL, rAAV.7M8.hOPN1SW prep 2 $1.9x10^{13}$ vg/mL.

Intravitreal Injections

Mice were anesthetized with an intraperitoneal injection of ketamine (110.25 mg/kg) and xylazine (11.025 mg/kg) diluted in sterile saline. The right eye was dilated with a topical solution of 1.0% atropine sulfate and both eyes were kept moist with GenTeal lubricant eye gel. The right eye was temporarily proptosed by fitting around the eye a half inch square of latex with a small incision in the center. The mice were stabilized in a stereotaxis (Stoelting) and positioned with the right eye pointing directing up. While viewing the eye under an Olympus SZX10 microscope, a small guide hole was created in the superior sclera with a sterile 30 gauge beveled needle. Virus injections were made using a 34 gauge beveled needle (World Precision Instruments, NF34BV) attached to a 10 ul Nanofil syringe (World Precision Instruments, NANOFIL and IO-KIT). A sterile 1% fluorescein solution was added to the virus (1:15) prior to loading the syringe so that a successful injection could be easily visualized. The injection needle was guided through the sclerotomy and into the vitreous chamber where the virus was injected. The syringe was controlled using a Micro4 microsyringe pump controller (World Precision Instruments, UMC3) to inject a volume of 2ul at a speed of 40ul/sec.

Electroretinogram

Mice were anesthetized with an intraperitoneal injection of ketamine (110.25 mg/kg) and xylazine (11.025 mg/kg) diluted in sterile saline. Both eyes were dilated by topical application of 1.0% atropine sulfate and were kept moist with GenTeal lubricant eye gel. Each animal was positioned on a water-heated Aluminum platform during the procedure. The active lead was an alligator clip electrode attached to a 1 cm DTL fiber which was draped across the cornea using the GenTeal as a conductive adhesive. A contact lens was pressed against the eye to create an optical interface. Platinum needle electrodes were used for both the reference and earth ground leads. The reference was placed subcutaneously between the ears and the ground electrode subcutaneously near the base of the tail.

A custom built chromatic flicker photometric ERG was used. The light source contained four LED wavelengths (450, 525, 635, and 660 nm) and was presented to the eye in a Maxwellian view configuration to maximize light intensity reaching the retina. Individual LED intensities were controlled via a high frequency (much higher than flicker frequency) pulse frequency modulation circuit such that the LED intensities behaved linearly and without shift in peak wavelength. A second circuit provided non-interrupt based control of the LED light driver to allow modification of any presentation parameter such as base frequency, number of cycles, duty cycle, and LED phase. ERG amplification was achieved with a differential amplifier from Warner Instrument Corp (DP-301), placed through an 8th order Butterworth switched capacitor low-pass filter (LPF), and then sampled at 50 kHz with a digital acquisition board from Measurement Computing (USB-2404). Finally, custom software communicated with the external control circuitry to set light intensities, program experiment parameters, display biosignal, and collect and present the incoming ERG averages to the screen.

For these experiments, the base frequency was set to 25 Hz (optimal for mouse to suppress rod response while allowing cone responses) and fluorescent room lights (250-300 Lux) were left on to further suppress rod response. Test and reference lights were 180 degrees out of phase with a 25% duty cycle. The number of cycles were typically set to no less than 75, where the first 20 were not included in the average trace. The DP-301 amplifier gain was set to 10,000 with the LPF knob set to 100 Hz and the high pass filter knob set to 10 Hz. This signal was then passed through the final switched capacitor LPF, which was programmed to 30 Hz. The 450 nm wavelength light was used as the reference and the 635 nm wavelength was used as the test light.

Immunohistochemistry

Mice were euthanized with a lethal dose of sodium pentobarbital and the sclera were lightly cauterized to mark orientation. The eyes were removed and fixed for 8-10 minutes in 4% paraformaldehyde (PFA) in PBS. The cornea and lens were removed and the eyecup was fixed in PFA for an additional 30 minutes. The retina was then dissected from the eyecup, washed in PBS, and then incubated overnight at 4°C with a solution containing 5% donkey serum (Jackson ImmunoResearch; Cat # 004-000-120), 1 mg/ml BSA (Jackson ImmunoResearch, West Grove, PA; Cat # 001-000-161), and 0.03% Triton X-100 in PBS (pH 7.4) to block non-specific labeling. The primary antibodies used in this study were rabbit anti red-green (L/M) opsin diluted 1:200 (Millipore, Billeria, CA; Cat # AB5405) and goat anti-S-opsin (Santa Cruz Biotechnology, Santa Cruz, CA; Cat # sc-14365) diluted 1:100. Note that there are no antibodies that distinguish between L and M opsins. Primary antibodies plus rhodamine-labeled peanut agglutinin (PNA, 5 µ g/ml; Vector Labs, Burlingame, CA; Cat # RL-1072) were incubated at 4°C for at least 2 days for whole mount tissue. Specimens were washed in PBS 3 times for 30 min each, then incubated

at 4°C overnight with DAPI (4 ′,6-diamidino-2-phenylindole,dihydrochloride 1:10,000; Invitrogen, Carlsbad, CA; Cat # D-21490) plus secondary antibodies. The secondary antibody for the L/M-opsin antibody was Alexa Fluor 488 labeled donkey anti-rabbit IgG(H + L) diluted 1:200 in antibody dilution buffer (Invitrogen; Cat # A21206) and the secondary antibody for the S-opsin antibody was Alexa Fluor 633 labeled donkey anti-goat IgG(H + L) diluted 1:200 (Invitrogen; Cat # A21082). This was followed by three 30-min PBS washes, 30 min of postfixation with 4% paraformaldehyde, and three more 30-min PBS washes. Finally, the retinas were placed on slides with 2% DABCO in glycerol and covered with cover slips. Samples were imaged on a Leica SP8 confocal microscope.

Results

At nine months of age, seven mice LIAIS and seven LVAVA mice were injected with 7M8.pMNTC.eGFP and seven LVAVA mice were injected with7M8.pMNTC.hOPN1SW in the right eye. Expression of virally delivered GFP was confirmed through in vivo fundus imaging using the Micron II (Phoenix Research Laboratories) retinal imaging microscope three months post injection. High expression levels were observed in all mice. Figure 4.1 shows sample fundus images from the mice with the highest and lowest levels of fluorescence. Even the least fluorescent retina shown in Figure 4.1B still has ample expression of GFP across a wide region in the retina.

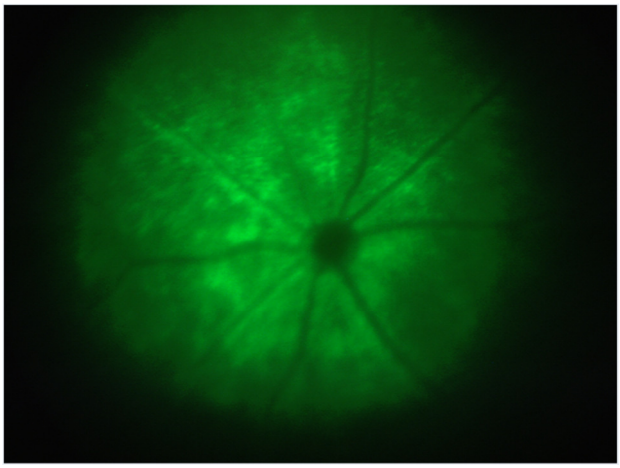
One mouse from each group was euthanized and the retinas were stained and imaged as described in Methods. The mouse retina contains a ventral-dorsal opsin expression gradient in which M opsin is expressed primarily in the dorsal retina (Applebury et al., 2000). Since the promoter used in the viral construct is expected to drive expression in M cones, we expected

high expression levels in dorsal retina and low expression in ventral retina. Confocal images from the dorsal retina of one LIAIS mouse injected with the GFP-encoding virus and one LVAVA mouse injected with the S opsin-encoding virus are shown in Figure 4.2. The image in Figure 4.1A was from the same mouse as Figure 4.2A. The expression of S opsin from Figure 4.2B is far lower than of GFP in Figure 4.2A, likely due to the 5 fold lower concentration of the S opsin-encoding virus. Still, cones that were infected show high levels of S opsin expression (Figure 4.2C).

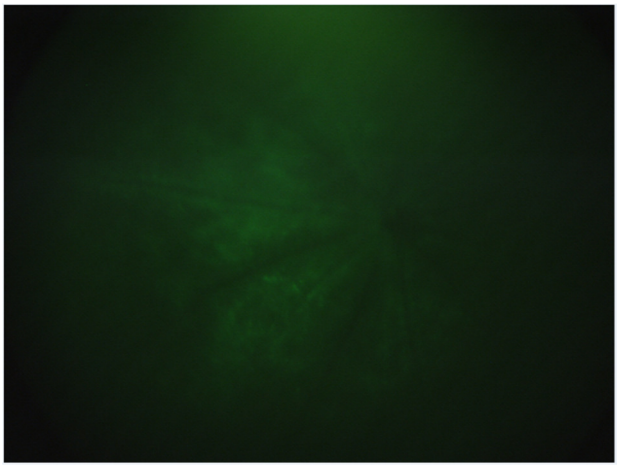
A third group of seven mice was injected with a second, higher titer prep of the virus containing the S opsin gene, 7M8.pMNTC. hOPN1SW prep 2. Injections were delivered intravitreally in the right eye at twelve weeks of age. Six weeks post injection, the mice were tested using flicker photometric electroretinography to measure S opsin function. We were able to record reliable ERG signals from four of the mice. A blue 450 nm LED at a single intensity was used as a reference and we measured the intensity of a green 525 nm LED and a red 635 nm LED needed to match the retinal response of the reference light. Results are plotted in Figure 4.3. The differences between the treated and untreated eye are highly significant ($p<0.001$, Wilcoxon Signed Rank test).

Figure 4.1 In vivo fluorescence imaging of mice injected with GFP-encoding virus

A



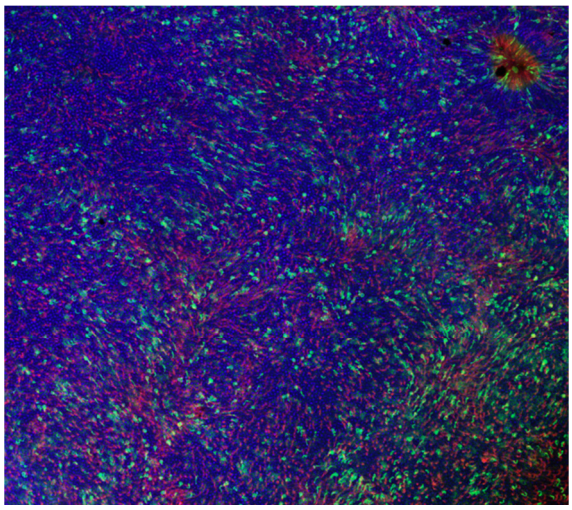
B



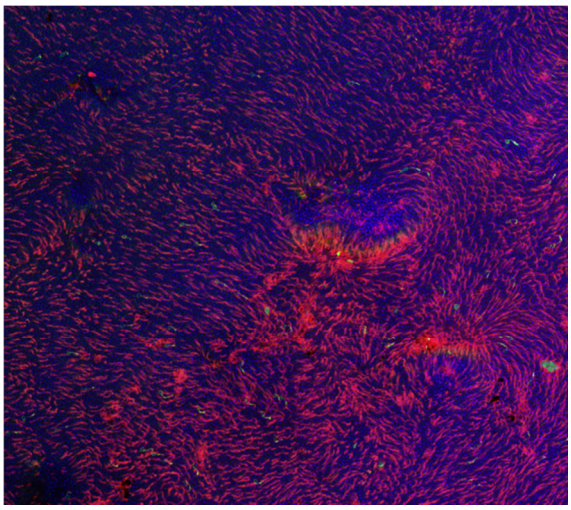
A) Sample fundus image of a highly fluorescent retina from an LIAIS mouse injected three months prior with the 7M8.pMNTC.GFP virus . B) Sample image of the least fluorescent retina from the same cohort of mice. Five of the seven retinas imaged had fluorescence levels like the one shown in A and the other two were like the retina shown in B.

Figure 4.2 Confocal images of retinas injected with GFP or S opsin encoding viruses

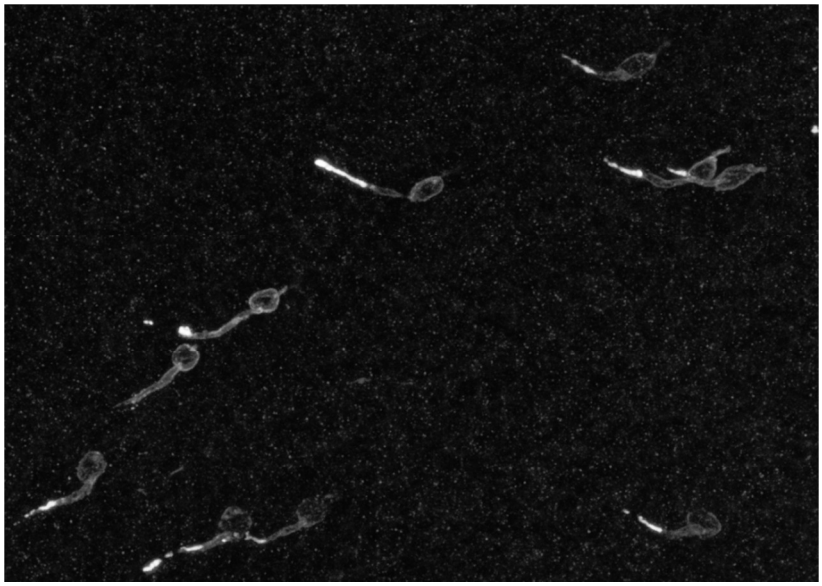
A



B

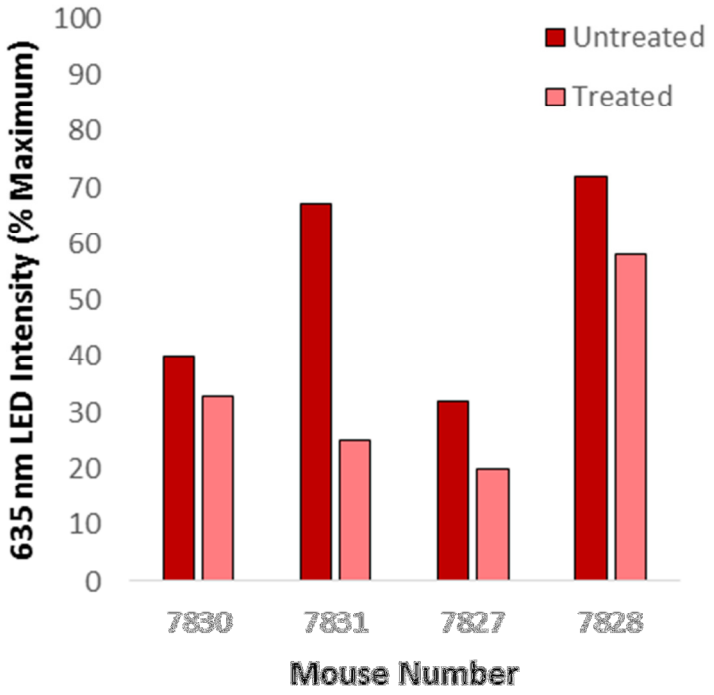


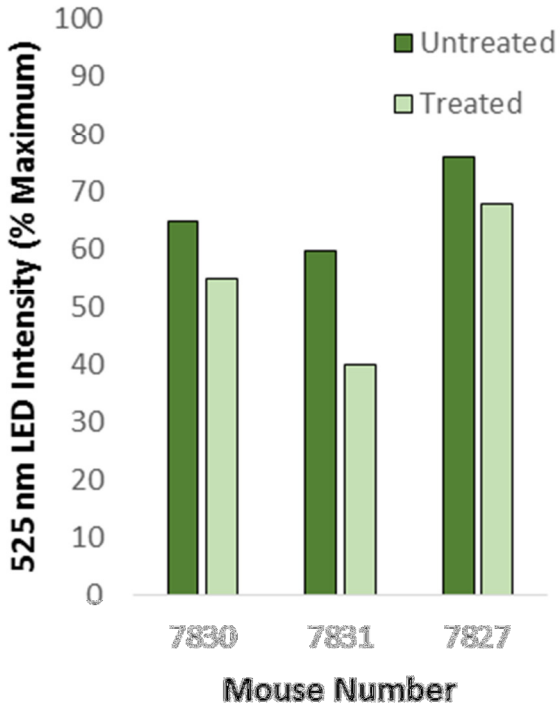
C



Maximum intensity projections of a Z stack from a region of dorsal retina. A) Blue = DAPI (cell nuclei), Red = PNA (cone sheaths), Green (GFP). B) Blue = DAPI (cell nuclei), Red = PNA (cone sheaths), Green (S opsin). C) S opsin expression from the same retina as in B.

Figure 4.3 Relative sensitivity to 525 nm and 635 nm light





Comparison of the treated and untreated eyes of each mouse six weeks following injection of a virus encoding an S opsin into the right eye.

Discussion

A major hurdle in using intraocular gene therapy to treat diseases of the photoreceptors in human patients has been that efficient infection by the virus required injection in close proximity to the target cells. Since the photoreceptor layer is in the outer retina furthest from the pupil, this required injection into the subretinal space, placing patients at risk of serious complications including retinal tears and detachments. A main motivation for this work was to create a virus that could be injected into the intravitreal space, traverse the inner limiting membrane and multiple cell layers to infect photoreceptors, and drive high levels of expression of opsins in specifically targeted cone populations.

With a combination of the modified AAV capsid 7M8 created by Dalkara and colleagues and an optimized expression cassette, we have achieved this aim. Here, as shown in Figure 4.3, we have demonstrated that GFP virus particles injected into the intravitreal space can successfully drive high expression, and highly specific expression, in distant photoreceptor cells. Moreover, we have shown via electroretinography that virally-introduced opsins are functional and can drive light responses in the retina.

The second aim of this work was to explore the potential of virally-mediated gene therapies to restore vision in people with diseases caused by mutant opsins. The mice used in these experiments had their native M opsin photopigment gene replaced by an engineered L opsin variant known to cause disease in humans. Prior work from our laboratory (Greenwald, 2013) has shown that mice with this opsin variant, called LVAVA, have lower ERG responses at all ages compared to control mice expressing a functional L opsin variant, LIAIS. We hypothesized that delivering a gene encoding a functional opsin to cones that natively express an LVAVA opsin would improve the light sensitivity of those cones and halt the degeneration of the

retina caused by the LVAVA opsin. To this end, we created two rAAV viruses encoding either a human S opsin or GFP under the control of an optimized expression cassette.

We tested functional restoration by the virally-delivered S opsin using flicker photometric electroretinography. Contribution of the S opsin to the light response of the retina would shift the retina's spectral sensitivity toward shorter wavelengths. We measured this effect by using a 450 nm light as a reference and finding the intensities of a 635 nm light and a 525 nm light required to match that standard response. We expected that in the treated eyes, a greater sensitivity to the blue light created by the S opsin would mean an increase in the intensity of the red light required to match it. Surprisingly, we instead found the opposite – consistently in all mice the treated eyes required a lower intensity of the red and green lights to equal the response to the standard blue light.

Future work on this project may illuminate the cause of the increase in red sensitivity we observed following introduction of an S opsin. First, the experiments described here compared an injected eye to an uninjected control eye. Any damage to the retina or to other parts of the eye resulting from the injection procedure itself will produce differences in responses between the control and test eye. A better control would be a sham injection of saline or of the GFP virus into the fellow eye. Moreover, our current in vivo ERG system requires exact alignment of the apparatus to ensure that maximum light intensity reaches the retina. Even slight mistakes in alignment can drastically alter results. Our protocol also included injecting the same eye in each animal and ERG testing the eyes in the same order. If we were systemically worse at aligning one eye compared to the other, or if prep quality was systemically worse in the second tested eye due to drying out of the eyes or shallower anesthesia, we may also see differences between treated and control eyes due to factors besides treatment.

An ex vivo ERG system, in which both retinas are dissected out and kept alive through perfusion with a physiological medium, will address some of these issues. Testing both retinas from the same animal simultaneously will remove inconsistencies from preparation variability. Correct alignment of the apparatus to focus light on the retina will be easily verified through visual inspection. No light will be lost to absorption or scattering by other parts of the eye. Tissue can be viable for recording for as long as several hours, allowing more thorough testing than is possible in living animals who begin to wake from anesthesia within twenty to thirty minutes.

Additionally, quantitation of the amount virally-introduced and endogenous opsin will be necessary to assess the potential for this type of gene therapy approach to cure disease in humans. It is possible that the toxic effect of the LVAVA variant is not caused by lack of functional opsin but by some deleterious activity of the mutant variant. If this is the case, merely introducing a functional opsin will not cure the disease. Rather, it would be necessary to knockdown expression of the LVAVA opsin in addition to providing a functional photopigment. The expression cassette used here is an optimized version of the human L/M promoter. It is our hope that driving the functional opsin gene with this enhanced version of the promoter will outcompete the endogenous promoter and result in lower expression of the native photopigment. We can test this hypothesis using real time quantitative PCR on mRNA harvested from injected eyes and uninjected eyes. This will allow us to compare virally-introduced S opsin levels to endogenous LVAVA opsin levels in injected eyes, and also to compare LVAVA opsin levels to an unrelated gene standard such as rhodopsin in both eyes to determine to what extent S opsin expression is increasing total opsin or displacing toxic LVAVA opsin.

Finally, to convincingly show that the rAAV treatment has improved the vision of the

treated mice, we need to test them in a visually-guided behavioral task. The ideal test is a variation on the mouse "virtual reality" maze described in (Harvey et al., 2009). In this test, the mouse runs on top of an air-supported spherical treadmill with its head held fixed in space using a head plate. An image of a virtual 3D environment is projected onto a large screen in front of the mouse from a digital light processing projector. The visual display is updated on the basis of the movements of the animal, measured as rotations of the spherical treadmill using an optical computer mouse. Rewards are delivered through a lick tube by a computer-controlled solenoid valve. Using this test gives precise control over all features of the visual stimulus and can easily measure aspects of visual function like contrast sensitivity or spatial acuity. We can create a stimulus with spectral content designed such that both objects and backgrounds appear identical to L cones but that can be differentiated by S opsin expressing cones.

While there is much more work to be done, the experiments reported here provide evidence that gene therapies using our 7M8-pMNTC recombinant AAV virus construct are a promising approach to creating cures for diseases of the cone opsins like those caused by toxic exon 3 variants.

Bibliography

Applebury, M. L., M. P. Antoch, L. C. Baxter, L. L. Y. Chun, J. D. Kalk, F. Farhangfar, K. Kage, M. G. Krzystolik, L. A. Lyass, and J. T. Robbins, 2000, The murine cone photoreceptor: A single cone type expresses both S and M opsins with retinal spatial patterning: Neuron, v. 27, p. 513-523.

Asenjo, A. B., J. Rim, and D. D. Oprian, 1994, Molecular determinants of human red/green color discrimination: Neuron, v. 12, p. 1131-1138.

Bailey, J. E., M. Neitz, D. Tait, and J. Neitz, 2004, Evaluation of an updated HRR color vision test: Visual Neuroscience, v. 21, p. 431-436.

Baraas, R. C., 2008, Poorer color discrimination by females when tested with pseudoisochromatic plates containing vanishing designs on neutral backgrounds: Vis Neurosci, v. 25, p. 501-5.

Baraas, R. C., J. Carroll, K. L. Gunther, M. Chung, D. R. Williams, D. H. Roster, and M. Neitz, 2007, Adaptive optics retinal imaging reveals S-cone dystrophy in tritan color vision deficiency: Journal of the Optical Society of America A, v. 24, p. 1438-1447.

Baraas, R. C., L. A. Hagen, E. W. Dees, and M. Neitz, 2012, Substitution of isoleucine for threonine at position 190 of S-opsin causes S-cone-function abnormalities: Vision Res, v. 73, p. 1-9.

Benson, N. C., J. R. Manning, and D. H. Brainard, 2014, Unsupervised learning of cone spectral classes from natural images: PLoS Comput Biol, v. 10, p. e1003652.

Birch, J., 1997, Efficiency of the Ishihara test for identifying red-green colour deficiency: Ophthalmic and Physiological Optics, v. 17, p. 403-408.

Birch, J., 2008a, Failure of concordance of the Farnsworth D15 test and the Nagel anomaloscope matching range in anomalous trichromatism: Vis Neurosci, v. 25, p. 451-3.

Birch, J., 2008b, Pass rates for the Farnsworth D15 colour vision test: Ophthalmic Physiol Opt, v. 28, p. 259-64.

Birch, J., 2010, Identification of red-green colour deficiency: sensitivity of the Ishihara and American Optical Company (Hard, Rand and Rittler) pseudo-isochromatic plates to identify slight anomalous trichromatism: Ophthalmic Physiol Opt, v. 30, p. 667-71.

Carroll, J., R. C. Baraas, M. Wagner-Schuman, J. Rha, C. A. Siebe, C. Sloan, D. M. Tait, S. Thompson, J. I. W. Morgan, J. Neitz, and M. Neitz, 2009, Cone photoreceptor mosaic disruption associated with Cys203Arg mutation in the M-cone opsin: Proceedings of the National Academy of Sciences of the United States of America, v. 106, p. 20948-20953.

Carroll, J., A. Dubra, J. C. Gardner, L. Mizrahi-Meissonnier, R. F. Cooper, A. M. Dubis, R. Nordgren, M. Genead, T. B. Connor, Jr., K. E. Stepien, D. Sharon, D. M. Hunt, E. Banin, A. J. Hardcastle, A. T. Moore, D. R. Williams, G. Fishman, J. Neitz, M. Neitz, and M. Michaelides, 2012, The effect of cone opsin mutations on retinal structure and the integrity of the photoreceptor mosaic: Invest Ophthalmol Vis Sci, v. 53, p. 8006-15.

Carroll, J., M. Neitz, H. Hofer, J. Neitz, and D. R. Williams, 2004, Functional photoreceptor loss revealed with adaptive optics: An alternate cause of color blindness: Proceedings of the National Academy of Sciences of the United States of America, v. 101, p. 8461-8466.

Cole, B. L., K. Y. Lian, and C. Lakkis, 2006, The new Richmond HRR pseudodisochromatic test of colour vision is better than the Ishihara test: Clinical and Experimental Optometry, v. 89, p. 73-80.

Cosstick, M., D. Robaei, K. Rose, E. Rochtchina, and P. Mitchell, 2005, Numerical confusion errors in ishihara testing: findings from a population-based study: Am J Ophthalmol, v. 140, p. 154-6.

Crone, R. A., 1959, Spectral sensitivity in color-defective subjects and heterozygous carriers: American Journal of Ophthalmology, v. 48, p. 213-238.

Dalkara, D., L. C. Byrne, R. R. Klimczak, M. Visel, L. Yin, W. H. Merigan, J. G. Flannery, and D. V. Schaffer, 2013, In vivo-directed evolution of a new adeno-associated virus for therapeutic outer retinal gene delivery from the vitreous: Sci Transl Med, v. 5, p. 189ra76.

Deeb, S. S., D. T. Lindsey, Y. Hibiya, E. Sanocki, J. Winderickx, D. Y. Teller, and A. G. Motulsky, 1992, Genotype-phenotype relationships in human red/green color-vision defects: molecular and psychophysical studies: American Journal of Human Genetics, v. 51, p. 687-700.

Desmet, F. O., D. Hamroun, M. Lalande, G. Collod-Béroud, M. Claustres, and C. Béroud, 2009, Human Splicing Finder: an online bioinformatics tool to predict splicing signals: Nucleic Acids Res, v. 37, p. e67.

Drummond-Borg, M., S. S. Deeb, and A. G. Motulsky, 1989, Molecular patterns of X-chromosome-linked color genes among 134 men of European ancestry: Proceedings of the National Academy of Sciences of the United States of America, v. 86, p. 983-987.

Farnsworth, D., 1947, The Farnsworth Dichotomous Test for Colour Blindness Panel D15 Test Manual, New York, Psychological Corporation.

Feig, K., and H. Ropers, 1978, On the incidence of unilateral and bilateral colour blindness in heterozygous females: Human Genetics, v. 41, p. 313-323.

Flannery, J. G., and M. Visel, 2013, Adeno-associated viral vectors for gene therapy of inherited retinal degenerations: Methods Mol Biol, v. 935, p. 351-69.

Gardner, J. C., G. Liew, Y. H. Quan, B. Ermetal, H. Ueyama, A. E. Davidson, N. Schwarz, N. Kanuga, R. Chana, E. R. Maher, A. R. Webster, G. E. Holder, A. G. Robson, M. E. Cheetham, J. Liebelt, J. B. Ruddle, A. T. Moore, M. Michaelides, and A. J. Hardcastle, 2014, Three different cone opsin gene array mutational mechanisms with genotype-phenotype correlation and functional investigation of cone opsin variants: Hum Mutat, v. 35, p. 1354-62.

Gobba, F., and A. Cavalleri, 2003, Color vision impairment in workers exposed to neurotoxic chemicals: Neurotoxicology, v. 24, p. 693-702.

Greenwald, S. H., 2013, Effects of deleterious opsin gene variants on cone photoreceptor structure, function, and viability, University of Washington.

Gunther, K. L., J. Neitz, and M. Neitz, 2006, A novel mutation in the short-wavelength sensitive cone pigment gene associated with a tritan color vision defect: Visual Neuroscience, v. 23, p. 403-409.

Harvey, C. D., F. Collman, D. A. Dombeck, and D. W. Tank, 2009, Intracellular dynamics of hippocampal place cells during virtual navigation: Nature, v. 461, p. 941-6.

Hayashi, S., H. Ueyama, S. Tanabe, S. Yamade, and K. Kani, 2001, Number and variations of the red and green visual pigment genes in Japanese men with normal color vision: Japanese Journal of Ophthalmology, v. 45, p. 60-67.

Hayashi, T., A. G. Motulsky, and S. S. Deeb, 1999, Position of a 'green-red' hybrid gene in the visual pigment array determines colour-vision phenotype: Nature Genetics, v. 22, p. 90-93.

Hecht, S., S. Shlaer, and M. H. Pirenne, 1941, ENERGY AT THE THRESHOLD OF VISION: Science, v. 93, p. 585-7.

Jacobs, G. H., J. Neitz, and J. F. Deegan, 1991, Retinal receptors in rodents maximally sensitive to ultraviolet light: Nature, v. 353, p. 655-6.

Jordan, G., S. Deeb, J. Bosten, and J. D. Mollon, 2010, The dimensionality of color vision in carriers of anomalous trichromacy: J Vision, v. 10, p. 12.

Jordan, G., and J. D. Mollon, 1993, A study of women heterozygous for colour deficiencies: Vision Research, v. 33, p. 1495-1508.

Jørgensen, A. L., S. S. Deeb, and A. G. Motulsky, 1990, Molecular genetics of X chromosome-linked color vision among populations of African and Japanese ancestry: High frequency of a shortened red pigment gene among Afro-Americans: Proceedings of the National Academy of Sciences of the United States of America, v. 87, p. 6512-6516.

Kainz, P. M., M. Neitz, and J. Neitz, 1996, Severity of deutan color defect can be predicted from L-pigment gene: Investigative Ophthalmology & Visual Science (Supplement), v. 37.

Kainz, P. M., M. Neitz, and J. Neitz, 1997, Molecular detection of female carriers of protan color vision defects: Investigative Ophthalmology & Visual Science (Supplement), v. 38, p. S1015.

Katz, B., 1995, The dyschromatopsia of optic neuritis: a descriptive analysis of data from the optic neuritis treatment trial: Trans Am Ophthalmol Soc, v. 93, p. 685-708.

Koliopoulos, J., P. Iordanides, G. Palimeris, and E. Chimonidou, 1976, Data concerning colour vision deficiencies amongst 29,985 young Greeks: Modern Problems in Ophthalmology, v. 17, p. 161-164.

Koliopoulos, J., and G. Palimeris, 1972, On acquired colour vision disturbances during treatment with ethambutol and indomethacin: Mod Probl Ophthalmol, v. 11, p. 178-84.

Komáromy, A. M., J. J. Alexander, A. E. Cooper, V. A. Chiodo, L. G. Glushakova, G. M. Acland, W. W. Hauswirth, and G. D. Aguirre, 2008, Targeting gene expression to cones with human cone opsin promoters in recombinant AAV: Gene Ther, v. 15, p. 1049-55.

Mancuso, K., W. W. Hauswirth, Q. Li, T. B. Connor, J. A. Kuchenbecker, M. C. Mauck, J. Neitz, and M. Neitz, 2009, Gene therapy for red-green colour blindness in adult primates: Nature, v. 461, p. 784-787.

McClements, M., W. I. Davies, M. Michaelides, T. Young, M. Neitz, R. E. Maclaren, A. T. Moore, and D. M. Hunt, 2013, Variations in opsin coding sequences cause X-linked cone dysfunction syndrome with myopia and dichromacy: Invest Ophthalmol Vis Sci.

McCulley, T. J., K. C. Golnik, B. L. Lam, and W. J. Feuer, 2006, The effect of decreased visual acuity on clinical color vision testing: Am J Ophthalmol, v. 141, p. 194-6.

Merbs, S. L., and J. Nathans, 1992a, Absorption spectra of human cone pigments: Nature, v. 356, p. 433-435.

Merbs, S. L., and J. Nathans, 1992b, Absorption spectra of the hybrid pigments responsible for anomalous color vision: Science, v. 258, p. 464-466.

Miyahara, E., 2008, Errors reading the Ishihara pseudoisochromatic plates made by observers with normal colour vision: Clin Exp Optom, v. 91, p. 161-5.

Mizrahi-Meissonnier, L., S. Merin, E. Banin, and D. Sharon, 2010, Variable retinal phenotypes caused by mutations in the X-linked photopigment gene array: Investigative Ophthalmology and Visual Science, v. 51, p. 3884-3892.

Nathans, J., T. P. Piantanida, R. L. Eddy, T. B. Shows, and D. S. Hogness, 1986a, Molecular genetics of inherited variation in human color vision: Science, v. 232, p. 203-210.

Nathans, J., D. Thomas, and D. S. Hogness, 1986b, Molecular genetics of human color vision: the genes encoding blue, green, and red pigments: Science, v. 232, p. 193-202.

Neitz, J., and M. Neitz, 2011, The Genetics of Normal and Defective Color Vision: Vision Research, v. 51, p. 633-651.

Neitz, J., M. Neitz, J. C. He, and S. K. Shevell, 1999, Trichromatic color vision with only two spectrally distinct photopigments: Nature Neuroscience, v. 2, p. 884-888.

Neitz, J., M. Neitz, and G. H. Jacobs, 1993, More than three different cone pigments among people with normal color vision: Vision Research, v. 33, p. 117-122.

Neitz, J., M. Neitz, and P. M. Kainz, 1996, Visual pigment gene structure and the severity of human color vision defects: Science, v. 274, p. 801-804.

Neitz, M., J. Carroll, A. Renner, H. Knau, J. S. Werner, and J. Neitz, 2004, Variety of genotypes in males diagnosed as dichromatic on a conventional clinical anomaloscope: Visual Neuroscience, v. 21, p. 205-216.

Neitz, M., and J. Neitz, 1995, Numbers and ratios of visual pigment genes for normal red-green color vision: Science, v. 267, p. 1013-1016.

Neitz, M., J. Neitz, and A. Grishok, 1995, Polymorphism in the number of genes encoding long-wavelength sensitive cone pigments among males with normal color vision: Vision Research, v. 35, p. 2395-2407.

Neitz, M., J. Neitz, and G. H. Jacobs, 1991, Spectral tuning of pigments underlying red-green color vision: Science, v. 252, p. 971-974.

O'Neill-Biba, M., S. Sivaprasad, M. Rodriguez-Carmona, J. E. Wolf, and J. L. Barbur, 2010, Loss of chromatic sensitivity in AMD and diabetes: a comparative study: Ophthalmic Physiol Opt, v. 30, p. 705-16.

Oda, S., H. Ueyama, S. Tanabe, Y. Tanaka, S. Yamade, and K. Kani, 2000, Detection of female carriers of congenital color-vision deficiencies by visual pigment gene analysis., Current Eye Research, p. 767-773.

Pearlman, A. L., J. Birch, and J. C. Meadows, 1979, Cerebral color blindness: an acquired defect in hue discrimination: Ann Neurol, v. 5, p. 253-61.

Perdriel, G., and P. J. Manent, 1982, [Drug-induced dyschromatopsias]: Annee Ther Clin Ophtalmol, v. 33, p. 289-98.

Rodriguez-Carmona, M., M. O'Neill-Biba, and J. L. Barbur, 2012, Assessing the severity of color vision loss with implications for aviation and other occupational environments: Aviat Space Environ Med, v. 83, p. 19-29.

Schwarz, J. M., D. N. Cooper, M. Schuelke, and D. Seelow, 2014, MutationTaster2: mutation prediction for the deep-sequencing age: Nat Methods, v. 11, p. 361-2.

Shaygannejad, V., K. Golabchi, A. Dehghani, F. Ashtari, S. Haghighi, M. Mirzendehdel, and M. Ghasemi, 2012, Color blindness among multiple sclerosis patients in Isfahan: J Res Med Sci, v. 17, p. 254-7.

Shoji, T., Y. Sakurai, H. Sato, E. Chihara, and M. Takeuchi, 2011, Do type 2 diabetes patients without diabetic retinopathy or subjects with impaired fasting glucose have impaired colour vision? The Okubo Color Study Report: Diabet Med, v. 28, p. 865-71.

Smith, J. W., and T. A. Layden, 1971, Color vision defects in alcoholism. II: Br J Addict Alcohol Other Drugs, v. 66, p. 31-7.

Ueyama, H., S. Hayashi, S. Tanabe, Y. Tanaka, T. Hayashi, S. S. Deeb, S. Yamade, and I. Ohkubo, 2001, Number and arrangement of the red and green visual pigment genes in color-normal Japanese males, Color Research & Application, p. S84-S88.

Ueyama, H., S. Muraki, S. Tanabe, S. Yamade, and H. Ogita, 2015a, A new subset of deutan color-vision defect associated with an L/M visual pigment gene array of normal order and −71C substitution in the Japanese population: Journal of Biochemistry.

Ueyama, H., S. Muraki, S. Tanabe, S. Yamade, and H. Ogita, 2015b, A new subset of deutan color-vision defect associated with an L/M visual pigment gene array of normal order and −71C substitution in the Japanese population, The Journal of Biochemistry.

Ueyama, H., S. Muraki-Oda, S. Yamade, S. Tanabe, T. Yamashita, Y. Shichida, and H. Ogita, 2012, Unique haplotype in exon 3 of cone opsin mRNA affects splicing of its precursor, leading to congenital color vision defect: Biochem Biophys Res Commun, v. 424, p. 152-7.

Verrelli, B. C., and S. A. Tishkoff, 2004, Signatures of selection and gene conversion associated with human color vision variation: American Journal of Human Genetics, v. 75, p. 363-375.

Waaler, G. H. M., 1927, Über die ErblichkeitsverhaItnisse der verschiedenen Arten von angeborener Rotgrünblindheit, Zeitschrift für induktive Abstammungs und Vererbungslehre, p. 279-233.

Wachtler, T., E. Doi, T. Lee, and T. J. Sejnowski, 2007, Cone selectivity derived from the responses of the retinal cone mosaic to natural scenes: J Vis, v. 7, p. 6.

Wald, G., 1967, The molecular basis of visual excitation: Nature, v. 219, p. 800-807.

Wald, G., 1968, The molecular basis of visual excitation: Science, v. 162, p. 230-239.

Weitz, C. J., Y. Miyake, K. Shinzato, E. Montag, E. Zrenner, L. N. Went, and J. Nathans, 1992a, Human tritanopia associated with two amino acid substitutions in the blue sensitive opsin: American Journal of Human Genetics, v. 50, p. 498-507.

Weitz, C. J., L. N. Went, and J. Nathans, 1992b, Human tritanopia associated with a third amino acid substitution in the blue sensitive visual pigment: American Journal of Human Genetics, v. 51, p. 444-446.

Winderickx, J., L. Battisti, A. G. Motulsky, and S. S. Deeb, 1992, Selective expression of human X chromosome-linked green opsin genes: Proceedings of the National Academy of Sciences of the United States of America, v. 89, p. 9710-9714.