

Acknowledgments

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[42] Spectral Sensitivities of Human Cone Visual Pigments Determined *in Vivo* and *in Vitro*

By ANDREW STOCKMAN, LINDSAY T. SHARPE, SHANNATH MERBS,
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Introduction

Human color vision is trichromatic. It depends on three cone photoreceptors, each of which responds univariantly to absorbed quanta with different spectral sensitivity. The three types are now conventionally referred to as S (short-wavelength sensitive), M (middle-wavelength sensitive), and L (long-wavelength sensitive), according to the relative spectral positions of their peak sensitivities (see Fig. 1A). In the older literature and in some genetics literature, they are more often referred to as blue, green, and red. The overlapping spectral sensitivities of the three cone types (see Fig. 1A) are determined by the molecular properties of the photopigment that each contains. By comparing their different quantal catches, the brain obtains information about the spectral composition of the light arriving at the photoreceptors. Each human cone pigment is encoded by a separate gene; those encoding the M and L cone pigments are arranged in a head-to-tail tandem array on the X chromosome.¹

That trichromacy is a property of the eye rather than of the physics of light was first formally postulated in 1802 by Thomas Young.² In 1860, James Clerk Maxwell described an instrument for producing and mixing monochromatic lights in defined proportions, and with this instrument Maxwell made the first careful, quantitative measurements of color matching and trichromacy.³ However, the color-matching data of normal trichromats, obtained under standard viewing conditions, cannot uniquely define the

¹ J. Nathans, D. Thomas, and D. S. Hogness, *Science* **232**, 193 (1986).

² T. Young, *Phil. Trans. R. Soc.* **92**, 20 (1802).

³ J. C. Maxwell, *Phil. Trans. R. Soc.* **150**, 57 (1860).

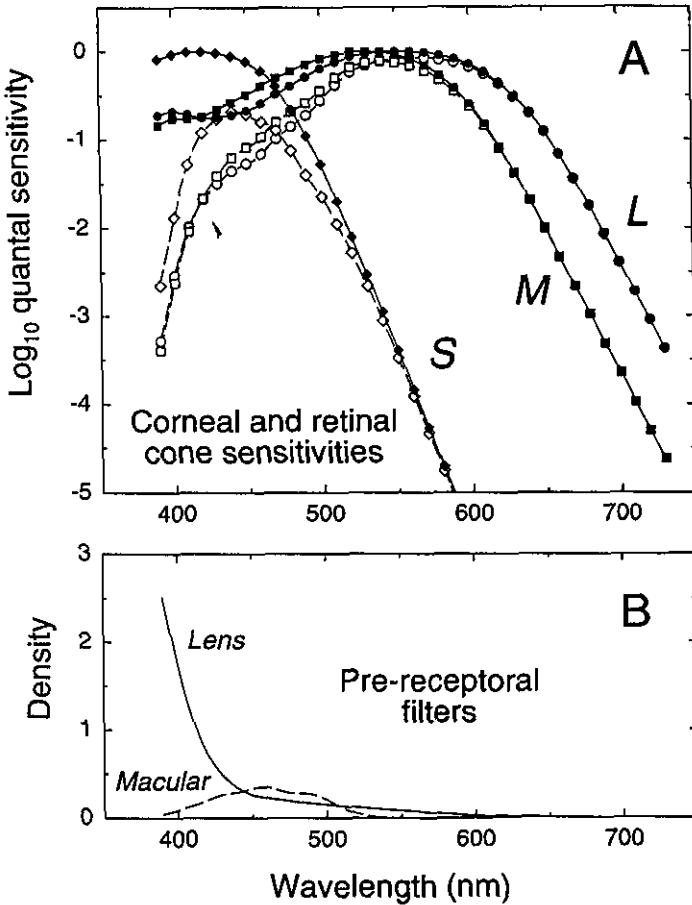


FIG. 1. Cone spectral sensitivities. (A) The spectral sensitivities of the L (circles) M (squares), and S (diamonds) cones measured at the cornea (open symbols, dashed lines) adjusted to the retinal level (filled symbols, solid lines) by removing the filtering effects of the macular and lens pigments. The sensitivities are linear transformations of color-matching functions guided by the spectral sensitivities of dichromats and S cone monochromats. The L cone spectral sensitivity takes into account diversity in the normal population. It is a weighted mixture of the two major polymorphic pigment variants L(S180) and L(A180) (see text) according to the ratio 63 to 37%. (B) Estimates of the optical density spectra of the macular (dashed line) and lens (solid line) pigments. [Adapted from Ref. 7.]

three fundamental sensitivity curves (with the exception of parts of the S curve⁴⁻⁶).

In the twentieth century, a number of strategies have been applied to determine the three human cone spectral sensitivities (for a review, see Ref. 7). One approach uses psychophysical techniques that isolate single cone sensitivities *in vivo* by exploiting the selective desensitization caused by either steady⁸⁻¹¹ or transient^{12,13} chromatic adaptation. With these techniques it is possible to isolate psychophysically the L and M cones of normal subjects throughout the spectrum^{5,13} (see dotted triangles, Fig. 7), and the S cones from short wavelengths to about 540 nm^{6,14} (see diamonds, Fig. 7). The cone spectral sensitivities can also be defined by using the constraints imposed by color matching or spectral sensitivity data obtained from three types of congenital, partially colorblind individuals, called dichromats, who lack one of the three cone types. Of these, those lacking L cones (protanopes) and M cones (deuteranopes) are common but those lacking S cones (tritanopes) are rare.¹⁵ König and Dieterici used this approach in 1893 to derive a set of cone sensitivity curves that are substantially correct in their shapes and locations along the wavelength axis,¹⁶ and this work has since been refined by many researchers.^{7,17-20} Relevant data may also be obtained from the much rarer congenital monochromats (e.g., S or blue cone monochromats) who lack two of the three cone types.⁶ Over the past several decades, the techniques of fundus reflectometry,²¹ microspectrophotome-

⁴ M. M. Bongard and M. S. Smirnov, *Doklady Akad. S.S.S.R.* **102**, 111 (1954).

⁵ A. Stockman, D. I. A. MacLeod, and N. E. Johnson, *J. Opt. Soc. Am.* **10**, 2491 (1993).

⁶ A. Stockman, L. T. Sharpe, and C. C. Fach, *Vision Res.* **39**, 2901 (1999).

⁷ A. Stockman and L. T. Sharpe, in "Color Vision: From Genes to Perception" (K. Gegenfurtner and L. T. Sharpe, eds.). Cambridge University Press, Cambridge, 1999.

⁸ W. S. Stiles, *Proc. R. Soc. London B* **127**, 64 (1939).

⁹ H. I. De Vries, *Physica* **14**, 367 (1948).

¹⁰ W. S. Stiles, *Science* **145**, 1016 (1964).

¹¹ G. Wald, *Science* **145**, 1007 (1964).

¹² P. E. King-Smith and J. R. Webb, *Vision Res.* **14**, 421 (1974).

¹³ A. Stockman, D. I. A. MacLeod, and J. A. Vivien, *J. Opt. Soc. Am.* **10**, 2471 (1993).

¹⁴ W. S. Stiles, *Coloq. Probl. Opt. Vis. (UIPAP, Madrid)* **1**, 65 (1953).

¹⁵ L. T. Sharpe, A. Stockman, H. Jagle, and J. Nathans, in "Color Vision: From Genes to Perception" (K. Gegenfurtner and L. T. Sharpe, eds.). Cambridge University Press, Cambridge, 1999.

¹⁶ A. König and C. Dieterici, *Z. Psychol. Physiol. Sinnesorg* **4**, 241 (1893).

¹⁷ F. H. G. Pitt, "Medical Research Council Special Report Series No. 200." His Majesty's Stationery Office, London, 1935.

¹⁸ S. Hecht, *Doc. Ophthal.* **3**, 289 (1949).

¹⁹ W. A. H. Rushton, D. S. Powell, and K. D. White, *Vision Res.* **13**, 1993 (1973).

²⁰ V. C. Smith and J. Pokorny, *Vision Res.* **15**, 161 (1975).

²¹ W. A. H. Rushton, *J. Physiol.* **176**, 24 (1965).

try,²² single-cell electrophysiology,²³ and electroretinography²⁴ have also been applied to the study of human cone pigment spectral sensitivities. The most recently developed approach to this problem is the *in vitro* study of recombinant cone pigments produced in tissue culture cells.^{25–28}

Each of these techniques has strengths and weaknesses. Work based on the perceptions of dichromats and monochromats assumes that their color vision is a “reduced” form of normal color vision^{3,29}; that is, that their surviving cones have the same spectral sensitivities as their counterparts in color-normal trichromats. However, it is now known that not all dichromats with alterations in the M or L cones conform to the reduction hypothesis, either because they have hybrid visual pigments or because they have multiple photopigment genes (Fig. 2^{30,31}). Only M/L (i.e., X chromosome-linked or red–green) dichromats with a single, normal visual pigment gene or with multiple genes that produce identical visual pigments conform completely to the reduction hypothesis. This genetic complexity calls into question the conclusions of previous studies in which the genotypes of the M/L dichromats were unknown.

Desensitization techniques used to separate cone responses psychophysically or in the electroretinogram are limited by the requirement for a minimum separation between the relevant spectral sensitivity curves. As a result, the isolation of cones containing L–M or M–L hybrid pigments—present in the approximately 6% of Caucasian males with X-linked anomalous trichromacy (a phenotype in which trichromatic color vision is present but reduced in discriminatory power¹⁵)—from the accompanying normal M or L cones has been difficult or impossible to achieve psychophysically owing to the similarities between the spectral sensitivities of these pigments.³² Microspectrophotometry and especially fundus reflectometry have been limited by a low signal-to-noise (*S/N*) ratio, and microspectrophotometry and single-cell electrophysiology are limited by the requirement for fresh

²² H. J. A. Dartnall, J. K. Bowmaker, and J. D. Mollon, *Proc. R. Soc. (London) Ser. B* **220**, 115 (1983).

²³ J. L. Schnapf, T. W. Kraft, and D. A. Baylor, *Nature (London)* **325**, 439 (1987).

²⁴ M. Neitz, J. Neitz, and G. H. Jacobs, *Vision Res.* **35**, 2095 (1995).

²⁵ D. D. Oprian, A. B. Asenjo, N. Lee, and S. L. Pelletier, *Biochemistry* **30**, 11367 (1991).

²⁶ S. L. Merbs and J. Nathans, *Nature (London)* **356**, 431 (1992).

²⁷ S. L. Merbs and J. Nathans, *Science* **258**, 464 (1992).

²⁸ A. B. Asenjo, J. Rim, and D. D. Oprian, *Neuron* **12**, 1131 (1994).

²⁹ A. König and C. Dieterici, *Sitz. Akad. Wiss. (Berlin)* 805 (1886).

³⁰ J. Nathans, T. P. Piantanida, R. L. Eddy, T. B. Shows, and D. S. Hogness, *Science* **232**, 302 (1986).

³¹ S. S. Deeb, D. T. Lindsey, Y. Hbiya, E. Sanocki, J. Winderickx, D. Y. Teller, and A. G. Motulsky, *Am. J. Hum. Genet.* **51**, 687 (1992).

³² T. P. Piantanida and H. G. Sperling, *Vision Res.* **13**, 2033 (1973).

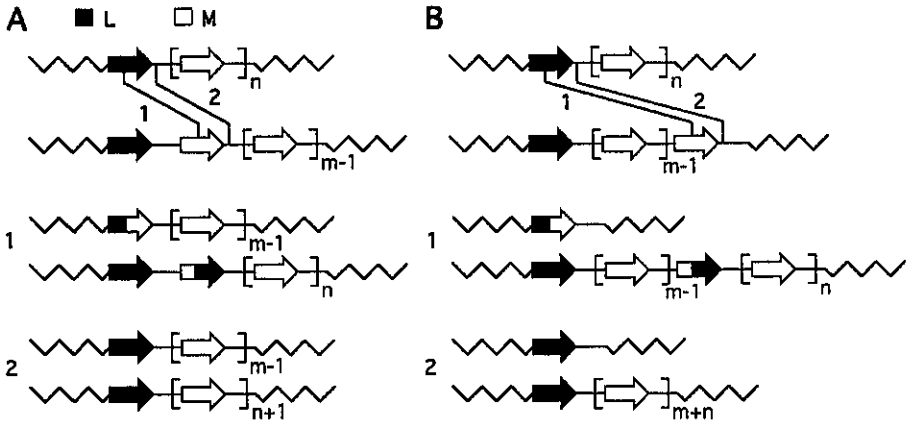


FIG. 2. Unequal recombination within the tandem array of L and M pigment genes responsible for the common anomalies of color vision. Each gene is represented by an arrow: the base corresponds to the 5' end and the tip to the 3' end. Filled arrows, L pigment genes; open arrows, M pigment genes. Unique flanking DNA is represented by zig-zag lines, and homologous intergenic DNA by straight lines. The total number of M pigment genes per array is indicated by m and n . For each recombination event, the reciprocal products are shown. (A) Unequal homologous recombination between two wild-type gene arrays, each containing one L pigment gene and a variable number of M pigment genes. In this example, recombination occurs within the first M pigment gene repeat unit. Intragenic and intergenic recombination events are indicated by 1 and 2, respectively. (B) The special case in which unequal homologous recombination occurs between the most 5' gene in one visual pigment gene array (an L pigment gene) and the most 3' gene in a second visual pigment gene array (an M pigment gene) thereby producing a single gene dichromat genotype. These single-gene recombination products would arise in (A) when $m = 1$. An intragenic recombination event (crossover 1) produces an array with a single 5' L-3' M hybrid gene resulting in a dichromatic (reduced) phenotype: either a classic protanopia (i.e., missing the L pigment, but retaining the normal M pigment) or an anomalous protanopia (i.e., missing the L pigment, but possessing a shifted M pigment). An intergenic recombination event (crossover 2) produces an array with a single L pigment gene also resulting in a dichromatic phenotype: a classic deuteranopia (i.e., missing the M pigment, but retaining the normal L pigment). When a 5' L-3' M hybrid gene is paired with a normal M pigment gene, an anomalous trichromatic phenotype, protanomalous trichromacy or protanomaly, results (i.e., a shifted L pigment is paired with a normal M pigment). In contrast, when a downstream 5' M-3' L hybrid gene is paired with a normal L pigment gene (see crossover 1 in panel (A), lower recombination product), deuteranomalous trichromacy or deuteranomaly results (i.e., a normal L pigment is paired with a shifted M pigment).

human retinal tissue. A strength of psychophysical, electrophysiological, and electroretinographic methods is that the signal amplification produced by the photoreceptor permits accurate measurements over a range of at least 4-5 log units in visual pigment sensitivity. Electroretinography appears to hold promise for future investigations as it is noninvasive and relatively

rapid, and unlike classic psychophysical testing it does not require a high level of cooperation and sustained attention from the subject.

Sensitivity measurements made by microspectrophotometry and single-photoreceptor electrophysiology are made transversely through the photoreceptor outer segment, and measurements of recombinant visual pigment absorbance in solution are made with the pigment molecules oriented randomly. In the living human eye, absorbance occurs axially along the outer segment, so that sensitivity is affected by waveguiding³³ and self-screening.³⁴ Measurements of recombinant pigment absorbance are further handicapped by being accurate to only within approximately 1 log unit of the peak sensitivity, thus encompassing only a limited range of wavelengths near the peak sensitivity (λ_{\max}). Recombinant pigments also differ from their *in situ* counterparts with respect to posttranslational modifications, local lipid environment, and the effects of detergent solubilization, which is known to produce blue shifts of several nanometers in some pigments.³⁵ However, this approach has the virtue that any visual pigment sequence can be created by site-directed mutagenesis, the recombinant pigment is studied free of other visual pigments, and the experiments do not require recruiting and screening of human subjects.

Some forms of sensitivity measurement, such as psychophysics and electroretinography, are made relative to light entering the eye at the cornea, whereas other forms of measurement, such as microspectrophotometry, electrophysiology, and visual pigment absorbance, are made relative to light at the isolated photoreceptor or photopigment. Consequently, before they can be compared, sensitivity curves must be adjusted to account for prereceptor absorption. Figure 1B shows the changes in cone spectral sensitivity caused by the lens and macular pigment, two pigments that lie between the cornea and the photoreceptors, and that absorb mainly short-wavelength light. Notice that, owing to these prereceptor filters the λ_{\max} values measured at the cornea are substantially longer in wavelength than those measured at the photoreceptor, particularly in the case of the S cones, the λ_{\max} of which shifts by more than 20 nm. Another consideration is that the effective photopigment optical density is higher in *in vivo* measurements, because light travels axially along the outer segment, than in *in vitro* measurement, in which light is passed transversely through the outer segment (such as in microspectrophotometry). For comparisons to be made, adjustments in photopigment optical density must be applied; but unlike the adjustments for the lens and macular pigment, such adjustments do not

³³ J. M. Enoch, *J. Opt. Soc. Am.* **51**, 1122 (1961).

³⁴ G. S. Brindley, *J. Physiol.* **122**, 332 (1953).

³⁵ G. Wald and P. K. Brown, *Science* **127**, 222 (1958).

TABLE I

ABSORBANCE SPECTRUM PEAKS (λ_{\max} + SD) OF HUMAN NORMAL AND HYBRID CONE PIGMENTS

| Genotype | <i>In vitro</i> | | | <i>In vivo</i> | | |
|--------------------|--|--------------------------------|-------------------------------------|-----------------------------------|----------------------------|--------------------------------|
| | Recombinant pigments (Refs. 26 and 27) | Recombinant pigments (Ref. 28) | Suction electrode (Refs. 62 and 63) | Microspectro-photometry (Ref. 22) | Psycho-physics (Ref. 43) | Electro-retinography (Ref. 24) |
| S | 426.3 ± 1.0 | 424.0 ^a | — | 419.0 ± 3.6 ^b | 418.9 ± 1.5 ^{b,c} | — |
| M(A180)/L1M2(A180) | 529.7 ± 2.0 | 532 ± 1.0 | 531 ^d | 530.8 ± 3.5 ^b | 527.8 ± 1.1 | 530 |
| L2M3(A180) | 529.5 ± 2.6 | 532 ± 1.0 | — | — | 528.5 ± 0.7 | 530 |
| L3M4(S180) | 533.3 ± 1.0 | 534 ± 1.0 | — | — | 531.5 ± 0.8 | — |
| L4M5(A180) | 531.6 ± 1.8 | — | — | — | 535.4 | — |
| L4M5(S180) | 536.0 ± 1.4 | 538 ± 1.0 | — | — | 534.2 | 537 |
| M2L3(A180) | 549.6 ± 0.9 | — | — | — | — | — |
| M2L3(S180) | 553.0 ± 1.4 | 559 ± 1.0 | — | — | — | — |
| M3L4 | 548.8 ± 1.3 | 555 ± 1.0 | — | — | — | — |
| M4L5 | 544.8 ± 1.8 | 551 ± 1.0 | — | — | — | — |
| L(A180) | 552.4 ± 1.1 | 556 ± 1.0 | 559 | — | 557.9 ± 0.4 | 558 |
| L(M2, A180) | — | — | — | — | 556.9 | — |
| L(M2, S180) | — | — | — | — | 558.5 | — |
| L(S180) | 556.7 ± 2.1 | 563 ± 1.0 | 564 | — | 560.3 ± 0.3 | 563 |

^a Value from Ref. 25.^b Gene sequence not determined.^c Value from Ref. 6.^d Value from Ref. 63.

affect the λ_{\max} value. When adjusted to the same level, the sensitivity curves obtained by the various methods are in relatively good agreement, especially near λ_{\max} , but some differences remain (Table I).⁷

One potential source of variability associated with measurements in the living eye is uncertainty regarding the lens and macular density corrections that should be made, because both vary considerably between individuals. Another source of variability within and between studies derives from person-to-person differences in cone pigment spectral sensitivities. First inferred,³⁶ and then later fully established³⁷ psychophysically, the most prominent of these differences derives from single-nucleotide polymorphisms that create variant M or L pigments in which spectral sensitivity may be shifted by several nanometers. The most common polymorphic

³⁶ J. Neitz and G. H. Jacobs, *Nature (London)* **323**, 623 (1986).³⁷ J. Winderickx, D. T. Lindsey, E. Sanocki, D. Y. Teller, A. R. Motulsky, and S. S. Deeb, *Nature (London)* **356**, 431 (1992).

variation occurs at codon 180 in the L pigment gene where site-directed mutagenesis experiments suggest that the presence of an alanine or a serine results in a shift to shorter or longer wavelengths, respectively, of approximately 4 nm²⁶ or 2–7 nm.²⁸ A second complication arises from variation in the number of M and L pigment genes between X chromosomes. In general, each X chromosome array has only a single L pigment gene, whereas the number of M pigment genes varies from one to at least five (Fig. 2).^{1,38–41} The presence of more than one M pigment gene, or in the case of deuteranomalous trichromats (subjects with an altered M cone sensitivity) more than one 5' M–3' L hybrid gene and/or M pigment gene, complicates the correlation of genotype and phenotype because evidence indicates that only a subset of the M pigment genes is expressed.^{40,42} There is currently no method for determining from the genotype which M or 5' M–3' L hybrid pigment genes are expressed in those individuals who carry multiple copies of these genes in their array. A partial solution to this genetic complexity can be achieved by studying male dichromats whose X chromosomes carry only a single visual pigment gene, an arrangement observed in approximately 1% of human X chromosomes (Fig. 2).⁴³ This simplified arrangement allows a straightforward correlation to be made between spectral sensitivity and visual pigment sequence, and it eliminates problems associated with dichromats who carry multiple genes that may differ subtly in spectral sensitivity.

In this chapter, we summarize the current status of the spectral sensitivity curves that underlie normal and anomalous human color vision, with an emphasis on *in vivo* psychophysical measurements in genetically well-characterized subjects and *in vitro* measurements with recombinant cone pigments.

Absorption Spectra of Recombinant Cone Pigments

The methods and results outlined in this section are from the work of Merbs and Nathans.^{26,27,44,45}

³⁸ M. Drummond-Borg, S. S. Deeb, and A. G. Motulsky, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 983 (1989).

³⁹ J. P. Macke and J. Nathans, *Invest. Ophthalmol. Vis. Sci.* **38**, 1040 (1997).

⁴⁰ T. Yamaguchi, A. G. Motulsky, and S. S. Deeb, *Hum. Mol. Genet.* **6**, 981 (1997).

⁴¹ S. Wolf, L. T. Sharpe, H. J. Schmidt, H. Knau, S. Weitz, P. Kioschis, A. Poustka, E. Zrenner, P. Lichter, and B. Wissinger, *Invest. Ophthalmol. Vis. Sci.* **40**, 1585 (1999).

⁴² J. Winderickx, L. Battisti, A. R. Motulsky, and S. S. Deeb, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 9710 (1992).

⁴³ L. T. Sharpe, A. Stockman, H. Jägle, H. Knau, G. Klausen, A. Reitner, and J. Nathans, *J. Neurosci.* **18**, 10053 (1998).

⁴⁴ S. L. Merbs and J. Nathans, *Photochem. Photobiol.* **56**, 869 (1992).

⁴⁵ S. L. Merbs and J. Nathans, *Photochem. Photobiol.* **58**, 706 (1993).

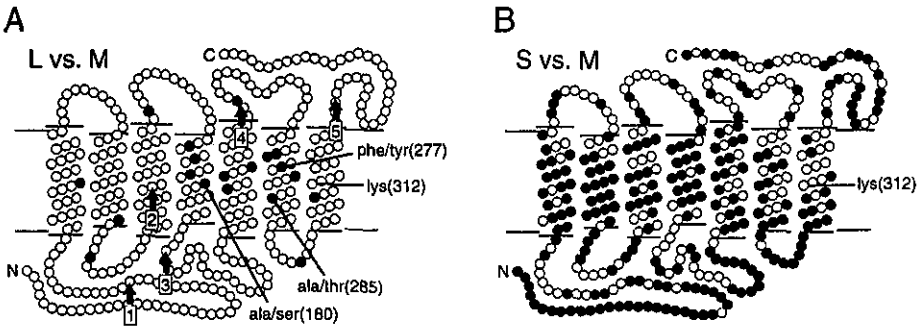


FIG. 3. Topographical model and pairwise comparison of human cone pigments showing amino acid identities (open circles) and differences (filled circles).¹ The seven α -helical segments are shown embedded within the membrane (horizontal lines). N and C denote the amino and carboxy termini, with the C terminus on the cytoplasmic side of the membrane. (A) L pigment versus M pigment. (B) S pigment versus M pigment. Ala/Ser(180) refers to the common L pigment polymorphism. Two other amino acid differences at codon positions 285 (Ala/Thr) and 309 (Phe/Tyr), which are relevant to the differential spectral tuning of the M and L pigments, are indicated, as well as the location of lysine at codon position 312, the site of covalent attachment of the 11-*cis*-retinal chromophore. The five intron positions in the L and M genes are indicated by numbered vertical arrows.

Cone Pigment Expression Constructs

Cone pigment expression vectors were constructed by inserting human cDNA clones hs37, hs2, and hs7, encoding, respectively, the S, M and L(A180) pigments (Fig. 3),¹ into the mammalian expression plasmid pCIS, which uses the cytomegalovirus (CMV) promoter and enhancer. Standard oligonucleotide-directed mutagenesis procedures were used to prepare single-amino acid substitutions. cDNAs encoding M-L hybrids were prepared by digesting either the M or L pigment cDNAs to varying extents with exonuclease III, followed by digestion with S1 nuclease. The resulting cDNA fragment was then used to prime synthesis on a single-stranded template containing L or M pigment cDNA, respectively. The partial heteroduplex products of this reaction were transformed into an *Escherichia coli* strain defective in mismatch repair and appropriate hybrids were identified by oligonucleotide hybridization and single-track sequencing. Prior to transfection, the entire insert was sequenced. To increase translation efficiency, the 5' untranslated region of the L and M pigment cDNAs was replaced with the last 10 base pairs (bp) of the bovine rhodopsin 5' untranslated region, a sequence known to give high levels of opsin expression with the pCIS vector.⁴⁶

⁴⁶ J. Nathans, C. J. Weitz, N. Agarwal, I. Nir, and D. S. Papermaster, *Vision Res.* 29, 907 (1989).

Production and Reconstitution of Recombinant Cone Pigments

Cone pigments were expressed in human embryonic kidney cell line 293S (ATCC CRL 1573) after transient transfection. In a typical transient transfection, twenty to forty 10-cm plates of 293S cells were transfected with 100 to 200 μg of the pCIS expression plasmid and 10 to 20 μg of pRSV-TAg [a simian virus 40 (SV40) T-antigen expression plasmid] by the calcium phosphate method. Sixty hours after transient transfection, the cells were collected by washing the plates with ice-cold phosphate-buffered saline (PBS) containing 5 mM ethylenediaminetetraacetic acid (EDTA). Cells were pelleted at 4° by centrifugation at 1000g for 10 min. Cell pellets were washed once with 25 ml of ice-cold PBS and then homogenized in 20 ml of ice-cold buffer A [50 mM *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid) (HEPES, pH 6.5), 140 mM NaCl, 3 mM MgCl₂, and 2 mM EDTA] containing 250 mM sucrose, aprotinin and leupeptin (10 $\mu\text{g}/\text{ml}$ each), 0.2 mM phenylmethylsulfonyl fluoride (PMSF), and 1 mM dithiothreitol (DTT) for 45 sec with a Polytron (Brinkmann, Westbury, NY) homogenizer at a setting of 5.5. The homogenate was layered onto 15 ml of 1.5 M sucrose in buffer A, and centrifuged at 4° in a swinging bucket rotor (SW28) at 105,000g for 30 min. Cell membranes were collected from the interface in a volume of 6 to 9 ml and additional DTT was added to increase the concentration by 1 mM. All further manipulations were performed at room temperature either under dim red light or in the dark. Cone pigment reconstitution was accomplished by incubation of the purified cell membranes for 30 min to 2 hr with a 20-fold molar excess of 11-*cis*-retinal added in 1–5 μl of ethanol. More than 95% of the free 11-*cis*-retinal was then removed by diluting the membranes in buffer A containing 4% (w/v) bovine serum albumin and pelleting the membranes in a swinging-bucket rotor (SW28) at 105,000g for 30 min at 4°. The membrane pellet was rinsed with buffer A and resuspended in buffer A containing 2% (w/v) 3-(3-cholamidopropyl)dimethylammonio-1-propane sulfonate (CHAPS). To remove insoluble material, the membrane-detergent mixture was centrifuged at either 10,000g for 5 min at 4° in a microcentrifuge, or at 86,000g for 10 min at 4° in a table-top ultracentrifuge.

Ultraviolet-Visible Absorption Spectroscopy

Absorption spectra were recorded using a Kontron Instruments (Milan, Italy) Uvikon 860 equipped with a water-jacketed cuvette holder. Before photobleaching, four absorption spectra were measured and averaged. The sample was photobleached with light from a 150-W fiber optic light that had been passed through an appropriate filter to maximize pigment bleaching and minimize isomerization of residual retinal (S pigment, 1 min through

a 420-nm short wavelength-cutoff filter; M pigment, 2 min through a 580-nm narrow-bandpass filter; and L pigment, 1 min through a 580-nm narrow-bandpass filter). After photobleaching, four absorption spectra were measured and averaged, and the difference absorption spectrum was calculated by subtracting the averaged postbleach curve from the averaged prebleach curve. For each S pigment difference spectrum, a 293S cell pellet was processed in parallel, and the resulting control membrane difference spectrum was subtracted from the S pigment difference spectrum to correct for the change in retinal absorbance that occurs when the >420-nm bleaching light is used. Some difference spectra, especially those of samples centrifuged at 10,000g in the last step, showed a downward sloping background at shorter wavelengths owing to an increase in light scattering as the spectra were collected. From those curves requiring background correction, a difference curve of 293S membranes (without added 11-*cis*-retinal), showing the absorbance change due to light scattering, was appropriately scaled and subtracted to equalize the absorbance values at 300 and 700 nm. An absorption spectrum of all-*trans*-retinal in 2% CHAPS, buffer A, was scaled and added to each S pigment difference curve to correct for the effect of the released all-*trans*-retinal (Fig. 4B).

The most significant sources of experimental variability in determining photobleaching difference spectra are baseline drift due to light scattering and distortions due to released all-*trans*-retinal or other retinal-based photoproducts. As seen in Fig. 4B, released all-*trans*-retinal significantly distorts the uncorrected S pigment photobleaching difference spectrum. The L, M, and L-M hybrid pigment spectra are not affected because retinal absorption is negligible at wavelengths above 500 nm. There is also some variability among experiments involving L, M, and L-M hybrid pigments in the 440-nm region of the photobleaching difference spectra, which most likely arises from photochemical events involving Schiff bases of 11-*cis*-retinal. Long-lived photoproducts appear not to accumulate to significant levels as determined by the minimal differences between spectra obtained during a 5-min period after photobleaching. Figure 4 shows the sum of multiple recombinant cone pigment spectra, giving a weighted-average curve for each pigment [six curves for the S pigment, two curves for the M pigment, seven curves for the L(A180) pigment, and seven curves for the L(S180) pigment].

Absorption Maxima Determined in Vitro

For each photobleaching difference absorption spectrum, the wavelength of maximal absorption (λ_{\max}) was determined by calculating the best fitting fifth-order polynomial to a 100-nm segment of the spectrum centered at the approximate peak sensitivity (Fig. 5). Table I lists the absorption

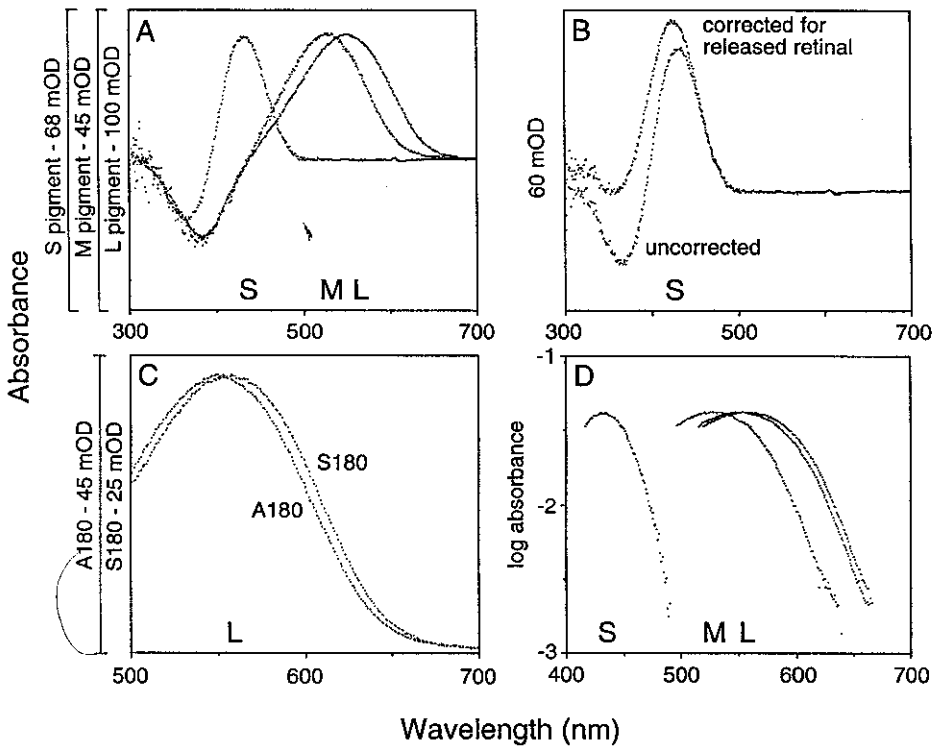


FIG. 4. Superimposed photobleaching difference absorption spectra of recombinant human cone pigments. (A) S, M, and L(A180) pigments. (B) S pigment, uncorrected and corrected for released all-*trans*-retinal. (C) L pigments, containing either alanine or serine at position 180. (D) S, M, L(A180), and L(S180) pigments (from left to right) plotted on a log scale. Only those regions of the spectra that are greater than 80% of the absorbance maximum on the short-wavelength side and greater than 5% of the absorbance maximum on the long-wavelength side are included. mOD, Optical density units $\times 10^{-3}$. [Adapted from Ref. 45.]

maxima of the normal human cone pigments and the 5' M-3' L and 5' L-3' M hybrid pigments that are commonly encountered in the human population. The hybrid pigments arise from recombination events within introns and therefore produce hybrids in which exons 1 to X ($X = 2, 3, 4$, or 5) derive from either an M or L pigment and exons $X + 1$ to 6 derive from either an L or M pigment, respectively (Fig. 2). As exons 1 and 6 are identical between M and L pigments, the crossover events that produce hybrid pigments are confined to introns 2, 3, and 4. Each hybrid pigment is referred to by an abbreviation that reflects the origin of its exons and, if exon 3 is derived from an L pigment, the identity of the polymorphic

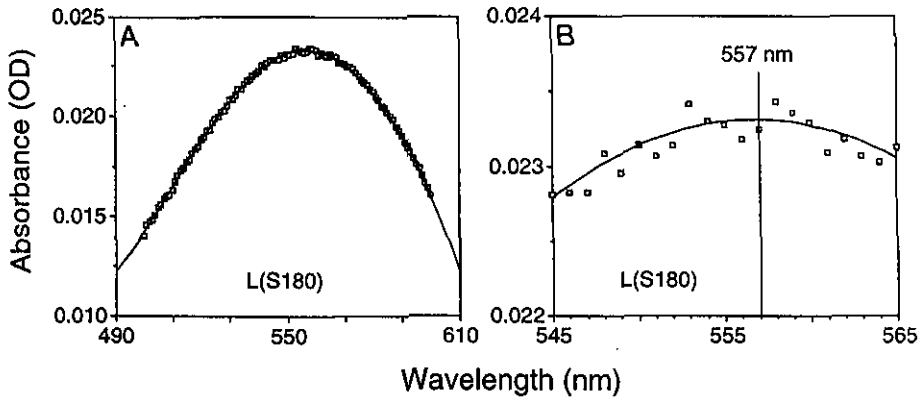


FIG. 5. The fifth-order polynomial calculated as the best fit to the 100-nm region of the spectrum centered about the approximate peak sensitivity of one absorption spectrum of the L(S180) cone pigment. (A) The best fitting polynomial superimposed on the raw data from 500 to 600 nm. (B) Expanded x axis and peak sensitivity determination (557 nm) from the local maximum of the fifth-order polynomial. OD, Optical density units. [Adapted from Ref. 45.]

residue (alanine or serine) at position 180 in the third exon. For example, L4M5(A180) is a hybrid pigment encoded by a gene in which exons 1–4 are derived from an L pigment gene, exons 5 and 6 are derived from an M pigment gene, and position 180 is occupied by alanine. L pigment genes are designated L(A180) or L(S180) to indicate the presence of alanine or serine, respectively, at position 180. Table I lists the absorption maxima determined by two research groups for recombinant pigments *in vitro*.^{26–28} Although the values reported by Asenjo *et al.*²⁸ are systematically 4 ± 2 nm greater than those reported by Merbs and Nathans,^{26,27} the two sets of data are in close agreement with respect to absorption differences between pigments. The systematic differences could arise from differences in the lipids, detergents, or buffers used by the different laboratories.

Psychophysical Determination of Cone Spectral Sensitivities

The methods and results outlined in this section are from the work of Sharpe and co-workers.^{6,43}

M, L, and 5' L–3' M Hybrid Cone Sensitivities: Ascertainment of Subjects

In vivo estimates of the M, L, and 5' L–3' M hybrid pigment sensitivities at the cornea can be obtained most simply by studying male dichromats

whose X chromosomes carry only a single visual pigment gene. Males with severe color vision deficiencies were recruited and screened by anomaloscopy, using the Rayleigh match.⁴⁷ Virtually all such subjects have defects in the M or L cones; S cone defects are far less common and are easily distinguished from M and L cone defects in preliminary screening tests. Prospective subjects had to behave as dichromats in the Rayleigh test; that is, they had to be able to match a spectral yellow light to a juxtaposed mixture of spectral red and green lights by adjusting the intensity of the yellow, regardless of the red-to-green ratio. This implies that quantal absorptions in a single photopigment are responsible for the matches. The choice of the wavelengths and intensities of the primary lights as well as the small field size (2–2.6° diameter) largely preclude absorptions in the S cones or rods from influencing the matches. Of 94 dichromat males identified by anomaloscope testing, 41 were found to carry a single L or L–M hybrid gene by whole genome Southern blot hybridization, and for these subjects the sequences of exons 2–5, which differ between L and M pigments, were determined by direct sequencing of polymerase chain reaction (PCR) products generated with flanking intron primers.⁴³

Each single-gene dichromat made repeated matches (3 to 5 times) in random order for 17 different red-to-green mixture ratios, by adjusting only the intensity of the yellow primary light. Their individual matching range slopes (i.e., the slopes of regression lines fitted to their yellow intensity settings for the 17 red–green mixtures) and intercepts (i.e., the yellow intensity required to match the red primary alone) were then determined by a least-squares criterion. From the slope of the regression line, the subjects were categorized as protanopes (missing the L pigment) or deuteranopes (missing the M pigment).^{15,48,49}

Flicker Photometry: Methodology

Foveal spectral sensitivities were determined in 37 single-gene dichromats by heterochromatic flicker photometry. A reference light of 560 nm was alternated at a rate of 16 or 25 Hz with a superimposed test light, the wavelength of which was varied in 5-nm steps from 400 to 700 nm. Subjects found the radiance of the test light that eliminated or “nulled” the perception of flicker produced by the alternation of the two lights. To saturate the rods and to desensitize the S cones, and thus prevent both from contributing to spectral sensitivity, the flickering stimuli were superimposed on a

⁴⁷ L. Rayleigh (J. W. Strutt), *Nature (London)* **25**, 64 (1881).

⁴⁸ J. Pokorny, V. C. Smith, G. Verriest, and A. J. L. G. Pinckers, “Congenital and Acquired Color Vision Defects.” Grune & Stratton, New York, 1979.

⁴⁹ G. Wyszecki and W. S. Stiles, “Color Science.” John Wiley & Sons, New York, 1982.

large, violet (430 nm) background with an intensity of $11.0 \log \text{ quanta sec}^{-1} \text{ deg}^{-2}$, which is a strong S cone stimulus and more than 1 log unit more radiant than the rod saturating level.

Because the S cones are desensitized by the background (and in any case make little or no contribution to flicker photometry^{50,51}) and the rods are saturated, the null should occur when the test and reference lights produce the same levels of activation in the remaining single class of L, de facto M, or L-M hybrid cone in each single-gene dichromat. The radiance of the test light required to null the reference light as a function of wavelength is therefore an estimate of the spectral sensitivity of the single longer wavelength cone type of each subject.

Flicker Photometry: Apparatus

A Maxwellian-view optical system produced the flickering test stimuli and the steady adapting field, all of which originated from a xenon arc lamp.⁴³ Two channels provide the 2° in visual diameter flickering test and reference lights. The wavelength of the reference light was always set to 560 nm, while that of the test light was varied from 400 to 700 nm in 5-nm steps. A third channel provided the 18° -diameter, 430-nm adapting fields. The images of the xenon arc were less than 1.5 mm in diameter at the plane of the observer's pupil (i.e., smaller than the smallest pupil diameter, so that changes in pupil size have no effect). Circular field stops placed in collimated portions of each beam defined the test and adapting fields as seen by the observer. Mechanical shutters driven by a computer-controlled square-wave generator were positioned in each channel near focal points of the xenon arc to produce the square-wave flicker seen by the subjects. Fine control over the luminance of the stimuli was achieved by variable, 2.0 log unit linear (Spindler & Hoyer, Göttingen, Germany) or 4.0 log unit circular (Rolyn Optics, Covina, CA) neutral density wedges positioned at image points of the xenon arc lamp, and by insertion of fixed neutral density filters in parallel portions of the beams. The position of the observer's head was maintained by a rigidly mounted dental wax impression.

The radiant fluxes of the test and adapting fields were measured at the plane of the observer's pupil with a calibrated radiometer (model 80X optometer; United Detector Technology, Baltimore, MD) or with a Pin-10 diode connected to a picoammeter (model 486; Keithley, Cleveland, OH). The fixed and variable neutral density filters were calibrated *in situ* for all test and field wavelengths.

⁵⁰ A. Eisner and D. I. A. MacLeod, *J. Opt. Soc. Am.* **70**, 121 (1980).

⁵¹ A. Stockman, D. I. A. MacLeod, and D. D. DePriest, *Vision Res.* **31**, 189 (1991).

L, M, and 5' L-3' M Hybrid Pigment Spectral Sensitivity Measurements

Corneal spectral sensitivity measurements were confined to the central 2° of the fovea. At the start of the spectral sensitivity experiment, the subject adjusted the intensity of the 560-nm reference flickering light until satisfied that the flicker was just at threshold. After five settings had been made, the mean threshold setting was calculated and the reference light was set 0.2 log unit above this value. The test light was then added to the reference light in counterphase. The subject adjusted the intensity of the flickering test light until the flicker perception disappeared or was minimized. This procedure was repeated five times at each wavelength. After each setting, the intensity of the flickering test light was randomly reset to a higher or lower intensity, so that the subject must readjust the intensity to find the best setting. The target wavelength was randomly varied in 5-nm steps from 400 to 700 nm. From two to six complete runs were carried out by each subject. Thus, each data point represents between 10 and 30 threshold settings.

Analysis of Flicker Photometry Data

Methods for elimination of clearly discrepant data are described in Sharpe *et al.*⁴³ In that study the cumulative rejection rate was about 6%. The λ_{\max} of the L, M, or L-M hybrid spectral sensitivity at the retina of each subject was estimated by fitting a photopigment template to their flicker photometry data corrected to the retinal level. The photopigment template was derived from the M and L cone spectral sensitivities of Stockman *et al.*,⁵ which are based on color-matching data and spectral sensitivity measurements made in dichromats and normal trichromats under conditions of selective desensitization (dotted triangles, Fig. 7). First, the M and L cone spectral sensitivities⁵ were individually corrected to the retinal level by removing the effects of macular and lens pigmentation [Eq. (1), below]. Next, they were corrected to photopigment optical density (or absorbance) spectra by adjusting them to infinitely dilute photopigment concentrations [Eq. (2), below].

Calculating Photopigment Spectra from Corneal Spectral Sensitivities and Vice Versa

The calculation of photopigment optical density spectra from corneal spectral sensitivities is, in principle, straightforward, provided that the appropriate values of (1) D_{peak} , the peak optical density of the photopigment, (2) k_{lens} , the scaling constant by which the lens density spectrum [$d_{\text{lens}}(\lambda)$, Fig. 1B, solid line] should be multiplied, and (3) k_{mac} , the scaling constant

by which the macular density spectrum [$d_{\text{mac}}(\lambda)$, Fig. 1B, dashed line] should be multiplied are known. Starting with the quantal cone spectral sensitivity [$S(\lambda)$], the effects of the lens pigment [$K_{\text{lens}}d_{\text{lens}}(\lambda)$] and the macular pigment [$k_{\text{mac}}d_{\text{mac}}(\lambda)$] are first removed, by restoring the sensitivity losses that they cause:

$$\log[S_r(\lambda)] = \log[S(\lambda)] + k_{\text{lens}}d_{\text{lens}}(\lambda) + k_{\text{mac}}d_{\text{mac}}(\lambda) \quad (1)$$

The functions $d_{\text{lens}}(\lambda)$ and $d_{\text{mac}}(\lambda)$ are the optical density spectra of the lens and macular pigment depicted in Fig. 1B.⁷ They are scaled to the densities that are appropriate for a 2° viewing field for an average observer (a peak macular density at 460 nm of 0.35, and a lens density at, e.g., 400 nm of 1.765; Stockman and Sharpe^{51a}). The values k_{mac} and k_{lens} are therefore 1 for the mean 2° spectral sensitivities, but should be adjusted for individual observers or small groups of observers, who are likely to have different lens and macular densities. Because macular pigment density decreases with retinal eccentricity, k_{mac} must also be adjusted for other viewing fields. $S_r(\lambda)$ is the cone spectral sensitivity at the retina, which by convention means in the absence of macular pigment absorption.

To calculate the photopigment optical density of the L cones scaled to unity peak [$S_{\text{OD}}(\lambda)$], from $S_r(\lambda)$:

$$S_{\text{OD}}(\lambda) = \frac{-\log_{10}[1 - S_r(\lambda)]}{D_{\text{peak}}} \quad (2)$$

D_{peak} , the peak optical density, was assumed to be 0.5, 0.5, and 0.4 for the L, M, and S cones, respectively. [$S_r(\lambda)$ should be scaled before applying Eq. (2), so that $S_{\text{OD}}(\lambda)$ peaks at one.] The optical densities of the cone photopigments are known to diminish with retinal eccentricity; so these values correspond only to the central 2° of the viewing field. Moreover, these calculations from corneal spectral sensitivities to retinal photopigment optical densities ignore changes in spectral sensitivity that may result from the structure of the photoreceptor or other ocular structures and pigments (unless they are incorporated in the lens or macular pigment density spectra).

The calculation of relative quantal corneal spectral sensitivities from photopigment or absorbance spectra is also straightforward, again if the appropriate values (D_{peak} , k_{lens} , and k_{mac}) are known. First, the spectral

^{51a} A. Stockman and L. T. Sharpe, *Vision Res.*, in press (1999).

sensitivity at the retina, $S_r(\lambda)$, is calculated from the normalized photopigment optical density spectrum, $S_{OD}(\lambda)$, by the inversion of Eq. (2)⁵²:

$$S_r(\lambda) = 1 - 10^{-D_{\text{peak}} S_{OD}(\lambda)} \quad (3)$$

Then, the filtering effects of the lens and macular pigments are added back:

$$\log[S(\lambda)] = \log[S_r(\lambda)] - k_{\text{lens}} d_{\text{lens}}(\lambda) - k_{\text{mac}} d_{\text{mac}}(\lambda) \quad (4)$$

Scales

A simple polynomial function was devised to describe the logarithm of the L, M, and 5' L-3' M hybrid photopigment spectra, after the L cone spectrum had been shifted horizontally along a $\log_{10}(\lambda)$ scale to align it with the M cone spectrum. In deriving this template, and analyzing the spectral sensitivity data, it was assumed that the family of L, M and 5' L-3' M hybrid photopigment spectra are invariant in shape when plotted as a function of $\log_{10}(\lambda)$.⁵³⁻⁵⁵ This simplification provides a straightforward means of analyzing the spectral sensitivity data, because the λ_{max} of each photopigment can then be estimated from a simple shift of the polynomial curve.⁵⁶

Attempts have been made previously to simplify cone photopigment spectra by finding an abscissa that produces spectra of a fixed spectral shape, whatever the photopigment λ_{max} . An early proposal was by Dartnall,⁵⁷ who described a "nomogram" or fixed template shape for photopigment spectra plotted as a function of wavenumber ($1/\lambda$, in units of cm^{-1}). Another proposal was that the spectra are shape invariant when plotted as a function of \log_{10} frequency or wavenumber [$\log_{10}(1/\lambda)$],^{53,54} which is equivalent to \log_{10} wavelength [$\log_{10}(\lambda)$] or normalized frequency ($\lambda_{\text{max}}/\lambda$). For this scale, Lamb has proposed a template.⁵⁵ Barlow has also proposed an abscissa of the fourth root of wavelength ($\lambda^{1/4}$).⁵⁸

Fitting of the retinal photopigment template to the corneal data was carried out by an iterative procedure that simultaneously (1) found the best-fitting shift of the template along the log wavelength scale, (2) adjusted

⁵² A Knowles and H. J. A. Dartnall, "The Eye," Vol. 2B: "The Photobiology of Vision." Academic Press, London, 1977.

⁵³ R. J. W. Mansfield, in "The Visual System" (A. Fein and J. S. Levine, eds.), p. 89. Alan R. Liss, New York, 1985.

⁵⁴ E. F. MacNichol, *Vision Res.* 26, 1543 (1986).

⁵⁵ T. D. Lamb, *Vision Res.* 35, 3083 (1995).

⁵⁶ D. A. Baylor, B. J. Nunn, and J. L. Schnapf, *J. Physiol.* 390, 145 (1987).

⁵⁷ H. J. A. Dartnall, *Br. Med. Bull.* 9, 24 (1953).

⁵⁸ H. B. Barlow, *Vision Res.* 22, 635 (1982).

the template to a peak photopigment optical density of 0.5, and (3) added back the effects of the best-fitting lens and macular pigment optical densities.⁴³ In one analysis the model was fitted at all measured wavelengths, and in a second analysis we carried out the fit only for measurements made at wavelengths ≥ 520 nm. Restricting the fit to ≥ 520 nm simplified the fitting procedure, because at those wavelengths macular pigment plays little role, and the lens is relatively transparent (having an average optical density of only 0.10 log unit at 520 nm that declines with wavelength). The ≥ 520 -nm fit served, in part, as a control for the full-spectrum fit, and in particular for the reliance on best-fitting macular and lens densities, which could, in principle, distort the λ_{\max} estimates. Given that small differences are expected between the two estimates, because the lens density assumed for the partial fit is the population mean density rather than the optimized individual density, the agreement between the two is extremely good.⁴³

Systematic errors in both fits would be expected if the various L, M, or L-M hybrid pigments are not shape invariant when plotted against $\log_{10}(\lambda)$ or if the peak photopigment optical density varies with genotype. Such errors would cause small shifts in the λ_{\max} estimates between genotypes, but would have little effect on estimates within genotypes. Individual differences in photopigment optical density within a genotype would increase the variability of the λ_{\max} estimates within that group.

Figure 6 shows representative examples of the heterochromatic flicker data (symbols) for nine different genotypes, five of which produce protanopia (Fig. 6A) and four of which produce deuteranopia (Fig. 6B). Cone photopigment λ_{\max} values obtained from subjects with identical visual pigment amino acid sequences show up to an ~ 3 -nm variation from subject to subject, presumably owing to a combination of inexact (or no) corrections for variation in preretinal absorption, variation in photopigment optical density, optical effects within the photoreceptor, and measurement error. This variation implies that spectral sensitivities must be averaged over multiple subjects with the same genotype to obtain accurate values for a given pigment.

Average values for each genotype, varying in the number of subjects from 1 to 19, are given in Table I (the complete data set for all single-gene dichromats can be seen in Sharpe *et al.*⁴³). Note that to allow comparisons with the *in vitro* estimates, the λ_{\max} values are for the psychophysical spectral sensitivities adjusted to the retinal level (see above). One limitation of single-gene dichromats in determining such estimations is that they do not carry 5' M-3' L hybrid genes, with the result that 5' M-3' L pigments can be studied psychophysically only in deuteranomalous trichromats.

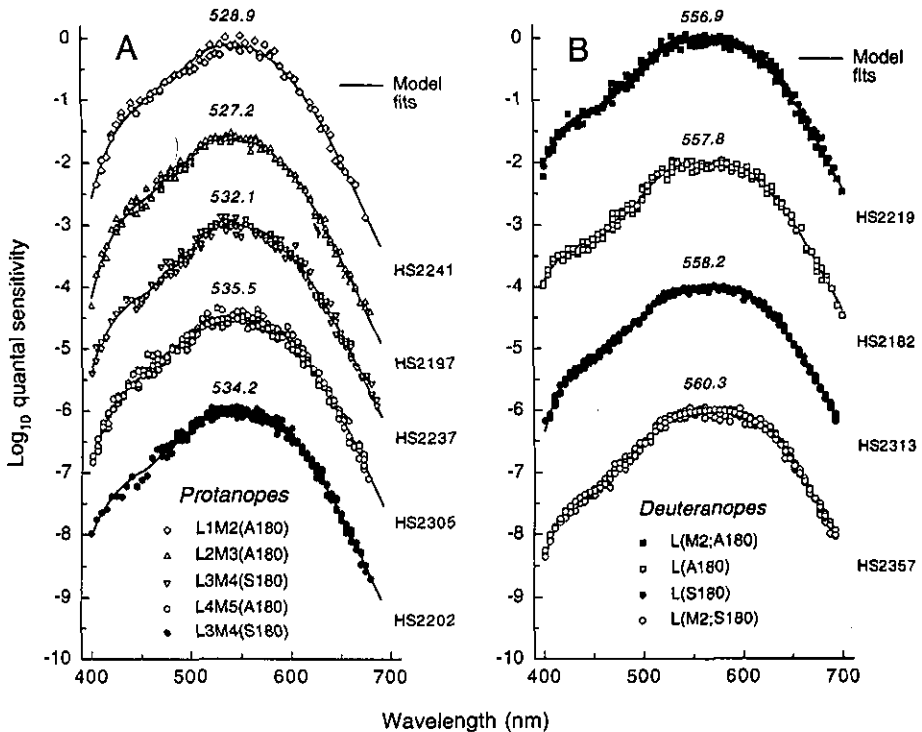


FIG. 6. Representative individual cone spectral sensitivity data (symbols) for nine genotypes found in single-gene dichromats; together with the fits (continuous lines) of the visual pigment template.⁴³ (A) Genotypes producing protanopia: L1M2(A180), L2M3(A180), L3M4(S180), L4M5(A180), and L4M5(S180). (B) Genotypes producing deuteranopia: L(M2; A180), L(A180), L(M2; S180), and L(S180). The λ_{max} of the fitted photopigment template is given above each curve. Data are from several single runs. (Note that the λ_{max} values are values for individual observers; therefore they do not necessarily correspond to the values averaged over different observers with the same genotype, as given in Table I.) Further details can be found in Ref. 43, in which the same subject code is used, including details of the gene sequences for each subject.

Psychophysical Determinations of S Cone Spectral Sensitivity

S cone spectral sensitivity was measured in five normal trichromats, using selective chromatic adaptation to suppress the M and L cones, and in three S (or blue) cone monochromats. The S cone monochromats are

known to lack M and L cone function on genetic grounds.⁵⁹ One of the three observers has two X chromosome photopigment genes but an upstream deletion in the region that controls their expression. The two other observers have a single X chromosome photopigment gene with a point mutation that results in a cysteine-to-arginine substitution at position 203 in the opsin.

The same experimental apparatus that was used for the M and L cone measurements (see above) was also used for the S cone measurements, with some important modifications. For measurements in normal subjects, a single 2°-diameter target field was presented in the center of an intense 16°-diameter, yellow (580 nm) background field of 12.10 log quanta sec⁻¹ deg⁻² (5.93 log photopic td or 5.47 log scotopic td), which was chosen to selectively adapt the M and L cones and saturate the rods, but have comparatively little direct effect on the S cones. The target was square-wave flickered at 1 Hz. A much lower temporal frequency was used for the S cone measurements, because S cone pathways are typically sluggish.^{22,60} For measurements in the S cone monochromats, the target was presented on an orange (620 nm) background of 11.24 log quanta sec⁻¹ deg⁻² (4.68 log photopic td or 3.36 log scotopic td), which was chosen to saturate the rods. The subject's task was to set the threshold for detecting the flicker as a function of target wavelength. Five settings at each target wavelength were made on each of four runs.

On long-wavelength backgrounds, S cone isolation could be achieved in normal subjects from short wavelengths (the lower limit of the measurements was 390 nm) to ~540 nm, and in the S cone monochromats throughout the spectrum. From 390 to 540 nm the data for the normal subjects and the S cone monochromats agree well, except that—consistent with their extrafoveal fixation—the monochromats have lower macular pigment and S cone photopigment optical densities. The mean centrally measured S cone thresholds, after individually adjusting the S cone monochromat data to normal density values, are shown in Fig. 7 (diamonds). The mean is based on normal and monochromat data up to 540 nm and on monochromat data alone at wavelengths longer than 540 nm.⁶

Absorption Maxima Determined Psychophysically

Figure 7 shows a compilation of the mean psychophysically determined corneal spectral sensitivities for S, M, L(A180), and L(S180) pigment-

⁵⁹ J. Nathans, I. H. Maumenee, E. Zrenner, B. Sadowski, L. T. Sharpe, R. A. Lewis, E. Hansen, T. Rosenberg, M. Schwartz, J. R. Heckenlively, E. Traboulsi, R. Klingaman, N. T. Bech-Hansen, G. R. LaRoche, R. A. Pagon, W. H. Murphey, and R. G. Weleber, *Am. J. Hum. Genet.* **53**, 987 (1993).

⁶⁰ G. S. Brindley, J. J. Du Croz, and W. A. H. Rushton, *J. Physiol.* **183**, 497 (1966).

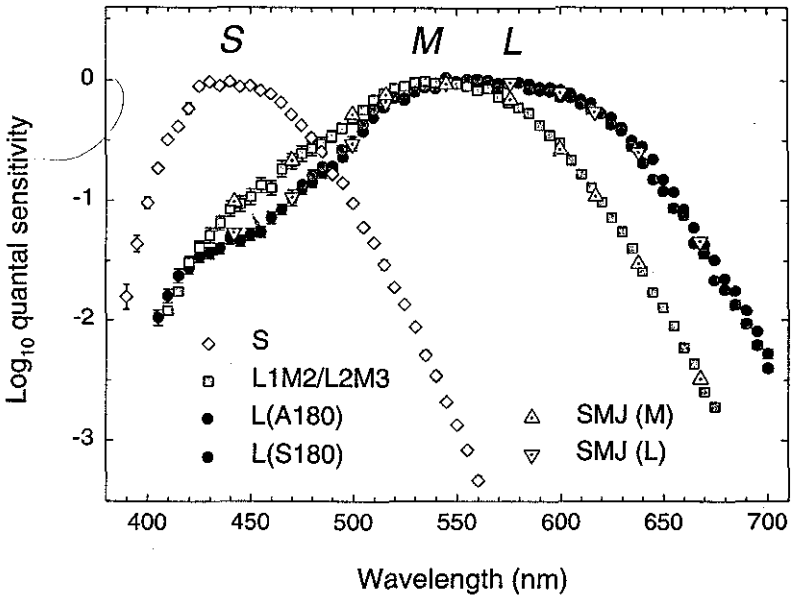


FIG. 7. Comparison of mean spectral sensitivity data: L cone data for 15 L(S180) subjects (black circles), 5 L(A180) subjects (gray circles), and M cone data for 9 protanopes (gray squares)⁴³; S cone data for five normal trichromats and three S (or blue) cone monochromats (white diamonds)⁶; L cone data (white dotted inverted triangles) for 12 normal subjects and 4 deuteranopes and M cone data (white dotted triangles) for 9 normal subjects and 2 protanopes measured using transient adaptation.⁵

containing cones.^{6,43} The M cone data were obtained from genotyped single- and multiple-gene dichromats carrying either an L1M2 hybrid gene, which encodes a de facto M pigment, an L2M3 hybrid gene, or either of these genes and one or more normal M pigment genes. The absorbance maximum of the L2M3 hybrid pigment does not differ significantly from that of the M pigment as determined psychophysically and by analysis of recombinant pigments (Table I). Therefore data from subjects carrying an L2M3 pigment can be combined with data from subjects carrying a L1M2 pigment, thereby improving the signal-to-noise ratio. Table I lists the λ_{\max} of the S pigment determined psychophysically from normal trichromats, using selective desensitization, and from S cone monochromats.

Comparison of Spectral Sensitivities Determined by Different Methods

The spectral sensitivities of the L, M, and some 5' L'-3' M hybrid pigments have also been studied in five dichromats, using electroretinogra-

phy²⁴ (Table I). The spectral sensitivity functions were measured twice for each subject and were corrected for preretinal absorption by the lens.⁴⁹ The λ_{\max} of each function was then determined by translation of a standard visual pigment absorption curve on a log wavenumber axis.⁶¹

Suction electrode (current) recordings from single human cones have also provided spectral sensitivities for the L(A180) and L(S180) pigments⁶² and for the M pigment.⁶³ In these measurements, spectral sensitivity was estimated by adjusting the intensity of light for as many as 20 wavelengths to produce a criterion response of about 25% of the maximum photocurrent. An eighth-order polynomial⁵⁶ was used to provide the estimates of λ_{\max} along a log wavenumber axis (see Table I).

For comparison, microspectrophotometric estimates (transverse absorbance measurements) of the S cone and M cone λ_{\max} values²² are given in Table I. The values are based on averages of 11 (S cone) and 49 (M cone) spectra records obtained from cone outer segments excised from seven human eyes. The genotype was not determined. The absorbance spectra were constructed from the raw data by plotting them on a $\lambda^{1/4}$ scale. Sixty-nine records were also obtained from L cone photoreceptors, but because their mean λ_{\max} value depends on an unknown mixture of L(S180) and L(A180) pigments, it is not tabulated.

As can be seen in Table I, the psychophysical, recombinant pigment, suction electrode, and ERG data are in rough agreement with respect to the λ_{\max} values of the normal human cone pigments: M(A180), L(A180), and L(S180). However, as noted in the introduction, the recombinant pigments and those obtained from suction electrode recordings differ from their *in vivo* counterparts with respect to waveguiding, self-screening, and local chemical environment. For instance, compared with the spectroscopy of recombinant pigments and suction electrode recordings, spectra obtained from psychophysics are slightly steeper at longer wavelengths, presumably owing to waveguiding effects. This can result in a slightly shorter λ_{\max} estimate.

The agreement between spectral sensitivities estimated *in vivo* and *in vitro* is also good for the L-M hybrid pigments. The data indicate that all of the hybrids derived from L and M pigments have spectral sensitivities between those of the two parental pigments, and that the spectral sensitivity of each hybrid depends on the position of the crossover and on the identity of other polymorphic amino acids, principally alanine or serine at position

⁶¹ G. H. Jacobs, J. Neitz, and K. Krogh, *J. Opt. Soc. Am.* **13**, 641 (1996).

⁶² T. W. Kraft, J. Neitz, and M. Neitz, *Vision Res.* **38**, 3663 (1998).

⁶³ T. W. Kraft, personal communication (1998).

180. For each exon, the set of amino acids normally associated with the L or M pigments produce, respectively, spectral shifts to longer or shorter wavelengths, thus producing a monotonic relationship between the λ_{\max} and the fraction of the hybrid pigment derived from the parental L or M pigment.

The primary determinants of the spectral shift are located in exon 5, as seen by the clustering of the λ_{\max} values of 5' L-3' M or 5' M-3' L hybrids within 10 nm of those of the normal M or L pigments, respectively (see Table I). A comparison of the spectral sensitivities of the L(A180) and L(S180) pigments with those of the L4M5(A180) and L4M5(S180) pigments indicates that L/M sequence differences in exon 5 result in a spectral shift of approximately 25 nm. Site-directed mutagenesis experiments have shown that threonine/alanine and tyrosine/phenylalanine differences at codons 285 and 309, respectively (see Fig. 3), account for essentially all of the effects of exon 5.^{28,45} These data are consistent with inferences based on a comparison of primate visual pigment gene sequences and cone spectral sensitivity curves.⁶⁴⁻⁶⁶

Considerable effort has been made to determine the effect of the serine/alanine polymorphism at position 180.^{24,26,28,37,67,68} All of the reported measurements have shown that serine-containing pigments are red shifted with respect to alanine-containing pigments, but the magnitude of the shift has been controversial. On the basis of anomaloscope matches, Sanocki *et al.* estimated that the substitution of alanine by serine in the L pigment or within 5' L-3' M hybrid pigments results in a red shift of λ_{\max} by 4.3, 3.5, and 2.6-2.7 nm for deuteranopes, protanopes, and normal trichromats, respectively.^{67,68} Neitz and Jacobs estimated a red shift of approximately 3 nm from Rayleigh match data obtained from 60 normal trichromats.⁶⁹ Red-shift estimates of 5-7 nm were obtained from five dichromats by electroretinography, but this interpretation was complicated by additional amino acid differences between the pigments.²⁴ *In vitro* measurements of recombinant pigments by Merbs and Nathans showed a red shift of 4.3-4.4 nm in the L pigment and in 5' L-3' M and 5' M-3' L hybrid pigments.^{26,27} In contrast, Asenjo *et al.* found a range of red shifts depending on the parental pigment: a 2-nm shift in a 5' L-3' M hybrid and in the M pigment,

⁶⁴ M. Neitz, J. Neitz, and G. H. Jacobs, *Science* **252**, 971 (1991).

⁶⁵ R. E. Ibbotson, D. M. Hunt, J. K. Bowmaker, and J. D. Mollon, *Proc. R. Soc. (London)* **B 247**, 145 (1992).

⁶⁶ A. J. Williams, D. M. Hunt, J. K. Bowmaker, and J. D. Mollon, *EMBO J.* **11**, 2039 (1992).

⁶⁷ E. Sanocki, D. T. Lindsey, J. Winderickx, D. Y. Teller, S. S. Deeb, and A. G. Motulsky, *Vision Res.* **33**, 2139 (1993).

⁶⁸ E. Sanocki, S. K. Shevell, and J. Winderickx, *Vision Res.* **34**, 377 (1994).

⁶⁹ J. Neitz and G. H. Jacobs, *Vision Res.* **30**, 621 (1990).

a 4-nm shift in a 5' M-3' L hybrid, and a 7-nm shift in the L pigment.²⁸ The spectral sensitivity curves obtained from single-gene dichromats show a mean separation of 2.5–2.9 nm, depending on which of two methods is used to calculate the spectral sensitivity curve, and whether individual or mean data are used.⁴³ These results are consistent with other psychophysical measures of the variability of the L cone λ_{\max} in the normal population.^{70,71}

Implications for Variant Color Vision and Derivation of Human Cone Fundamentals

The absorption differences that distinguish closely related L–M hybrid pigments and A180 and S180 variants most likely account for the observation that anomalous trichromats differ greatly in the location and range of their Rayleigh matching points.^{15,48,72} These data support a model of anomalous trichromacy in which any one of many M-like or L-like anomalous pigments can be paired with one of the polymorphic versions of the more similar normal pigment.^{27,73} As the spectral sensitivities of the normal or the anomalous pigments shift, the midpoint of the Rayleigh match will shift, and as the separation between the spectral sensitivities of the normal and anomalous pigments increases or decreases, the better or poorer will be the subject's chromatic discrimination.

The existence of polymorphisms among normal M and L pigment genes, most especially the A180/S180 polymorphism, means that a single set of cone fundamentals (i.e., the basic cone spectral sensitivities of trichromatic color vision) will accurately describe the color vision of only a subset of normal trichromats, and that in the construction of an average set of fundamentals it is important that the weighting of polymorphic types within the test population match that in the general population. Thus, the *in vivo* determination of the cone fundamentals requires an analysis of the spectral sensitivity curves for subjects whose visual pigment gene sequences reveal which of the various possible pigments they possess.⁷

In conclusion, by building on advances in molecular biology and exploiting high-precision *in vivo* and *in vitro* techniques, significant progress has been made toward the goal of fully cataloging the rich diversity of cone photopigments that underlie normal and anomalous human color vision.

⁷⁰ M. A. Webster, *J. Opt. Soc. Am.* **9**, 1419 (1992).

⁷¹ M. A. Webster and D. I. A. MacLeod, *J. Opt. Soc. Am.* **5**, 1722 (1988).

⁷² J. D. Mollon, in "Color Vision Deficiencies XIII" (C. R. Cavonius, ed.), p. 3. Kluwer Academic, Dordrecht, 1997.

⁷³ J. Neitz, M. Neitz, and P. Kainz, *Science* **274**, 801 (1996).