United States Patent	12390146
Kind Code	B2
Date of Patent	August 19, 2025
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Reagents and methods for modulating cone photoreceptor activity

Abstract

The present invention provides reagents and methods for modulating cone photoreceptor activity, and devices for assessment of cone photoreceptor activity.

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Appl. No.: **17/500426**

Filed: **October 13, 2021**

Prior Publication Data

Document Identifier	Publication Date
US 20220183613 A1	Jun. 16, 2022

Related U.S. Application Data

continuation parent-doc US 15939674 20180329 US 11160488 child-doc US 17500426
continuation parent-doc US 14837448 20150827 ABANDONED child-doc US 15939674
continuation parent-doc US 14075415 20131108 US 9198595 20151201 child-doc US 14837448
continuation parent-doc US 13395609 ABANDONED WO PCT/US2010/048964 20100915 child-doc US 14075415
us-provisional-application US 61242587 20090915

Publication Classification

Int. Cl.: **C12N15/86** (20060101); **A61B5/00** (20060101); **A61B5/398** (20210101); **A61K48/00** (20060101); **A61N5/06** (20060101); **C07K14/005** (20060101); **C12N7/00** (20060101); **C12N15/85** (20060101)

U.S. Cl.:

CPC **A61B5/398** (20210101); **A61B5/0036** (20180801); **A61B5/0048** (20130101); **A61B5/4836** (20130101); **A61B5/4848** (20130101); **A61B5/6821** (20130101); **A61B5/7225** (20130101); **A61K48/0058** (20130101); **A61K48/0075** (20130101); **A61N5/0622** (20130101); **C07K14/005** (20130101); **C12N7/00** (20130101); **C12N15/85** (20130101); **C12N15/86** (20130101); A61N2005/0652 (20130101); A61N2005/0661 (20130101); A61N2005/0663 (20130101); C12N2750/14132 (20130101); C12N2750/14143 (20130101); C12N2799/025 (20130101); C12N2830/008 (20130101); C12N2830/42 (20130101); C12N2840/44 (20130101)

Field of Classification Search

CPC: C07K (14/005); C12N (7/00); C12N (15/85); C12N (15/86)

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Background/Summary

RELATED APPLICATIONS (1) This application is a continuation of U.S. patent application Ser. No. 15/939,674, filed Mar. 29, 2018, which is a continuation of U.S. patent application Ser. No. 14/837,448 filed Aug. 27, 2015, which is a continuation of U.S. patent application Ser. No. 14/075,415 filed Nov. 8, 2013 now U.S. Pat. No. 9,198,595 issued Dec. 1, 2015, which is a continuation of U.S. patent application Ser. No. 13/395,609 filed Mar. 12, 2012, which is a U.S. National Phase of International Application No. PCT/US2010/048964, filed Sep. 15, 2010, which claims priority to U.S. Provisional Application No. 61/242,587, filed Sep. 15, 2009, the disclosures of which are hereby incorporated by reference in their entireties.

BACKGROUND
(1) Classic visual deprivation experiments have led to the expectation that neural connections established during development would not appropriately process an input that was not present from birth. Therefore, it was believed that treatment of congenital vision disorders would be ineffective unless administered to the very young.
SUMMARY OF THE INVENTION
(2) In a first aspect, the present invention provides methods for cone cell gene therapy in a primate, comprising administering to the eye of a primate in need of cone cell gene therapy a recombinant gene delivery vector comprising:
(3) (a) a promoter region, wherein the promoter region is specific for retinal cone cells; and
(4) (b) a gene encoding a therapeutic, wherein the gene is operatively linked to the promoter region;
(5) wherein in vivo expression of the therapeutic in cone cells of the primate serves to treat the primate in need of cone cell gene therapy.
(6) The method of this aspect of the invention can be used, for example, to treat a cone cell disorder, including but not limited to color blindness, blue cone monochomacy, achromatopsia, incomplete achromatopsia, rod-cone degeneration, retinitis pigmentosa (RP), macular degeneration, cone dystrophy, blindness, Stargardt's Disease, and Leber's congenital amaurosis. In one embodiment, the methods restore visual capacity in the primate; in another embodiment, the

primate is able to visualize new colors as a result of the therapy. In another embodiment, the primate has a vision disorder in which its photoreceptors are healthy. In a further embodiment, the primate is an adult primate.
(7) In another aspect, the present invention provides isolated nucleic acid expression vector comprising:
(8) (a) a promoter region, wherein the promoter region is specific for primate retinal cone cells; and
(9) (b) a gene encoding a therapeutic, wherein the gene is operatively linked to the promoter region. In various embodiments, the vectors further comprise an enhancer element upstream of the promoter, wherein the gene is operatively linked to the enhancer element, and/or an intron comprising a splice donor/acceptor region, wherein the intron is located downstream of the promoter region and is located upstream of the gene. The vectors can be used, for example, in the methods of the invention.
(10) In another aspect, the present invention provides color multi-focal electroretinogram (mf-ERG) comprising:
(11) (a) an electroretinogram (ERG) comprising (i) a recording electrode that is (A) designed for placement on at least one of a cornea and a sclera of at least one eye of a subject and (B) arranged to output at least one signal generated by the at least one eye; and (ii) a computing system communicatively coupled to the recording electrode, the computing system comprising (A) at least one processor and (B) data storage containing instructions executable by the at least one processor to carry out a set of functions, the set of functions including processing and saving the at least one signal generated by the at least one eye;
(12) (b) a retinal stimulator comprising matched light sources selected from the group consisting of red, green, blue, and ultraviolet light sources, wherein the matched light sources are connected to the ERG and in operation can be independently frequency modulated at rates between about 1 Hz and about 60 Hz, inclusive, wherein the stimulator in operation is capable of stimulating a retinal field of a subject throughout an operating radius of at least about 70 degrees;
(13) (c) one or more constant current integrated circuit chips arranged to drive the stimulator; and
(14) (d) a pulse-frequency modulator connected to the retinal stimulator, wherein in operation the pulse-frequency modulator is capable of controlling individual stimulator segments while keeping relative spectral content of the light constant. In various preferred embodiments, the matched light sources are paired red and green light sources;
(15) triplets of red, green, and blue light sources; or quartets of red, green, blue, and ultraviolet light sources. In another preferred embodiment the retinal stimulator comprises a concave surface comprising a series of trapezoidal-shaped circuit boards placed edge-to-edge, wherein the concave surface positions the matched light sources so in operation they are held equidistantly from and pointing toward a single focal point where a subject's pupil can be positioned.

Description

BRIEF DESCRIPTION OF THE FIGURES
(1) FIG. **1**. rAAV2/5 vector produced functional L-opsin in primate retina. a) Molecular map; TR=terminal repeats; LCR=locus control region; PP=proximal promoter; SD/SA=splice donor/acceptor; RHLOPS=recombinant human L opsin cDNA; PA.sub.1=polyadenylation signal. b) Red light Multi-focal electroretinogram (Mf-ERG) stimulus. c) mf-ERG 40 weeks after two injections (yellow circles) of a mixture of L-opsin- and green fluorescent protein (GFP)-coding viruses. Grey lines show borders of highest response; for comparison, inset=mfERG 16 weeks post-injection; there was no reliable signal from L-opsin, unchanged from baseline. High responses in far peripheral retina were measured reliably and may have originated from offshoot of one of the injections. d) Fluorescence photographs from a similar retinal area as c; grey lines from c were

copied in d. e) Confocal microscopy revealed a mosaic pattern of GFP expression in 5-12% of cones. Because GFP-coding virus was diluted to ⅓ compared to L-opsin virus, an estimated 15-36% of cones in behaviourally tested animals express L-opsin. f) Mf-ERG from a behaviourally tested animal 70 weeks after 3 injections of L-opsin virus.

(2) FIG. **2**. Pre-therapy colour vision and possible treatment outcomes. a) Colour vision stimuli examples. b) Pre-therapy results, monkey 1. Hues tested are represented as dominant wavelengths (DWs) rather than u′, v′ coordinates. If a hue could not be reliably distinguished at even the highest saturation, the extrapolated threshold approached infinity. c) Pre-therapy results, monkey 2. d)-e), Possible experimental outcomes: Monkeys could have a relative increase in long-wavelength sensitivity, but remain dichromatic (dashed lines, d); theoretical colour spectrum appearances for a dichromat and a possible “spectral shift” are shown. Alternatively, dichromatic monkeys could become trichromatic. Results from a trichromatic female control monkey are plotted (dashed line, e; error bars=SEM and n varied from 7-11).

(3) FIG. **3**. Gene therapy produced trichromatic colour vision. a) Time course of thresholds for the blue-green confusion colour, DW=490 nm (circles), and a yellowish colour, DW=554 nm (squares). A logarithmic scale was used to fit high thresholds for DW=490 nm; significant improvement occurred after 20 weeks. Enclosed data points=untreated dichromatic monkey thresholds, DW=490 nm (triangle) and DW=554 nm (diamond). b)-c) Comparison of pre-therapy (open circles, solid line) and post-therapy thresholds (solid dots, dashed line). Enclosed data points are DW=490 nm thresholds when tested against a red-violet background (DW=−499 nm); pink triangles=trichromatic female control thresholds. Error bars=SEM; n varied from 7-11.

(4) FIG. **4**. a) The geodesic dome was created by placing trapezoidal-shaped circuit boards edge-to-edge. This structure holds the light emitting diodes (LEDs) so they converge on a single focal point. b) The circuit board takes the incoming control signals from the Retis-can mf-ERG and reroutes and modifies them to work with the new dome. The most frequent integrated circuit on the board are the constant current devices. c) The spectral composition of the red LED. d) The spectral composition of the green LED. e) The spectral sensitivity curves for the human M- (solid line) and L- (dashed line) cone photoreceptors. f) The activation of M-opsin (solid line) and the L-opsin (dashed line) in response to both the red and green LEDs.

(5) FIG. **5**. Circles and dashed line represent the red LED out-puts in microwatts as a function of intensity; triangles and solid line represent the green LED outputs. Each data point represents an average of 3 measurements. Error bars are three standard deviations (99.7% confidence interval). To measure linearity, r2 values were computed for both the red and green LEDs.

(6) FIG. **6**. The signal to noise ratio (SNR) for as a function of degrees of eccentricity for a typical (averaged) subject and for the highest subject recorded.

(7) FIG. **7**. a) Gerbil mf-ERG data in response to the L-cone isolating red stimulus. Locations of the retina that show a large amount of activity in response to stimulation by the red LEDs are indicated in red, while those areas that show the lowest amount of activation are indicated in blue (see scale). Gray lines show borders of the region where the mfERG response was highest. b) GFP fluorescence fundus image from the gerbil, scaled to the appropriate size, overlaid on the red-light mf-ERG data. The gray lines from (a) were copied into (b) to illustrate that areas of increased mf-ERG response corresponded to the same locations where robust GFP fluorescence was present. c) Red-light mf-ERG data from the squirrel monkey, which received two injections of the virus mixture, one superiorly and one inferiorly. d) A montage of GFP fluorescence images from the squirrel monkey, scaled to the appropriate size, overlaid on the mfERG data.

(8) FIG. **8**. The circles represent ERG response from LEDs that were moved on a linear path while the subject fixated forward. By 30 degrees, the signal is already less than half. In contrast, the triangles represent ERG response from LEDs that were fixed on a boom and rotated so that the LEDs always pointed at the pupil. Under this experimental protocol, −3 dB occurs first at 60 degrees.

DETAILED DESCRIPTION OF THE INVENTION

(9) In a first aspect, the present invention provides methods for cone cell gene therapy in a primate, comprising administering to the eye of a primate in need of cone cell gene therapy a recombinant gene delivery vector comprising:

(10) (a) a promoter region, wherein the promoter region is specific for retinal cone cells; and

(11) (b) a gene encoding a therapeutic, wherein the gene is operatively linked to the promoter region;

(12) wherein in vivo expression of the therapeutic in cone cells of the primate serves to treat the primate in need of cone cell gene therapy.

(13) Cone cells are photoreceptor cells in the retina of the eye that function best in relatively bright light. The cone cells gradually become sparser towards the periphery of the retina. The methods of the present invention can be used for treatment of any condition that can be addressed, at least in part, by gene therapy of retinal cone photoreceptor cells. The inventors have demonstrated effective treatment of congenital vision disorders in adult primates, a result that is completely unexpected in the art.

(14) In one preferred embodiment, the gene therapy serves to treat a cone cell disorder. As used herein, a “cone cell disorder” is any disorder impacting retinal cone cells, including but not limited to color blindness, blue cone monochomacy, achromatopsia, incomplete achromatopsia, rod-cone degeneration, retinitis pigmentosa (RP), macular degeneration, cone dystrophy, blindness, Stargardt's Disease, and Leber's congenital amaurosis.

(15) The gene encoding a therapeutic to be expressed in the cone cells can comprise or consist of any gene or cDNA that encodes a polypeptide or RNA-based therapeutic (siRNA, antisense, ribozyme, shRNA, etc.) that can be used as a therapeutic for treating a cone cell disorder. In a preferred embodiment, the primate is of the Parvorder Catarrhini. As is known in the art, Catarrhini is one of the two subdivisions of the higher primates (the other being the New World monkeys), and includes Old World monkeys and the apes, which in turn are further divided into the lesser apes or gibbons and the great apes, consisting of the orangutans, gorillas, chimpanzees, bonobos, and humans. In a further preferred embodiment, the primate is a human.

(16) A “promoter” is a DNA sequence that directs the binding of RNA polymerase and thereby promotes RNA synthesis, i.e., a minimal sequence sufficient to direct transcription. Any suitable promoter region can be used in the gene therapy vectors, so long as it specifically promotes expression of the gene in retinal cone cells. In a preferred embodiment, the promoter specifically promotes expression of the gene in primate retinal cone cells; more preferably in Catarrhini retinal cone cells; even more preferably in human retinal cone cells. As used herein, “specifically” means that the promoter predominately promotes expression of the gene in retinal cone cells compared to other cell types, such that at least 80%, and preferably 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 97%, 98%, 99%, 99.5%, or more of expression of the gene after delivery of the vector to the eye will be in cone cells. Exemplary suitable promoter regions include the promoter region for any cone-specific gene, such as the L opsin promoter (SEQ ID NO:1), the M opsin promoter (SEQ ID NO:2), and the S opsin promoter (SEQ ID NO:3), or portions thereof suitable to promote expression in a cone-specific manner. Any suitable method for identifying promoter sequences capable of driving expression in primate cone cells can be used to identify such promoters, as will be understood by those of skill in the art based on the teachings herein.

(17) In a preferred embodiment, the gene delivery vector further comprises an enhancer element upstream of the promoter, wherein the gene is operatively linked to the enhancer element. Enhancers are cis-acting elements that stimulate transcription of adjacent genes. Any suitable enhancer element can be used in the gene therapy vectors, so long as it enhances expression of the gene when used in combination with the promoter. In a preferred embodiment, the enhancer element is specific for retinal cone cells; more preferably, it is specific for primate retinal cone cells; more preferably in Catarrhini retinal cone cells; even more preferably in human retinal cone

cells. As used herein, “specifically” means that the enhancer predominately enhances expression of the gene in retinal cone cells compared to other cell types, such that at least 80%, and preferably 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 97%, 98%, 99%, 99.5%, or more of expression of the gene after delivery of the vector to the eye will be in cone cells. Exemplary suitable enhancer regions comprise or consist of the enhancer region for any cone-specific gene, such as the L/M minimal opsin enhancer (SEQ ID NO: 51), L/M enhancer elements of 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1100, 1200, 1300, 1400, 1500, 1600, 1700, 1800, 1900, 2000, or more nucleotides that comprise one or more copies of the L/M minimal opsin enhancer, and the full L/M opsin enhancer (SEQ ID NO:4), or other portions thereof suitable to promote expression in a cone-specific manner. Any suitable method for identifying enhancer sequences capable of driving expression in primate cone cells can be used to identify such enhancers, as will be understood by those of skill in the art based on the teachings herein.

(18) The length of the promoter and enhancer regions can be of any suitable length for their intended purpose, and the spacing between the promoter and enhancer regions can be any suitable spacing to promote cone-specific expression of the gene product. In various preferred embodiments, the enhancer is located 0-1500; 0-1250; 0-1000; 0-750; 0-600; 0-500; 0-400; 0-300; 0-200; 0-100; 0-90; 0-80; 0-70; 0-60; 0-50; 0-40; 0-30; 0-20; or 0-10 nucleotides upstream of the promoter. The promoter can be any suitable distance upstream of the encoded gene.

(19) In a further preferred embodiment that can be combined with any other embodiment in any aspect of the present invention, the enhancer comprises or consists of a sequence selected from the group consisting of the L/M minimal opsin enhancer (SEQ ID NO: 51), L/M enhancer elements of 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1100, 1200, 1300, 1400, 1500, 1600, 1700, 1800, 1900, 2000, or more nucleotides that comprise one or more copies of the L/M minimal opsin enhancer, and the full L/M opsin enhancer (SEQ ID NO:4), or other portions thereof suitable to promote expression in a cone-specific manner, and the promoter comprises or consists of a sequence selected from the group consisting of L opsin promoter (SEQ ID NO: 1), the M opsin promoter (SEQ ID NO:2), and the S opsin promoter (SEQ ID NO:3).

(20) In a further preferred embodiment, the gene delivery vector further comprises an intron comprising a splice donor/acceptor region, wherein the intron is located downstream of the promoter region and is located upstream of the gene. Any intron can be used, so long as it comprises a splice donor/acceptor region recognized in primate cone cells, so that the intron can be spliced out of the resulting mRNA product. In one embodiment, the intron comprises or consists of an SV40 intron according to SEQ ID NO:5. In various preferred embodiments, the 3′ end of the intron is 0-20; 0-15; 0-10; 0-9; 0-8; 0-7; 0-6; or 0-5 nucleotides upstream of the gene, and its 5′ end is 0-20; 0-15; 0-10; 0-9; 0-8; 0-7; 0-6; or 0-5 nucleotides downstream of the proximal promoter region.

(21) The gene is operatively linked to the promoter region and the enhancer element, such that the promoter and enhancer elements are capable of driving expression of the gene or cDNA in cone cells of the subject.

(22) The gene encoding a therapeutic to be expressed in the cone cells can be any gene or cDNA that encodes a polypeptide or RNA-based therapeutic (siRNA, antisense, ribozyme, shRNA, etc.) that can be used as a therapeutic for treating a cone cell disorder, or as a means to otherwise enhance vision, including but not limited to promoting tetrachromatic color vision. In various preferred embodiments, the gene encodes a therapeutic protein selected from the group consisting of

(23) (a) SEQ ID NO: 7 (SEQ ID NO: 6) Homo sapiens opsin 1 (cone pigments), short-wave-sensitive (OPN1SW), mRNA NCBI Reference Sequence: NM_001708.2;

(24) (b) SEQ ID NO: 9 (SEQ ID NO: 8) Homo sapiens opsin 1 (cone pigments), medium-wave-sensitive (OPN1MW), mRNA NCBI Reference Sequence: NM_000513.2;

(25) (c) SEQ ID NO: 11 (SEQ ID NO: 10) Homo sapiens opsin 1 (cone pigments), long-wave-

sensitive (OPN1LW), mRNA NCBI Reference Sequence: NM_020061.4;
(26) (d) SEQ ID NO: 13 (SEQ ID NO: 12) ATP binding cassette retina gene (ABCR) gene (NM_000350);
(27) (e) SEQ ID NO: 15 (SEQ ID NO: 14) retinal pigmented epithelium-specific 65 kD protein gene (RPE65) (NM_000329);
(28) (f) SEQ ID NO: 17 (SEQ ID NO: 16) retinal binding protein 1 gene (RLBP1) (NM_000326);
(29) (g) SEQ ID NO: 19 (SEQ ID NO: 18) peripherin/retinal degeneration slow gene, (NM_000322);
(30) (h) SEQ ID NO: 21 (SEQ ID NO: 20) arrestin (SAG) (NM_000541);
(31) (i) SEQ ID NO: 23 (SEQ ID NO: 22) alpha-transducin (GNAT1) (NM_000172);
(32) (j) SEQ ID NO: 24 guanylate cyclase activator 1A (GUCA1A) (NP_000400.2);
(33) (k) SEQ ID NO: 25 retina specific guanylate cyclase (GUCY2D), (NP_000171.1); (l) SEQ ID NO: 26 & 27 alpha subunit of the cone cyclic nucleotide gated cation channel (CNGA3) (NP_001073347.1 or NP_001289.1);
(34) (m) SEQ ID NO: 28 Human cone transducin alpha subunit (incomplete achromotopsia);
(35) (n) SEQ ID NO: 29 cone cGMP-specific 3′,5′-cyclic phosphodiesterase subunit alpha′, protein (cone dystrophy type 4);
(36) (o) SEQ ID NO: 30 retinal cone rhodopsin-sensitive cGMP 3′,5′-cyclic phosphodiesterase subunit gamma, protein (retinal cone dystrophy type 3A);
(37) (p) SEQ ID NO: 31 cone rod homeobox, protein (Cone-rod dystrophy);
(38) (q) SEQ ID NO: 32 cone photoreceptor cyclic nucleotide-gated channel beta subunit, protein (achromatopsia);
(39) (r) SEQ ID NO: 33 cone photoreceptor cGMP-gated cation channel beta-subunit, protein (total color blindness, for example, among Pingelapese Islanders);
(40) (s) SEQ ID NO: 35 (SEQ ID NO: 34) retinitis pigmentosa 1 (autosomal dominant) (RP1);
(41) (t) SEQ ID NO: 37 (SEQ ID NO: 36) retinitis pigmentosa GTPase regulator interacting protein 1 (RPGRIP1);
(42) (u) SEQ ID NO: 39 (SEQ ID NO: 38) PRP8;
(43) (v) SEQ ID NO: 41 (SEQ ID NO: 40) centrosomal protein 290 kDa (CEP290);
(44) (w) SEQ ID NO: 43 (SEQ ID NO: 42) IMP (inosine 5′-monophosphate) dehydrogenase 1 (IMPDH1), transcript variant 1;
(45) (x) SEQ ID NO: 45 (SEQ ID NO: 44) aryl hydrocarbon receptor interacting protein-like 1 (AIPL1), transcript variant 1;
(46) (y) SEQ ID NO: 47 (SEQ ID NO: 46) retinol dehydrogenase 12 (all-trans/9-cis/11-cis) (RDH12);
(47) (z) SEQ ID NO: 49 (SEQ ID NO: 48) Leber congenital amaurosis 5 (LCAS), transcript variant 1; and
(48) (aa) exemplary OPN1LW/OPN1MW2 polymorphs (compared to OPN1LW (L opsin) polypeptide sequence; the amino acid to the left of the number is the residue present in the L opsin sequence; the number is the reside number in L opsin, and the reside to the right of the number is the variation from L opsin. Polymorphs according to these embodiments may comprise one or more of the amino acid substitutions in Table 1 below:
(49) TABLE-US-00001 TABLE 1 (i) Thr65Ile (ii) Ile111Val (iii) Ser116Tyr (iv) Leu153Met (v) Ile171Val (vi) Ala174Val (vii) Ile178Val (viii) Ser180Ala (ix) Ile230Thr (x) Ala233Ser (xi) Val236Met (xii) Ile274Val (xiii) Phe275Leu (xiv) Tyr277Phe (xv) Val279Phe (xvi) Thr285Ala (xvii) Pro298Ala (xviii) Tyr309Phe.
(50) The proteins recited in (a)-(c) and (aa) are all involved in color vision. The exemplary polymorphs include ones at positions 65, 116, 180, 230, 233, 277, 285, and 309 that affect the spectra of the pigments in cone cells expressing them. Positions 274, 275, 277, 279, 285, 298 and 309 together distinguish L opsin from M opsin.

(51) The proteins recited (d)-(z) are exemplary eye disease-associated gene, such as in retinitis pigmentosa (polypeptides “e”-“l”, “s”-“y”), incomplete achromatopsia (polypeptide “m”), Stargardt's (polypeptide “d”); Leber congenital amaurosis (polypeptide “z”); cone dystrophy, such as cone dystrophy type 4 (polypeptide “n”); retinal cone dystrophy; for example, retinal cone dystrophy type 3A (polypeptide “o”) ; Cone-rod dystrophy (polypeptide “p”); achromatopsia (polypeptide “q’); and total color blindness, for example, among Pingelapese Islanders (polypeptide “r”).
(52) Exemplary nucleic acids encoding these polypeptides are shown by SEQ ID NO in parenthesis. Thus, in a further preferred embodiment, the genes comprise or consist of a nucleic acid sequence according to one or more of the nucleic acid sequences recited above. In a further preferred embodiment, the vector comprises the sequence shown in SEQ ID NO: 50
(53) Any suitable gene therapy vector that can be used for cone cell delivery can be used in the methods of the present invention; the vector may comprise single or double stranded nucleic acid; preferably single stranded or double stranded DNA. In a preferred embodiment that can be combined with any of the above embodiments, the gene delivery vector comprises a recombinant adeno-associated virus (AAV) gene delivery vector. Prior to the present invention, rAAV vectors had not been shown capable of transducing primate cone cells. In this embodiment, the gene delivery vector is bounded on the 5′ and 3′ end by functional AAV inverted terminal repeat (ITR) sequences. By “functional AAV ITR sequences” is meant that the ITR sequences function as intended for the rescue, replication and packaging of the AAV virion. Hence, AAV ITRs for use in the vectors of the invention need not have a wild-type nucleotide sequence, and may be altered by the insertion, deletion or substitution of nucleotides or the AAV ITRs may be derived from any of several AAV serotypes. The rAAV vector may be derived from an adeno-associated virus serotype, including without limitation, AAV-1, AAV-2, AAV-3, AAV-4, AAV-5, AAV-6, AAV-7, AAV-8, etc. Preferred AAV vectors have the wild type REP and CAP genes deleted in whole or part, but retain functional flanking ITR sequences. In a further preferred embodiment, the AAV vector comprises rAAV.sup.2/.sub.5, a “pseudotyped” version of AAV2 created by using rep from AAV2 and cap from A AV5 or AAV2 , A AV3, AAV4, AAV6, AAV7, AA V8 together with a plasmid containing a vector based on AAV2. Preferably, the rAAV is replication defective, in that the AAV vector cannot independently further replicate and package its genome. For example, when cone cells are transduced with rAAV virions, the gene is expressed in the transduced cone cells, however, due to the fact that the transduced cone cells lack AAV rep and cap genes and accessory function genes, the rAAV is not able to replicate.
(54) Recombinant AAV (rAAV) virions encapsidating the vectors recited above for use in transducing cone cells may be produced using standard methodology. In one embodiment, an AAV expression vector according to the invention is introduced into a producer cell, followed by introduction of an AAV helper construct, where the helper construct includes AAV coding regions capable of being expressed in the producer cell and which complement AAV helper functions absent in the AAV vector. This is followed by introduction of helper virus and/or additional vectors into the producer cell, wherein the helper virus and/or additional vectors provide accessory functions capable of supporting efficient rAAV virus production. The producer cells are then cultured to produce rAAV. These steps are carried out using standard methodology. Replication-defective AAV virions encapsulating the recombinant AAV vectors of the instant invention are made by standard techniques known in the art using AAV packaging cells and packaging technology. Examples of these methods may be found, for example, in U.S. Pat. Nos. 5,436,146; 5,753,500, 6,040,183, 6,093,570 and 6,548,286, expressly incorporated by reference herein in their entirety. Further compositions and methods for packaging are described in Wang et al. (US 2002/0168342), also incorporated by reference herein in its entirety.
(55) Any suitable method for producing viral particles for delivery can be used, including but not limited to those described in the examples that follow. Any concentration of viral particles suitable

to effectively transducer cone cells can be administered to the eye. In one preferred embodiment, viral particles are delivered in a concentration of at least 10.sup.10 vector genome containing particles per mL; in various preferred embodiments, the viral particles are delivered in a concentration of at least 7.5×10.sup.10; 10.sup.11; 5×10.sup.11; 10.sup.12; 5×10.sup.12; 10.sup.13; 1.5×10.sup.13; 3×10.sup.13; 5×10.sup.13; 7.5×9×10.sup.13; or 9×10.sup.13 vector genome containing particles per mL. Similarly, any total number of viral particles suitable to provide appropriate transduction of retinal cone cells can be administered to the primate's eye. In various preferred embodiments, at least 10.sup.10; 5×10.sup.10; 10.sup.11; 5×10.sup.11; 10.sup.12; 1.5×10.sup.12; 3×10.sup.12; 5×10.sup.12; 7.5×10.sup.12; 10.sup.13; 1.5×10.sup.13; or 2.7'10.sup.13 viral particles are injected per eye. Any suitable number of administrations of the vector to the primate eye can be made. In one embodiment, the methods comprise a single administration; in other embodiments, multiple administrations are made over time as deemed appropriate by an attending clinician.

(56) The viral stock for delivery to the primate eye can be treated as appropriate for delivery. The viral stock can be combined with pharmaceutically-acceptable carriers, diluents and reagents useful in preparing a formulation that is generally safe, non-toxic, and desirable, and includes excipients that are acceptable for primate use. Such excipients can be solid, liquid, semisolid, or, in the case of an aerosol composition, gaseous. Examples of such carriers or diluents include, but are not limited to, water, saline, Ringer's solutions, dextrose solution, and 5% human serum albumin. Supplementary active compounds can also be incorporated into the formulations. Solutions or suspensions used for the formulations can include a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial compounds such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating compounds such as ethylenediaminetetraacetic acid (EDTA); buffers such as acetates, citrates or phosphates; detergents such as Tween 20 to prevent aggregation; and compounds for the adjustment of tonicity such as sodium chloride or dextrose. The pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide.

(57) In a further preferred embodiment that can be combined with any other embodiment, the methods of the invention restore visual capacity in the primate. As used herein, “restoring visual capacity” means that some benefit to vision is provided, including but not limited to a reduction or slowing of vision loss; improved visual acuity; decrease in abnormal sensitivity to bright lights; and/or an increase in one or more visual attributes, such as improved color perception (ie: monochromatic to dichromatic vision; dichromatic to trichromatic vision; trichromatic to tetrachromatic vision; etc.). The primate is preferably of the Parvorder Catarrhini, and more preferably is a human.

(58) In a further preferred embodiment that can be combined with all of the above embodiments, the primate suffers from color blindness, and the primate is able to visualize new colors as a result of the therapy. In this embodiment, it is preferred that the enhancer (if present) comprises or consists of a sequence selected from the group consisting of the L/M minimal opsin enhancer (SEQ ID NO: 51), L/M enhancer elements of 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1100, 1200, 1300, 1400, 1500, 1600, 1700, 1800, 1900, 2000, or more nucleotides that comprise one or more copies of the L/M minimal opsin enhancer, and the full L/M opsin enhancer (SEQ ID NO:4), or other portions thereof suitable to promote expression in a cone-specific manner, and the promoter comprises or consists of a sequence selected from the group consisting of L opsin promoter (SEQ ID NO: 1), the M opsin promoter (SEQ ID NO:2), and the S opsin promoter (SEQ ID NO:3), while the gene encodes one or more polypeptides comprising or consisting of a sequence selected from the group consisting of SEQ ID NO: 7 (OPN1SW), SEQ ID NO: 9 (OPN1MW), SEQ ID NO: 11 (OPN1LW), mRNA; and exemplary OPN1LW/OPN1MW2 polymorphs as described in Table 1 above. It is further preferred that the vector comprises a rAAV vector as described above.

(59) The color blindness may be acquired or inherited, and can be full (monochromatic) or partial. In a preferred embodiment, the primate has partial color blindness selected from the group consisting of red-green and blue-yellow color blindness. The partial color blindness can comprise, for example, dichromacy or anomalous trichromasy. These methods result in the primate improved color perception (ie: monochromatic to dichromatic vision; dichromatic to trichromatic vision; trichromatic to tetrachromatic vision; etc.). The primate is preferably of the Parvorder Catarrhini, and more preferably is a human.
(60) As described in detail below, the methods may be used to improve color perception in primates from dichromatic to trichromatic. Dichromats who are missing either the L- or the M-photopigment fail to distinguish from grey:colours near the so-called ‘spectral neutral point’ located in the bluegreen region of color space (near dominant wavelength of 490 nm) and complementary colors near the ‘extra-spectral neutral point’ in the red-violet region (near dominant wavelength of 499 nm). Co-expressing the L-opsin transgene within a subset of endogenous M-cones shifted their spectral sensitivity to respond to long wavelength light, thus producing two distinct cone types absorbing in the middle-to-long wavelengths, as required for trichromasy. These results demonstrate that gene therapy changed the spectral sensitivity of a subset of the cones, and the results further demonstrate the unexpected result that adult monkeys gained new color vision capacities because of the gene therapy.
(61) In a further preferred embodiment of all of the above embodiments, the primate has a vision disorder in which its photoreceptors are healthy, such as color blindness. As used herein, “healthy” means that the cells being treated are functioning but simply do not provide for the desired color perception, in contrast to gene therapy in which the target cells are degenerating or dying. The studies reported herein are the first to use gene therapy in primates to address a vision disorder in which all photoreceptors are intact and healthy, making it possible to assess the full potential of gene therapy to restore visual capacities. The methods of the present invention thus will allow many opportunities for functions to be added or restored in the eye.
(62) In a further preferred embodiment that can be combined with any of the other embodiments herein, the primate is an adult primate, such as an adult human (ie: at least 16 years of age; preferably at least 18 years of age or 21 years of age). Classic visual deprivation experiments have led to the expectation that neural connections established during development would not appropriately process an input that was not present from birth. Therefore, it was believed that treatment of congenital vision disorders would be ineffective unless administered to the very young. The present study thus provides significantly unexpected results in curing a visual disorder in an adult primate.
(63) Those of skill in the art will readily appreciate, based on the teachings herein, the variety of treatment modalities that can be accomplished using the methods of the invention. In one embodiment, the gene encodes ABCR and is administered to the eye of a primate with Stargardt disease. In other embodiments, the gene encodes:
(64) one or more of polypeptides “e”-“l” and “s”-“y” in Table 1, and is administered to the eye of a primate with retinitis pigmentosa;
(65) polypeptide “m” in Table 1, and is administered to the eye of a primate with incomplete achromatopsia;
(66) polypeptide “z” in Table 1, and is administered to the eye of a primate with Leber congenital amaurosis;
(67) polypeptide “n” in Table 1, and is administered to the eye of a primate with cone dystrophy, such as cone dystrophy type 4;
(68) polypeptide “o” in Table 1, and is administered to the eye of a primate with retinal cone dystrophy, for example, retinal cone dystrophy type 3A;
(69) polypeptide “p” in Table 1, and is administered to the eye of a primate with cone-rod dystrophy;

(70) polypeptide “q” in Table 1, and is administered to the eye of a primate with achromatopsia; and/or
(71) polypeptide “r” in Table 1, and is administered to the eye of a primate with total color blindness, for example, a native of the Pingelapese Islands.
(72) Any suitable means for delivery of the gene therapy vector to the eye can be used, including but not limited to administering in a contact lens fluid, contact lens cleaning and rinsing solutions, eye drops, surgical irrigation solutions, ophthalmological devices, injection, iontophoresis, topical instillation on the eye, and topical instillation. The topical instillation can be administered, for example, in the form of a liquid solution, a paste, of a hydrogel. The topical instillation can be embedded, for example, in a foam matrix or supported in a reservoir. The injection into the primate eye can be, for example, an intracameral injection, an intracorneal injection, a subconjonctival injection, a subtenon injection, a subretinal injection, an intravitreal injection, and an injection into the anterior chamber.
(73) The primate's progress in response to the treatment may be monitored by any suitable means. In embodiments where the methods are used to treat color blindness, monitoring or progress may comprise, for example, use of standard color vision tests, or the wide-field color multifocal electroretinogram (mf-ERG) system described below to detect spectral sensitivity shifts in the primate's vision. Thus, in another aspect, the present invention provides methods for use of the electroretinogram disclosed herein for monitoring changes in vision perception, such as color perception, of a primate undergoing gene therapy to treat a visual disorder. All embodiments of the methods disclosed above can be combined with all embodiments of the electroretinogram disclosed below.
(74) In a second aspect, the present invention provides isolated nucleic acid expression vectors comprising:
(75) (a) a promoter region, wherein the promoter region is specific for primate retinal cone cells; and
(76) (b) a gene encoding a therapeutic, wherein the gene is operatively linked to the promoter region.
(77) All terms in this second aspect have the same meaning as disclosed above for the first aspect of the invention. Similarly, all embodiments and combinations thereof disclosed above in the first aspect of the invention can be used in this second aspect of the invention. The inventors have demonstrated effective treatment of congenital vision disorders in adult primates using the recombinant vectors of the invention, a result that is completely unexpected in the art.
(78) Any suitable promoter region can be used in the isolated nucleic acid expression vector, so long as it specifically promotes expression of the gene in retinal cone cells. In a preferred embodiment, the promoter specifically promotes expression of the gene in Catarrhini retinal cone cells; even more preferably in human retinal cone cells. Exemplary suitable promoter regions include the promoter region for any cone-specific gene, such as the L opsin promoter (SEQ ID NO: 1), the M opsin promoter (SEQ ID NO: 2), and the S opsin promoter (SEQ ID NO: 3), or portions thereof suitable to promote expression in a cone-specific manner.
(79) In a preferred embodiment, the isolated nucleic acid expression vector further comprises an enhancer element upstream of the promoter, wherein the gene is operatively linked to the enhancer element. Any suitable enhancer element can be used in the gene therapy vectors, so long as it enhances expression of the gene when used in combination with the promoter. In a preferred embodiment, the enhancer element is specific for retinal cone cells; more preferably, it is specific for primate retinal cone cells; more preferably in Catarrhini retinal cone cells; even more preferably in human retinal cone cells. Exemplary suitable enhancer regions comprise or consist of the enhancer region for any cone-specific gene, such as the L/M minimal opsin enhancer (SEQ ID NO: 51), L/M enhancer elements of 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1100, 1200, 1300, 1400, 1500, 1600, 1700, 1800, 1900, 2000, or more nucleotides that comprise one or more

copies of the L/M minimal opsin enhancer, and the full L/M opsin enhancer (SEQ ID NO: 4), or other portions thereof suitable to promote expression in a cone-specific manner.

(80) The length of the promoter and enhancer regions can be of any suitable length for their intended purpose, and the spacing between the promoter and enhancer regions can be any suitable spacing to promote cone-specific expression of the gene product. In various preferred embodiments, the enhancer is located 0-1500; 0-1250; 0-1000; 0-750; 0-600; 0-500; 0-400; 0-300; 0-200; 0-100; 0-90; 0-80; 0-70; 0-60; 0-50; 0-40; 0-30; 0-20; or 0-10 nucleotides upstream of the promoter. The promoter can be any suitable distance upstream of the encoded gene.

(81) In a further preferred embodiment that can be combined with any other embodiment in any aspect of the present invention, the enhancer comprises or consists of a sequence selected from the group consisting of the L/M minimal opsin enhancer (SEQ ID NO: 51), L/M enhancer elements of 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1100, 1200, 1300, 1400, 1500, 1600, 1700, 1800, 1900, 2000, or more nucleotides that comprise one or more copies of the L/M minimal opsin enhancer, and the full L/M opsin enhancer (SEQ ID NO: 4), or other portions thereof suitable to promote expression in a cone-specific manner, and the promoter comprises or consists of a sequence selected from the group consisting of L opsin promoter (SEQ ID NO: 1), the M opsin promoter (SEQ ID NO: 2), and the S opsin promoter (SEQ ID NO: 3).

(82) In a further preferred embodiment, the isolated nucleic acid expression vector further comprises an intron comprising a splice donor/acceptor region, wherein the intron is located downstream of the promoter region and is located upstream of the gene. Any intron can be used, so long as it comprises a splice donor/acceptor region recognized in primate cone cells, so that the intron can be spliced out of the resulting mRNA product. In one embodiment, the intron comprises or consists of an SV40 intron according to SEQ ID NO: 5. In various preferred embodiments, the 3′ end of the intron is 0-20; 0-15; 0-10; 0-9; 0-8; 0-7; 0-6; or 0-5 nucleotides upstream of the gene, and its 5′ end is 0-20; 0-15; 0-10; 0-9; 0-8; 0-7; 0-6; or 0-5 nucleotides downstream of the proximal promoter region.

(83) The gene is operatively linked to the promoter region and the enhancer element, such that the promoter and enhancer elements are capable of driving expression of the gene or cDNA in cone cells of the subject.

(84) The gene encoding a therapeutic to be expressed in the cone cells can be any gene or cDNA that encodes a polypeptide or RNA-based therapeutic (siRNA, antisense, ribozyme, shRNA, etc.) that can be used as a therapeutic for treating a cone cell disorder, or as a means to otherwise enhance vision, including but not limited to promoting tetrachromatic color vision. In various preferred embodiments, the gene encodes a therapeutic protein comprising or consisting of those disclosed above in the methods of the first aspect of the invention.

(85) In a further preferred embodiment, the vector comprises the sequence shown in SEQ ID NO: 50, which details the vector used in at least some of the examples that follow.

(86) In a further preferred embodiment that can be combined with any of the above embodiments, the gene delivery vector comprises a recombinant adeno-associated virus (AAV) gene delivery vector. In a further preferred embodiment, the AAV vector comprises rAAV2/5. Preferably, the rAAV is replication defective, in that the AAV vector cannot independently further replicate and package its genome. For example, when cone cells are transduced with rAAV virions, the gene is expressed in the transduced cone cells, however, due to the fact that the transduced cone cells lack AAV rep and cap genes and accessory function genes, the rAAV is not able to replicate.

(87) In a third aspect, the present invention provides a formulation comprising packaged viral particles containing the nucleic acid expression vectors of the second aspect of the invention. In one preferred embodiment, viral particles are present in a concentration of at least 10.sup.10 vector genome containing particles per mL; in various preferred embodiments, the viral particles are delivered in a concentration of at least 7.5×10.sup.10; 10.sup.11; 5×10.sup.11; 10.sup.12; 5×10.sup.12; 10.sup.13; 1.5×10.sup.13; 3×10.sup.13; 5×10.sup.13; 7.5×9×10.sup.13; or

9'10.sup.13 vector genome containing particles per mL. The formulation may further comprise pharmaceutically-acceptable carriers, diluents and reagents as described above in the first aspect of the invention. The formulation may be in the form of a liquid solution, a paste, a hydrogel, or may be embedded within a substrate, including but not limited to a foam matrix or supported in a reservoir.

(88) In a fourth aspect, the present invention provides recombinant host cells transfected or transduced with the nucleic acid expression vector of the second aspect of the invention. The cells may be of any type that can be transfected with the expression vector. In one embodiment where the expression vector is a rAAV vector, the cells comprise producer cells transduced with a replication incompetent rAAV expression vector according to the second aspect of the invention, form which viral particles can be obtained by introduction of an AAV helper construct as described above and as is well known in the art.

(89) In a fifth aspect, the present invention provides a color multifocal electroretinogram system, comprising:

(90) (a) an electroretinogram (ERG) comprising (i) a recording electrode that is (A) designed for placement on at least one of a cornea and a sclera of at least one eye of a subject and (B) arranged to output at least one signal generated by the at least one eye; and (ii) a computing system communicatively coupled to the recording electrode, the computing system comprising (A) at least one processor and (B) data storage containing instructions executable by the at least one processor to carry out a set of functions, the set of functions including processing and saving the at least one signal generated by the at least one eye;

(91) (b) a retinal stimulator comprising matched light sources selected from the group consisting of red, green, blue, and ultraviolet light sources, wherein the matched light sources are connected to the ERG and in operation can be independently frequency modulated at rates between about 1 Hz and about 60 Hz, inclusive, wherein the stimulator in operation is capable of stimulating a retinal field of a subject throughout an operating radius of at least about 70 degrees;

(92) (c) one or more constant current integrated circuit chips arranged to drive the stimulator; and

(93) (d) a pulse-frequency modulator connected to the retinal stimulator, wherein in operation the pulse-frequency modulator is capable of controlling individual stimulator segments while keeping relative spectral content of the light constant.

(94) The electroretinograms of the present invention can be used, for example, in characterizing the topography of expression of the different opsin transgenes in the eyes of living subjects treated with gene therapy, and thus can be used with the gene therapy methods of the invention disclosed above.

(95) As used here, a “matched” light source is one that includes light stimulus of different wavelengths, wherein the number of pixels is approximately the same, or is the same, at each wavelength. One non-limiting example is a matched light source stimulus containing 1024 doublet pixels each containing a red (653 nm, half-bandwidth 22 nm) and a green (527 nm, half-bandwidth 33 nm) LED, with a resulting matched light source with 2,048 paired green and red LEDs.

(96) In various preferred embodiments, the stimulator in operation is capable of stimulating a retinal field of a subject throughout an operating radius of at least about 80, 90, 100, 110, 120, 130, 140, 150, or more degrees.

(97) In one preferred embodiment, the matched light sources are paired red and green light sources. In another preferred embodiment, the matched light sources are triplets of red, green, and blue light sources. In a further preferred embodiment, the matched light sources are quartets of red, green, blue, and ultraviolet light sources.

(98) Any suitable matched light source can be used. In one preferred embodiment, the matched light sources comprise matched light emitting diodes (LEDs).

(99) In another preferred embodiment, that can be combined with any of the embodiments herein, the retinal stimulator comprises a concave surface comprising a series of trapezoidal-shaped circuit

boards placed edge-to-edge, wherein the concave surface positions the matched light sources so in operation they are held equidistantly from and pointing toward a single focal point where a subject's pupil can be positioned. This embodiment helps to limit SNR fall-off in peripheral retinal regions. Any suitable concave surface can be used; in a preferred embodiment, the concave surface comprises a geodesic dome.

(100) In a further embodiment that can be combined with any of the above embodiments, the set of functions executable by the processor further comprises coding and decoding topographical regions on the recording electrode using a cyclic summation technique.

(101) In a further preferred embodiment that can be combined with any of the embodiments herein, the ERG further comprises an amplifier; and wherein the computing system is communicatively coupled to the amplifier.

(102) Further embodiments and details of the color multifocal electroretinogram system are provided in the Examples that follow.

(103) In a further aspect, the present invention provides methods for determining a location of functioning opsin expression in a subject, comprising use of the mf-ERG of any embodiment or combination of embodiments of the fifth aspect of the invention, wherein the recording electrode is placed on at least one of a cornea and a sclera of at least one eye of a subject; stimulating the subject's retinal field with the retinal stimulator; and determining responses of different areas of the subject's retina to different stimulation frequencies to generate a map of retinal responses, wherein the map provides a location of functioning opsin expression in a subject. In one preferred embodiment, the subject has been treated according to the gene therapy methods for color blindness disclosed above according to any embodiment or combination of embodiments of the first aspect of the invention.

(104) Unless the context clearly dictates otherwise, embodiments in one aspect of the invention may be used in other aspects of the invention, and can be combined with each other.

EXAMPLE 1

(105) Red-green colour blindness, which results from the absence of either the long- (L) or middle- (M) wavelength-sensitive visual photopigments, is the most common single locus genetic disorder. Here, the possibility of curing colour blindness using gene therapy was explored in experiments on adult monkeys that had been colour blind since birth. A third type of cone pigment was added to dichromatic retinas, providing the receptoral basis for trichromatic colour vision. This opened a new avenue to explore the requirements for establishing the neural circuits for a new dimension of colour sensation. Classic visual deprivation experiments.sup.1 have led to the expectation that neural connections established during development would not appropriately process an input that was not present from birth. Therefore, it was believed that treatment of congenital vision disorders would be ineffective unless administered to the very young. Here, however, addition of a third opsin in adult red-green colour-deficient primates was sufficient to produce trichromatic colour vision behaviour. Thus, trichromacy can arise from a single addition of a third cone class and it does not require an early developmental process. This provides a positive outlook for the potential of gene therapy to cure adult vision disorders.

(106) Gene therapy was performed on adult squirrel monkeys (*Saimiri sciureus*) that were missing the L opsin gene. In this species, some females have trichromatic colour vision while males are red-green colour blind.sup.2. Serotype 2/5 recombinant adeno-associated virus (rAAV) containing a human L-opsin gene under control of the L/M opsin enhancer and promoter (FIG. **1***a*) was delivered to the photoreceptor layer via subretinal injections. Transcriptional regulatory elements were chosen to direct expression preferentially in M cones, but not short- (S) wavelength-sensitive cones or rods.sup.3. To provide the receptoral basis for trichromacy, animals received three 100 μL injections (containing a total of 2.7×10.sup.13 viral particles) in each eye which produced a relatively uniform, third submosaic of approximately 15-36% of M cones that coexpressed the transgene (FIG. **1***e, f*).

(107) Prior to treatment, monkeys were trained to perform a computer-based colour vision test, the Cambridge Colour Test.sup.4,5, which was modified for use with animals.sup.6 (FIG. **2***a*). Dichromats who are missing either the L- or M-photopigment fail to distinguish from grey: colours near the so-called "spectral neutral point" located in the blue-green region of colour space (near dominant wavelength (DW) 490 nm) and complementary colours near the "extra-spectral neutral point," in the red-violet region (near DW=-499 nm). While trichromats have four main hue percepts—blue, yellow, red, and green—dichromats have only two percepts, nominally blue and yellow. Before treatment, two dichromatic monkeys completed three colour vision tests consisting of 16 hues (FIG. **2***b, c*). Four-to-six months was required to test all 16 hues; thus, baseline results represent testing conducted for more than a year. As predicted, prior to treatment monkeys had low thresholds (averaging <0.03 units in u′, v′ colour space) for colours that represent blues and yellows to their eyes, but always failed to discriminate the blue-green (DW=490 nm) and red-violet hues (DW=−499 nm) with thresholds extrapolated from psychometric functions being orders of magnitude higher (FIG. **2***b, c*). Results were highly repeatable, with no improvement between the first and third tests, making us confident that animals would not spontaneously improve in the absence of treatment.

(108) Co-expressing the L-opsin transgene within a subset of endogenous M-cones shifted their spectral sensitivity to respond to long wavelength light, thus producing two distinct cone types absorbing in the middle-to-long wavelengths, as required for trichromacy. The spectral sensitivity shift was readily detected using a custom-built wide-field colour multifocal electroretinogram (mf-ERG) system (FIG. **1***b, c, f*) (see ref 7 for details). In preliminary experiments, validity of the colour mf-ERG was tested using an animal that had received a mixture of the L-opsin-coding virus plus an identical virus, except that a green fluorescent protein (GFP) gene replaced the L-opsin gene. As reported previously, faint GFP fluorescence was first detected at 9 weeks post-injection, and it continued to increase in area and intensity through 24 weeks.sup.8. While faint signs of GFP were first detectable at 9 weeks, L-opsin levels sufficient to produce suprathreshold mf-ERG signals were still not present at 16 weeks post-injection (FIG. **1***c*, inset). After GFP fluorescence became robust, the red light mf-ERG, which indicates responses from the introduced L-opsin, showed highly elevated response amplitudes in two areas (FIG. **1***c*) corresponding to locations of subretinal injections (FIG. **1***d*).

(109) The two dichromatic monkeys who participated in behavioural tests of colour vision were treated with only L-opsin-coding virus. While the elongated pattern produced by two injections in FIG. **1***c* and *d* allowed mf-ERG validation, the treatment goal was to produce a homogeneous region, as resulted from 3 injections shown in f, where the highest mf-ERG response covered about 80° of central retina, roughly the area for which humans have good red-green discrimination. These results demonstrate that gene therapy changed the spectral sensitivity of a subset of the cones. A priori, there were two possibilities for how a change in spectral sensitivity might change colour vision behaviour: 1) animals may have an increase in sensitivity to long-wavelength light, but if the neural circuitry for extracting colour information from the nascent "M+L cone" submosaic was absent, they would remain dichromatic, the hallmark of which is having two hues that are indistinguishable from grey (FIG. **2***d*). The spectral neutral point for individuals that have only S- and M-cones, (e.g. monkeys 1 and 2 pre-therapy), occurs near dominant wavelength (DW)=495 nm. At the limit, an increase in spectral sensitivity would shift the monkeys' neutral point toward that of individuals with only S and L cones, near DW=505 nm (dashed blue lines, FIG. **2***d*). 2) The second, more engaging possibility was that treatment would be sufficient to expand sensory capacity in monkeys, providing them with trichromatic vision. In this case, the animals' post-therapy results would appear similar to FIG. **2***e*, obtained from a trichromatic female control monkey.

(110) Daily testing continued after treatment. After about 20 weeks post-injection (arrow, FIG. **3***a*), the trained monkeys' thresholds for blue-green and red-violet (DWs=490 and −499 nm,

respectively, FIG. **3***b*, *c*) improved, reducing to an average of 0.08 units in u′, v′ colour space, indicating that they gained trichromatic vision. This time point corresponded to the same period in which robust levels of transgene expression were reported in the squirrel monkey.sup.8. A trichromatic female monkey and untreated dichromatic monkeys were tested in parallel. As expected, the female had low thresholds for all colours, averaging <0.03 units in u′, v′ colour space, but the untreated dichromats always failed to discriminate DWs=490 nm (triangle, FIG. **3***a*) and −499 nm, indicating a clear difference between treated and untreated monkeys.

(111) Early experiments in which we obtained negative results served as “sham controls,” demonstrating that acquiring a new dimension of colour vision requires a shift in spectral sensitivity that results from expression of an L pigment in a subset of M cones. Using similar subretinal injection procedures, we delivered fewer viral particles of an L-opsin-coding rAAV2/5 virus with an extra 146 base pair (bp) segment near the splice donor/acceptor site that had been carried over from the cloning vector and that was absent in the GFP-coding rAAV2/5 virus. The 146 bp segment contained an ATG and a duplicate mRNA start site that may have interfered with expression (see Full Methods online). Three monkeys received injections of this vector, containing an average of 1.7×10.sup.12 virus particles per eye, and no reliable changes in spectral sensitivity were measured using the ERG. One animal was also tested behaviourally and his colour vision was unchanged from baseline 1 year after injection. In subsequent experiments reported here, we removed the extra 146 bp segment and also increased the amount of viral particles delivered per eye by approximately 16-fold, to 2.7×10.sup.13. Negative results from earlier injections demonstrated that the subretinal injection procedure itself does not produce changes in the ERG or in colour vision.

(112) The change in spectral sensitivity measured with the mf-ERG is necessary but not sufficient to produce a new colour vision capacity. For example, individuals with L but no M cones (termed deuteranopes) have a relatively enhanced sensitivity to red light but they are still as dichromatic as individuals with M but no L cones (protanopes), in that they are unable to distinguish particular “colours” from grey. To verify that the behavioural change observed in animals expressing the L pigment transgene was not purely a shift in spectral sensitivity (see FIG. **2***d*), monkey 1 was also tested on DWs=496 and 500 nm, and monkey 2 was tested on DWs 496 and 507 nm. Together, these DWs span the possible confusion points for deuteranopes and protanopes and for any intermediate dichromatic forms that could arise from expressing combinations of L and M pigments. As shown in FIG. **3***b* and *c*, both monkeys' measured thresholds for these additional hues were similar to their thresholds for DW=490 nm, demonstrating they now lacked a spectral neutral point and have become truly trichromatic. Furthermore, treated monkeys were able to discriminate blue-green (DW=490 nm) when it was tested against a red-violet background (DW=−499 nm), instead of the grey background, indicating that the monkeys' newly-acquired “green” and “red” percepts were distinct from one another. The treated monkeys' improvement in colour vision has remained stable for over 2 years and we plan to continue testing the animals to evaluate long term treatment effects.

(113) Classic experiments in which visual deprivation of one eye during development caused permanent vision loss.sup.1 led to the idea that inputs must be present during development for the formation of circuits to process them. From the clear change in behaviour associated with treatment, compared both between and within subjects, we conclude that adult monkeys gained new colour vision capacities because of gene therapy. These startling empirical results provide insight into the evolutionary question of what changes in the visual system are required for adding a new dimension of colour vision. Previously, it seemed possible that a transformation from dichromacy to trichromacy would require evolutionary/developmental changes, in addition to acquiring a third cone type. For example, L and M opsin-specific genetic regulatory elements might have been required to direct the opsins into distinct cone types.sup.9 that would be recognized by L and M cone-specific retinal circuitry.sup.10, and to account for cortical processing, multi-stage

circuitry.sup.11 might have evolved specifically for the purpose of trichromacy. However, our results demonstrate that trichromatic colour vision behaviour requires nothing more than a third cone type. As an alternative to the idea that the new dimension of colour vision arose by acquisition of a new L vs. M pathway, it is possible that it exploited the pre-existing blue-yellow circuitry. For example, if addition of the third cone class split the formerly S vs. M receptive fields into two types with differing spectral sensitivities, this would obviate the need for neural rewiring as part of the process of adopting new colour vision.

(114) Some form of inherent plasticity in the mammalian visual system can be inferred from the acquisition of novel colour vision, as was also demonstrated in genetically engineered mice.sup.12; however, the point has been made that such plasticity need not imply that any rewiring of the neural circuitry has occurred.sup.13. Similarly, given the fact that new colour vision behaviour in adult squirrel monkeys corresponded to the same time interval as the appearance of robust levels of transgene expression, we conclude that rewiring of the visual system was not associated with the change from dichromatic to trichromatic vision.

(115) Treated adult monkeys unquestionably respond to colours previously invisible to them. The internal experiences associated with the dramatic change in discrimination thresholds measured here cannot be determined; therefore, we cannot know whether the animals experience new internal sensations of “red” and “green.” Nonetheless, we do know that evolution acts on behaviour, not on internalized experiences, and we suggest that gene therapy recapitulated what occurred during evolution of trichromasy in primates. These experiments demonstrate that a new colour vision capacity, as defined by new discrimination abilities, can be added by taking advantage of pre-existing neural circuitry and, internal experience aside, full colour vision could have evolved in the absence of any other change in the visual system except the addition of a third cone type.

(116) Gene therapy trials are underway for Leber's congenital amaurosis.sup.14-16. Thus far, treatment has been administered to individuals who have suffered retinal degeneration from the disease. The experiments reported here are the first to use gene therapy in primates to address a vision disorder in which all photoreceptors are intact and healthy, making it possible to assess the full potential of gene therapy to restore visual capacities. Treatment allowing monkeys to see new colours in adulthood provides a striking counter-example to what occurs under conditions of monocular deprivation. For instance, it is impossible to restore vision in an adult who had grown up with a unilateral cataract. Future technologies will allow many opportunities for functions to be added or restored in the eye. While some changes may produce outcomes analogous to monocular deprivation, we predict that others, like gene therapy for red-green colour blindness, will provide vision where there was previously blindness.

Methods Summary for Example 1

(117) Viral vector. CHOPS2053 was a 2.1 kb fragment containing the locus control region (LCR) and proximal promoter (PP) upstream of the human X-chromosome opsin gene array.sup.9,17. These elements (also known as pR2.1) have been shown to target transgene expression to mammalian L/M cones.sup.3,18. RHLOPS was a 1.2 kb fragment containing recombinant human L opsin cDNA. A clone of the human L opsin cDNA.sup.19, known as hs7, was generously provided by J. Nathans. The QuickChange kit (Stratagene) was used to convert codon 180 so that it would encode a human L pigment maximally sensitive to 562 nm.sup.20. The virus was made using the genome from rAAV serotype 2 and the capsid from serotype 5, and the preparation had 9×10.sup.13 DNase-resistant vector genome containing particles per mL. To prevent vector aggregation, 0.014% Tween 20 was added to the final vector preparation. A total of 2.7×10.sup.13 viral particles was injected per eye.

(118) An earlier version of the L-opsin coding rAAV2/5 used in previous unsuccessful experiments contained an extra 146 base pair segment between the splice donor/acceptor site and the translational start codon of the L-opsin gene that had been carried over from the cloning vector. Because we were concerned that this fragment may have interfered with transgene expression, a

second version of L-opsin rAAV2/5 in which the extra 146 bp had been removed was used in later experiments described here. In addition to modifying the vector, we also increased the amount of viral particles delivered per eye by approximately 16-fold, from 1.7×10.sup.12 to 2.7×10.sup.13. Thus, we cannot conclude from this set of experiments what exact titer of viral particles was required to produce the effects on color vision behaviour, or exactly what effects, if any, the extra 146 bp had on transgene expression in earlier unsuccessful attempts.

(119) The single-stranded DNA genome of conventional rAAV vectors, including rAAV2/5 used here, is devoid of Rep coding sequences. Thus, the vector genome is stabilized predominantly in an episomal form; however, the potential for integration exists.sup.21. According to NIH guidelines, the viral vector used here is rated biosafety level 1 (BSL1), and animal biosafety level 1 (ABSL1) meaning no special precautions were required in handling the virus or animals treated with the virus. Following treatment, squirrel monkeys had an increase in AAV antibody titers, ranging from 4-12 fold. Antibody titers remained unchanged in untreated control animals who were housed with treated animals.

(120) Subretinal Injections. Subretinal injections were performed by a vitreo-retinal surgeon (T. B. C.) using a KDS model 210 syringe pump under a stereomicroscope. A 500 μL Hamilton Gastight (#1750TTL) Luer Lock syringe was connected to 88.9 cm of 30 gauge teflon tubing with male Luer Lock adapters at both ends (Hamilton 30TF double hub), which was then connected to a 30 gauge Becton Dickinson Yale regular bevel cannula (ref #511258) that was manually bent to produce a 135° angle 1.5 mm from the tip. All components were sterilized prior to use. The syringe and tubing were filled with sterile lactated Ringers solution to produce a dead volume of approximately 210 μL. Just prior to injection, 300 μL of rAAV was withdrawn using a rate of 100 μL/min.

(121) Squirrel monkeys were anesthetized using intramuscular injections of ketamine (15 mg/kg) and xylazine (2 mg/kg); atropine (0.05 mg/kg) was also given to reduce airway secretions. The eye was dilated with 2-3 drops of tropicamide (1%) and treated with 1 drop each of betadine (5%), vigamox (0.5%), and proparacaine (1%). Subconjunctival injection of 0.1 mL of lidocaine (2%) was given and the anterior portion of the eye was exposed by performing a temporal canthotomy followed by limited conjuntival peritomy. Eyelids were held open with a speculum designed for premature infants. A temporal sclerotomy was made 1 mm posterior to the limbus with a 27 gauge needle, through which the injection cannula was inserted. Three subsequent 100 μL injections were made at different subretinal locations using an infusion rate of 1060 μL/min. Post-procedure, 0.05 mL each of decadron (10 mg/mL), kenalog (40 mg/mL), and cephazolin (100 mg/mL) were injected subconjunctivaly; 1 drop each of betadine (5%) and vigamox (0.5%) and a 0.6 cm strip of tobradex (0.3% tobramycin, 0.1% dexamethasone) ointment were applied topically; 10-20 mL of subcutaneous fluids (sterile lactated Ringers) were also given. Subsequent administration of steroids and analgesics were administered as needed post-procedure for potential inflammation or discomfort.

(122) Confocal Microscopy. The animal in FIG. **1***c* and *d* succumbed to respiratory illness, unrelated to gene therapy, approximately 2 years and 3 months post-injection. The retina was fixed in 4% paraformaldehyde in phosphate buffered saline (PBS), and rinsed in PBS with 10% and 30% sucrose. It was sequentially incubated with 10% Normal Donkey Serum, rabbit monoclonal antibody to M/L opsin (Chemicon AB5405), and a Cy3 (red) conjugated donkey anti-rabbit antibody (Jackson Immunoresearch). Confocal images were analyzed using ImageJ (rsbweb.nih.gov). In the middle panel of FIG. **1***e*, magenta dots mark cone locations, and the red anti-M/L-opsin antibody staining was removed to show GFP-expressing (green) cells more clearly.

(123) Behavioural Colour Vision Assessment. A three-alternative forced-choice paradigm in which position and saturation of the stimulus was randomized between trials was used. Monkeys had to discriminate the location of a coloured patch of dots that varied in size and brightness, surrounded by similarly varying grey dots. When animals touched the coloured target, a positive tone sounded

and a juice reward was given; the next stimulus appeared immediately. (The squirrel monkey shown in FIG. **2***c* is drinking a reward from a previous trial.) If the wrong position was chosen, a negative tone sounded, and a 2-3 sec "penalty time" occurred before the next trial.

(124) For each hue, monkeys were tested on up to 11 different saturations ranging from 0.01 to 0.11 in u′, v′ colour space (CIE 1976) and a threshold was calculated, which was taken as the saturation required to reach a criterion of 57% correct, the value determined to be significantly greater than chance (33% correct, P=0.05); see ref. 6 for full details.

References for Example 1

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Example 2: Description and Validation of new LED-based, Wide-Field, Color mf-ERG

(126) The electroretinogram (ERG) is an electrophysiologic recording technique used to measure electrical activity of the entire retina. The electrochemical potential of retinal cells change in response to light, which in turn induces voltage on an electrode placed on the cornea and/or sclera. The first ERGs were recorded by a Swedish physiologist working in the mid-1800's in amphibian

retina. Since this time the technique has been widely incorporated in clinical practice as a diagnostic tool. Marriage of physiology to engineering has led to a variety of stimuli and analysis paradigms which can tease out specific cellular responses or region specific information. The latter has been motivated by the fact that the topographical organization of the retina plays an important role in disease with different diseases being characterized by different affected retinal areas. Early attempts to evaluate the function of specific retinal regions using the ERG illuminated only a small patch of retina, however, such “focal ERGs” have the drawback that light reflected from the focal area onto other retinal regions produces an ERG response that cannot be uncoupled from that produced by the region of interest. The other problem is that obtaining any type of a topographical map of retinal function using the mono-focal approach is prohibited by the time required to obtain ERGs serially from many different retinal regions. Both problems have been solved with the development of the multifocal- (mf-) ERG, pioneered by Erich Sutter in the early 1990s (Sutter, 1991). Mf-ERGs perform a series of individual focal ERG experiments simultaneously by taking advantage of either (1) correlation techniques or (2) frequency encoding. In this way, a complete topographical map of electrical responses over a large retinal region is obtained in a relatively short period of time.

(127) The typical ERG apparatus for mf-ERGs stimulates a hexagonal patch of retina with a 20 degree radius and uses video display. More recently a display that employs white light emitting diodes (LEDs) was designed for use with a frequency encoding method for obtaining mf-ERGs. Having a stimulus that could reach further into the periphery would be useful in the early detection of retinal diseases that progress from peripheral to central retina. Additionally, individuals with normal trichromatic color vision express three distinct photopigments, or opsins, in separate classes of cone photoreceptor: short- (S-), middle- (M-), and long- (L-) wavelength sensitive. The S-cones are maximally sensitive to short wavelengths of light near 420 nm; M-cones have their maximal response near 530 nm; and the L-cones are most sensitive near 560 nm. Thus, an mf-ERG stimulus containing LEDs of different wavelengths would have applications in characterizing the topography of expression of the different opsin transgenes in the eyes of living subjects treated with gene therapy.

(128) In particular, in gene therapy treatments administering recombinant adeno-associated virus (rAAV) carrying a human L-opsin gene M-opsin gene or S-opsin gene to primates that have two cone types to produce trichromatic color vision. As such, a non-invasive objective method is needed to determine the locations of functioning opsin expression in vivo.

(129) Here we describe a wide-field color mf-ERG capable of stimulating a radius greater than 70 degrees. The wide field mf-ERG has a colored LED-based stimulus that incorporates a new design capable of maintaining viable signal-to-noise ratio (SNR) out into the far peripheral retina.

(130) mf-ERG and Stimulus Panel

(131) An LEDs as a light source was chosen because new advancements in LED technology allow for a large number of focused photons to be emitted from a point source. Additionally, LEDs are available in a variety of single peak narrow-bandwidth packages. The stimulus contained 1024 doublet pixels each containing a red (653 nm, half-bandwidth 22 nm, FIG. **2**.**1***c*) and a green (527 nm, half-bandwidth 33 nm, FIG. **2**.**1***d*) LED. Thus, the new display had 2,048 paired green and red LEDs. LEDs have inherent manufacturing variations in their breakdown resistance. Since the amount of current is proportional to the number of photons per unit time, applying a constant voltage across the LEDs would result in varying photon output. To circumvent these issues and ensure repeatability and linearity, constant current integrated circuit chips (Allegro A6276) were used to drive the LEDs (FIG. **2**.**1***b*). These devices are designed to maintain constant current despite fluctuations in anode voltage. To prevent variation in peak wavelength over varied intensities, a pulse frequency modulation (PFM) signal was used (Swanson, Ueno, Smith, & Pokorny, 1987).

(132) Using red and green LEDs, it is possible to isolate responses of L- or M-cones using silent substitution. Integrating the spectral composition of the green LED with the spectral sensitivity

curves of the human M- and L-photopigments (FIG. **2**.**1***e*) yields that approximately equal quanta are caught by both photopigments (FIG. **2**.**1***f*). In contrast, the red LED is six times more effective at stimulating the L photopigment than it is the M (FIG. **2**.**1***f*). Isolation of responses that are due to transduction from an introduced L-opsin transgene, M-opsin transgene or S-opsin transgene are straightforward in primates because red, blue or green LEDs can be chosen that are much more effective for the L-opsin M-opsin or S-opsin transgene respectively than for the endogenous pigments of the untreated dichromatic primates. In the case of Squirrel monkeys, they have S-cones maximally sensitive near 430 nm, and they can express any of several variants of middle-to-long wavelength opsin including L or M. The monkey used in validation experiments had M-cones sensitive near 532 nm, in addition to his S-cones. Thus, following the administration of the L-opsin transgene via subretinal injection, the mf-ERGs of squirrel monkey were predicted to show elevations in mf-ERG amplitude to red light in regions corresponding areas of the retina transduced, and distal areas would show progressively lower amplitudes to the L-cone isolating stimulus.

(133) Resolution of the stimulator was 1024 LED over a larger stimulating area of about 150 degrees of visual field. Sensitivity of the measurement can be increased by summing more retinal responses per unit area and by using ultra-bright LEDs. Other new techniques that were used to prevent peripheral SNR fall-off included the use of trapezoidal shaped printed circuit boards that when placed edge-to-edge created a geodesic dome (FIG. **2**.**1***a*). The advantage of this design was that LEDs were held equidistantly from and pointing toward a single focal point where the subject's pupil was positioned. Any variation in directionality caused by imperfect sphericity was corrected by aiming each LED individually. In the typical usage, LEDs from areas of the dome shaped stimulator were summed so that there were 37 individual segments, which together subtended the 150° of visual angle, thus allowing a wide-field functional map of an area covering almost the entire retina to be produced.

(134) There are two mathematical methodologies available to code and decode the topographical regions on the recording electrode: One is a cross-correlation technique called m-sequence and the other is a frequency encoding technique called cyclic summation. Cyclic summation is preferred in our application because it has been empirically shown to provide higher signal-to-noise ratio than m-sequence (Lindenberg, Horn, & Korth, 2003). Also, cyclic summation cannot be done using any kind of conventional CRT or LCD video display. Cyclic summation requires independent control of every segment of the display. Conventional video displays update the entire screen with every video frame typically at 60 Hz. In cyclic summation, each segment being analyzed is updating at a slightly different frequency. Typically, the “center frequency” is 30 Hz but each segment fractionally different from 30 Hz, i.e., 30.00 Hz, 30.10 Hz, 30.20 . . . etc. In the analysis, responses to different areas of the retina to different segment frequencies are used to generate a map of retinal responses which are read out as electrophysiological “activity.” For example, after gene therapy using an L opsin gene in a primate retina containing only M and S cones, areas of retina that express the newly introduced L opsin will have higher electrical activity in response to the red LED light relative to the green light than areas of the retina not expressing the transgene. This allows the effectiveness of the gene therapy in terms of areas responding their time course to be monitored with an objective measure.

(135) In practice, we define the following parameters; f.sub.c=center frequency, T=total time, Q=number of segments, and n=0 .. . (Q−1). The number of cycles per segment is given by

(136) $\text{cycles}_n = f_c$ .Math. $T - [\frac{Q-1}{2} + n]$. (2.1)

(137) Then, f.sub.n represents the frequencies at which each segment is encoded into the stimulus is

(138) $f_n = \frac{\text{cycles}_n}{T}$. (2.2)

Finally, by windowing and summing the recorded retinal waveform, defined as w(t), region specific

signal, Activity.sub.n, can be extracted in equation 2.3,

(139) $\text{Activity}_n = \text{.Math.}_{n=0}^{(Q-1)} \text{.Math.}_{m=0}^{(\text{cycles}_n - 1)} w\left(\frac{m}{f_n}\right) \text{.Math.} \frac{(m+1)}{f_n}$. (2.3)

For our purposes, f.sub.c=30 Hz, T=40 seconds, and Q=37. In the retina, an additional advantage of the cyclic summation method are that the intensity and temporal frequency of the LEDs can be specified to isolate cone photoreceptor responses and silence rod photoreceptor responses.

Repeatability, Linearity, and SNR

Measurements for repeatability and linearity were made using a UDT S370 Optometer (UDT Instruments, San Diego, CA). LED outputs were measured in microwatts (uW) at seven different intensity settings, in random order. Three complete sets of data were taken on three separate days. All measurements were taken after the instrument became equilibrated with the ambient room temperature, which reflects normal operation of the instrument.

(140) Signal-to-noise ratio was measured using a human subject by first placing an opaque material in front of the stimulus and running successive mf-ERGs. Voltages received during the blocked trials were taken as the noise of the instrument. Signal was then measured by performing mf-ERGs on four human subjects with normal trichromatic color vision. To compare SNR as a function of eccentricity, signal and noise were broken into different eccentric rings. Best subject and the average of all subjects were calculated for each ring. Tests involving human subjects were done in accordance with the principles embodied in the Declaration of Helsinki.

(141) Viral Vector and Subretinal Injections

(142) To validate whether the instrument operated as expected, a mixture of two recombinant adeno-associated viruses was injected sub-retinally in a gerbil (*Meriones unguiculatus*) and a squirrel monkey (*Saimiri sciureus*). One virus, rAAV.CHOPS2053.GFP, carried a gene for green fluorescent protein (GFP) and the other virus, rAAV.CHOPS2053.RHLOPS, coded for human L-opsin. The L-opsin virus was identical to the GFP virus except that a Not I restriction fragment containing the coding sequence for green fluorescent protein was replaced with a 1.2 kb Not I restriction fragment containing recombinant human L opsin cDNA. The opsin gene encoded a human L pigment that is predicted from the deduced amino acid sequence to be maximally sensitive to 562 nm light. In order to provide a method for visualizing transduced cone photoreceptors using immunohistochemistry in future experiments, the sequence of the human L opsin transgene was changed so that the last 12 amino acids matched the known epitope for the monoclonal antibody OS-2. This antibody was previously shown to specifically label S or UV cones in mammalian and primate retina. The C-terminal 12 amino acids of human S opsin were demonstrated to be the epitope. The substitution of the C-terminal 12 amino acids of S-opsin into L-opsin is not predicted to change the spectral sensitivity of the L-opsin trans-gene product.

(143) Subretinal injections were performed. Briefly, gerbils were anesthetized using a combination of Ketamine (50 mg/kg) and Xylazine (2 mg/kg), and it received two 5 uL subretinal injections of a 1:1 (volume:volume) mixture of rAAV.CHOPS2053.GFP and rAAV.CHOPS2053.RHLOPS that were placed in the superior retinal area. A color mf-ERG was then performed on this animal at 6 months post-injection. Squirrel monkeys was anesthetized using 15 mg/kg ketamine and 2 mg/kg xylazine, and they received two 100 uL subretinal injections of a virus mixture containing 110 uL of rAAV.CHOPS2053.GFP and 220 uL of rAAV.CHOPS2053.RHLOPS. Both injections were positioned near the fovea, with the first injection placed superiorly, and the second injection placed in the inferotemporal region of the retina. A color mf-ERG was performed on this animal about 42 weeks, or 10.5 months post-injection. All of these procedures were conducted in accordance with the experimental animal care and usage guidelines of the United States National Institutes of Health.

(144) Fundus Exams

(145) Both the gerbil and squirrel monkey had fundus images taken at multiple time points post-

injection to observe expression of the GFP transgene over time. Fundus images were obtained using the fluorescein angiography mode of the RetCam II digital imaging system (Massie Laboratories, Pleasanton, CA. For the gerbil, images were taken with a lens designed for detecting retinopathy in premature infants, which provides a 130° field of view, and for the monkey, a high magnification 30° lens was used. Thus, multiple fundus images from the squirrel monkey were pieced together into a montage, in order to show a comparable retinal area.

(146) Results

(147) Measures of repeatability, linearity, and signal-to-noise ratio (SNR) were performed to evaluate the wide-field color ERG system. More properly, given n=2 . . . 8, m=½.sup.n, and t=[0, ˜3.5, 7] days, then intensity, I.sub.m(t), is said to be repeatable if

(148) $s = 3$ .Math. $\sqrt{\frac{1}{2} \text{ .Math. } \sum_{t=1}^{3} \left(I_m(t) - \bar{I_m}(t)\right)^2}$ ≤ .Math. (2.4)

where ε is defined as

ε=0.05.Math.max(photon.sub.m). (2.5)

The instrument was said to be linear if Pearson's R.sup.2 was greater than 0.95. And finally, signal (in SNR) was is calculated by averaging response from four subjects, and noise was taken as the residual signal while an opaque material blocked stimulus.

(149) Repeatability and linearity are demonstrated in FIG. **2.2**. Each data point is an average of optometer measurements for the particular intensity setting taken on three separate days; error bars are the standard deviations with a 99.7% confidence interval. Pearson's R correlation was calculated and the coefficient of determination (R.sup.2) values is shown. The red LEDs shared 0.9987 total variance and the green LEDs shared 0.9996 total variance demonstrating system is linear and repeatable over time. SNR values are shown in Table 2.1. The SNR was high for the inner segments of the stimulus panel; it decreased in more peripheral regions but remained high enough to allow measurements into the far periphery.

(150) Because the GFP-coding virus and the L-opsin-coding virus were injected together at the same locations, fluorescence fundus images could be registered with mf-ERG data from the same animal in order to validate the redesigned wide-field color mf-ERG system. Areas of high GFP expression corresponded well to areas of high mf-ERG voltage in response to the L-cone isolating red stimulus (FIG. **2.3**). Animals were insensitive to the red 653 nm wave-length light prior to injection, verifying that the signal measured in animals post-injection is true signal coming from the introduced L-opsin transgene product. In the squirrel monkey, the red-light mf-ERG data showed high voltage in the regions that corresponded to the fluorescence fundus images.

(151) To evaluate whether the concave surface of the newly developed stimulator produces ERG amplitudes that are relatively constant with retinal eccentricity an experiment was performed in which flicker ERG responses were measured under two different conditions. In FIG. **2.4**, the circles represent ERG responses from an LED traveling down a linear path while the subject fixated forward. On the same graph, triangles represent ERG responses from an LED traveling on a rotating boom while the subject fixated forward. The boom held the LEDs perpendicular to the cornea. Results from this experiment demonstrate the increase in signal given by the convex stimulator, compared to a traditional flat-panel LCD screen or CRT monitor. The curved stimulus design, in addition to the use of ultra-bright LEDs as the light source, increased the SNR sufficiently high in the far periphery to ensure viable signals were recorded from cone photoreceptors.

(152) In the conventional mf-ERG, the signal amplitudes are greatly reduced in the periphery because the illumination from a flat screen falls off roughly as the cosine with increasing eccentricity. In our design, the redesigned stimulus had concave structure holding the LEDs such that the inner surface pointed perpendicular to the stimulated retinal area. In addition, similar to traditional mf-ERG stimulators, the number of LEDs in each segment was increased with eccentricity to compensate for the decrease in cone density.

(153) If 40% of cones express the transgene the increase in red sensitivity should be that expected from a spectral ERG in which 20% of the total ERG contribution is from L opsin while 80% is from M opsin. This is consistent with the observed red sensitivity in both monkeys and gerbils.
(154) This is the first time that measurements from cones in the far peripheral retina have been achieved. Results from these experiments indicate that the wide-field color mf-ERG system is a valid technique for measuring the topography of opsin expression in living subjects, and it will serve as an important tool for evaluating success of gene therapy in humans.
References for Example 2
(155) Sutter, E. E. (1991). The Fast m-Transform: A Fast Computation of Cross-Correlations with Binary m-Sequences. *SIAM Journal on Computing,* 20(4), 686-694. Swanson, W. H., Ueno, T., Smith, V. C., & Pokorny, J. (1987). Temporal modulation sensitivity and pulse-detection thresholds for chromatic and luminance perturbations. *Journal of the Optical Society of America A—Optics, Image Science and Vision,* 4(10), 13. Lindenberg, T., Horn, F. K., & Korth, M. (2003). Cyclic summation versus m-sequence technique in the multifocal ERG. *Graefes Archive of Clinical Experimental Ophthalmology.,* 241(6), 505-510.

Claims

1. An isolated nucleic acid expression vector comprising: (a) a promoter region comprising an M opsin promoter comprising the nucleic acid sequence of SEQ ID NO: 2, wherein the promoter region is specific for primate retinal cone cells; and (b) a gene encoding a therapeutic protein comprising the amino acid sequence of SEQ ID NO: 11, wherein the gene is operatively linked to the promoter region.
2. The isolated nucleic acid expression vector of claim 1, wherein the expression vector is a recombinant adeno-associated virus (AAV) gene delivery vector.
3. The isolated nucleic acid expression vector of claim 1, wherein the therapeutic protein, when expressed in cone cells of a primate having color blindness and/or blue cone monochromacy (BCM), is capable of: 1) enabling the primate to visualize and to discriminate between red and green colors; and/or 2) restoring visual capacity in the primate.
4. The isolated nucleic acid expression vector of claim 3, wherein the visual capacity is selected from the group consisting of: a reduction or slowing of vision loss; improved visual acuity; and a decrease in abnormal sensitivity to bright lights.
5. The isolated nucleic acid expression vector of claim 1, further comprising an enhancer element upstream of the M opsin promoter, wherein the enhancer element comprises the nucleic acid sequence of SEQ ID NO: 51, and wherein the gene is operatively linked to the enhancer element.
6. The isolated nucleic acid expression vector of claim 1, further comprising an intron comprising a splice donor/acceptor region, wherein the intron is located downstream of the promoter region and is located upstream of the gene.
7. A formulation comprising packaged viral particles comprising the nucleic acid expression vectors of claim 1.
8. The formulation of claim 7, wherein the formulation is used to treat a cone cell disorder.
9. The formulation of claim 8, wherein the cone cell disorder is selected from the group consisting of color blindness, blue cone monochomacy, achromatopsia, incomplete achromatopsia, rod-cone degeneration, retinitis pigmentosa (RP), macular degeneration, cone dystrophy, blindness, Stargardt's Disease, and Leber's congenital amaurosis.
10. A recombinant host cells transfected or transduced with the nucleic acid expression vector of claim 1.