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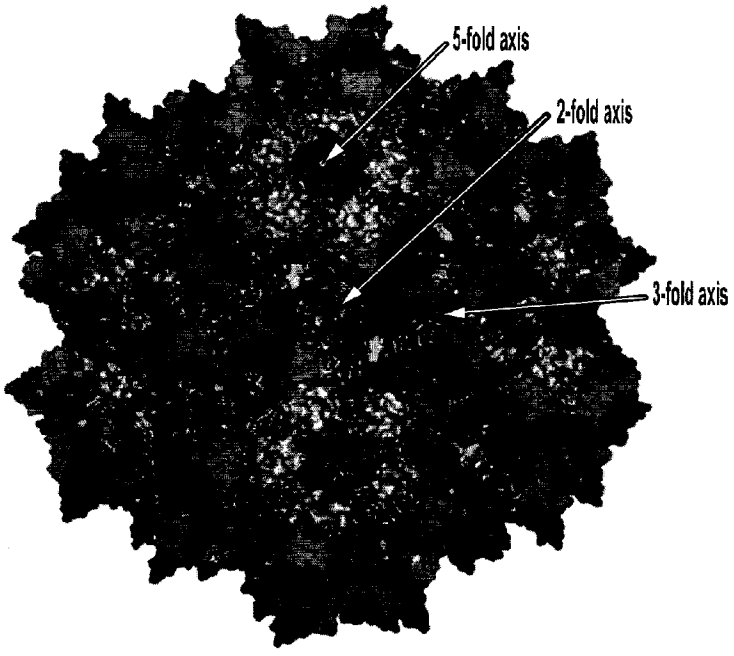

(57) Abrégé/Abstract:
Adeno associated viral (AAV) particles are emerging as a useful vehicle for gene delivery to various organs and tissues, one of them being the retina. Provided here are variant AAV (e.g., variant serotype 2 (AAV2)) capsid proteins and variant capsid protein containing particles with enhanced ability to transduce retinal cells.

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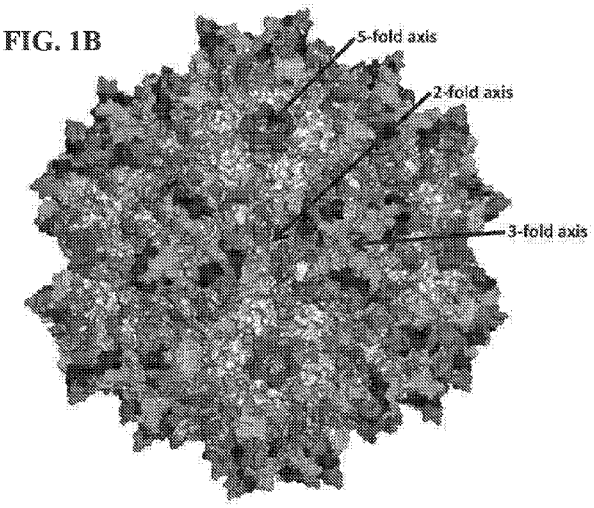
(54) Title: MODIFIED AAV CAPSID PROTEINS AND USES THEREOF

FIG. 1B



(57) Abstract: Adeno associated viral (AAV) particles are emerging as a useful vehicle for gene delivery to various organs and tissues, one of them being the retina. Provided here are variant AAV (e.g., variant serotype 2 (AAV2)) capsid proteins and variant capsid protein containing particles with enhanced ability to transduce retinal cells.

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MODIFIED AAV CAPSID PROTEINS AND USES THEREOF

BACKGROUND

Adeno associated viral (AAV) particles are emerging as a useful vehicle for gene delivery. While different AAV serotypes have particular organ tropism that can be taken advantage of to target gene-based therapies to a target organ (see e.g., Surace et al., Vision Res. 2008, 48(3):353-9; Zincarelli et al., Mol Ther. 2008,16(6):1073-80), the increased efficiency in AAV for targeting certain organs or tissues would be of great benefit. An example of such tissue is the retina.

SUMMARY

The organ or tissue tropism of AAV particles depends highly, if not entirely, on the make-up of the particle surface, or the capsid. AAV serotype 2 (AAV2) has a tropism for and is used to deliver genes to the retina (see e.g., Vandenberghe et al., Gene Ther. 2012, 19(2):162-8). The AAV2 capsid is made up of three proteins, VP1, VP2 and VP3. Provided herein are compositions and methods for variant (e.g., modified) AAV (e.g., AAV2) capsid proteins and particles that have an improved efficiency to transduce retinal cells (e.g., photoreceptors, retinal ganglion cells and retinal neural cells). This disclosure is based, at least in part, on the identification of AAV2 (AAV2) variant proteins (e.g., modified AAV2 capsid proteins) and recombinant particles comprising the modified capsid proteins that have a greater efficiency to transduce retinal cells compared to rAAV2 particles comprising wild-type capsid proteins, using *in vivo* screening of a AAV2 capsid library containing capsid variants with amino acid substitutions or mutations in the capsid proteins of AAV2 in a mouse model and a macaque model.

In some embodiments, provided herein are variant (e.g., modified) recombinant adeno-associated virus (rAAV) serotype 2 (AAV2) capsid proteins comprising sequences DGE and/or DF in variable region (VR) V (VRV), and any one or more of the following sets of sequences and/or substitutions:

(a) EDATENXIXXDR (as set forth in SEQ ID NO: 4) in VRVII,

(b) NA in VRI; and SAAGADXAXDS (as set forth in SEQ ID NO: 5) in VRVII,

(c) NA in VRI; and EDATENXIXXDR (as set forth in SEQ ID NO: 4) in VRVII,

(d) SAAGADXAXDS (as set forth in SEQ ID NO: 5) substitution in VRVII,

(e) NA in VRI; and SAAGADXAXDS (as set forth in SEQ ID NO: 5) in VRVII,

(f) a Q to A substitution in loop I; and EDATENXIXXDR (as set forth in SEQ ID NO: 4) in VRVII,

(g) a Q to A substitution in loop I; a K to T substitution in VRV; and EDATENXIXXDR (as set forth in SEQ ID NO: 4) in VRVII, and

(h) a S to W substitution at position 267; and EDATENXIXXDR (as set forth in SEQ ID NO: 4) in VRVII. X may be any amino acid (e.g., alanine, arginine, asparagine, aspartic acid, cysteine, glutamine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan or valine).

In some embodiments, provided herein is a variant (e.g., modified) recombinant AAV2 capsid protein comprising sequences DGE and/or DF in VRV. In some embodiments, provided herein is a variant recombinant AAV2 capsid protein comprising sequences DGE and/or DF in VRV, and NA in VRI.

In some embodiments, provided herein is a variant (e.g., modified) recombinant AAV2 capsid protein comprising any one of the following sets of sequences and/or substitutions:

(a''') NA in VRI; a F at position 444; and DEAXSEXKXTXR (as set forth in SEQ ID NO: 7) in VRIV,

(b''') Q325K in VRII; Y444F; S452A, T454N and T455V in VRIV; and RXXDD (as set forth in SEQ ID NO: 8) in VRVI,

(c''') Q263A in VRI; K490T, S492P, E499D and Y500F in VRV; and E530D in VRVI,

(d''') NA in VRI; Y444F; P451A, T454N, T455V and R459T in VRIV; and RXXDD (as set forth in SEQ ID NO: 8) in VRVI,

(e''') E530D in VRVI,

(f''') QDXE (as set forth in SEQ ID NO: 9), and substitutions Y500F and T503P in VRV, and

(g''') EA in VRI; T491V and Y500F in VRV; and AAADDXEXDG (as set forth in SEQ ID NO: 10) in VRVII. X may be any amino acid (e.g., alanine, arginine, asparagine, aspartic acid, cysteine, glutamine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan or valine).

In some embodiments, amino acids denoted by X are amino acids in wild-type AAV2 sequence as set forth in SEQ ID NO: 1. For example, sequence EDATENXIXXDR, as set forth in SEQ ID NO: 4, is homologous to amino acids 545 to 556 in VRVII of wild-type AAV2 VP1 protein as set forth in SEQ ID NO: 1. Therefore, in some embodiments, sequence EDATENXIXXDR may be sequence EDATENNIDIDR. Similarly, in some embodiments, sequence RXXDD (SEQ ID NO: 8) is sequence RDDDD.

In some embodiments, a variant rAAV2 capsid protein comprises the sequences DGE and/or DF in VRV, and sequence EDATENXIXXDR (as set forth in SEQ ID NO: 4) in VRVII. In some embodiments, a variant rAAV2 capsid protein comprises the sequences DGE and/or DF in VRV, and NA in VRI; and SAAGADXAXDS (as set forth in SEQ ID NO: 5) in VRVII.

This disclosure is also partly based on further improvement of the performance of rAAV2 capsid variants having greater than wild-type efficiency to transduce retinal cells by introducing more amino acid substitutions based on rational capsid design. Accordingly, also provided herein, in some embodiments, are variant rAAV2 capsid proteins further comprising amino acid substitutions that are rationally designed. Any one of the variant rAAV2 capsid protein disclosed herein may further comprise substitution Y444F. In some embodiments, a variant rAAV2 capsid protein comprises sequences DGE and/or DF in VRV, any one of the sequences and/or substitutions in sets (a''') to (c''') and (e''') to (h''') as described above, and substitution Y444F. In some embodiments, any one of the variant rAAV2 capsid protein disclosed herein may further comprise substitution Y730F. In some embodiments, a variant (e.g., modified) recombinant AAV2 capsid protein comprising sequences DGE and/or DF in VRV further comprise one or more of the following substitutions: Y252F, Y272F, Y444F, Y700F, Y704F, Y730F and T491V. In some embodiments, a variant (e.g., modified) recombinant AAV2 capsid protein comprising sequences DGE and/or DF in VRV and NA in VRI further comprise one or more of the following substitutions: Y252F, Y272F, Y444F, Y700F, Y704F, Y730F and T491V. In some embodiments, any one of the variant rAAV2

capsid proteins disclosed herein further comprises the substitutions Y272F, Y444F, Y730F and T491V.

Any one of the variant (e.g., modified) rAAV2 capsid proteins disclosed herein may further comprise substitution Y252F. Any one of the modified rAAV2 capsid proteins disclosed herein may further comprise substitution Y272F. Any one of the variant rAAV2 capsid protein disclosed herein may further comprise substitution Y500F. Any one of the variant rAAV2 capsid protein disclosed herein may further comprise substitution Y700F. Any one of the variant rAAV2 capsid protein disclosed herein may further comprise substitution Y704F. In some embodiments, any one of the variant rAAV2 capsid protein disclosed herein may further comprise substitution T491V, if a valine does not exist at that position already. In some embodiments, a variant rAAV2 capsid protein comprises any one of the sets (a'''), (b'''), (c''') and (e''') of sequences and/or substitutions as described above, and further comprises the substitution Y500F.

In some embodiments any one the modified capsids disclosed herein may contain insertions of 6 to 8 amino acids at positions 587 or 588 of VP1, VP2 and VP3.

In some embodiments, a modified AAV2 capsid protein is a VP3 protein. In some embodiments, a modified AAV2 capsid protein is a VP2 protein. In some embodiments, a modified AAV2 capsid protein is a VP1 protein.

In some aspects, provided herein are rAAV particles that comprise any of the modified AAV2 capsid proteins disclosed herein. In some embodiments, a variant rAAV2 particle comprises a nucleic acid comprising inverted terminal repeats (ITRs). In some embodiments of any one of the variant rAAV2 particles disclosed herein comprises a nucleic acid comprising a gene of interest.

In some embodiments, a nucleic acid comprised in a variant rAAV2 particle is single-stranded. In some embodiments, a nucleic acid comprised in a variant rAAV2 particle is double-stranded.

In some aspects, provided herein is a composition comprising a plurality of any one of the variant rAAV2 particles disclosed herein. In some embodiments, a compositions of rAAV particles further comprises a pharmaceutically acceptable carrier.

In some aspects, provided here are also methods of using any one of the particles disclosed herein to transduce retinal cells with a gene. In some embodiments, a method of transducing a photoreceptor cell and/or retinal ganglion cell with a gene of interest comprises providing to the photoreceptor cell any one of the compositions disclosed herein. In some embodiments, AAV2 particles provided to the photoreceptor cells and/or retinal ganglion

cells comprise the gene of interest. In some embodiments, a composition is provided to the photoreceptor cell and/or retinal ganglion cell via an intravitreal injection to the subject carrying the photoreceptor and/or retinal ganglion cell. In some embodiments, a compositions is provided to the photoreceptor cell and/or retinal ganglion cell via a subretinal injection to the subject carrying the photoreceptor cell and/or retinal ganglion cell. In some embodiments a compositions is provided to the photoreceptor cell and/or retinal ganglion cell via a subILM injection to the subject carrying the photoreceptor cell and/or retinal ganglion cell (see e.g., Hum Gene Ther. 2016 Aug;27(8):580-97).

Provided herein is also a method of transducing an ependymal cell or a Purkinje cell with a gene of interest. In some embodiments, the method comprises providing to the ependymal cell or the Purkinje cell a composition comprising a plurality of recombinant AAV2 particles comprising a variant recombinant AAV2 capsid protein, wherein the capsid protein comprises the sequences DGE and/or DF in VRV, and NA in VRI; and SAAGADXAXDS (as set forth in SEQ ID NO: 5) in VRVII. In some embodiments, a composition is provided to the ependymal cell or the Purkinje cell via an intraventricular injection to the subject carrying the ependymal cell or the Purkinje cell.

In some embodiments, a subject is a mammal. In some embodiments, a mammal is a human. In some embodiments, a gene of interest encodes a therapeutic protein. A therapeutic protein may be an antibody or antibody fragment, a peptibody, a growth factor, a hormone, a membrane protein, a cytokine, a chemokine, an activating or inhibitory peptide acting on cell surface receptors or ion channels, a cell-permeant peptide targeting intracellular processes, an enzyme, a nuclease or other protein used for gene editing. In some embodiments a gene of interest encodes an RNA, such as a ribozyme RNA, shRNA, or miRNA for regulating gene expression, or a guide RNA for gene editing.

In some embodiments, provided herein are variant (e.g., modified) recombinant adeno-associated virus (rAAV) serotype 2 (AAV2) capsid proteins comprising (a') XX in variable region I (VRI); QDXE in variable region V (VRV); Y500F; and T503P, (b') XX in VRI; Y444F; SD, ID, and/or NXM in variable region IV (VRIV); S492A; DF in VRV; and DG in variable region VI (VRVI), (c') XX and/or X in VRI; Y444F; T450D; T454S; MXTXR in VRIV; T491V; Y500F; and E531D, (d') NA in VRI; DGE and DF in variable VRV; and Q545E, (e') DAXXT in VRI; Y444F; AXMXKXH (SEQ ID NO: 30) in VRIV; YN in VRV; Y500F; K507T; and DXR in VRIV, (f') Y444F; GAXNMXTXAXR (SEQ ID NO: 31) in VRIV; TXP and DF in VRV; and E530D, (g') XX in VRI; T491V; Y500F; and AAADDXEXDG (SEQ ID NO: 10) in variable region VII (VRVII), (h') XX in VRI; E530D;

and AGRADIXXXS (SEQ ID NO: 33) in VRVII, or (i') XX and/or X in VRI; QDXE in VRV; Y500F; T503P; and SAAGADXAXDS (SEQ ID NO: 5) in VRVII, wherein X may be any amino acid (e.g., alanine, arginine, asparagine, aspartic acid, cysteine, glutamine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan or valine). In some embodiments, any one or more Xs (e.g., all Xs) are wild-type amino acid(s) present in the corresponding position(s) in a wild-type AAV2 capsid protein.

In some embodiments, the variant (e.g., modified) recombinant AAV2 capsid protein comprises: (a'') QS, NA, EA, DA, AS, AA, DT, NS, GA, GS, RS, TA, TS, ES, GT, QA, or TT in VRI; QDXE in VRV; Y500F; and T503P, (b'') QS, NT, ES, GS, NA, AS, AA, GA or DS in VRI; Y444F; SD, ID, and/or NXM in VRIV; S492A; DF in VRV; and DG in VRVI, (c'') QSGAS (SEQ ID NO: 46), NAGAS (SEQ ID NO: 47), TTGAT (SEQ ID NO: 48), EAGAS (SEQ ID NO: 49), TTGAS (SEQ ID NO: 50) or GAGAS (SEQ ID NO: 51) in VRI, (d'') QS, EA, QA, NA, AS or ES in VRI; T491V; Y500F; and AAADDXEXDG (SEQ ID NO: 10) in VRVII, (e'') QS, DS, NA, AS, DA or AT in VRI; E530D; and AGRADIXXXS (SEQ ID NO: 33) in VRVII, or (f'') QSGAS (SEQ ID NO: 46), NAGAS (SEQ ID NO: 47), ASGAS (SEQ ID NO: 52), GAGAS (SEQ ID NO: 51), TAGAS (SEQ ID NO: 53), QTGAS (SEQ ID NO: 54) or TTGAS (SEQ ID NO: 50) in VRI; QDXE in VRV; Y500F; T503P; and SAAGADXAXDS (SEQ ID NO: 5) in VRVII.

This disclosure is also partly based on further improvement of the performance of rAAV2 capsid variants having greater than wild-type efficiency to transduce retinal cells by introducing more amino acid substitutions based on rational capsid design. Accordingly, also provided herein, in some embodiments, are variant rAAV2 capsid proteins further comprising amino acid substitutions that are rationally designed. Any one of the variant rAAV2 capsid protein disclosed herein may further comprise substitution Y444F. In some embodiments, a variant rAAV2 capsid protein comprises any one of the sequences and/or substitutions in sets (a') to (i') or (a'') to (f'') as described above, and substitution Y444F. In some embodiments, any one of the variant rAAV2 capsid protein disclosed herein may further comprise substitution Y730F. In some embodiments, a variant rAAV2 capsid protein comprises any one of the sequences and/or substitutions in sets (a') to (i') or (a'') to (f'') as described above, and substitution Y730F. In some embodiments, any one of the variant rAAV2 capsid protein disclosed herein may further comprise substitution Y272F. In some embodiments, a variant rAAV2 capsid protein comprises any one of the sequences and/or substitutions in sets (a') to (i') or (a'') to (f'') as described above, and substitution Y272F. In some embodiments, any

one of the variant rAAV2 capsid protein disclosed herein may further comprise substitution T491V, if a valine does not exist that position already. In some embodiments, a variant rAAV2 capsid protein comprises any one of the sequences and/or substitutions in sets (a') to (i') or (a'') to (f'') as described above, and substitution T491V. In some embodiments, any one of the variant rAAV2 capsid protein disclosed herein may further comprise substitution Y500F. In some embodiments, a variant rAAV2 capsid protein comprises any one of the sets (a') to (i') or (a'') to (f'') of sequences and/or substitutions as described above, and further comprises the substitution Y500F.

In some embodiments, a variant rAAV2 capsid protein is a VP3 protein. In some embodiments, a variant rAAV2 capsid protein is a VP2 protein. In some embodiments, a variant rAAV2 capsid protein is a VP1 protein.

In some aspects, provided herein are rAAV particles that comprise any of the variant rAAV2 capsid proteins disclosed herein. In some embodiments, a variant rAAV2 particle comprises a nucleic acid comprising inverted terminal repeats (ITRs). In some embodiments of any one of the variant rAAV2 particles disclosed herein comprises a nucleic acid comprising a gene of interest.

In some embodiments, a nucleic acid comprised in a variant rAAV2 particle is single-stranded. In some embodiments, a nucleic acid comprised in a variant rAAV2 particle is double-stranded. In some embodiments, a nucleic acid comprised in a variant rAAV2 particle is a self-complementary rAAV genome (e.g., an scAAV2 genome).

In some aspects, provided herein is a composition comprising a plurality of any one of the variant rAAV2 particles disclosed herein. In some embodiments, a compositions of rAAV particles further comprises a pharmaceutically acceptable carrier.

In some aspects, provided here are also methods of using any one of the particles disclosed herein to transduce retinal cells with a gene, e.g., a gene of interest. In some embodiments, a method of transducing a photoreceptor cell and/or retinal ganglion cell with a gene of interest comprises providing to the photoreceptor cell any one of the compositions disclosed herein. In some embodiments, AAV2 particles provided to the photoreceptor cells and/or retinal ganglion cells comprise the gene of interest. In some embodiments, a composition is provided to the photoreceptor cell and/or retinal ganglion cell via an intravitreal injection to the subject carrying the photoreceptor and/or retinal ganglion cell. In some embodiments, a composition is provided to the photoreceptor cell and/or retinal ganglion cell via a subretinal injection to the subject carrying the photoreceptor cell and/or retinal ganglion cell.

In some embodiments, a subject is a mammal. In some embodiments, a mammal is a human. In some embodiments, a gene of interest encodes a therapeutic protein. A therapeutic protein may be, e.g., an antibody or antibody fragment, a peptibody, a growth factor, a hormone, a membrane protein, a cytokine, a chemokine, an activating or inhibitory peptide acting on cell surface receptors or ion channels, a cell-permeant peptide targeting intracellular processes, an enzyme, a nuclease or other protein used for gene editing.

Certain peptide sequences inserted at the heparin binding domain of AAV (e.g., AAV2) are known to enhance transduction efficiency. See e.g., Körbelin et al. (EMBO Mol Med. 2016 Jun 1;8(6):609-25), Michelfelder et al. (PLoS One. 2009;4(4):e5122. doi: 10.1371/journal.pone.0005122), and Körbelin et al. (Mol Ther. 2016 Jun;24(6):1050-1061. doi: 10.1038/mt.2016.62). Accordingly, in some embodiments any one the variant capsids disclosed herein may contain insertions of 6 to 8 amino acids at positions 587 or 588 of VP1, VP2 and VP3. In some embodiments, any one of the variant rAAV (e.g., variant rAAV2) capsid protein disclosed here further comprises a peptide. In some embodiments, a peptide may be any one of the peptides disclosed in Körbelin et al. (EMBO Mol Med. 2016 Jun 1;8(6):609-25), Michelfelder et al. (PLoS One. 2009;4(4):e5122. doi: 10.1371/journal.pone.0005122), and Körbelin et al. (Mol Ther. 2016 Jun;24(6):1050-1061. doi: 10.1038/mt.2016.62). In some embodiments, any one of the variant rAAV (e.g., variant rAAV2) capsid protein disclosed here further comprises one or more of any one of the following peptides: LALGETTRPA (SEQ ID NO: 66), NRGTEWD (SEQ ID NO: 67), ADGVQWT (SEQ ID NO: 68), GEARISA (SEQ ID NO: 69), SGNSGAA (SEQ ID NO: 70), ESGLSQS (SEQ ID NO: 71), EYRDSSG (SEQ ID NO: 72), DLGSARA (SEQ ID NO: 73), PRSADLA (SEQ ID NO: 74), PRSTSDP (SEQ ID NO: 75), and ESGHGYF (SEQ ID NO: 76). In some embodiments of any one of the variant rAAV (e.g., variant rAAV2) capsid proteins disclosed herein, a peptide is inserted between amino acid positions 587 and 588. In some embodiments of any one of the variant rAAV (e.g., variant rAAV2) capsid proteins disclosed herein, one or more of LALGETTRPA (SEQ ID NO: 66), NRGTEWD (SEQ ID NO: 67), ADGVQWT (SEQ ID NO: 68), GEARISA (SEQ ID NO: 69), SGNSGAA (SEQ ID NO: 70), ESGLSQS (SEQ ID NO: 71), EYRDSSG (SEQ ID NO: 72), DLGSARA (SEQ ID NO: 73), PRSADLA (SEQ ID NO: 74), PRSTSDP (SEQ ID NO: 75), and ESGHGYF (SEQ ID NO: 76) lies between amino acids 585 and 588. In some embodiments of any one of the variant rAAV (e.g., variant rAAV2) capsid proteins disclosed herein, one or more of LALGETTRPA (SEQ ID NO: 66), NRGTEWD (SEQ ID NO: 67), ADGVQWT (SEQ ID NO: 68), GEARISA (SEQ ID NO: 69), SGNSGAA (SEQ ID NO: 70), ESGLSQS (SEQ ID

NO: 71), EYRDSSG (SEQ ID NO: 72), DLGSARA (SEQ ID NO: 73), PRSADLA (SEQ ID NO: 74), PRSTSDP (SEQ ID NO: 75), and ESGHGYF (SEQ ID NO: 76) lies between amino acids 587 and 588.

In some embodiments, any one of the variant recombinant AAV2 capsid proteins disclosed herein further comprises one or more of any one of the following substitutions: Y252F, Y272F, Y444F, Y700F, Y704F, Y730F and T491V, or any combination thereof (e.g., any combination of 2, 3, 4, 5, or 6 thereof, or all 7 thereof).

BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present disclosure, which can be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein. It is to be understood that the data illustrated in the drawings in no way limit the scope of the disclosure.

FIGs. 1A-1C show characteristics of an example AAV capsid library. **FIG. 1A** shows the structure of wild-type AAV2 protein with variable loops. **FIG. 1B** shows the structure of wild-type AAV2 capsid. **FIG. 1C** depicts the CAPLIB-7 capsid library, which shows input plasmid and capsid diversity.

FIG. 2 depicts how the CAPLIB-7 AAV capsid library was screened in mice for transducing retinal cells. Three rounds of screens were performed, wherein Nrl-GFP mice were intravitreally injected with the combinatorial AAV library and AAV variants identified based on prevalance.

FIG. 3 shows results after 3 rounds of screening in Nrl-GFP mice following intravitreal injections of AAV capsid library. Variants are shown in order of prevalence, the top-most being the most prevalent.

FIG. 4 shows how the CAPLIB-7 AAV2 capsid library was screened in non-human primate (NHP) for transducing retinal cells, specifically photoreceptor cells (PRs) and retinal ganglion cells (RGCs). Sortable cell populations were created in primate retina including photoreceptors (PR) via subretinal injection of AAV5-GRK1-GFP and retinal ganglion cells (RGC) by direct injection of TRITC-Dextran-Biotin into the lateral geniculate nucleus (LGN) and retrograde transport. The capsid library was delivered during the in-life phase by intravitreal (Ivt) injection.

FIG. 5 shows the most prevalent AAV2 variants identified from the primate screening.

FIGs. 6A-6C. FIG. 6A shows fundus images captured 3 weeks post-intravitreal injection of Sc-smCBA-mCherry carrying AAV2 variant Va particles in Nrl-GFP mice. **FIG. 6B** shows representative fluorescent activated cell sorting (FACS) scatterplots of retinal cells from Nrl-GFP mice intravitreally injected with AAV2(QuadYF+T-V) or AAV2-Va. **FIG. 6C** shows the quantification of transduction rates in Nrl-GFP mice as determined by FACS. Values are the average of 6 eyes per vector. Black bars represent rod photoreceptors and grey bars represent non rod, neural retinal cells.

FIGs. 7A-7B show mCherry expression in brain sections from a mouse intraventricularly injected (3rd ventricle) with AAV2-Va particles carrying Sc-smCBA-mCherry. **FIG. 7A** shows expression of mCherry in sections containing ependymal cells. **FIG. 7B** shows expression of mCherry in sections containing Purkinje cells.

FIGs. 8A-8C. FIG. 8A shows fundus images captured 3 weeks post-intravitreal injection of Sc-smCBA-mCherry carrying AAV2 variant Vb particles in Nrl-GFP mice. **FIG. 8B** shows representative fluorescent activated cell sorting (FACS) scatterplots of retinal cells from Nrl-GFP mice intravitreally injected with AAV2(QuadYF+T-V) or AAV2-Vb. **FIG. 8C** shows the quantification of transduction rates in Nrl-GFP mice as determined by FACS. Values are the average of 6 eyes per vector. Black bars represent rod photoreceptors and grey bars represent non rod, neural retinal cells.

FIG. 9 shows transduction efficiency of AAV2-V2 variant in ARPE19 retinal epithelium cells. Cells were infected at a multiplicity of infection (MOI) of 10,000.

FIGs. 10A-10C show transduction efficiency for AAV2 variant V2. FIG. 10A shows mCherry fluorescence in mouse retinas as observed using funduscopy. **FIG. 10B** shows representative FACS scatterplots of Nrl-GFP mice intravitreally injected with AAV2-V2 or AAV2(QuadYF+T-V). The mice were sacrificed 4 weeks post injection. **FIG. 10C** shows transduction efficiency relative to AAV2(quadY-F+T-V). Mice were sacrificed at 4 weeks post injection with 1.2e12 vg/ml of Sc-smCBA-mCherry. Values are the average of 6 eyes per vector.

FIG. 11 shows transduction efficiency of AAV2-V3 in ARPE19 cells. Cells were infected at a multiplicity of infection (MOI) of 10,000.

FIGs. 12A-12C show transduction efficiency for AAV2 variant V3. **FIG. 12A** shows mCherry fluorescence in mouse retinas as observed using funduscopy. **FIG. 12B** shows representative FACS scatterplots of Nrl-GFP mice intravitreally injected with AAV2-V3 or

AAV2(QuadYF+T-V). The mice were sacrificed 4 weeks post injection. **FIG. 12C** shows transduction efficiency relative to AAV2(quadY-F+T-V). Mice were sacrificed at 4 weeks post injection with 1.2e12 vg/ml of Sc-smCBA-mCherry. Values are the average of 6 eyes per vector.

FIG. 13 shows fundus images of retinas provided Va and Vb AAV2 capsid variants having additional T to F and/or T to V substitutions. YF represents Y444F and Y730F mutations; YF+TV represents Y272F, Y444F and Y730F, and T291V mutations.

FIG. 14 shows quantification of FACS data illustrating transduction rates of Va and Vb AAV2 capsid variants having additional T to F and/or T to V substitutions as defined in FIG. 13 in Nrl-GFP mice.

FIG. 15 shows the treatment procedure for Macaque (subretinal AAV5-GRK1-GFP + LGN microruby) and Mouse (Nrl-GFP).

FIG. 16 shows the distribution of major variants within recovered tissues after 2 rounds of screening in primate. The X axis represents different cell typesand location within the retina. PR: photoreceptor; RGC: retinal ganglion cell: RPE: retinal pigment epithelium; A: central/macula; BC: peripheral retina.

FIG. 17 shows major variants and the location substitutions by VR.

FIG. 18 shows quantification of transduction efficiencies. The bar represents the level of rod transduction exhibited by vectors AAV2(Y-F+T-V) and AAV-7m8

FIG. 19 shows fundoscopy and V2 4 weeks post Ivt injection with 2e9 vector genomes and raw mCherry fluorescence.

FIG. 20 shows the evaluation of relative transduction and transgene expression efficiencies of capsid variants in macaque and mouse retina utilizing barcoded vectors.

FIG. 21 shows RGC labeled animals (1 and 2) at 2 weeks post Ivt injection of barcoded vectors.

FIG. 22 shows PR labeled animal 3 at 20 days post Ivt barcoded vectors. Enhanced transduction of barcoded vectors evident proximal to the retinotomy for the submacular injection of AAV5-GFP. mCherry expression present in the periphery outside the field of view in the OD and OS.

FIG. 23 shows PR labeled animal 4 at 20 days post Ivt barcoded vectors. Enhanced transduction of barcoded vectors is evident proximal to the retinotomy for the submacular injection of AAV5-GFP. mCherry expression is present in the periphery outside the field of view in the OD.

FIG. 24 shows round three of screening results. Sequences corresponding to SEQ ID NOs: 45, and 36-44 from top to bottom.

FIG. 25 shows further round three of screening results. Sequences corresponding to SEQ ID NOs: 45, 25, 12, 24, 56-59, 14, 11, 60-64 in the upper panel from top to bottom and SEQ ID NOs: 45, 36, 65, 39, 37, 40, 41, 38, 43 and 44 in the lower panel from top to bottom.

DETAILED DESCRIPTION

AAV-derived vectors are promising tools for human gene therapy applications because of reduced pathogenicity compared to other vectors, episomal localization, and stable transgene expression. AAV particles show huge promise for the delivery of therapeutic genes to the eye, and particularly the retina (Pierce et al. Cold Spring Harb Perspect Med. 2015, 5(9):a017285; Schon et al., Eur J Pharm Biopharm. 2015 95(Pt B):343-52; Barnard et al., Cold Spring Harb Perspect Med. 2014, 5(3):a017293; Trapani et al., Prog Retin Eye Res. 2014,43:108-28; Carvalho and Vandenberghe, Vision Res. 2015,111(Pt B):124-33; Dalkara and Sahel, C R Biol. 2014, 337(3):185-92; Petrs-Silva and Linden, Clin Ophthalmol. 2014;8:127-36). Improving the transduction efficiency of AAV particles having tropism for retinal cells would therefore be of great benefit. AAV of serotype 2 is already known to have tropism for certain ocular cells, e.g., retinal cells. Accordingly, provided herein are variants of wild-type AAV (e.g., AAV2) particles having substitutions in the capsid proteins, compositions of such particles and methods of using these compositions to transduce one or more particular cell type (e.g., photoreceptors, retinal ganglion cells, neural retinal cells, Purkinje cells and ependymal cells) relative to the transduction efficiency in the same cell type of a corresponding rAAV that does not have any of the capsid variants (for example relative to a corresponding rAAV2 that has wild type AAV2 capsid proteins).

AAV structure and capsid proteins

The AAV genome is built of single-stranded deoxyribonucleic acid (ssDNA), which is either positive- or negative-sensed. At each end of the DNA strand is an inverted terminal repeat (ITR). Between the ITRs are two open reading frames (ORFs): rep and cap. The rep ORF is composed of four overlapping genes encoding Rep proteins required for the AAV life cycle. The cap ORF contains overlapping nucleotide sequences of capsid proteins: VP1, VP2 and VP3, which interact together to form a capsid of an icosahedral symmetry.

The capsid proteins, which are controlled by the same promoter, designated p40, are translated from the same mRNA. The molecular weights of VP1, VP2 and VP3 are 87, 72

and 62 kiloDaltons, respectively. The AAV capsid is composed of 60 capsid protein subunits, VP1, VP2, and VP3, that are arranged in an icosahedral symmetry in a ratio of 1:1:10.

SEQ ID NO: 1 corresponds to an example of a wild-type AAV2 VP1 amino acid sequence. The AAV2 VP2 and VP3 capsid proteins correspond to amino acids 138 to735 and 204 to 735 of VP1, respectively. SEQ ID NOs: 2 and 3 corresponds to examples of wild-type AAV2 VP2 and AAV2 VP3 amino acid sequences.

wild-type AAV2 VP1 amino acid sequence:

MAADGYLPDWLEDTLSEGIRQWWKLKPGPPPPKPAERHKDDSRGLVLPGYKYLGPF
NGLDKGEPVNEADAAALEHDKAYDRQLDSGDNPYLKYNHADAEFQERLKEDTSFG
GNLGRAVFQAKKRVLEPLGLVEEPVKTAPGKKRPVEHSPVEPDSSSGTGKAGQQPA
RKRLNFGQTGDADSVPDPQPLGQPPAAPSGLGTNTMATGSGAPMADNNEGADGVG
NSSGNWHCDSTWMGDRVITTSTRTWALPTYNNHLYKQISSQSGASNDNHYFGYSTP
WGYFDFNRFHCHFSPRDWQRLINNNWGFRPKRLNFKLFNIQVKEVTQNDGTTTIAN
NLTSTVQVFTDSEYQLPYVLGSAHQGCLPPFPADVFMVPQYGYLTLNNGSQAVGRS
SFYCLEYFPSQMLRTGNNFTFSYTFEDVPFHSSYAHSQSLDRLMNPLIDQYLYYLSRT
NTPSGTTTQSRLQFSQAGASDIRDQSRNWLPGPCYRQQRVSKTSADNNNSEYSWTG
ATKYHLNGRDSLVNPGPAMASHKDDEEKFFPQSGVLIFGKQGSEKTNVDIEKVMITD
EEEIRTTNPVATEQYGSVSTNLQRGNRQAATADVNTQGVLPGMVWQDRDVYLQGPI
WAKIPHTDGHFHPSPLMGGFGLKHPPPQILIKNTPVPANPSTTFSAAKFASFITQYSTG
QVSVEIEWELQKENSKRWNPEIQYTSNYNKSVNVDFTVDTNGVYSEPRPIGTRYLTR
NL (SEQ ID NO: 1)

wild-type AAV2 VP2 amino acid sequence:

MAPGKKRPVEHSPVEPDSSSGTGKAGQQPARKRLNFGQTGDADSVPDPQPLGQPPA
APSGLGTNTMATGSGAPMADNNEGADGVGNSSGNWHCDSTWMGDRVITTSTRTW
ALPTYNNHLYKQISSQSGASNDNHYFGYSTPWGYFDFNRFHCHFSPRDWQRLINNN
WGFRPKRLNFKLFNIQVKEVTQNDGTTTIANNLTSTVQVFTDSEYQLPYVLGSAHQG
CLPPFPADVFMVPQYGYLTLNNGSQAVGRSSFYCLEYFPSQMLRTGNNFTFSYTFED
VPFHSSYAHSQSLDRLMNPLIDQYLYYLSRTNTPSGTTTQSRLQFSQAGASDIRDQSR
NWLPGPCYRQQRVSKTSADNNNSEYSWTGATKYHLNGRDSLVNPGPAMASHKDDE
EKFFPQSGVLIFGKQGSEKTNVDIEKVMITDEEEIRTTNPVATEQYGSVSTNLQRGNR
QAATADVNTQGVLPGMVWQDRDVYLQGPIWAKIPHTDGHFHPSPLMGGFGLKHPP

PQILIKNTPVPANPSTTFSAAKFASFITQYSTGQVSVEIEWELQKENSKRWNPEIQYTS
NYNKSVNVDFTVDTNGVYSEPRPIGTRYLTRNL (SEQ ID NO: 2)

wild-type AAV2 VP3 amino acid sequence:

MATGSGAPMADNNEGADGVGNSSGNWHCDSTWMGDRVITTSTRTWALPTYNNHL
YKQISSQSGASNDNHYFGYSTPWGYFDFNRFHCHFSPRDWQRLINNNWGFRPKRLN
FKLFNIQVKEVTQNDGTTTIANNLTSTVQVFTDSEYQLPYVLGSAHQGCLPPFPADVF
MVPQYGYLTLNNGSQAVGRSSFYCLEYFPSQMLRTGNNFTFSYTFEDVPFHSSYAHS
QSLDRLMNPLIDQYLYYLSRTNTPSGTTTQSRLQFSQAGASDIRDQSRNWLPGPCYR
QQRVSKTSADNNNSEYSWTGATKYHLNGRDSLVNPGPAMASHKDDEEKFFPQSGV
LIFGKQGSEKTNVDIEKVMITDEEEIRTTNPVATEQYGSVSTNLQRGNRQAATADVN
TQGVLPGMVWQDRDVYLQGPIWAKIPHTDGHFHPSPLMGGFGLKHPPPQILIKNTPV
PANPSTTFSAAKFASFITQYSTGQVSVEIEWELQKENSKRWNPEIQYTSNYNKSVNVD
FTVDTNGVYSEPRPIGTRYLTRNL (SEQ ID NO: 3)

Variant recombinant AAV proteins

The tissue tropism and transduction efficiency of AAV particles is determined by the nature of amino acid residues exposed at the surface of the capsid (Wu et al., J Virol. 2006, 80(22):11393-7). Therefore, manipulating the amino acids of the capsid proteins provides an opportunity to fine tune the tissue tropism of the particle and also improve transduction efficiency. However, certain manipulations, e.g., substitutions of amino acids, of the capsid protein can cause it to mis-fold or not form a capsid at all. To circumvent issues of protein mis-folding and capsid mis-forming, the recombinant AAV2 (rAAV2) variant proteins and particles disclosed herein were identified from a variant AAV2 capsid library that was built by making substitutions in only the variable loops of the capsid protein. Herein, "variable loops" are also referred to as "variable regions". AAV2 has 9 variable regions, numbered from VRI to VRIX. FIG. 1A shows the structure of wild-type AAV2 protein with the variable loops. Marsic et al. (Mol Ther. 2014, 22(11):1900-9) describes how such an AAV2 capsid library was made as well as its characteristics.

Screening of an AAV2 capsid library in a mouse model as well as a macaque model led to the identification of AAV2 variant proteins that possess enhanced efficiency to transduce retinal cells (e.g., PRs, RGCs and neural retinal cells) compared to the transduction efficiency of wild-type AAV2 capsid proteins.

Accordingly, provided herein are rAAV2 capsid proteins comprising substitutions, relative to the wild-type AAV2 VP1 sequence (e.g., as set for in SEQ ID NO: 1). In some embodiments, an amino acid substitution in any one of the variant AAV2 capsid proteins disclosed herein lies in a variable region as defined by wild-type AAV2 VP1 protein. It should be understood that any positioning of an amino acid as described herein is with respect to the sequence of the wild-type AAV2 VP1 sequence as set forth in SEQ ID NO: 1. The amino acids corresponding to various variable regions of AAV2 VP1 are as shown in Table 1.

Table 1: AAV2 capsid protein variable regions and corresponding amino acids

Variable Region	Corresponding Amino Acids
VRI	263-265
VRII	325-330
VRIII	381-384
VRIV	450-466
VRV	490-503
VRVI	527-532
VRVII	545-556
VRVIII	585-596
VRIX	704-713

In some embodiments, a variant rAAV (e.g., variant rAAV2) capsid protein has one or more amino acid substitutions in any one variable region (e.g., VRI, VRII, VRIII, VRIV, VRV, VRVI, VRVII, VRVIII or VRIX). In some embodiments, a variant rAAV (e.g., variant rAAV2) capsid protein has one or more amino acid substitutions in more than one variable region (e.g., VRI and VRII, VRI and VRVII, VRV and VRVII, VRV and VRI and VRVII or VRIV and VRII). It should be understood that variant rAAV (e.g., variant rAAV2) capsid proteins as disclosed herein can have one or more amino acid substitutions in any combination of more than one variable regions and is not limited to the examples provided above or elsewhere herein.

In some embodiments, a variant rAAV (e.g., variant rAAV2) capsid protein comprises any one or more of the amino acid substitutions shown in the sequences or substitutions in Table 2. For example, in some embodiments, a variant AAV2 capsid protein has the sequence DGE in variable region VRV. In some embodiments, a variant AAV2

capsid protein has the sequence DF in variable region VRV. In some embodiments, a variant AAV2 capsid protein has the sequences DGE and DF in variable region VRV. In some embodiments, a variant AAV2 capsid protein has the sequences DGE and DF in variable region VRV, and the sequence NA in VRI. In some embodiments, a DGE exists at amino acid positions 492-494. In some embodiments, a DF exists at amino acid positions 499-500. It is to be understood that the positions listed in Table 2 are only one of many possible amino acid positions and are non-limiting. For example, a DGE sequence may exist anywhere in variable region VRV (e.g., 490-492, 495-497, 496-500, or 500-503). All the amino acid substitutions disclosed anywhere herein can be combined with one or more of any of the other amino acid substitutions disclosed herein. For example, a DGE sequence at amino acid positions 496-500 could be combined with a DF sequence at amino acid positions 499 and 500 to result in a DGEDF sequence (SEQ ID NO: 32) in VRV.

In some embodiments, a variant rAAV (e.g., variant rAAV2) capsid protein has an amino acid listed in the second column of Table 2. In some embodiments, a variant rAAV (e.g., variant rAAV2) has an amino acid sequence that corresponds to a sequence found in FIG. 34. In some embodiments, such an amino acid can exist at a position that is offset from the position denoted in Table 2. In some embodiments, the width of the offset is up to 5 amino acids (e.g., 1, 2, 3, 4 or 5 amino acids) in either direction (upstream and downstream) for the position denoted in Table 2. For example, while a proline is designated at position 492 in VRV, a proline may exist at any position from 490 to 497 (please see S to P substitution at position 492 in VRV). In some embodiments, an amino acid listed in the second column of Table 2 is in a variant rAAV capsid protein of a serotype other than AAV2, e.g., in a homologous variable region of AAV 1, 3, 3B, 4, 5, 6, 7, 8, 9, 10, 11, 12 or 13.

In some embodiments, amino acids denoted by X are amino acids in wild-type AAV2 sequence as set forth in SEQ ID NO: 1. For example, sequence EDATENXIXXDR, as set forth in SEQ ID NO: 4, is homologous to amino acids 545 to 556 in VRVII of wild-type AAV2 VP1 protein as set forth in SEQ ID NO: 1. Therefore, in some embodiments, sequence EDATENXIXXDR (SEQ ID NO: 4) may be sequence EDATENNIDIDR (SEQ ID NO: 34). Similarly, in some embodiments, sequence RXXDD (SEQ ID NO: 8) is sequence RDDDD (SEQ ID NO: 35). In some embodiments, amino acids denoted by X are amino acids in other AAV serotypes (e.g., 1, 3, 3B, 4, 5, 6, 7, 8, 9, 10, 11, 12 or 13) at homologous positions.

Table 2: Amino acid substitutions or sequences in variant rAAV (e.g., variant rAAV2) capsid proteins

Variable Region	Amino Acids sequences and/or substitutions*	Possible positions	Corresponding SEQ ID NO
VRI	NA	263-264	
	EA	263-264	
	DA	263-264	
	XX	263-264	
	Q to A	263	
	X	267	
	Q to A	263	
	S to T	267	
	S to X	267	
	S to W	267	
	QSGAS	263-267	46
	NAGAS	263-267	47
	TTGAT	263-267	48
	EAGAS	263-267	49
	TTGAS	263-267	50
	GAGAS	263-267	51
	ASGAS	263-267	52
	TAGAS	263-267	53
	QTGAS	263-267	54
VRII	Q to K	325	
VRIV	DEAXSEXKXTXR	450-461	7
	Y to F	444	
	SD	450-451	
	T to D	450	
	P to A	451	
	GAXNMXTXAXR	451-461	31
	S to A	452	
	ID	454-455	
	T to N	454	
	T to S	454	
	AXMXKXH	455-461	30
	T to V	455	
	Q to M	457	
	R to N	459	

	R to T	459	
	Q to R	461	
	Q to M	461	
VRV	K to T	490, 507	
	T to V	491	
	QD	491-492	
	S to A	492	
	S to P	492	
	YN	492-493	
	DGE	492-494	
	D to E	494	
	DF	499-500	
	QDXE	491-494	9
	E to D	499	
	Y to F	500	
	T to P	503	
	K to T	507	
VRVI	RXXDD	527-531	8
	RXXDXR	527-532	55
	DG	530-531	
	K to R	527, 532	
	E to D	530, 531	
VRVII	EDATENXIXXDR	545-556	4
	Q to E	545	
	SAAGADXAXDS	546-556	5
	SGREGDAEXXD	546-556	6
	AAADDXEXDG	547-556	10
	AGRADIXXXS	547-556	33
	D to E	553	
	D to A	553	
	K to S	556	
	DG	555-556	
	DS	555-556	

*amino acids designated by "X" may be any known amino acid

Some non-limiting examples of variant AAV2 capsid proteins are shown in FIGs. 3 and 5. In some embodiments, a variant AAV2 capsid protein has the sequence as set forth in any one of SEQ ID NOs: 11 to 23 (see FIG. 3) or SEQ ID NOs: 24-28 (see FIG. 5) or 36-44 (see FIG. 24) or 56-65 (see FIG. 25). For example, a variant AAV2 capsid protein may have the sequence as set forth by SEQ ID NOs: 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 36, 37, 38, 39, 40, 41, 42, 43, 44, 56, 57, 58, 59, 60, 61, 62, 63, 64 or 65. In some embodiments, a variant AAV2 capsid protein has the sequence as set forth in SEQ ID NO: 11. In some embodiments, a variant AAV2 capsid protein has the sequence as set forth in SEQ ID NO: 12. In some embodiments, a variant AAV2 capsid protein has the sequence SEQ ID NO: 24. In some embodiments, a variant AAV2 capsid protein has the sequence SEQ ID NO: 25.

In some embodiments, a variant AAV2 capsid protein has sequences DGE and DF in VRV and sequence as set forth in SEQ ID NO: 4 in VRVII. In some embodiments, a variant AAV2 capsid protein has sequence NA in VRI, sequences DGE and DF in VRV, and sequence as set forth in SEQ ID NO: 5 in VRVII. In some embodiments, the variant recombinant AAV2 capsid protein comprises: (a) QS, NA, EA, DA, AS, AA, DT, NS, GA, GS, RS, TA, TS, ES, GT, QA, or TT in VRI; QDXE in VRV; Y500F; and T503P, (b) QS, NT, ES, GS, NA, AS, AA, GA or DS in VRI; Y444F; SD, ID, and/or NXM in VRIV; S492A; DF in VRV; and DG in VRVI, (c) QSGAS (SEQ ID NO: 46), NAGAS (SEQ ID NO: 47), TTGAT (SEQ ID NO: 48), EAGAS (SEQ ID NO: 49), TTGAS (SEQ ID NO: 50) or GAGAS (SEQ ID NO: 51) in VRI, (d) QS, EA, QA, NA, AS or ES in VRI; T491V; Y500F; and AAADDXEXDG (SEQ ID NO: 10) in VRVII, (e) QS, DS, NA, AS, DA or AT in VRI; E530D; and AGRADIXXXS (SEQ ID NO: 33) in VRVII, or (f) QSGAS (SEQ ID NO: 46), NAGAS (SEQ ID NO: 47), ASGAS (SEQ ID NO: 52), GAGAS (SEQ ID NO: 51), TAGAS (SEQ ID NO: 53), QTGAS (SEQ ID NO: 54) or TTGAS (SEQ ID NO: 50) in VRI; QDXE in VRV; Y500F; T503P; and SAAGADXAXDS (SEQ ID NO: 5) in VRVII. In some embodiments, a variant AAV2 capsid protein has one or more substitutions in Table 2 for the VRIV region. In some embodiments, a variant AAV2 capsid protein has one or more substitutions in Table 2 for the VRVII region.

After identifying the variant rAAV (e.g., variant rAAV2) capsid proteins with enhanced retinal transduction efficiency using screening in mice and macaque models, further modifications were made to improve transduction efficiency. For example, a method for quantifying relative transduction of photoreceptors by recombinant Adeno Associated Virus (rAAV) vectors in Rho-GFP mice has been used to identify a rationally designed capsid

variant, AAV2(quadY-F+T-V), capable of outer retinal transduction following intravitreal injection (Kay et al., PLoS One. 2013, 8(4):e62097). Accordingly, in some embodiments any one of the AAV2 variant proteins described herein may further comprise any one of the following amino acid substitutions: Y272F, Y444F, Y500F, Y730F, and T491V, or a combination of thereof. For example, a variant AAV2 capsid protein has a sequence as set forth in any one of SEQ ID NOs: 1-28, and if it does not already, has a phenylalanine at one or more of the positions 272, 444, 500 and 730. In another example, a variant AAV2 capsid protein comprises the substitutions Y272F, Y444F, Y500F and Y730F. In another example, a variant AAV2 capsid protein comprises the substitutions Y272F and Y444F.

In some embodiments, a variant AAV2 capsid protein has a sequence as set forth in any one of SEQ ID NOs: 1-28, and if it does not already, has a valine at position 491. For example, a variant rAAV (e.g., variant rAAV2) capsid protein may comprise sequences DGE and DF in VRV, the sequence as set forth in SEQ ID NO: 4 in VRVII and a Y444F substitution. In some embodiments, any one of the AAV2 variant proteins described herein may further comprise any one of the following amino acid substitutions: Y252F, Y700F, and Y704F, or a combination thereof.

In some embodiments, any one of the variant rAAV (e.g., variant rAAV2) capsid proteins disclosed herein is a variant VP1 protein (e.g., a variant AAV2 VP1 protein). In some embodiments, any one of the variant rAAV (e.g., variant AAV2) capsid proteins disclosed herein is an AAV VP2 protein (e.g., a variant AAV2 VP2 protein). In some embodiments, any one of the variant rAAV (e.g., variant AAV2) capsid proteins disclosed herein is an AAV VP3 protein (e.g., a variant AAV VP3 protein). It is to be understood that any of the variants can be in a VP1, VP2, or VP3 protein.

It is to be understood that any one of the variant rAAV (e.g., variant rAAV2) capsid proteins disclosed herein may have any one single amino acid substitution described herein, or any combination of amino acid substitutions described herein. For example, a variant rAAV (e.g., variant rAAV2) capsid protein may have sequence RXXDD (as set forth in SEQ ID NO: 8) as the only substitutions, or it might have additional amino acid substitutions (e.g., NA in VRI; Y444F; P451A, T454N, T455V and/or R459T in VRIV).

Contemplated herein are also variant rAAV capsid proteins of serotypes other than serotype 2. In some embodiments, any one of the amino acid substitutions described herein are in a variable region of the capsid protein of a serotype other than serotype 2 that is homologous to the variable region of AAV2. In some embodiments, a variant rAAV capsid protein of a serotype other than serotype 2 is of any serotype other than AAV2 (e.g., 1, 3, 3B,

Date Reçue/Date Received 2020-10-29

4, 5, 6, 7, 8, 9, 10, 11, 12 or 13). In some embodiments, a variant rAAV capsid protein of a serotype other than serotype 2 is of a closely related serotype (e.g., AAV1 or AAV6). see: PCT Application Publication Number WO2015121501A1.

Nucleic acids encoding variant rAAV capsid proteins

Provided herein are also nucleic acids that encode any one of the variant rAAV capsid proteins disclosed herein. In some embodiments, a nucleic acid encoding a variant rAAV capsid protein is comprised in a plasmid.

Recombinant AAV particles

Provided herein are variant rAAV (e.g., variant rAAV2) particles. In some embodiments, a particle is an empty particle (e.g., one that does not contain a nucleic acid vector comprising a gene of interest). In some embodiments, an AAV2 particle contains a nucleic acid vector comprising a gene of interest. As used herein, "a gene of interest" is a gene that encodes a RNA or protein of interest.

In some embodiments, a rAAV2 particle containing any one of the variant rAAV (e.g., variant rAAV2) capsid proteins disclosed herein comprises ITRs and/or rep ORF of serotype 2. In some embodiments, a rAAV2 particle is a pseudotyped rAAV particle, which comprises (a) a capsid comprised of capsid proteins derived from serotype 2, and (b) a nucleic acid vector comprising ITRs from another serotype (e.g., AAV1, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, or AAV10). For example, a particle may have ITRs of serotype 5 and a capsid of serotype 2. Such a pseudotyped rAAV particle would be designated AAV5/2.

A protein of interest may be a detectable marker or a therapeutic protein. A detectable marker is a molecule that can be visualized (e.g., using a naked eye or under a microscope). In some embodiments, the detectable marker is a fluorescent molecule, a bioluminescent molecule, or a molecule that provides color (e.g., β-galactosidase, β-lactamases, β-glucuronidase and spheriodenone). In some embodiments, a detectable marker is a fluorescent protein or functional peptide or functional polypeptide thereof.

In some embodiments, a gene of interest encodes a therapeutic protein and is referred to as a "therapeutic gene." A therapeutic gene may provide a therapeutic effect in a cell, tissue or organ to which it is delivered. For example, a therapeutic gene delivered to the intravitreal space of an eye (or two eyes) may benefit the photoreceptor cells of the retina of the eye (or two eyes) to which the gene was delivered. In some embodiments, a therapeutic

gene provides a therapeutic benefit to a cell, tissue or organ other than the one to which it is delivered. For example, a gene delivered to the brain may reach the retina of the eyes via the optic nerve and benefit one or more type of retinal cell (e.g., retinal ganglion cells). In some embodiments, a therapeutic gene encodes an antibody, a peptibody, a growth factor, a clotting factor, a hormone, a membrane protein, a cytokine, a chemokine, an activating or inhibitory peptide acting on cell surface receptors or ion channels, a cell-permeant peptide targeting intracellular processes, a thrombolytic, an enzyme, a bone morphogenetic proteins, a nuclease or other protein used for gene editing, an Fc-fusion protein, an anticoagulant, a nuclease, guide RNA or other nucleic acid or protein for gene editing. In some embodiments, a gene of interest encodes a therapeutic RNA, e.g., a small interfering RNA.

In some embodiments, a nucleic acid vector comprised in a rAAV2 particle comprises one or more of the following: (a) one or more heterologous nucleic acid regions comprising a gene of interest, and (b) one or more regions comprising inverted terminal repeat (ITR) sequences (e.g., wild-type ITR sequences or engineered ITR sequences) flanking the one or more nucleic acid regions (e.g., heterologous nucleic acid regions). In some embodiments, a nucleic acid vector in a rAAV particle comprises one or more nucleic acid regions comprising a control sequence that facilitates expression of the heterologous nucleic acid region (e.g., a promoter). In some embodiments, a nucleic acid vector in a rAAV2 particle comprises one or more nucleic acid regions comprising a sequence that facilitates integration of the heterologous nucleic acid region (optionally with the one or more nucleic acid regions comprising a sequence that facilitates expression) into the genome of the subject.

Non-limiting examples of expression control sequences include promoters, insulators, silencers, response elements, introns, enhancers, initiation sites, termination signals, and poly(A) tails. Any combination of such control sequences is contemplated herein (e.g., a promoter and an enhancer).

In some embodiments, one or more promoters may be operably linked to a coding nucleotide sequence in the heterologous nucleic acid. A promoter is "operably linked" to a nucleotide sequence when the promoter sequence controls and/or regulates the transcription of the nucleotide sequence. A promoter may be a constitutive promoter, tissue-specific promoter, an inducible promoter, or a synthetic promoter.

For example, constitutive promoters of different strengths can be used. A nucleic acid vector described herein may include one or more constitutive promoters, such as viral promoters or promoters from mammalian genes that are generally active in promoting transcription. Non-limiting examples of constitutive viral promoters include the Herpes

Simplex virus (HSV), thymidine kinase (TK), Rous Sarcoma Virus (RSV), Simian Virus 40 (SV40), Mouse Mammary Tumor Virus (MMTV), Ad E1A cytomegalovirus (CMV) promoters. Non-limiting examples of constitutive mammalian promoters include various housekeeping gene promoters, as exemplified by the β-actin promoter (e.g., chicken β-actin promoter) and human elongation factor-1 α (EF-1α) promoter. In some embodiments, chimeric viral/mammalian promoters may include a chimeric CMV/chicken beta actin (CBA, CB or CAG) promoters.

Inducible promoters and/or regulatory elements may also be contemplated for achieving appropriate expression levels of the protein or polypeptide of interest. Non-limiting examples of suitable inducible promoters include those from genes such as cytochrome P450 genes, heat shock protein genes, metallothionein genes, and hormone-inducible genes, such as the estrogen gene promoter. Another example of an inducible promoter is the tetVP16 promoter that is responsive to tetracycline.

Tissue-specific promoters and/or regulatory elements are also contemplated herein. In some embodiments, it may be beneficial to combine a variant rAAV (e.g., variant rAAV2) particle as disclosed herein, with a promoter that also targets the same cells, tissue, or organ as the variant rAAV (e.g., variant rAAV2) particle. For example, a variant rAAV (e.g., variant rAAV2) particle that targets photoreceptor cells of the retina might encapsidate a nucleic acid comprising a promoter that also targets photoreceptor cells or the retina as a whole. In some embodiments, a cell-type-specific promoter targeting the retina is human rhodopsin kinase promoter (hGRK1). Non-limiting examples of hGRK1 promoter can be found in Beltran et al., 2010, Gene Ther. 17:1162, Zolotukhin et al., 2005, Hum Gene Ther. 16:551, and Jacobson et al., Mol Ther. 13:1074. In some embodiments, a retina-specific promoter is a Pleiades Mini-promoter (for example Ple155). In some embodiments, a retina - specific promoter is glial fibrillary acidic protein promoter. Other non-limiting examples of promoters that can be used as retinal cell-type-specific promoters include red opsin promoter "PR2.1" (which targets M and L cones), chimeric 'IRBPe-GNAT2' promoter (which targets all cones), IRBP promoter (which targets rods), Grm6-SV40 enhancer/promoter (which targets bipolar cells), Thy1 (which targets RGCs), other Pleiades promoters, rod opsin promoter (which targets rods), cone arrestin promoters (which targets all cones), VMD2 or Bestrophin promoter (which targets RPE cells).

Several promoters are publically available or described. For example, Ple155 promoter is available through Addgene plasmid repository (Addgene plasmid # 29011, addgene.org/29011/) and is described in Scalabrino et al. (Hum Mol Genet. 2015,

24(21):6229-39). Ye et al. (Hum Gene Ther.; 27(1):72-82) describes a shorter version of this promoter called PR1.7. A Thy1 promoter construct is also available through Addgene plasmid repository (Addgene plasmid # 20736, addgene.org/20736/). A GRM6 promoter construct is also available through Addgene plasmid repository (Addgene plasmid # 66391, addgene.org/66391/). Guziewicz et al. (PLoS One. 2013 Oct 15;8(10):e75666) and Esumi et al (J Biol Chem. 2004, 279(18):19064-73) provide examples of the use of VMD2 promoter. Dyka et al. (Adv Exp Med Biol. 2014; 801: 695–701) describes cone specific promoters for use in gene therapy, including IRBP and IRBPe-GNAT2 promoter. The use of PR2.1 promoter has been demonstrated in Komáromy et al. (Gene Ther. 2008 Jul;15(14):1049-55) and its characterization in Karim et al. (Tree Physiol. 2015 Oct;35(10):1129-39). Aartsen et al. (PLoS One, 5(8):e12387) describes the use of GFAP promoter to drive GFP expression in Muller glial cells. Other examples of Muller glia specific promoters are RLBP1 and GLAST (Vázquez-Chona, Invest Ophthalmol Vis Sci. 2009, 50(8):3996-4003; Regan et al., Journal of Neuroscience, 2007, 27(25): 6607-6619).

Synthetic promoters are also contemplated herein. A synthetic promoter may comprise, for example, regions of known promoters, regulatory elements, transcription factor binding sites, enhancer elements, repressor elements, and the like.

It is to be understood that a promoter may be a fragment of any one of the promoters disclosed herein, or one that retains partial promoter activity (e.g., 10-90, 30-60, 50-80,80-99 or 90-99.9% of the activity) of a whole promoter.

Any nucleic acid vector described herein may be encapsidated by a viral capsid. In some embodiments a *cap* gene is modified to express a fusion protein comprising a detectable marker and VP proteins of AAV serotype 2. In some embodiments, a peptide is inserted into the capsid protein either at position 587/588 or at the C-terminus of VP2. In some embodiments, the nucleic acid vector is circular. In some embodiments, the nucleic acid vector is single-stranded. In some embodiments, the nucleic acid vector is double-stranded. In some embodiments, a double-stranded nucleic acid vector may be, for example, a self-complementary vector that contains a region of the nucleic acid vector that is complementary to another region of the nucleic acid vector, initiating the formation of the double-strandedness of the nucleic acid vector.

Method of making rAAV particles

Various methods of producing rAAV particles and nucleic acid vectors are known (see, e.g., Zolotukhin et al. Production and purification of serotype 1, 2, and 5 recombinant

adeno-associated viral vectors. Methods 28 (2002) 158–167; and U.S. Patent Publication Numbers US20070015238 and US20120322861; and plasmids and kits available from ATCC and Cell Biolabs, Inc.). In some embodiments, a vector (e.g., a plasmid) comprising a gene of interest may be combined with one or more helper plasmids, e.g., that contain a rep gene (e.g., encoding Rep78, Rep68, Rep52 and Rep40) and a cap gene (encoding VP1, VP2, and VP3, including a modified VP region as described herein), and transfected into a recombinant cells, called helper or producer cells, such that the nucleic acid vector is packaged or encapsidated inside the capsid and subsequently purified.

Non-limiting examples of mammalian helper cells include HEK293 cells, COS cells, HeLa cells, BHK cells, or CHO cells (see, e.g., ATCC® CRL-1573™, ATCC® CRL-1651™, ATCC® CRL-1650™, ATCC® CCL-2, ATCC® CCL-10™, or ATCC® CCL-61™). A non-limiting example of an insect helper cells is Sf9 cells (see, e.g., ATCC® CRL-1711™). A helper cell may comprises rep and/or cap genes that encode the Rep protein and/or Cap proteins. In some embodiments, the packaging is performed *in vitro* (e.g., outside of a cell).

In some embodiments, a nucleic acid vector (e.g., a plasmid) containing the gene of interest is combined with one or more helper plasmids, e.g., that contain a *rep* gene of a first serotype and a *cap* gene of the same serotype or a different serotype, and transfected into helper cells such that the rAAV particle is packaged. In some embodiments, the one or more helper plasmids include a first helper plasmid comprising a rep gene and a cap gene, and a second helper plasmid comprising one or more of the following helper genes: E1a gene, E1b gene, E4 gene, E2a gene, and VA gene. For clarity, helper genes are genes that encode helper proteins E1a, E1b, E4, E2a, and VA. Helper plasmids, and methods of making such plasmids, are known in the art and commercially available (see, e.g., pDF6, pRep, pDM, pDG, pDP1rs, pDP2rs, pDP3rs, pDP4rs, pDP5rs, pDP6rs, pDG(R484E/R585E), and pDP8.ape plasmids from PlasmidFactory, Bielefeld, Germany; other products and services available from Vector Biolabs, Philadelphia, PA; Cellbiolabs, San Diego, CA; Agilent Technologies, Santa Clara, Ca; and Addgene, Cambridge, MA; pxx6; Grimm et al. (1998), Novel Tools for Production and Purification of Recombinant Adeno associated Virus Vectors, Human Gene Therapy, Vol. 9, 2745-2760; Kern, A. et al. (2003), Identification of a Heparin-Binding Motif on Adeno-Associated Virus Type 2 Capsids, Journal of Virology, Vol. 77, 11072-11081.; Grimm et al. (2003), Helper Virus-Free, Optically Controllable, and Two-Plasmid-Based Production of Adeno-associated Virus Vectors of Serotypes 1 to 6,

Molecular Therapy,Vol. 7, 839-850; Kronenberg et al. (2005), A Conformational Change in the Adeno-Associated Virus Type 2 Capsid Leads to the Exposure of Hidden VP1 N Termini, Journal of Virology, Vol. 79, 5296-5303; and Moullier, P. and Snyder, R.O. (2008), International efforts for recombinant adeno-associated viral vector reference standards, Molecular Therapy, Vol. 16, 1185-1188). Plasmids that encode wild-type AAV coding regions for specific serotypes are also know and available. For example pSub201 is a plasmid that comprises the coding regions of the wild-type AAV2 genome (Samulski et al. (1987), J Virology, 6:3096-3101).

ITR sequences and plasmids containing ITR sequences are known in the art and are commercially available (see, e.g., products and services available from Vector Biolabs, Philadelphia, PA; Cellbiolabs, San Diego, CA; Agilent Technologies, Santa Clara, Ca; and Addgene, Cambridge, MA; and Gene delivery to skeletal muscle results in sustained expression and systemic delivery of a therapeutic protein. Kessler PD, Podsakoff GM, Chen X, McQuiston SA, Colosi PC, Matelis LA, Kurtzman GJ, Byrne BJ. Proc Natl Acad Sci U S A. 1996 Nov 26;93(24):14082-7; and Curtis A. Machida. Methods in Molecular Medicine™. Viral Vectors for Gene Therapy Methods and Protocols. 10.1385/1-59259-304-6:201 © Humana Press Inc. 2003. Chapter 10. Targeted Integration by Adeno-Associated Virus. Matthew D. Weitzman, Samuel M. Young Jr., Toni Cathomen and Richard Jude Samulski; U.S. Pat. Nos. 5,139,941 and 5,962,313).

Genebank reference numbers for sequences of AAV serotypes 1, 2, 3, 3B, 4, 5, 6, 7, 8, 9, 10, 11, 12, and 13 are listed in patent publication WO2012064960.

A non-limiting method of rAAV particle production method is described next. One or more helper plasmids are produced or obtained, which comprise rep and cap ORFs for the desired AAV serotype and the adenoviral VA, E2A (DBP), and E4 genes under the transcriptional control of their native promoters. In some embodiments, the one or more helper plasmids comprise *rep* genes, *cap* genes, and optionally one or more of the adenoviral VA, E2A (DBP), and E4 genes under the transcriptional control of their native promoters. In some embodiments, the one or more helper plasmids comprise cap ORFs (and optionally rep ORFs) for the desired AAV serotype and the adenoviral VA, E2A (DBP), and E4 genes under the transcriptional control of their native promoters. The cap ORF may also comprise one or more modifications to produce a modified capsid protein as described herein. As an example, HEK293 cells (available from ATCC®) are transfected via CaPO4-mediated transfection, lipids or polymeric molecules such as Polyethylenimine (PEI) with the helper

plasmid(s) and a plasmid containing a nucleic acid vector. The HEK293 cells are then incubated for at least 60 hours to allow for rAAV particle production. Alternatively, the HEK293 cells are transfected via methods described above with AAV-ITR containing one or more genes of interest, a helper plasmid comprising genes encoding Rep and Cap proteins, and co-infected with a helper virus. Helper viruses are viruses that allow the replication of AAV. Examples of helper virus are adenovirus and herpesvirus.

Alternatively, in another example, Sf9-based producer stable cell lines are infected with a single recombinant baculovirus containing the nucleic acid vector. As a further alternative, in another example HEK293 or BHK cell lines are infected with a HSV containing the nucleic acid vector and optionally one or more helper HSVs containing rep and cap ORFs as described herein and the adenoviral VA, E2A (DBP), and E4 genes under the transcriptional control of their native promoters. The HEK293, BHK, or Sf9 cells are then incubated for at least 60 hours to allow for rAAV particle production. The rAAV particles can then be purified using any method known in the art or described herein, e.g., by iodixanol step gradient, CsCl gradient, chromatography, or polyethylene glycol (PEG) precipitation.

Methods for large-scale production of AAV using a herpesvirus-based system are also known. See for example, Clement et al. (Hum Gene Ther. 2009, 20(8):796-806). Methods of producing exosome-associated AAV, which can be more resistant to neutralizing anti-AAV antibodies, are also known (Hudry et al., Gene Ther. 2016, 23(4):380-92; Macguire et al., Mol Ther. 2012, 20(5):960-71).

Methods for producing and using pseudotyped rAAV vectors are also known in the art (see, e.g., Duan et al., J. Virol., 75:7662-7671, 2001; Halbert et al., J. Virol., 74:1524-1532, 2000; Zolotukhin et al., Methods, 28:158-167, 2002; and Auricchio et al., Hum. Molec. Genet., 10:3075-3081, 2001).

Compositions

Various formulations have been developed to facilitate rAAV particle use. For example, for administration of an injectable aqueous solution of rAAV particles, the solution may be suitably buffered, if necessary, and the liquid diluent first rendered isotonic with sufficient saline or glucose. In some embodiments, a composition as provided herein comprises a plurality of any one of the variant rAAV (e.g., variant rAAV2) particles disclosed herein. In some embodiments, a composition comprises pluralities of more than one of the variant rAAV (e.g., variant rAAV2) particles disclosed herein. In some

embodiments, "administering" or "administration" means providing a material to a subject in a manner that is pharmacologically useful.

Accordingly, in some embodiments, a composition of variant rAAV particles comprises a pharmaceutically acceptable carrier. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the rAAV particle is administered. Such pharmaceutical carriers can be sterile liquids (e.g., water, oils, saline solutions, aqueous dextrose and glycerol solutions), suspending agents, preserving agents (e.g., methyl-, ethyl-, and propyl-hydroxy-benzoates), and pH adjusting agents (such as inorganic and organic acids and bases). In some embodiments, carriers include buffered saline solutions (e.g., phosphate buffered saline, HEPES-buffered saline). USP grade carriers and excipients are particularly useful for delivery of rAAV particles to human subjects. Such compositions may further optionally comprise a liposome, a lipid, a lipid complex, a microsphere, a microparticle, a nanosphere, or a nanoparticle, or may be otherwise formulated for administration to the cells, tissues, organs, or body of a subject in need thereof. Methods for making such compositions are well known and can be found in, for example, Remington: The Science and Practice of Pharmacy, 22nd edition, Pharmaceutical Press, 2012.

In some embodiments, a composition comprising any one of the rAAV particles disclosed herein comprises Balanced Salt Solution (BSS) supplemented with 0.014% Tween 20 (polysorbate 20). In some embodiments, a composition comprising any one of the rAAV particles disclosed herein comprises 100 mM sodium citrate, 10 mM Tris, pH 8.0, supplemented with 0.001% Pluronic F-68.

Typically, compositions may contain at least about 0.1% of the therapeutic agent (e.g., rAAV particle) or more, although the percentage of the active ingredient(s) may be varied and may conveniently be between about 1 or 2% and about 70% or 80% or more of the weight or volume of the total formulation. Naturally, the amount of therapeutic agent(s) (e.g., rAAV particle) in each therapeutically-useful composition may be prepared is such a way that a suitable dosage will be obtained in any given unit dose of the compound. Factors such as solubility, bioavailability, biological half-life, route of administration, product shelf life, as well as other pharmacological considerations will be contemplated by one skilled in the art of preparing such pharmaceutical formulations, and as such, a variety of dosages and treatment regimens may be desirable.

The pharmaceutical forms of rAAV particle compositions suitable for injectable use include sterile aqueous solutions or dispersions. In some embodiments, the form is sterile and fluid to the extent that easy syringability exists. In some embodiments, the form is stable

under the conditions of manufacture and storage and is preserved against the contaminating action of microorganisms, such as bacteria and fungi. In some embodiments, the form is sterile. The carrier can be a solvent or dispersion medium containing, for example, water, saline, ethanol, polyol (e.g., glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and/or vegetable oils. Proper fluidity may be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants.

Preparation of compositions for administration to a subject are known in the art. For example, a dosage may be dissolved in 1 ml of isotonic NaCl solution and either added to 1000 ml of hypodermoclysis fluid or injected at the proposed site of infusion, (see for example, "Remington's Pharmaceutical Sciences" 15th Edition, pages 1035-1038 and 1570-1580). Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject. Moreover, for human administration, preparations should meet sterility, pyrogenicity, and the general safety and purity standards as required by, e.g., FDA Office of Biologics standards.

Methods of transducing cells

Any one of the rAAV particles, or compositions comprising any one of the rAAV particles disclosed herein can be used to transduce a cell, tissue or organ. In some embodiments, a cell, tissue or organ that is transduced using any one of the variant rAAV (e.g., variant rAAV2) particles disclosed herein is transduced with a gene of interest that may be a therapeutic gene or one that is desired to study. In some embodiments, a cell, tissue or organ is transduced in an *in vitro* setting wherein the cell, tissue or organ is incubated or perfused with a media. A cell may be one of many cells cultured under certain conditions, or part of an organ that is harvested, part of an organoid, or an organism.

In some embodiments, a cell, tissue or organ is transduced *in vivo*, for example, for the purposes of treating a disease. In some embodiments, such a rAAV particle comprises a gene of interest that encodes a therapeutic protein or RNA. In some embodiments, provided herein is a method of transducing a cell or tissue of an eye (or two eyes) or brain. In some embodiments, a specific tissue in the eye (or two eyes) or brain in targeted. For example, the retina or one or more cell type of the retina may be targeted (e.g., photoreceptors (PR), retinal ganglion cells (RGC), bipolar cells, trabecular meshwork, retinal pigment epithelium (RPE) cells, amacrine cells, astrocytes, horizontal cell, microglia, or Muller glia).

Some non-limiting examples of retinal diseases that may be treated using any one of the compositions provided herein include age-related macular degeneration, choroidermia, color blindness, Leber's congenital amaurosis, reitinitis pigmentosa, Stargardt's disease, Acromatopsia, Blue cone monochromacy, Cone-rod dystrophy, congenital stationary night-blindness, Leber's hereditary Optic Neuropathy and Glaucoma. Some non-limiting examples of syndromic diseases where the retina and other neurons such as brain and sensory organs such as the ear may be treated using any one of the compositions provided herein include Bardet-Biedl syndrome, Glycogen storage diseases, Ceroid lipofuscinosis, Canavan disease, Friedreich's ataxia, Pompe's and Usher's syndrome. Accordingly, any one of the variant rAAV particles as disclosed herein or compositions comprising any one of the variant rAAV particles as disclosed herein, can be used to target the inner ear.

In some embodiments, a composition comprising any one or more of the variant rAAV (e.g., variant rAAV2) particles disclosed herein is provided to photoreceptor cells (PRs). In some embodiments, a composition comprising any one or more of the variant rAAV (e.g., variant rAAV2) particles disclosed herein is provided to retinal ganglion cells (RGCs). In some embodiments, a composition comprising variant rAAV (e.g., variant rAAV2)particles is provided to a PR and/or RGC via an intravitreal injection to the subject carrying the PR and/or RGC. In some embodiments, a composition is provided via subretinal injection. In some embodiments, a composition is provided via subILM injection. Other non-limiting examples of routes to administrate a composition as disclosed herein to the eye (or two eyes) of a subject include intracameral, periocular and subconjunctival injections. In some embodiments, a composition may be injected into the lateral geniculate nucleus of a subject. Such a method may be used to target RGCs. In some embodiments, a composition may be administered topically to an eye or two eyes of a subject (e.g., in eye drops).

In some embodiments, the tissue of the brain that is targeted comprises Purkinje cells or ependymal cells. The Purkinje cells project to the deep cerebellar nuclei and are the only output cells of the cerebellar cortex. Conditions involving Purkinje cells include ataxia telangiectasia and Niemann Pick disease type C, as well as cerebellar essential tremor. Purkinje cells can also be damaged in Alzheimer's disease and by rabies virus. Purkinje cells also play a role in degenerative diseases of the cerebellum (Ferrer et al., Clin Neuropathol. 1988, 7(1):22-8).

Ependymal cells make up the ependyma, which is the thin epithelial lining of the ventricular system of the brain and the central canal of the spinal cord. Ependymal cells play an important role in the production and regulation of CSF, and act as reservoir cells in the

forebrain, which can be activated after stroke and as in vivo and in vitro stem cells in the spinal cord. As such, these cells can be used to supply beneficial molecules to other cells in contact with CSF. For example, ependymal cells can be used to provide growth factors to other cells by transducing them with a gene that encodes one or more growth factors.

In some embodiments, a method of transducing an ependymal or Purkinje cell with a gene of interest involves providing to the ependymal cell or the Purkinje cell any one of the compositions provided herein. In some embodiments, such a composition is administered to a subject via intraventricular injection. In some embodiments, a variant rAAV particle that is used to transduce Purkinje and/or ependymal cells with a gene of interest comprises sequences DGE and DF in VRV, NA in VRI; and SEQ ID NO: 5 in VRVII. In some embodiments, a composition is administered to a subject via intrathecal injection, intracisternal injection, intracranial (e.g., thalamic, intracerebroventricular or ventral tegmental) injection.

In some embodiments, a subject in which a cell, tissue or organ is transduced is a vertebrate animal (e.g., a mammal or reptile). In some embodiments, a mammalian subject is a human, a non-human primate, a dog, a cat, a hamster, a mouse, a rat, a pig, a horse, a cow, a donkey or a rabbit. Non-limiting examples of non-human primate subjects include macaques (e.g., cynomolgus or rhesus macaques), marmosets, tamarins, spider monkeys, owl monkeys, vervet monkeys, squirrel monkeys, baboons, gorillas, chimpanzees, and orangutans. In some embodiments, a subject is a model for a particular disease or used to study the pharmacokinetics and/or pharmacokinetics of a protein or siRNA encoded by a gene of interest.

To "treat" a disease as the term is used herein, means to reduce the frequency or severity of at least one sign or symptom of a disease or disorder experienced by a subject. The compositions described above or elsewhere herein are typically administered to a subject in an effective amount, that is, an amount capable of producing a desirable result. The desirable result will depend upon the active agent being administered. For example, an effective amount of rAAV particles may be an amount of the particles that are capable of transferring an expression construct to a host cell, tissue or organ. A therapeutically acceptable amount may be an amount that is capable of treating a disease, e.g., Leber's congenital amaurosis. As is well known in the medical and veterinary arts, dosage for any one subject depends on many factors, including the subject's size, body surface area, age, the particular composition to be administered, the active ingredient(s) in the composition, time

and route of administration, general health, and other drugs being administered concurrently.

EXAMPLES

Example 1: AAV capsid library

An AAV capsid library was created to encompass as much of the 'natural' variation of existing Parvoviruses (see FIGs. 1A-1C). The capsid library was built with an AAV2 *cap* backbone using a structure informed approach. Diversification was restricted to the variable loops of the AAV capsid protein, which increases the likelihood of creating variants that assemble and package properly. The AAV capsid library was then screened in mice and non-human primates (see Examples 2 and 3, respectively) to identify the most prevalent AAV variants, which there subsequently validated and characterized.

Example 2: Mouse screen

The AAV capsid library (FIGs. 1A-1C) was screened in mice as shown in FIG. 2. The transgenic mice used for screening express enhanced green fluorescent protein (EGFP) under the control of neural retina leucine zipper (*nrl*) gene promoter specifically in rod photoreceptors (PRs).

Capsid variants contained a self-complementary AAV genome carrying the truncated CBA promoter driving mCherry (sc-smCBA-mCherry) expression. Transduction was quantified *in vitro* using ocular cell lines.

The AAV library was intravitreally injected into Nrl-GFP mice. The GFP positive photoreceptors were sorted by FACS. Total DNA from photoreceptors was isolated and PCR for AAV capsid genes carried out to construct an enriched library. After three rounds of screening, a subset of the most prevalent variants was identified (FIG. 3). As shown in FIG. 3, the first most prevalent AAV2 capsid variant had around a 32% relative frequency and the second most prevalent AAV2 capsid variant had around a 21% relative frequency. These heavily enriched variants were selected for further analysis.

Example 3: NHP screen

The AAV capsid library (FIGs. 1A-1C) was also screened in macaques (*Macacca fascicularis*) in order to identify AAV variants that target PRs and RGCs after intravitreal (Ivt) injection.

Sortable cell populations were created in primate retina using a method described in U.S. Patent Publication No. US201662296056. This method is also described in Choudhury, et al., Front Neurosci. 2016,10:551. Briefly, macaque PRs and RGCs were fluorescently labelled by sub retinal injection of AAV5-GRK1-GFP and retrograde transport of MICRO-RUBY™ (TRITC-Dextran-Biotin) from the lateral geniculate nucleus (LGN), respectively. As shown in FIG. 4, the capsid library was delivered subsequent to the injection into the LGN during the in-life phase by Ivt injection. Retinas were anatomically separated into different regions and cells from each region underwent fluorescent activated cell sorting (FACS) (see FIG. 4).

FIG. 5 shows the most prevalent AAV2 capsid variants that were isolated after two rounds of screening the capsid library in macaques. The four most prevalent AAV variants V1 (which is the same as Vb) to V4 (which is the same as Va) were selected for validation and further analysis, all of which displayed substantially improved transduction *in vitro* compared to wild-type AAV2 capsid. Interestingly, variants V1 (which is the same as Vb) to V4 (which is the same as Va), were also identified as the second (Vb) and most prevalent (Va) variants in the mouse screen.

Example 4: Evaluation of transduction profiles of AAV2 variant Va

After the most prevalent AAV2 variants were identified by screening in mouse and macaque models, as described above, the most prevalent variants were vectorized and tested for efficiency to transduce retinal cells.

AAV2 variant Va was found to be the most prevalent in the mouse screen and the fourth most prevalent in the macaque screen. FIGs. 6A-6C show the transduction profile of Va after Nrl-GFP mice were injected intravitreally with 1μl of $2e^{12}$ vg/ml of Sc-smCBA-mCherry packaged in a Va variant AAV2 capsid. Three weeks after the injection, transduction was evaluated by funduscopy (see FIG. 6A) and FACS (see FIGs. 6B and 6C). An AAV2 variant known to have enhanced transduction efficiency in retinal cells, AAV2(quadY-F+T-V), was included as a control. It can be seen in FIG. 6A that compared to the AAV2(quadY-F+T-V), the AAV2 Va variant particle carrying the gene for mCherry was able to transduce just as many, if not a higher number of retinal cells and with just as much expression per cell, if not greater expression per cell.

Four weeks after injection of the Va variant AAV2 particles, the mice were sacrificed, and retinal cells dissociated and sorted for GFP expression and mCherry expression. The PE-Texas Red channel in the cytometer was used to detect mCherry expression. In FIG. 6B, the

top right quadrant corresponds to the population of rod photoreceptors transduced by rAAV vector (GFP+ and mCherry+) and the bottom right quadrant corresponds to non rod, neural retinal cells transduced by rAAV vector (mCherry+ only).

FIG. 6C shows transduction rates for AAV2 variant Va when either administered to mice by intravitreal injection or subretinal injection. Mice were sacrificed 4 weeks after injection with the AAV2 variant particles. $2x10^9$ vg was injected. Compared to when the virus particles were delivered intravitreally, subretinally administered AAV2-Va was able to transduce a higher number of non rod, neural retinal cells. The levels of transduction achieved in both rod PRs and non rod, neural retinal cells after subretinal injection were comparable to those achieved with 2.5 times more of the wild-type AAV2 virus.

In addition to testing for transduction of retinal cells, experiments were done to assess the ability of the AAV2 variant Va capsid to transduce ependymal and Purkinje cells. Mice were injected with $4x10^9$ vg of virus particles carrying Sc-smCBA-mCherry. Four weeks thereafter, the mice were sacrifices and sections of the brain were prepared. As shown in FIG. 7A, AAV2-Va promotes the transduction of ependymal cells, which are responsible for secreting CSF and are an attractive target for neuroprotective gene therapy. As shown in FIG. 7B, AAV2-Va particles were also able to transduce Purkinje cells.

Example 5: Evaluation of transduction profiles of AAV2 variant Vb

AAV2 variant Vb was found to be the second most prevalent in the mouse screen and the most prevalent in the macaque screen. FIGs. 8A-8C show the transduction profile of Vb after Nrl-GFP mice were injected intravitreally with 1µl of $2e^{12}$ vg/ml of Sc-smCBA-mCherry packaged in a Vb variant AAV2 capsid. Three weeks after the injection, transduction was evaluated by funduscopy (see FIG. 8A) and FACS (see FIGs. 8B and 8C). Compared to the AAV2(quadY-F+T-V), which has the mutations Y272F, Y444F, Y500F, Y730F and T491V, the AAV2 Vb variant particle carrying the gene for mCherry was able to transduce a higher number of retinal cells and with a higher expression per cell (FIG. 8A).

Four weeks after injection of the Vb variant AAV2 particles, the mice were sacrificed, and retinal cells dissociated and sorted for GFP expression and mCherry expression. The PE-Texas Red channel in the cytometer was used to detect mCherry expression. In FIG. 8B, the top right quadrant corresponds to the population of rod photoreceptors transduced by rAAV vector (GFP+ and mCherry+) and the bottom right quadrant corresponds to non rod, neural retinal cells transduced by rAAV vector (mCherry+ only).

FIG. 8C shows transduction rates for AAV2 variant Va when either administered to mice by intravitreal injection or subretinal injection. Mice were sacrificed 4 weeks after injection with the AAV2 variant particles. $2x10^9$ vg was injected. The levels of transduction achieved in both rod PRs and non rod, neural retinal cells after subretinal injection were comparable to those achieved with 2.5 times more of the wild-type AAV2 virus.

Example 6: Evaluation of transduction profiles of AAV2 variant V2

The transduction efficiency was measured in ARPE19 cells and the results can be seen in FIG. 9. Compared to AAV2(quadY-F+T-V) variant virus, AAV2-V2 variant virus was able to result in mCherry expression levels that were approximately 7 times higher.

When tested in mice in a manner similar to how AAV2 variants Va and Vb, it was found that the transduction efficiency in mouse retina of AAV-V2 as observed by funduscopy was much higher compared to the control AAV2(quadY-F+T-V) (FIG. 10A). A Characteristic FACs plot for retinal cells transduced with AAV2-V2 is shown in FIG. 10B. The transduction efficiency relative to AAV2(quadY-F+T-V) is shown in FIG. 10C. As can be seen, the AAV2-V2 variant outperforms the AAV2(quadY-F+T-V) variant virus.

Example 7: Evaluation of transduction profiles of AAV2 variant V3

The transduction efficiency was measured in ARPE19 cells and the results can be seen in FIG. 11. Compared to AAV2(quadY-F+T-V) variant virus, AAV2-V3 variant virus was able to result in mCherry expression levels that were approximately 5 times higher.

When tested in mice in a manner similar to how AAV2 variants Va, Vb and V2, it was found that the transduction efficiency in mouse retina of AAV2-V3 as observed by funduscopy was much higher compared to the control AAV2(quadY-F+T-V) (FIG. 12A). A Characteristic FACs plot for retinal cells transduced with AAV2-V3 is shown in FIG. 12B. The transduction efficiency relative to AAV2(quadY-F+T-V) is shown in FIG. 12C. As can be seen, the AAV2-V3 variant outperforms the AAV2(quadY-F+T-V) variant virus.

Example 8: Rationally designed variants

Since it is known that certain mutations enhance the efficiency of AAV particles to transduce retinal cells, these mutations were superimposed onto the variants identified by the screening in mouse and macaque models to have greater retinal transduction capacity to

further improve their performance. FIG. 13 shows transduction profiles using funduscopy of AAV2 variants Va and Vb having additional Y to F, and T to V substitutions. Va-YF represents a variant with the sequence of variant Va with additional phenylalanines at positions 444 and 730. Similarly, Vb-YF represents a variant with the sequence of variant Vb with additional phenylalanines at positions 444 and 730. AAV2 variant Vb-YF-TV represents a variant with the sequence of variant Vb with additional F at positions 272, 444 and 730, and a valine at positon 491. It is clear from the fluorescence of mCherry in the fundus images that these substitutions greatly enhance the transduction efficiency (FIG. 13). Quantification of FACS data also shows that these additional mutations greatly improve the efficiency of the AAV2 capsid variants to transduce retinal cells (FIG. 14).

Example 9: Additional AAV Capsid Variants that Promote Efficient Transduction of Retina by Intravitreal Injection

Adeno-associated virus (AAV) variants were isolated from a highly diverse AAV capsid library, CAPLIB-7, described in Example 1 by three rounds of *in vivo* selection performed in nonhuman primate (NHP). Selection initially involved creating an NHP with sortable photoreceptors (via subretinal injection of AAV5-GRK1-GFP) and retinal ganglion cells (via injection of a retrograde tracer dye into the lateral geniculate nucleus). Following creation of sortable cells, intravitreal injection of the capsid library into NHP was performed. This was followed by separate isolation of NHP photoreceptors (PR) and retinal ganglion cells (RGC), subsequent recovery of capsid variants individually from each cell type, and regeneration of separate PR and RGC sublibraries. Subsequent screens were then done in parallel with NHPs receiving RGC sub-library and RGCs being isolated and vice versa for PR sub-library. After the second round of selection in primate, a number of novel capsid variants were identified. When a subset of these variants were isolated and vectorized with a reporter construct they were shown to have increased transduction efficiencies in cell culture. When vectors were intravitreally injected into mice, transduction efficiencies were greatly improved over AAV2 and in most cases were better than quadYF+T-V. Subsequent to this a third round of screening in primate was performed and additional capsid variants were identified. These additional capsid variants are disclosed herein, many of which were not observed in the first two rounds of selection. The new capsid variants fall into 2 broad groups 1) Capsid variants that have increased their relative abundance in both PRs and RGCs from the 2nd to 3rd round of screening and 2) capsid variants that display a distributional bias towards either retinal ganglion cells (RGC) or photoreceptors (PR). Group 1 variants include

P3-8, Vb, P3-3 and P3-4. Group 2 variants include P3-RGC1, P3-RGC2, and P3-RGC3 which displayed enrichment in primate retinal ganglion cells and low abundance in photoreceptors, and P3-PR1, P3-PR2, and P3-PR3 which conversely were substantially enriched in photoreceptors over retinal ganglion cells (FIGs. 24 and 25).

Example 10:

The methodology for screening capsid libraries in primate retina was as follows. It relied on the ability to selectively "sort" retinal cells while maintaining the integrity of the nucleic acids contained within the cells, and was accomplished by expression of green fluorescent protein in photoreceptors via subretinal delivery of AAV5-GRK1-GFP and/or retrograde labeling of retinal ganglion cells (RGCs) by injection of fluorescent dye into the lateral geniculate nucleus (LGN).

Round 3 screening results were assessed and variants enriched in photoreceptors and RGCs were identified (FIG. 24 and 25). Variants emerged with "biased" distribution between photoreceptors and RGCs.

Certain variants described herein were further enhanced by rational design. Va, Vb and V3 were modified to incorporate additional tyrosine to phenylalanine and threonine to valine mutations previously identified to enhance retinal transduction. Va (Y444+730F) was tested. Va (Y272+444+730F)+T491V was also created but packaged with poor efficiency (n=3). Vb (Y444+730F) and Vb (Y272+444+730F)+T491V were also tested. V3(Y272+500+730F)+T491V was also tested.

The transduction of mouse retina was characterized following Ivt injection. Capsid variants were vectorized to contain a self-complementary AAV with smCBA promoter driving mCherry. They were packaged at small scale, 2 cell stack, with iodixanol gradient purification. They were intravitreally injected at moderate dose, 2e9 vg in 1ul into Nrl-GFP mice (N=6 or more for each variant). Transgene expression was evaluated 4 weeks post injection by fundoscopy for mCherry fluorescence (in life) and by FACS of dissociated neural retina (RPE removed) to quantify the percentage of rod photoreceptor expressing mCherry (GFP-mCherry double positive cells). This is identical to published methodology for quantifying transduction efficiencies (Boye et al. J Virol. 2016 Mar 28;90(8):4215-31).

Capsid variants identified display substantially improved transduction of mouse retina following Ivt injection, relative to parent capsid AAV2. Rational design-guided mutagenesis

further enhanced transduction in capsid variants Va and Vb. Five capsid variants outperform benchmark vectors. IHC indicates capsids variants display broad cell tropism.

Table 3. Capsid variants selected for transduction in primate retina using "barcoded" reporter construct. Results are shown in FIG. 16.

Capsid variant	Type	Ivt rod transdxn/ AAV2(quadYF+T-V)
AAV2	benchmark	0.3X*
AAV2(trpYF)	benchmark	not tested
AAV2(quadYF+T-V)	benchmark	1.0X
Va	Library	0.6X
Vb	Library	1.5X
Vb(Y444+730F)	Library + rational des.	3.5X
Vb(Y272+444+730F)+T491V	Library + rational des.	**4.4X**
V2	Library	3.4X
V3	Library	2.6X
P3-RGC1 (P2-V6)	Library	1.5X
P3-PR3	Library	1.6X
DGE-DF (AKA 'V1V4 VR-V')	Library	2.5X
AAV-7m8	benchmark	1.8X

*Value based on previous experiments comparing AAV2 to other AAV2 capsid variants in the same mouse model and methodology, Boye et al. 2016 J. Virology.

Relative transduction and transgene expression efficiencies of capsid variants in macaque and mouse retina were evaluated utilizing barcoded vectors. Methods are shown in FIGs. 15 to 19. Vector constructs with CBA promoter driving mCherry that were identical except for a unique 5 nucleotide "barcode" (FIG. 20) were packaged individually in the selected capsid variants. The location of the barcode allows identification of DNA (vector genome) and RNA (transgene expression) associated with each capsid variant following recovery from tissue/cells. Barcoded vectors were manufactured by triple transfection and purified by successive double iodixanol density gradients followed by ion-exchange chromatography (FPLC, Q-column). Vectors were assessed for: purity by protein gel, endotoxin by Endosafe PTS (Charles River), spec. less than 5 Eu/mL, full to empty ratio by electron microscopy, and spec. >50% full capsids. Several vectors were remade due to aggregation of capsids as observed on EM and by loss of genome titer following freeze thaw

cycle. These vectors were put into a high salt buffer of BSS-tween supplemented with 150mM NaCl. All vector preparations utilized in the barcoded pool passed specifications.

"Barcoded" vector pools: two "pooled" mixes were made: 1.0X mix, total concentration of 3e12 vg/ml, with each variant at approx. 2.3e11 vg/ml, and 0.1X mix, total concentration of 3e11 vg/ml, with each variant at approx. 2.3e10 vg/ml. Both barcoded vector pools diluted into BSS tween buffer. 1.0X pool was calculated to be 398 mOsm vs 300 mOsm physiologic due to the inclusion of vector preps eluted in high salt. It was noted that significant dilution of vector occurred upon Ivt injection. Pools were created separately (i.e., 0.1X pool is not a 1:10 dilution of the 1.0X pool).

Barcoded experimental plan for NHPs: two *M. fascicularis* (cynomolgus monkey) had RGCs labeled for isolation by FACS. They received a single 100ul intravitreal injection of barcoded pool. One eye received 1.0X pool, the other eye 0.1X pool. 3 weeks after Ivt injection of barcoded vectors, they received LGN injection of "green" dye, and were sacrificed 1 week later (4 weeks following barcoded vector injection). Two NHPs had PRs were labeled for isolation by FACS. Multiple subretinal blebs of AAV5-GRK1-GFP labelled photoreceptors. Three days later, they received 100ul Ivt injection of barcoded pool (same as above). Six weeks after Ivt injection of barcoded vectors, the animals were sacrificed. The sacrifice was originally scheduled for 4 weeks following Ivt of barcode but was delayed by approximately a week and a half. All NHPs pre-screened for anti-AAV2 NAb. Selected animals appeared naïve.

Table 4. NHP information.

Animal # (and name/ID)	cell type labeled	Right eye	Left eye	DOB (approx age)
1 (Joseph/AH568L)	RGCs	1.0X barcode	0.1X barcode	12/23/2013 (3.7 yrs)
2 (Gus-Gus/BB328F)	RGCs	1.0X barcode	0.1X barcode	3/17/2010 (7.5 yrs)
3 (Rasheed/MR88G)	Photoreceptors	0.1X barcode	1.0X barcode	3/8/2008 (9.5 yrs)
4 (Sid/GB3X)	Photoreceptors	1.0X barcode	0.1X barcode	8/2/2010 (7.0 yrs)

RGC labeled animals were imaged as follows: 1 week pre-injection, color fundus only, 2 days post Ivt injection of barcode, color fundus only, 2 and 3 weeks post Ivt barcode, color fundus + mCherry fluorescence, 4 weeks post Ivt barcode and 6-7 days post LGN injection of tracer, FITC + mCherry fluorescence. PR labeled animals imaged: 4 days pre subretinal injection of AAV5-GFP (PR labeling), 9 days post subretinal AAV5-GFP, color fundus only, 23 days post subretinal AAV5-GFP and 20 days post Ivt barcode, color fundus + FITC + mCherry fluorescence 4 and 5 weeks post subretinal AAV5-GFP and Ivt barcode,

color fundus + FITC + mCherry fluorescence, 6 weeks post subretinal AAV5-GFP and Ivt barcode, FITC + mCherry fluorescence. The results of the imaging are shown in FIGs. 21, 22, and 23.

OTHER EMBODIMENTS

All of the features disclosed in this specification may be combined in any combination. Each feature disclosed in this specification may be replaced by an alternative feature serving the same, equivalent, or similar purpose. Thus, unless expressly stated otherwise, each feature disclosed is only an example of a generic series of equivalent or similar features.

From the above description, one skilled in the art can easily ascertain the essential characteristics of the present disclosure, and without departing from the spirit and scope thereof, can make various changes and modifications of the disclosure to adapt it to various usages and conditions. Thus, other embodiments are also within the claims.

EQUIVALENTS

While several inventive embodiments have been described and illustrated herein, those of ordinary skill in the art will readily envision a variety of other means and/or structures for performing the function and/or obtaining the results and/or one or more of the advantages described herein, and each of such variations and/or modifications is deemed to be within the scope of the inventive embodiments described herein. More generally, those skilled in the art will readily appreciate that all parameters, dimensions, materials, and configurations described herein are meant to be exemplary and that the actual parameters, dimensions, materials, and/or configurations will depend upon the specific application or applications for which the inventive teachings is/are used. Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific inventive embodiments described herein. It is, therefore, to be understood that the foregoing embodiments are presented by way of example only and that, within the scope of the appended claims and equivalents thereto, inventive embodiments may be practiced otherwise than as specifically described and claimed. Inventive embodiments of the present disclosure are directed to each individual feature, system, article, material, kit, and/or method described herein. In addition, any combination of two or more such features, systems, articles, materials, kits, and/or methods, if such features, systems, articles, materials,

kits, and/or methods are not mutually inconsistent, is included within the inventive scope of the present disclosure.

All definitions, as defined and used herein, should be understood to control over dictionary definitions, definitions in documents incorporated by reference, and/or ordinary meanings of the defined terms.

The indefinite articles "a" and "an," as used herein in the specification and in the claims, unless clearly indicated to the contrary, should be understood to mean "at least one."

The phrase "and/or," as used herein in the specification and in the claims, should be understood to mean "either or both" of the elements so conjoined, i.e., elements that are conjunctively present in some cases and disjunctively present in other cases. Multiple elements listed with "and/or" should be construed in the same fashion, i.e., "one or more" of the elements so conjoined. Other elements may optionally be present other than the elements specifically identified by the "and/or" clause, whether related or unrelated to those elements specifically identified. Thus, as a non-limiting example, a reference to "A and/or B", when used in conjunction with open-ended language such as "comprising" can refer, in one embodiment, to A only (optionally including elements other than B); in another embodiment, to B only (optionally including elements other than A); in yet another embodiment, to both A and B (optionally including other elements); etc.

As used herein in the specification and in the claims, "or" should be understood to have the same meaning as "and/or" as defined above. For example, when separating items in a list, "or" or "and/or" shall be interpreted as being inclusive, i.e., the inclusion of at least one, but also including more than one, of a number or list of elements, and, optionally, additional unlisted items. Only terms clearly indicated to the contrary, such as "only one of" or "exactly one of," or, when used in the claims, "consisting of," will refer to the inclusion of exactly one element of a number or list of elements. In general, the term "or" as used herein shall only be interpreted as indicating exclusive alternatives (i.e., "one or the other but not both") when preceded by terms of exclusivity, such as "either," "one of," "only one of," or "exactly one of." "Consisting essentially of," when used in the claims, shall have its ordinary meaning as used in the field of patent law.

As used herein in the specification and in the claims, the phrase "at least one," in reference to a list of one or more elements, should be understood to mean at least one element

selected from any one or more of the elements in the list of elements, but not necessarily including at least one of each and every element specifically listed within the list of elements and not excluding any combinations of elements in the list of elements. This definition also allows that elements may optionally be present other than the elements specifically identified within the list of elements to which the phrase "at least one" refers, whether related or unrelated to those elements specifically identified. Thus, as a non-limiting example, "at least one of A and B" (or, equivalently, "at least one of A or B," or, equivalently "at least one of A and/or B") can refer, in one embodiment, to at least one, optionally including more than one, A, with no B present (and optionally including elements other than B); in another embodiment, to at least one, optionally including more than one, B, with no A present (and optionally including elements other than A); in yet another embodiment, to at least one, optionally including more than one, A, and at least one, optionally including more than one, B (and optionally including other elements); etc.

It should also be understood that, unless clearly indicated to the contrary, in any methods claimed herein that include more than one step or act, the order of the steps or acts of the method is not necessarily limited to the order in which the steps or acts of the method are recited.

In the claims, as well as in the specification above, all transitional phrases such as "comprising," "including," "carrying," "having," "containing," "involving," "holding," "composed of," and the like are to be understood to be open-ended, i.e., to mean including but not limited to. Only the transitional phrases "consisting of" and "consisting essentially of" shall be closed or semi-closed transitional phrases, respectively, as set forth in the United States Patent Office Manual of Patent Examining Procedures, Section 2111.03. It should be appreciated that embodiments described in this document using an open-ended transitional phrase (e.g., "comprising") are also contemplated, in alternative embodiments, as "consisting of" and "consisting essentially of" the feature described by the open-ended transitional phrase. For example, if the disclosure describes "a composition comprising A and B", the disclosure also contemplates the alternative embodiments "a composition consisting of A and B" and "a composition consisting essentially of A and B".

CLAIMS

1. A variant recombinant adeno-associated virus (rAAV) capsid protein comprising DGE at positions 492-494, DF at positions 499 and 500, and SAAGADXAXDS (SEQ ID NO: 5) at positions corresponding to amino acids 546-556 of AAV2 VP1 capsid protein

wherein each X corresponds to amino acids of a wild-type AAV2 VP1 capsid sequence as set forth in SEQ ID NO: 1, or homologous amino acids of a wild-type VP1 capsid sequence of an AAV serotype other than AAV2.

2. A variant recombinant adeno-associated virus (rAAV) capsid protein comprising DGE at positions 492-494, DF at positions 499 and 500, and EDATENXIXXDR (SEQ ID NO: 4) at positions corresponding to amino acids 545-556 of AAV2 VP1 capsid protein;

wherein each X corresponds to amino acids of a wild-type AAV2 VP1 capsid sequence as set forth in SEQ ID NO:1, or homologous amino acids of a wild-type VP1 capsid sequence of an AAV serotype other than AAV2.

3. The rAAV capsid protein of claim 1, wherein the capsid protein is of serotype 2 and wherein the capsid protein comprises a sequence of SEQ ID NO: 12.

4. The rAAV capsid protein of claim 1 or 2 further comprising NA at positions 263 and 264 in variable region (VR) I.

5. The rAAV capsid protein of claim 1 or 2, further comprising phenylalanines at positions corresponding to amino acids 444 and 730 of the AAV2 VP1 capsid sequence.

6. The rAAV capsid protein of any one of claims 1, 2 and 5, further comprising a valine at a position corresponding to 491 of the AAV2 VP1 capsid sequence.

7. The rAAV capsid protein of any one of claims 1, 2 and 4-6, wherein the capsid protein is of serotype 2.

8. The rAAV capsid protein of claim 1 further comprising substitutions corresponding to T491Q and T503P in variable region V of the AAV2 VP1 capsid sequence.

Date Reçue/Date Received 2020-10-29

9. The rAAV capsid protein of claim 1 or 2 further comprising an alanine at a position corresponding to 263 of the AAV2 VP1 capsid sequence.

10. The rAAV capsid protein of any one of claims 1, 2 and 5, further comprising a phenylalanine at a position corresponding to 272 of the AAV2 VP1 capsid sequence.

11. The rAAV capsid protein of claim 2, wherein the capsid protein is of serotype 2 and comprises a sequence of SEQ ID NO: 11.

12. The rAAV capsid protein of claim 2 or 9 further comprising a threonine at a position corresponding to 507 of the AAV2 VP1 capsid sequence.

13. The rAAV capsid protein of claim 2 further comprising a tryptophan at a position corresponding to 267 of the AAV2 VP1 capsid sequence.

14. The rAAV capsid protein of claim 9 further comprising a threonine at a position corresponding to 490 of the AAV2 VP1 capsid sequence.

15. The rAAV capsid protein of any one of claims 1, 2, 4-6, 8 and 9, wherein the capsid protein is of a serotype other than serotype 2 and wherein each X corresponds to homologous amino acids of the variable region of a wild-type VP1 capsid sequence of a serotype other than serotype 2.

16. The variant rAAV capsid protein of claim 15, wherein the variant rAAV capsid protein is of serotype 1, 3, 3B, 4, 5, 6, 7, 8, 9, 10, 11, 12, or 13.

17. The variant rAAV capsid protein of claim 15, wherein the variant rAAV capsid protein is of serotype 1 or 6.

18. A variant recombinant AAV (rAAV) particle comprising the rAAV capsid protein of any one of claims 1-17.

19. The variant recombinant AAV particle of claim 18, wherein the particle is an AAV2 particle.

Date Reçue/Date Received 2020-10-29

20. The variant recombinant AAV2 particle of claim 19, further comprising a nucleic acid comprising a gene of interest.

21. The variant recombinant AAV particle of claim 20, wherein the nucleic acid is single stranded.

22. The variant recombinant AAV particle of claim 20, wherein the nucleic acid is double stranded.

23. A composition comprising a plurality of the variant recombinant AAV2 particles of any one of claims 19-22 and a pharmaceutically acceptable carrier.

24. An *in vitro* method of transducing a type of retinal cell with a gene of interest, the method comprising providing to the retinal cell the composition of claim 23, wherein the AAV2 particles in the composition comprise the gene of interest.

25. The method of claim 24, wherein the retinal cell is a photoreceptor, trabecular meshwork endothelial cell or fibroblast cell, retinal ganglion cell, bipolar cell, Muller cell, amacrine cell, astrocyte, horizontal cell or retinal pigment epithelial cell.

26. The method of claim 24 or 25, wherein the gene of interest encodes a therapeutic protein.

27. The method of claim 26, wherein the therapeutic protein is an antibody or antibody fragment, a peptibody, a growth factor, a hormone, a membrane protein, a cytokine, a chemokine, an activating or inhibitory peptide acting on cell surface receptors or ion channels, a cell-permeant peptide targeting intracellular processes, an enzyme, a nuclease or another protein used for gene editing.

28. Use of the variant rAAV particle of any one of claims 18-22 or the composition of claim 23 as a medicament.

29. Use of the variant rAAV particle of any one of claims 20-22 or the composition of claim 23 for administration to one or more retinal cells of a subject.

Date Reçue/Date Received 2020-10-29

30. The use of claim 29, wherein the variant particle or composition is for use as an intravitreal injection.

31. The use of claim 29, wherein the variant particle or composition is for use as a subretinal injection.

32. The use of any one of claims 29-31, wherein the one or more retinal cells is a photoreceptor, trabecular meshwork endothelial cell or fibroblast cell, retinal ganglion cell, bipolar cell, Muller cell, amacrine cell, astrocyte, horizontal cell or retinal pigment epithelial cell.

33. The use of any one of claims 29-32, wherein the gene of interest encodes a therapeutic protein.

34. The use of claim 33, wherein the therapeutic protein is an antibody or antibody fragment, a peptibody, a growth factor, a hormone, a membrane protein, a cytokine, a chemokine, an activating or inhibitory peptide acting on cell surface receptors or ion channels, a cell-permeant peptide targeting intracellular processes, an enzyme, a nuclease or another protein used for gene editing.

Date Reçue/Date Received 2020-10-29

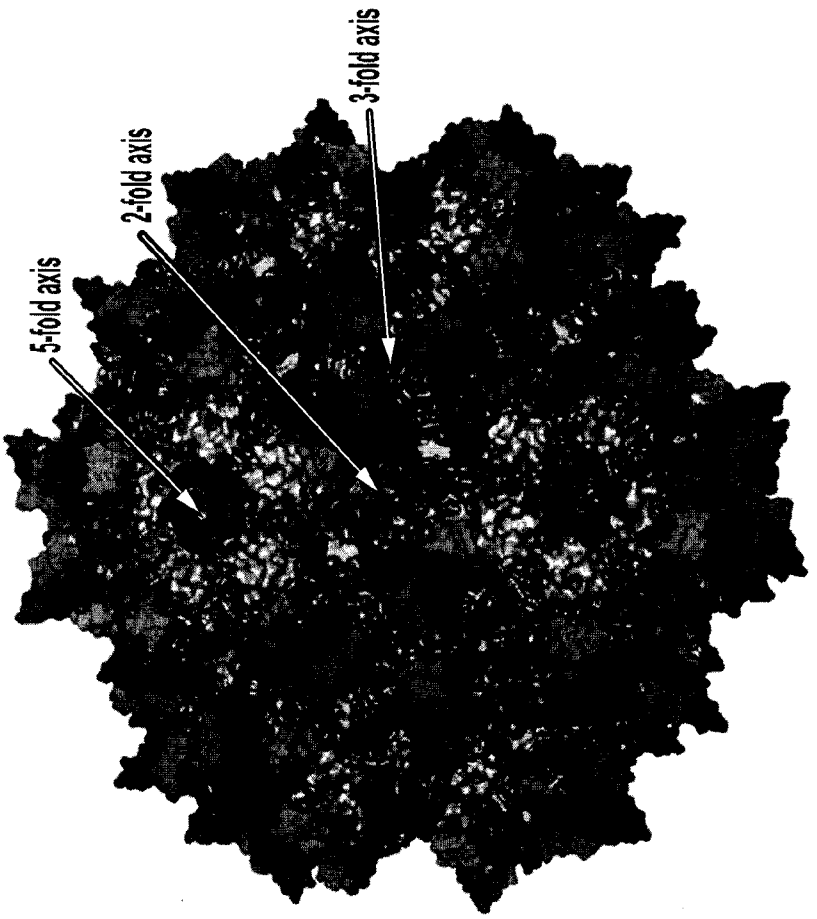
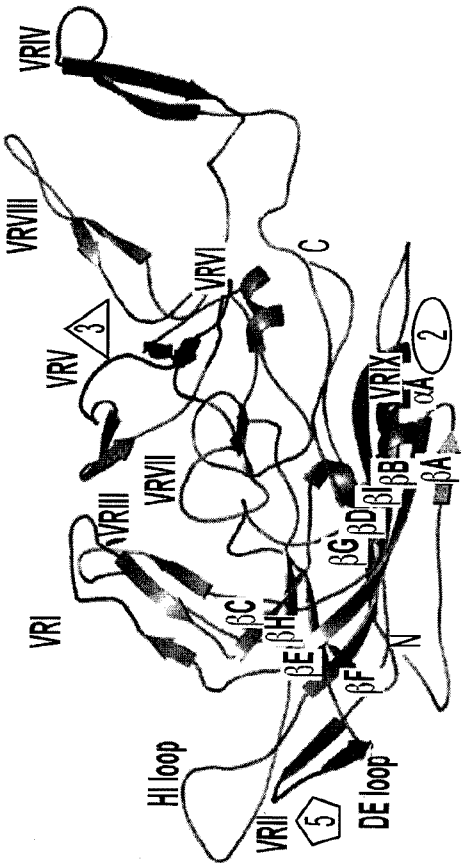

5-fold axis
2-fold axis
3-fold axis

FIG. 1B


VRIV
VRVIII
VRVI
C
VRV
3
VRIII
VRVII
VRIX
αA
2
βA
βB
βI
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βG
βC
βH
βE
βF
N
VRI
HI loop
VRII
5
DE loop

FIG. 1A

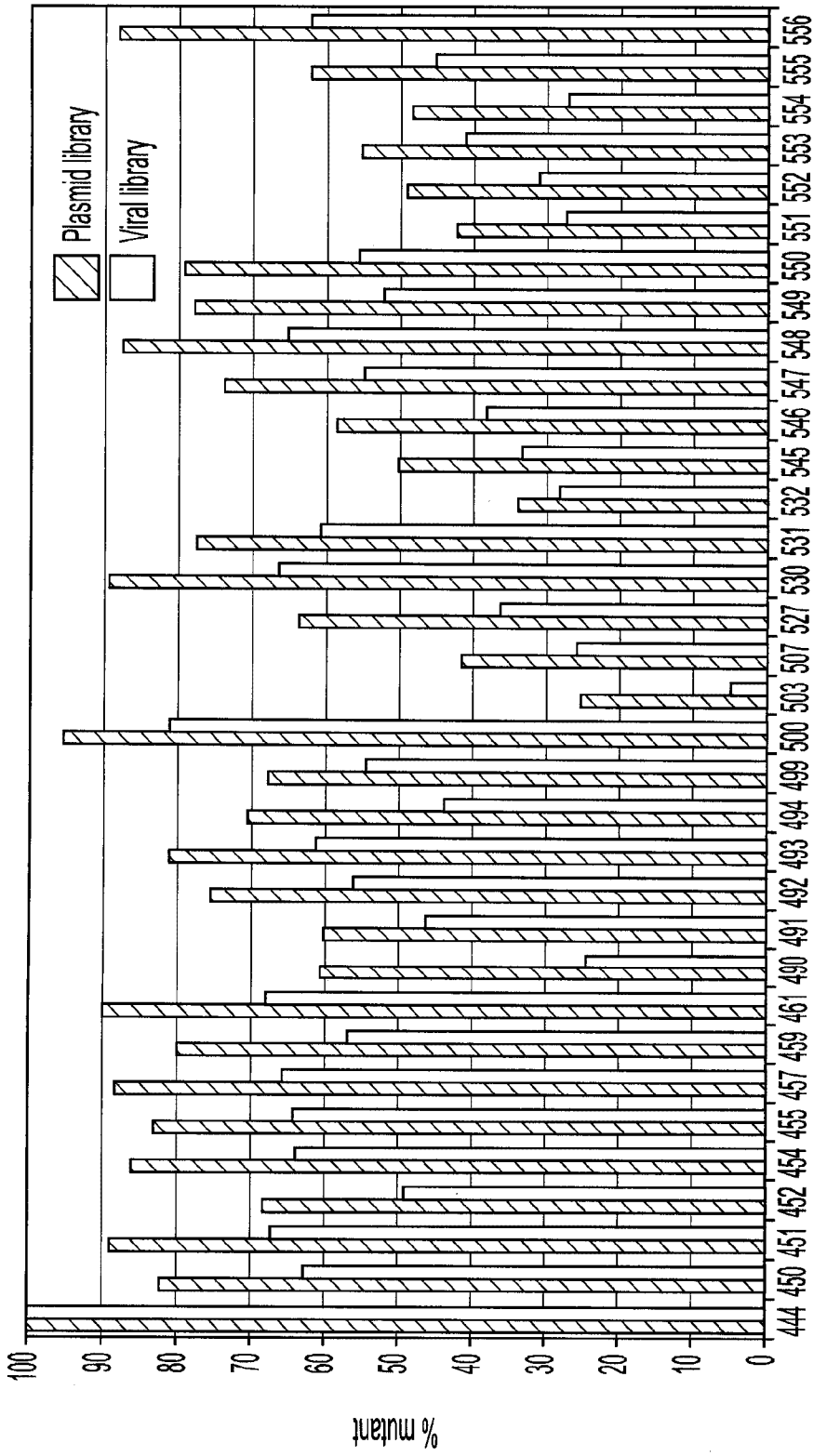

Plasmid library
Viral library
% mutant
100
90
80
70
60
50
40
30
20
10
0
444 450 451 452 454 455 457 459 461 490 491 492 493 494 499 500 503 507 527 530 531 532 545 546 547 548 549 550 551 552 553 554 555 556
Amino acid position

FIG. 1C

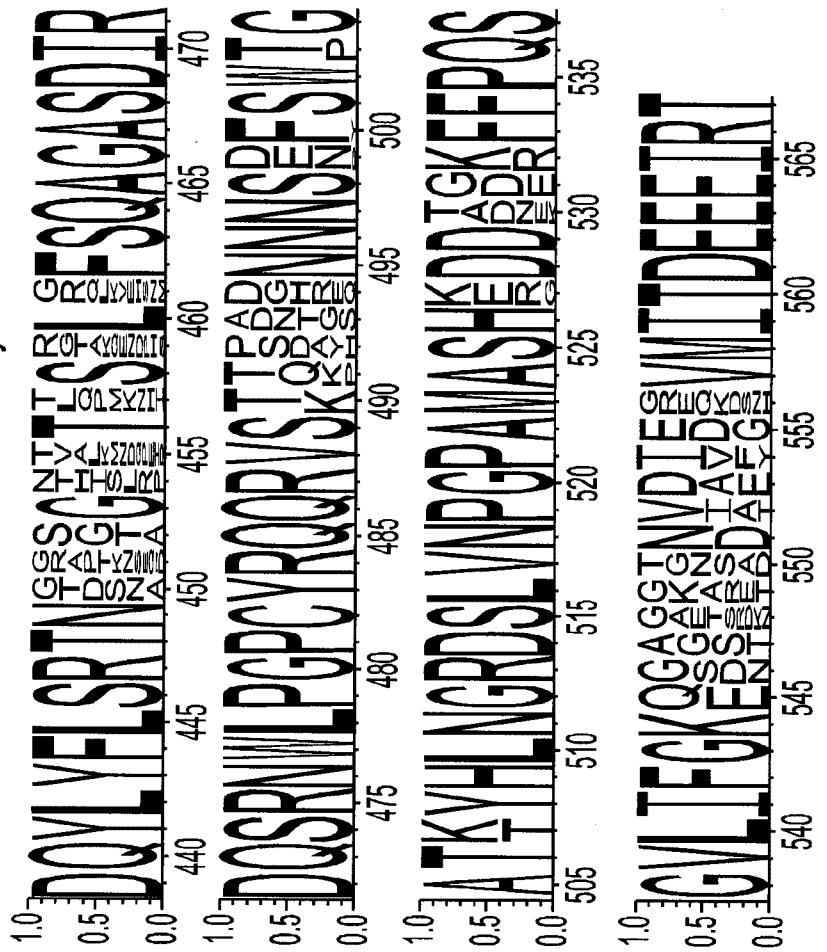


FIG. 1C continued

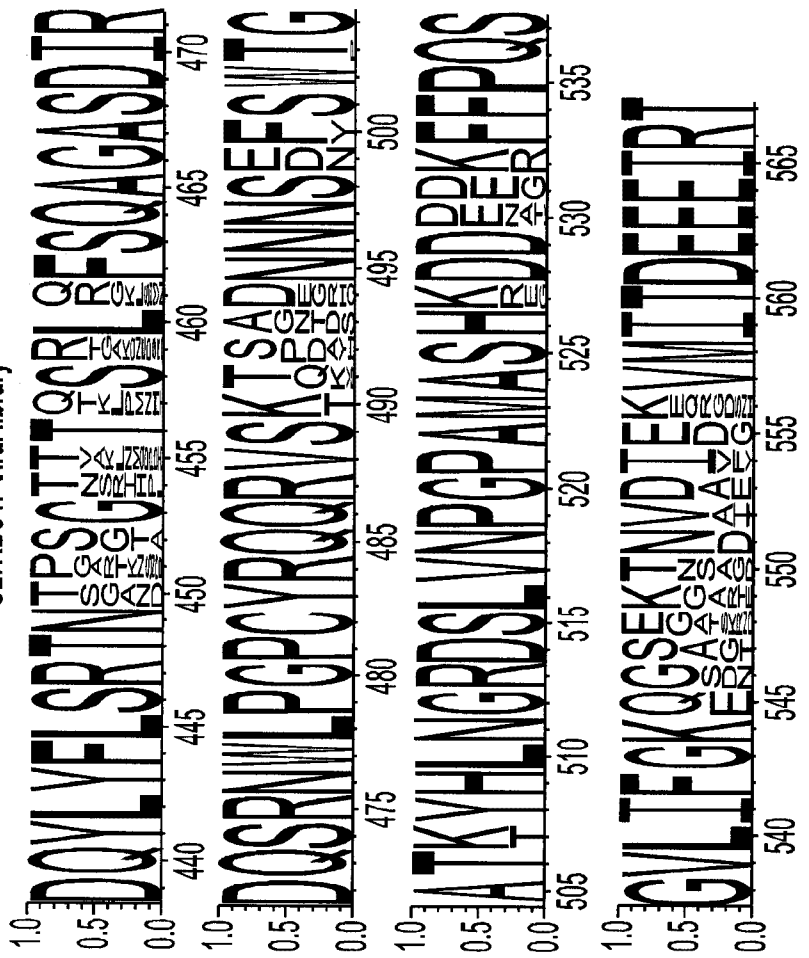


FIG. 1C continued

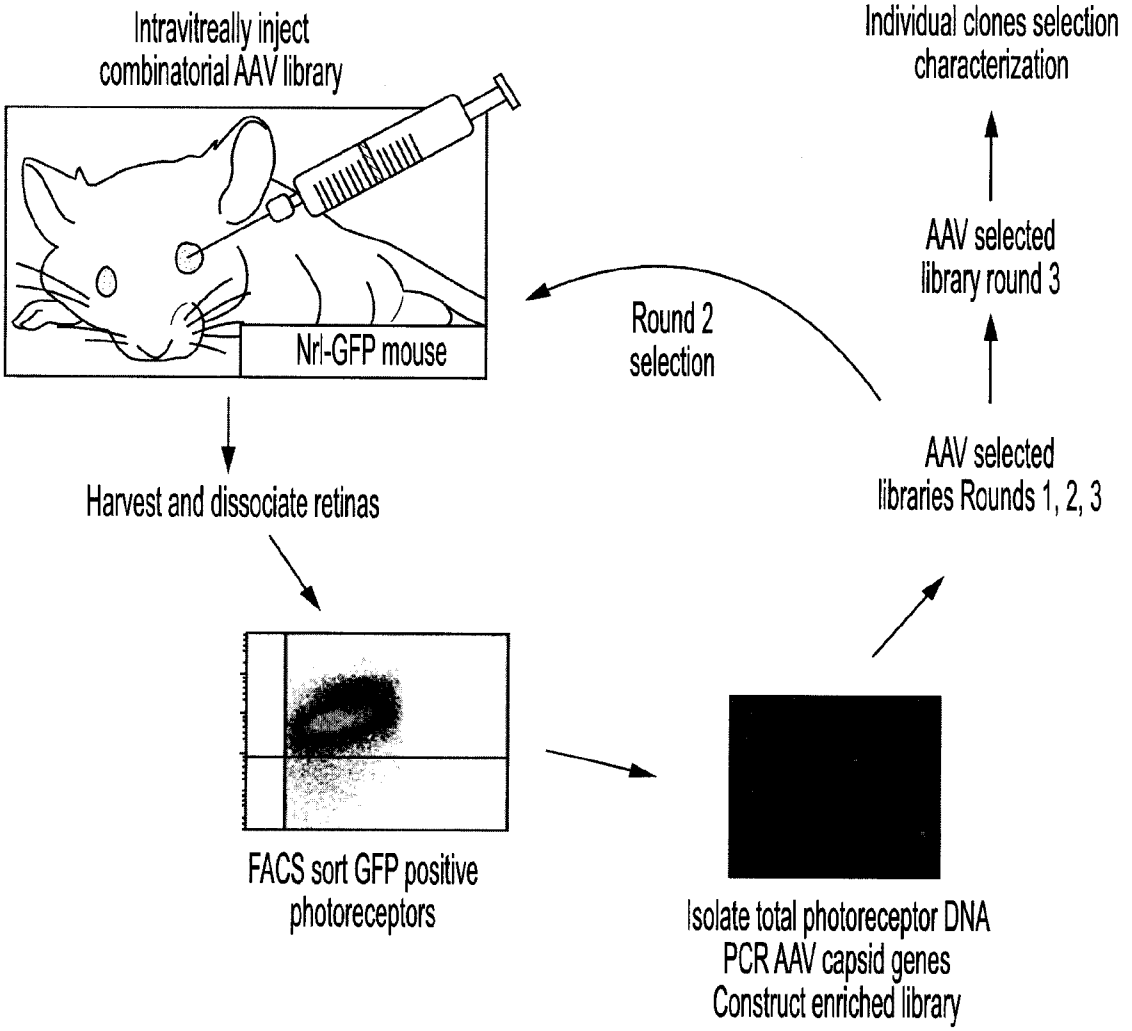
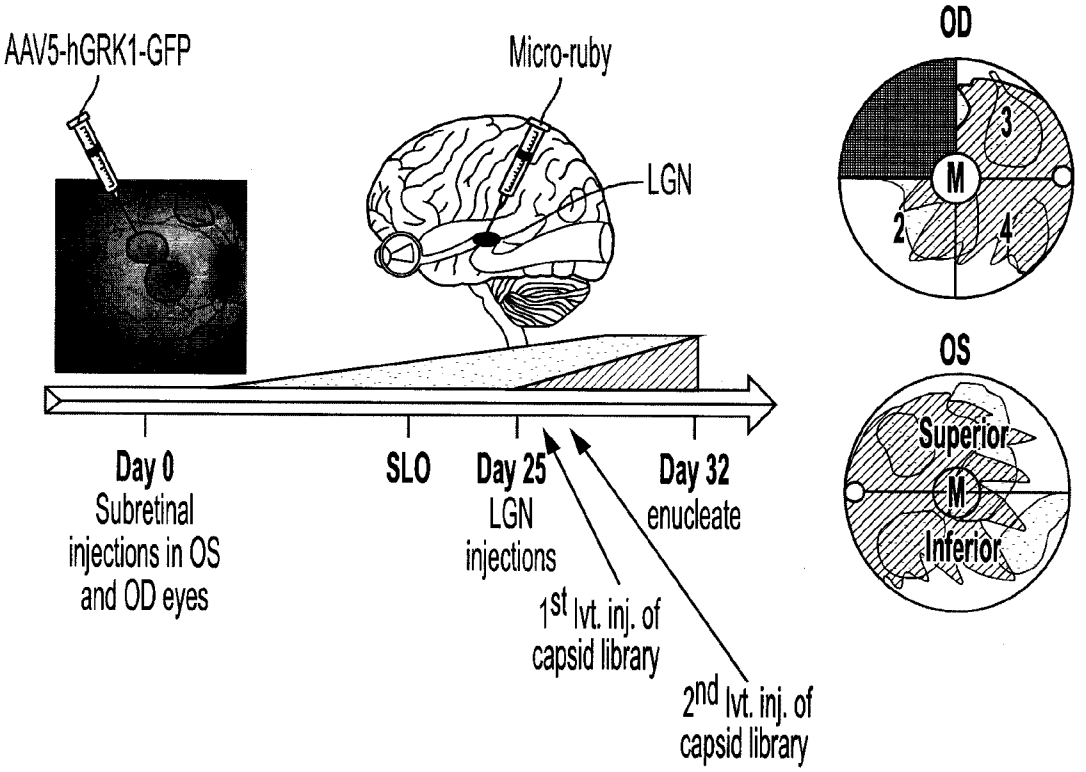

Intravitreally inject
combinatorial AAV library
Nrl-GFP mouse
Harvest and dissociate retinas
FACS sort GFP positive
photoreceptors
Isolate total photoreceptor DNA
PCR AAV capsid genes
Construct enriched library
AAV selected
libraries Rounds 1, 2, 3
Round 2
selection
AAV selected
library round 3
Individual clones selection
characterization

FIG. 2

	263	325	444	490	527	545	cn	%
Wild-type (SEQ ID NO: 29)	QSGAS	Q	YLSRTNTPSGTTTQSRLQ	KTSADNNNSEYSWTGATK	KDDEEK	QGSEKTNVDIEK		
Variant Va (SEQ ID NO: 11)	.....	.	.....	..DGE....DF.....	.....	EDATEN.I..DR	9	32.1
Variant Vb (SEQ ID NO: 12)	NA...	.	.....	..DGE....DF.....	.....	.SAAGAD.A.DS	6	21.4
Variant Vc (SEQ ID NO: 13)	NA...	.	.....	..DGE....DF.....	.....	EDATEN.I..DR	2	7.1
Variant Vd (SEQ ID NO: 14)	.....	.	.....	..DGE....DF.....	.....	.SAAGAD.A.DS	2	7.1
Variant Ve (SEQ ID NO: 15)	.....	.	F.....	..DGE....DF.....	.....	.....	1	3.6
Variant Vf (SEQ ID NO: 16)	.....	.	.....	..DGE....DF.....	.....	.....	1	3.6
Variant Vg (SEQ ID NO: 17)	.....	.	F.....	.....	.....	.....	1	3.6
Variant Vh (SEQ ID NO: 18)	NA...	.	F.....DEA.SE.K.T.R	.....	.....	.....	1	3.6
Variant Vi (SEQ ID NO: 19)	NA...	.	.....	..DGE....DF.....	.....	.SGREGDAE..D	1	3.6
Variant Vj (SEQ ID NO: 20)	A....	.	.....	..DGE....DF.....	.....	EDATEN.I..DR	1	3.6
Variant Vk (SEQ ID NO: 21)	A....	.	.....	T.DGE....DF.....	.....	EDATEN.I..DR	1	3.6
Variant Vl (SEQ ID NO: 22)	....W	.	.....	..DGE....DF.....	.....	EDATEN.I..DR	1	3.6
Variant Vm (SEQ ID NO: 23)	.....	K	F.....A..NV.....	.....	R..DD.	.....	1	3.6

FIG. 3


AAV5-hGRK1-GFP
Micro-ruby
LGN
OD
3
M
2
4
OS
Superior
M
Inferior
Day 0
Subretinal
injections in OS
and OD eyes
SLO
Day 25
LGN
injections
Day 32
enucleate
1st Ivt. inj. of
capsid library
2nd Ivt. inj. of
capsid library

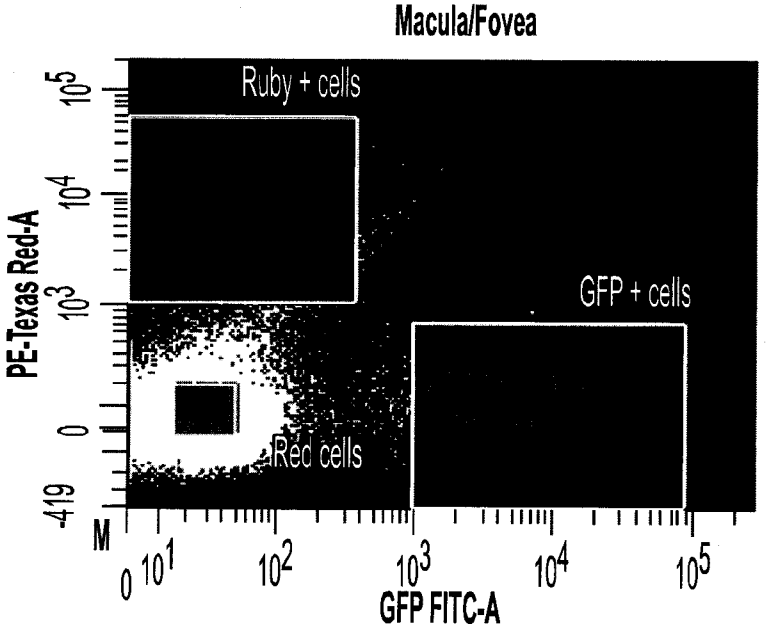
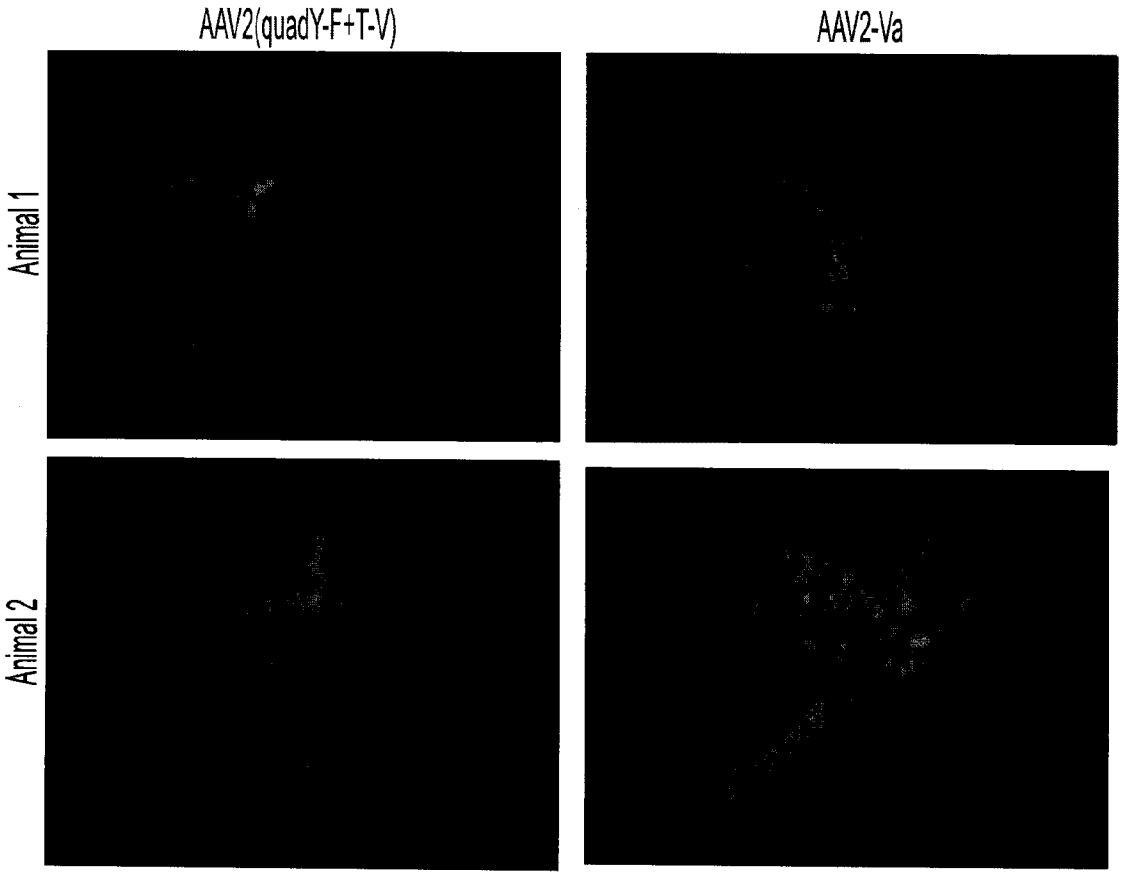

Macula/Fovea
Ruby + cells
GFP + cells
Red cells
PE-Texas Red-A
GFP FITC-A
M
0
-419
10^1
10^2
10^3
10^4
10^5

FIG. 4

	263	444	490	527	545
Wild-type (SEQ ID NO: 29)	QSGAS	YLSRTNTPSGTTTQSRLQ	KTSADNNNSEYSWTGATK	KDDEEK	QGSEKTNVDIEK
Variant V1 (SEQ ID NO: 12)	NA...	.....	..DGE....DF.....	.....	.SAAGAD.A.DS
Variant V2 (SEQ ID NO: 24)	*A....*	.....	*T.P.....DF.....*	*...D..*	.....
Variant V3 (SEQ ID NO: 25)	NA...	F.....A..NV...T..	.....	R..DD.	.....
Variant V4 (SEQ ID NO: 11)	.....	.....	***..DGE....DF.....***	.....	***EDATEN.I..DR***
Variant V5 (SEQ ID NO: 26)	.....	.....	.....	*...D..*	.....
Variant V6 (SEQ ID NO: 27)	.....	.....	***.QD.E.....F..P....***	.....	.....
Variant V7 (SEQ ID NO: 28)	***EA***...	.....	***.V.....F.....***	.....	***..AAADD.E.DG***

FIG. 5



Fundus images captured 3 weeks post injection

FIG. 6A

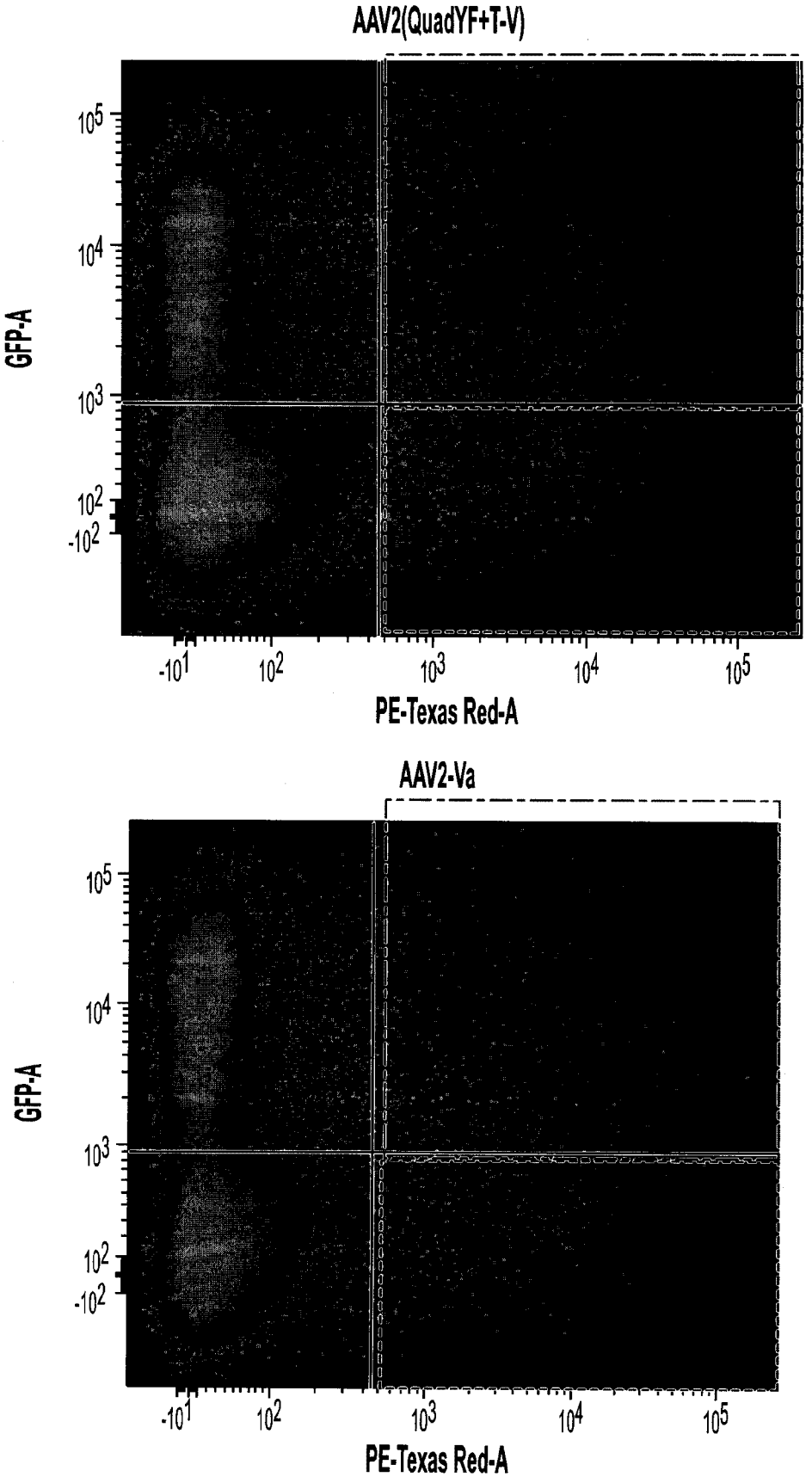


FIG. 6B

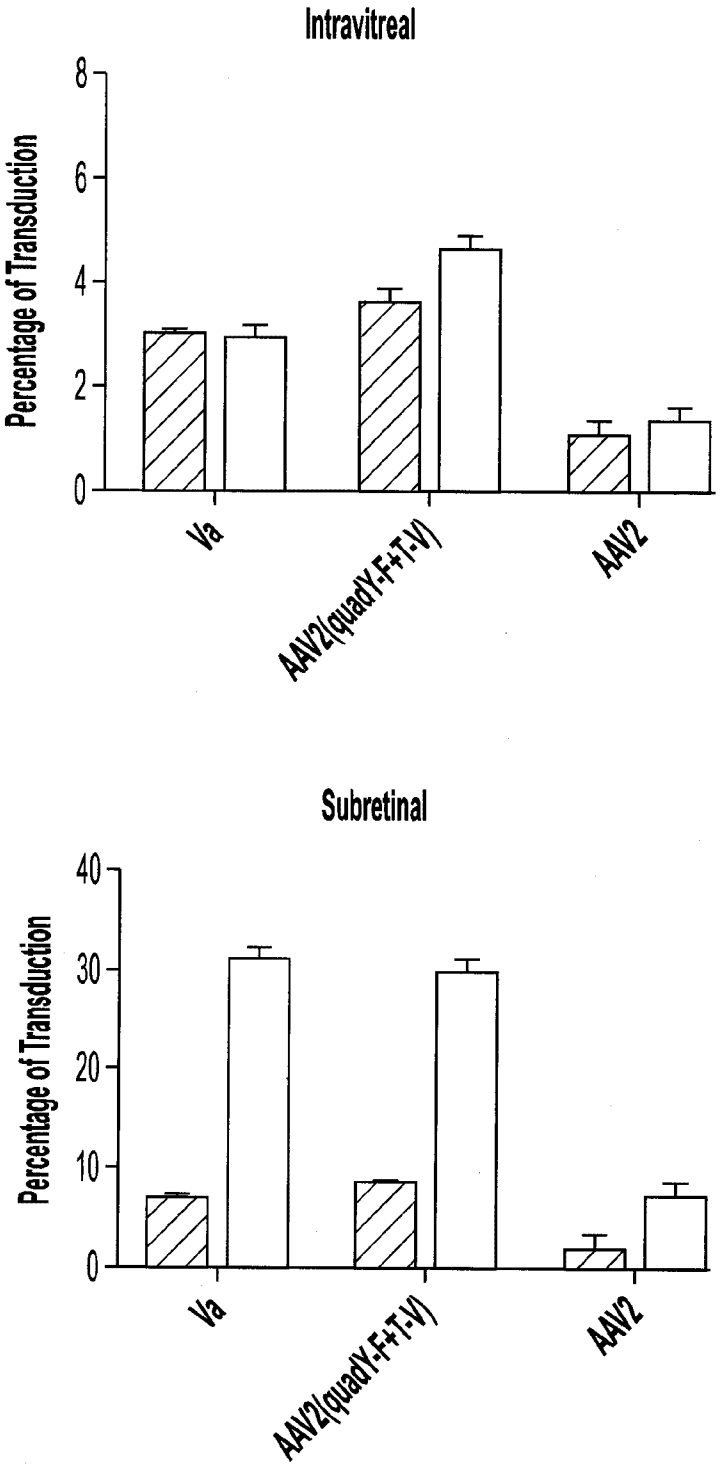

Intravitreal
Percentage of Transduction
8
6
4
2
0
Va
AAV2(quadY-F+T-V)
AAV2
Subretinal
Percentage of Transduction
40
30
20
10
0
Va
AAV2(quadY-F+T-V)
AAV2

FIG. 6C

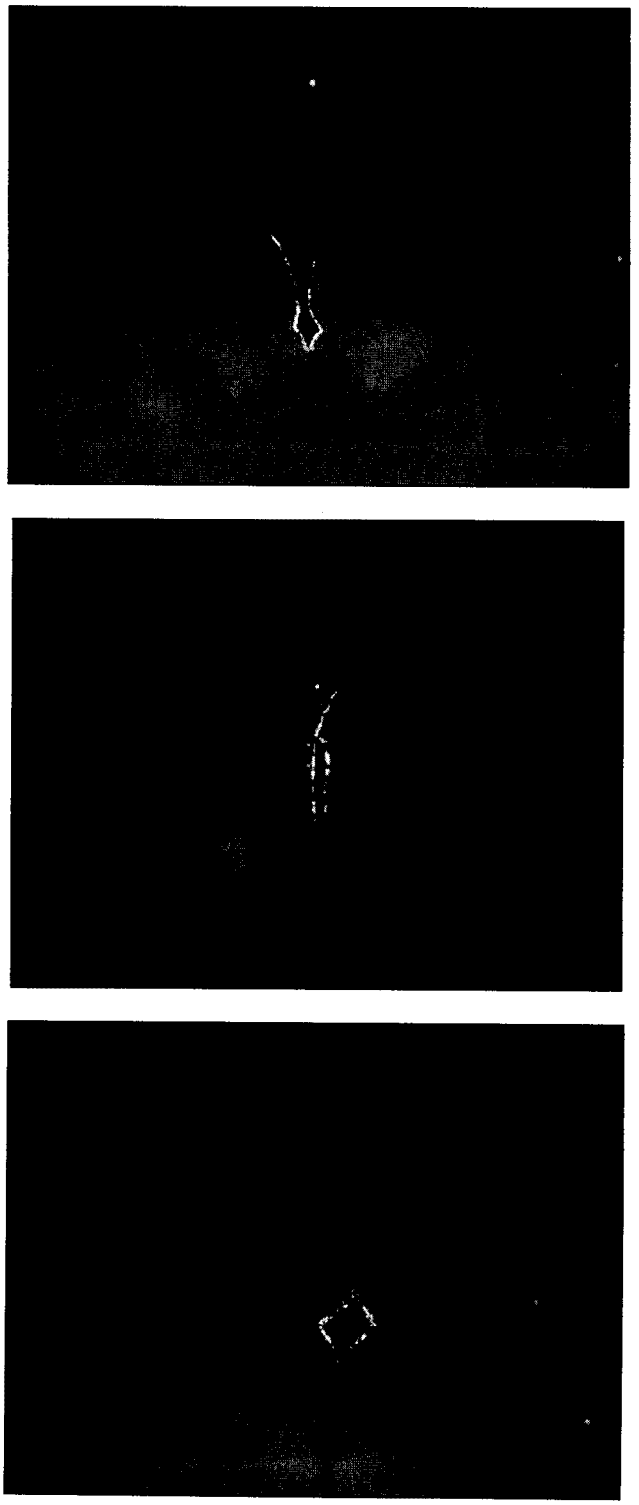


FIG. 7A

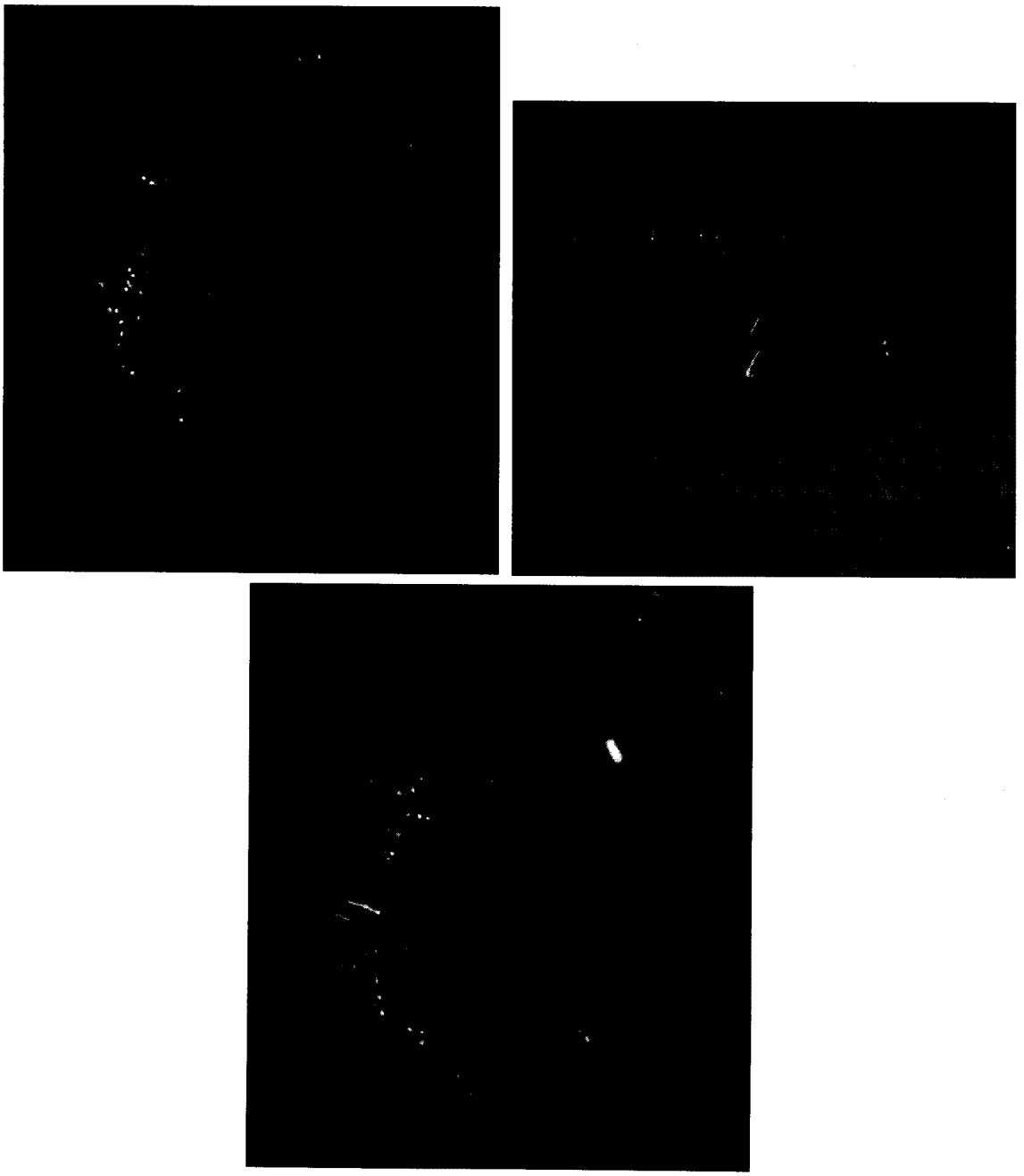


FIG. 7B

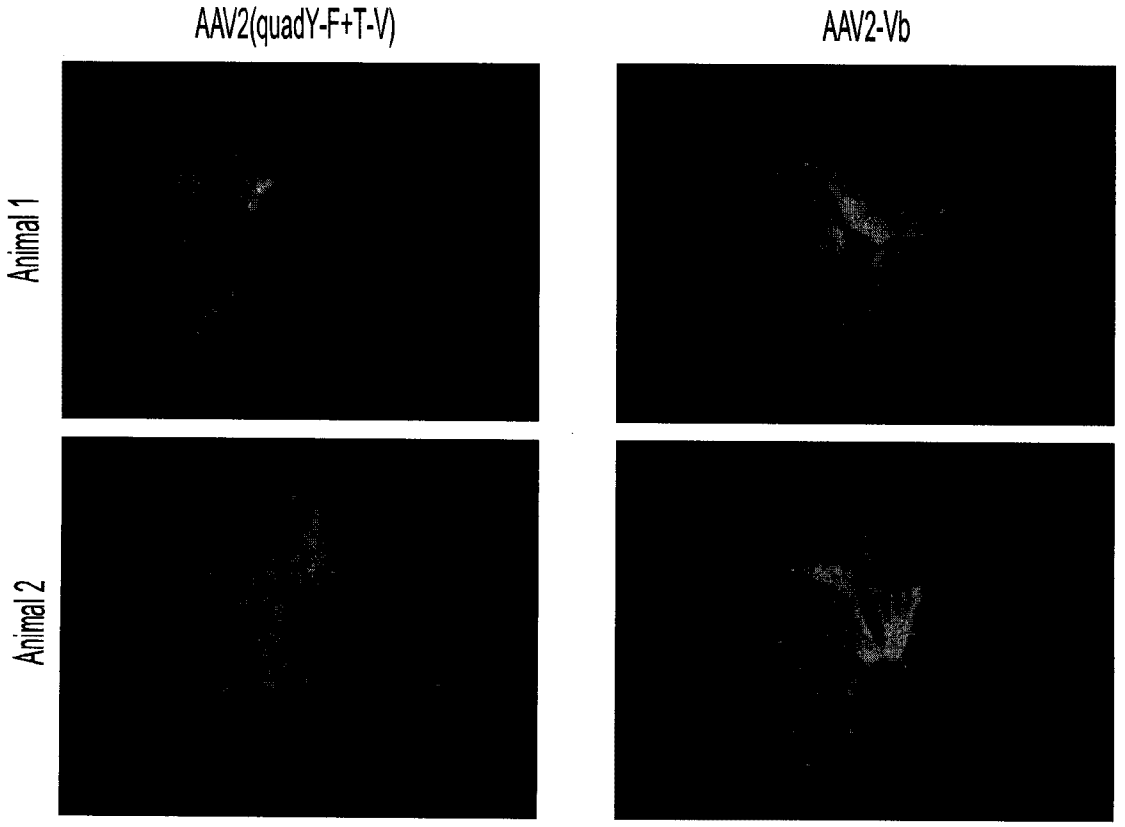

AAV2(quadY-F+T-V)
AAV2-Vb
Animal 1
Animal 2
Fundus images captured 3 weeks post injection

FIG. 8A

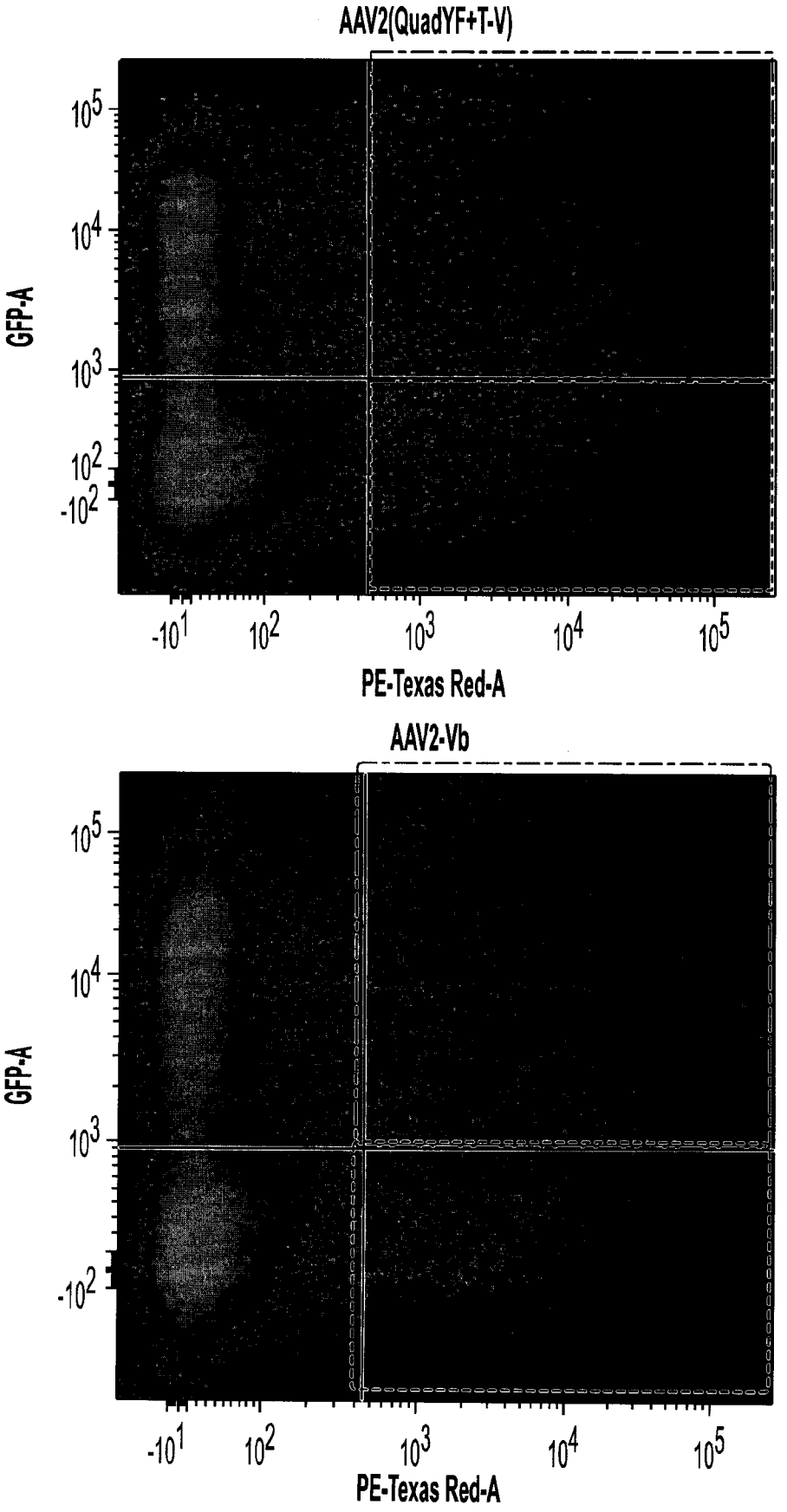

AAV2(QuadYF+T-V)
GFP-A
10^5
10^4
10^3
10^2
-10^2
-10^1
10^2
10^3
10^4
10^5
PE-Texas Red-A
AAV2-Vb
GFP-A
10^5
10^4
10^3
-10^2
-10^1
10^2
10^3
10^4
10^5
PE-Texas Red-A

FIG. 8B

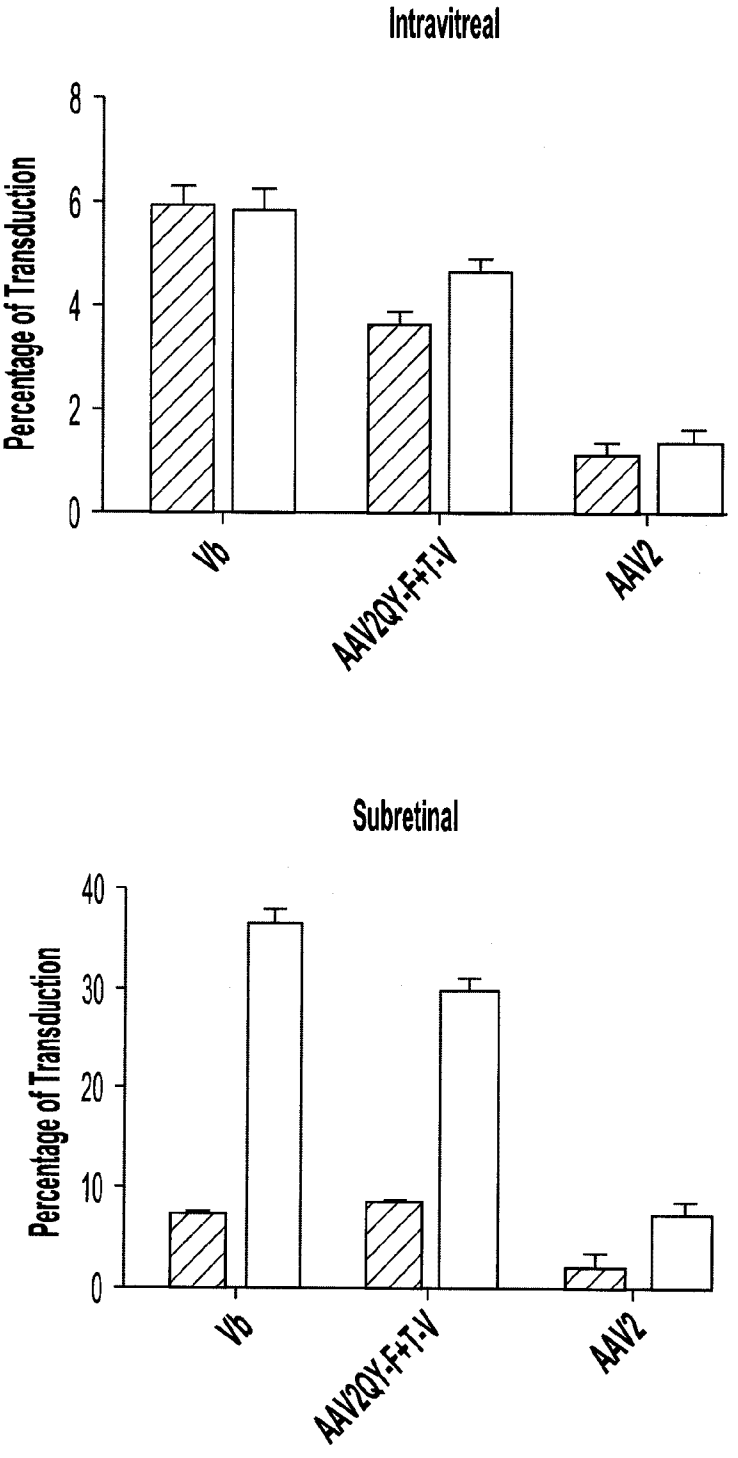

Intravitreal
Percentage of Transduction
8
6
4
2
0
Vb
AAV2QY-F+T-V
AAV2
Subretinal
Percentage of Transduction
40
30
20
10
0
Vb
AAV2QY-F+T-V
AAV2

FIG. 8C

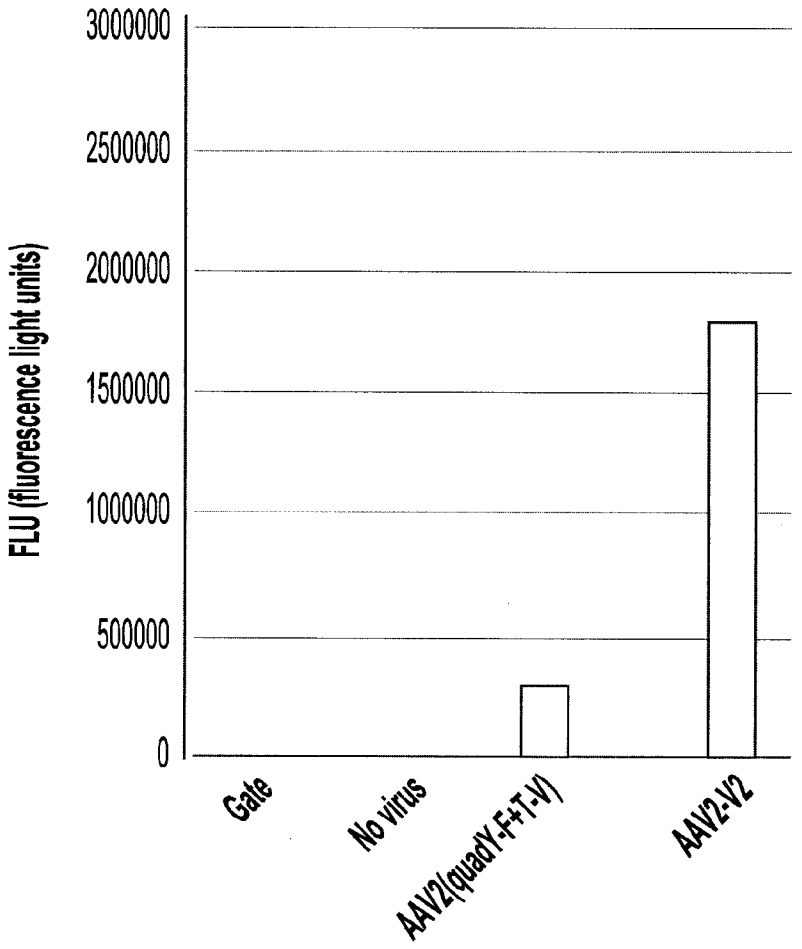

3000000
2500000
2000000
1500000
1000000
500000
0
FLU (fluorescence light units)
Gate
No virus
AAV2(quadY-F+T-V)
AAV2-V2

FIG. 9

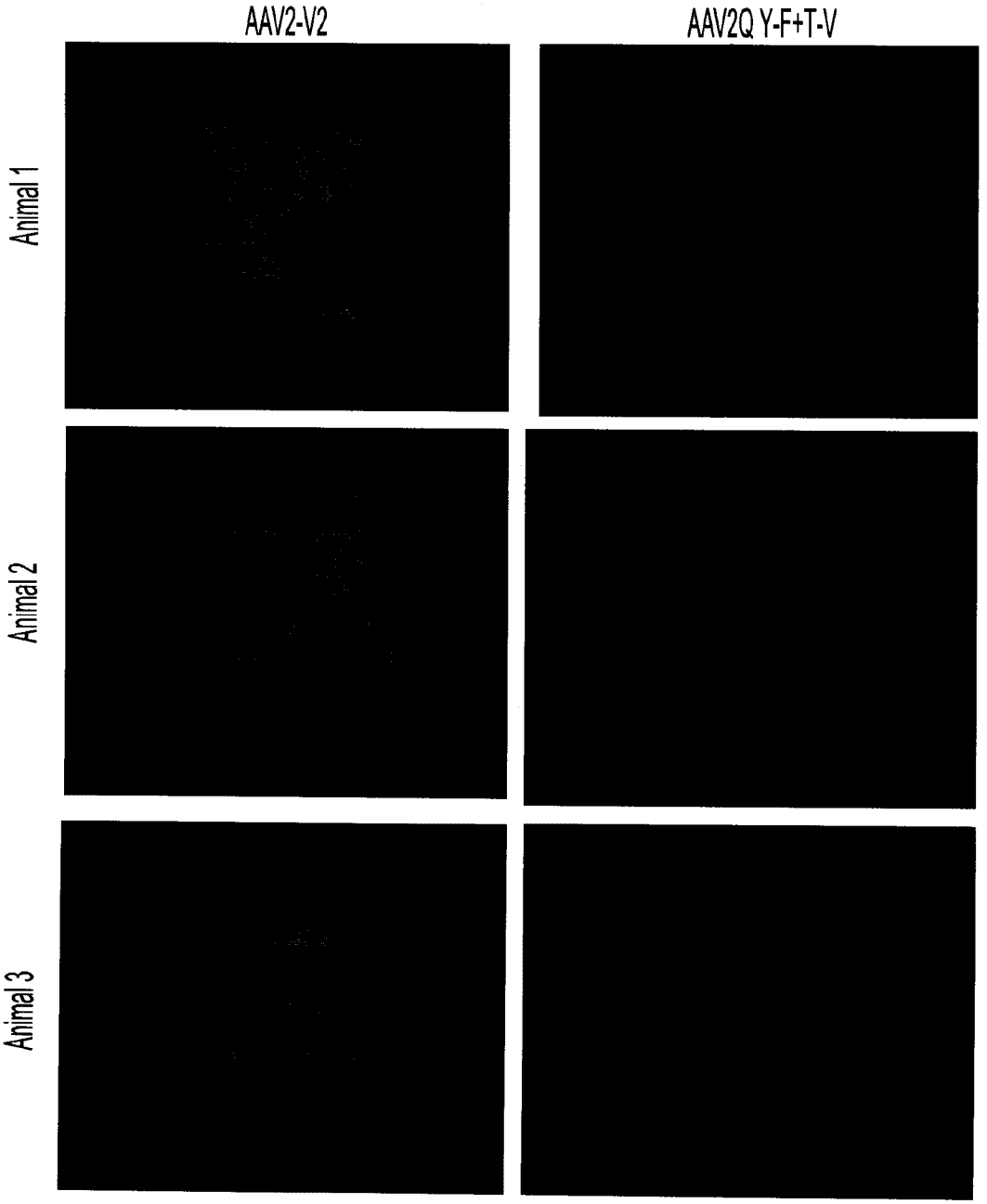


FIG. 10A

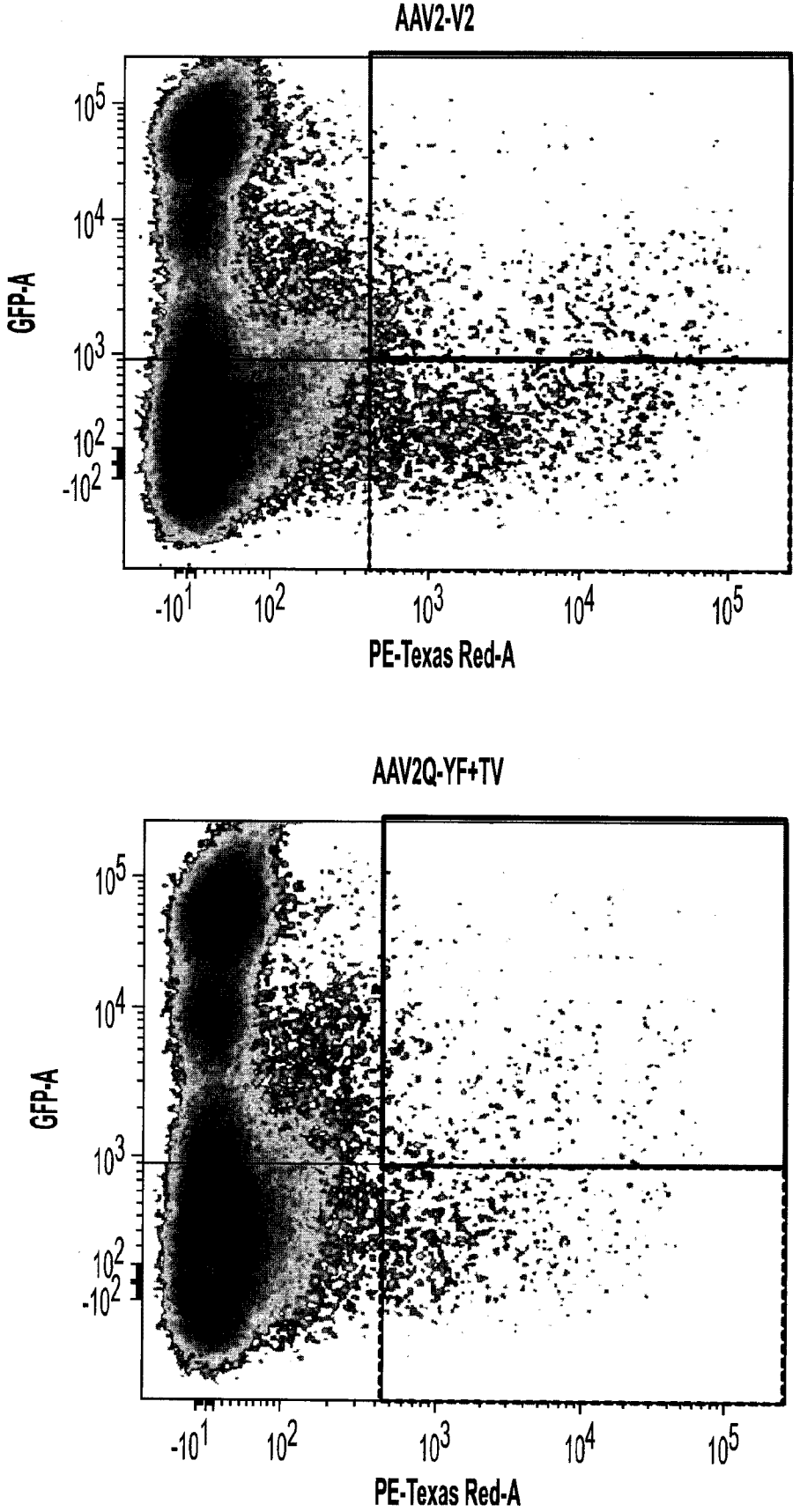
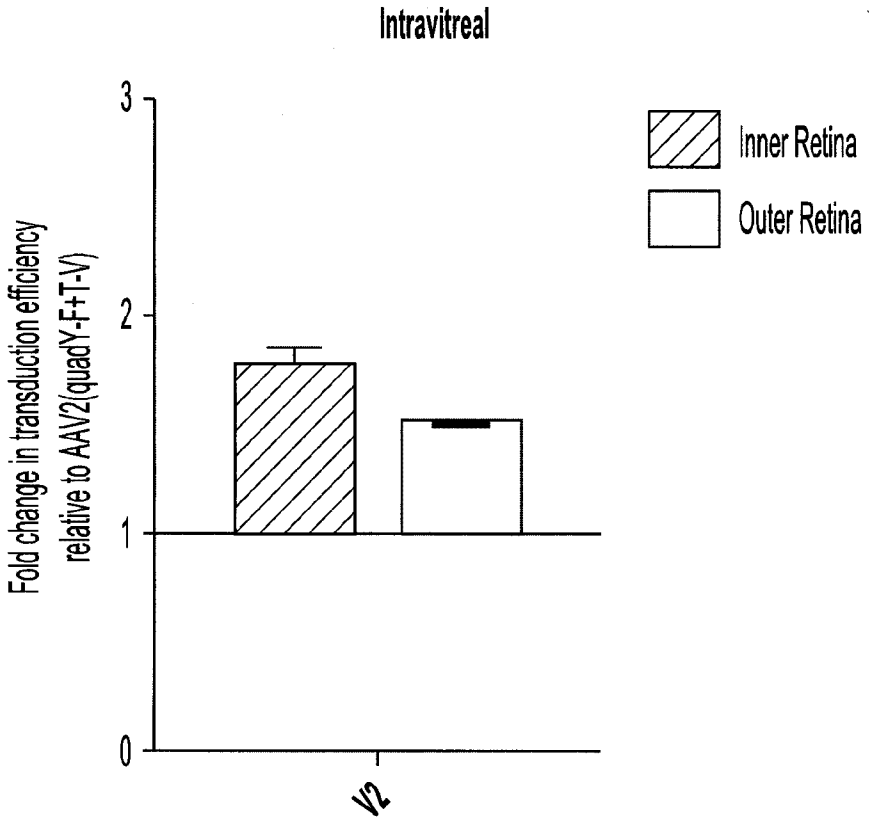

AAV2-V2
GFP-A
10^5
10^4
10^3
10^2
-10^2
-10^1
10^2
10^3
10^4
10^5
PE-Texas Red-A
AAV2Q-YF+TV
GFP-A
10^5
10^4
10^3
10^2
-10^2
-10^1
10^2
10^3
10^4
10^5
PE-Texas Red-A

FIG. 10B



Titer: 1.2E+12	Inner Retina	Outer Retina
V2	1.76 fold	1.5 fold
AAV2QY-F+T-V	1 fold	1 fold

FIG. 10C

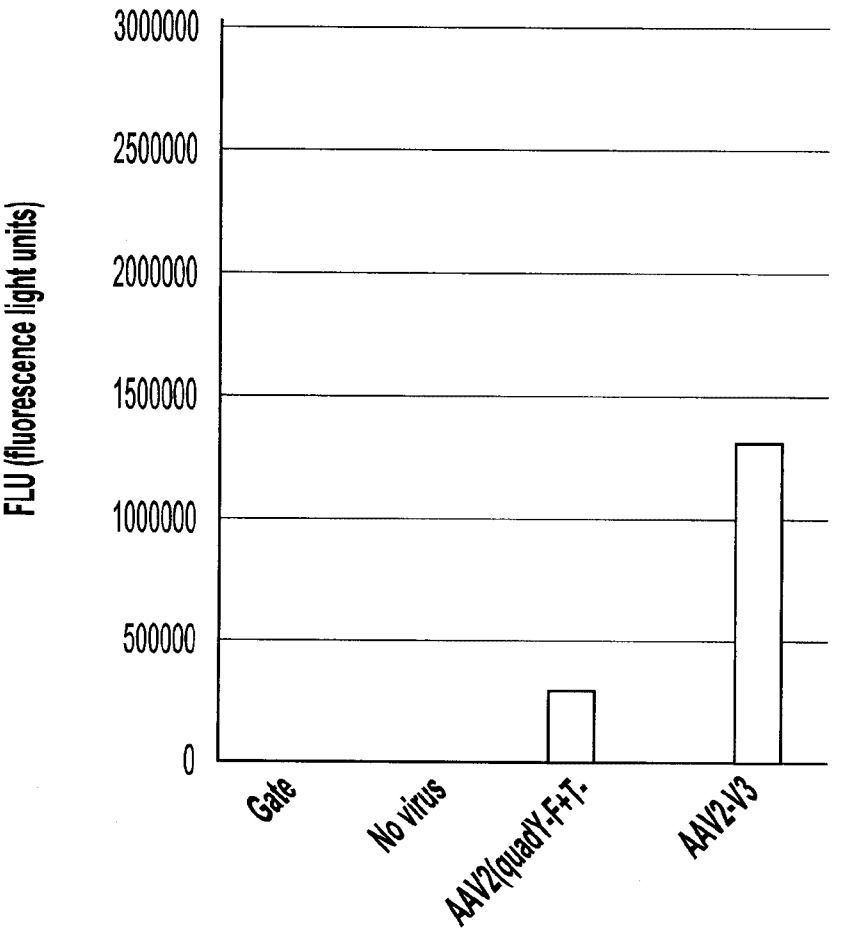

3000000
2500000
2000000
1500000
1000000
500000
0
FLU (fluorescence light units)
Gate
No virus
AAV2(quadY-F+T-
AAV2-V3

FIG. 11

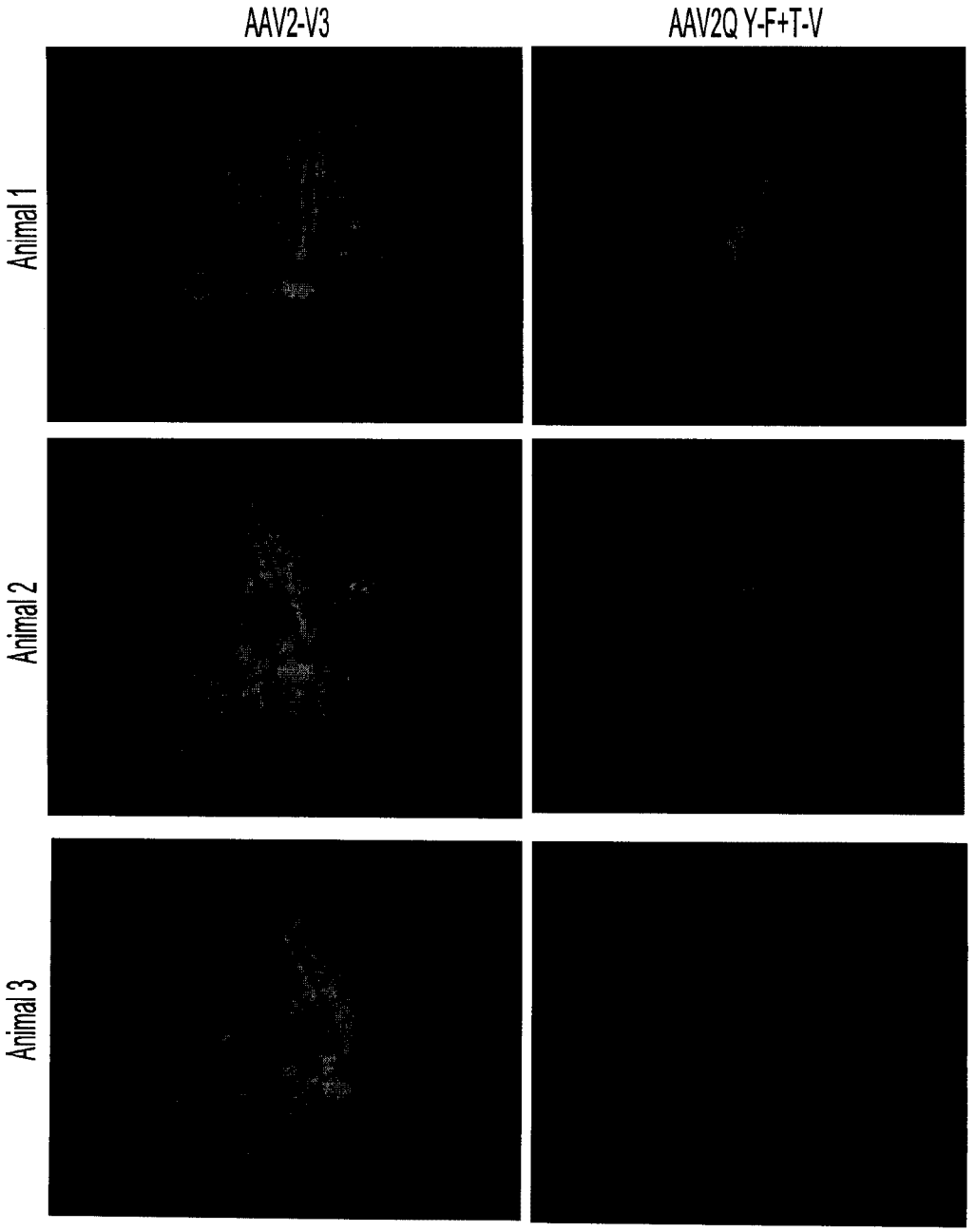


FIG. 12A

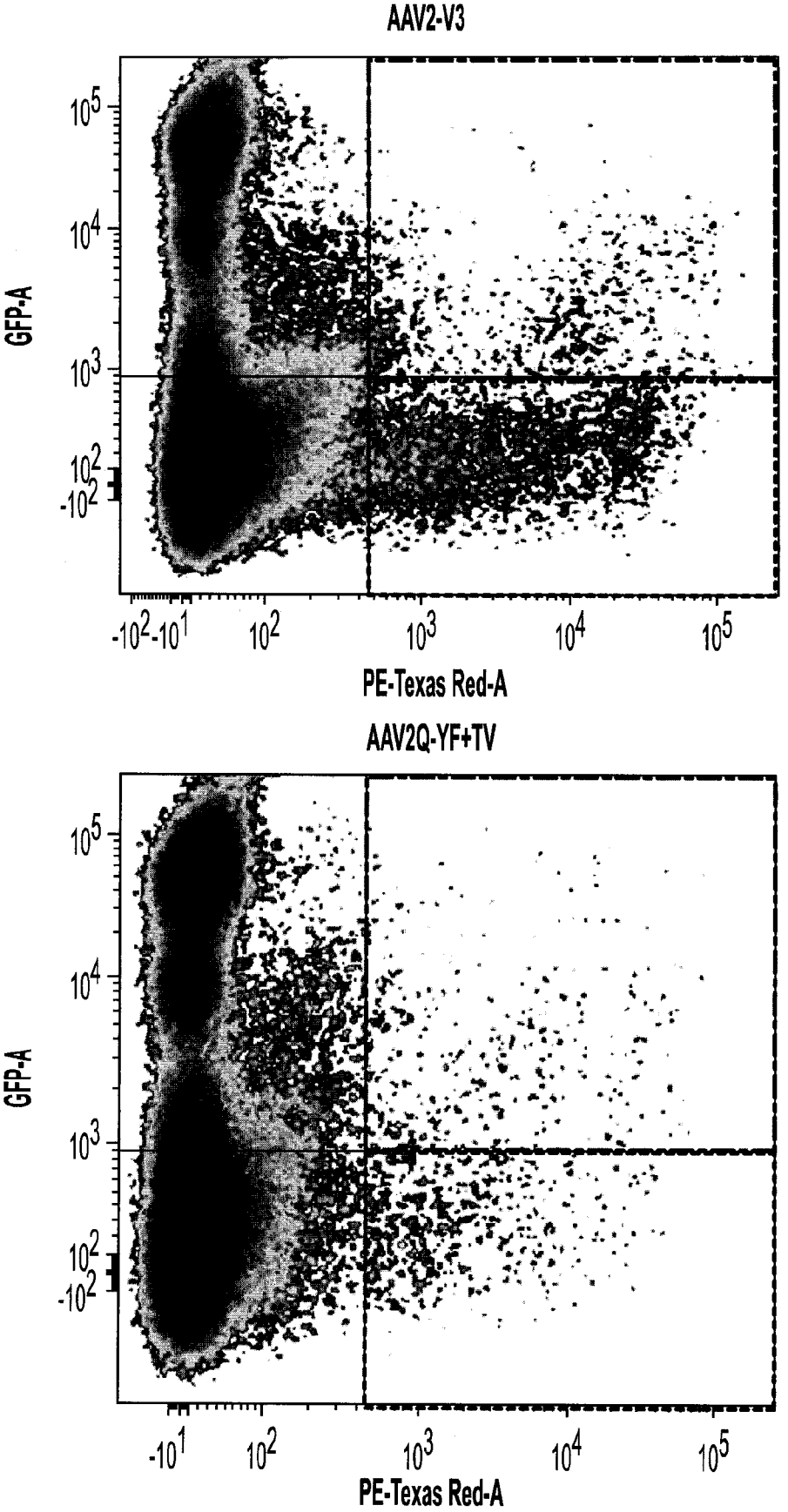
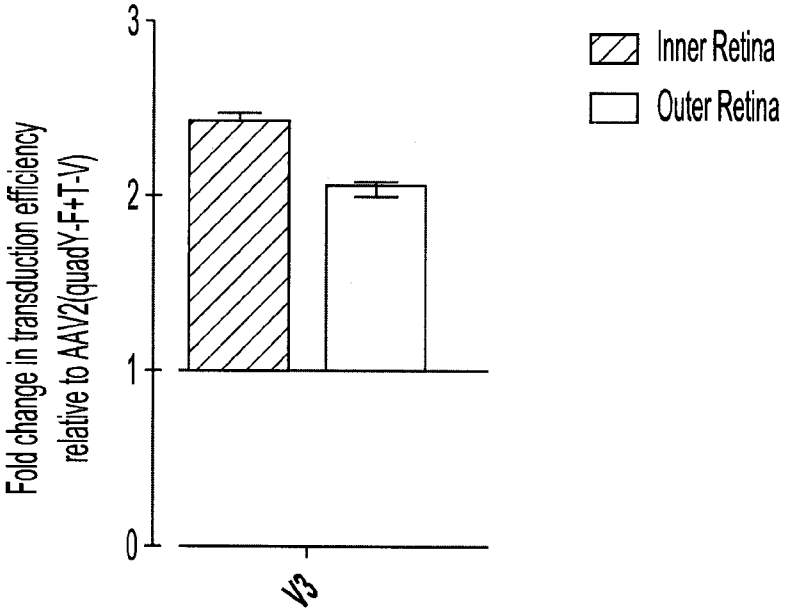


FIG. 12B



Titer: 1.2E+12	Inner Retina	Outer Retina
V3	2.4 fold	2.03 fold
AAV2QY-F+T-V	1 fold	1 fold

FIG. 12C

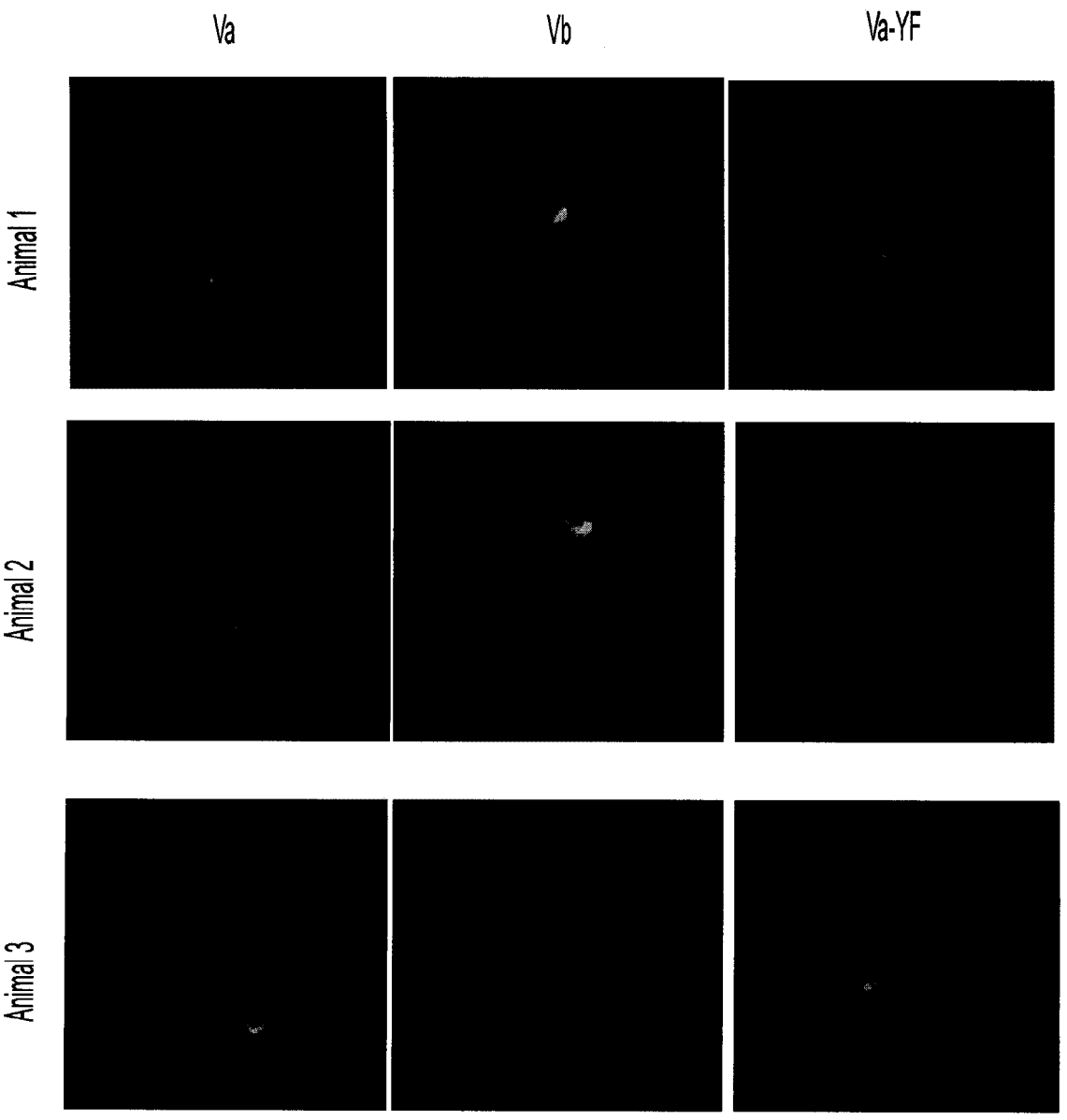


FIG. 13

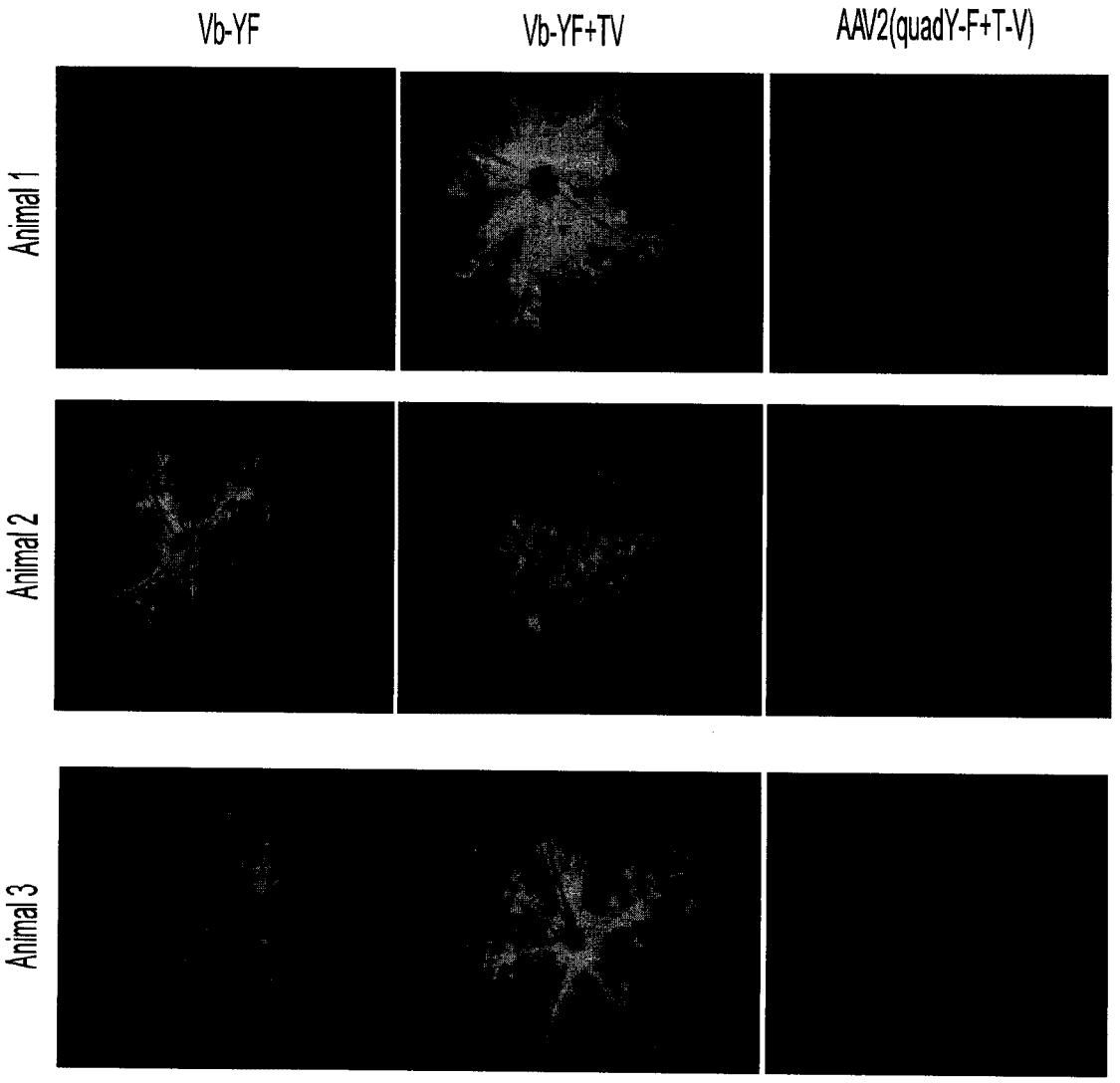
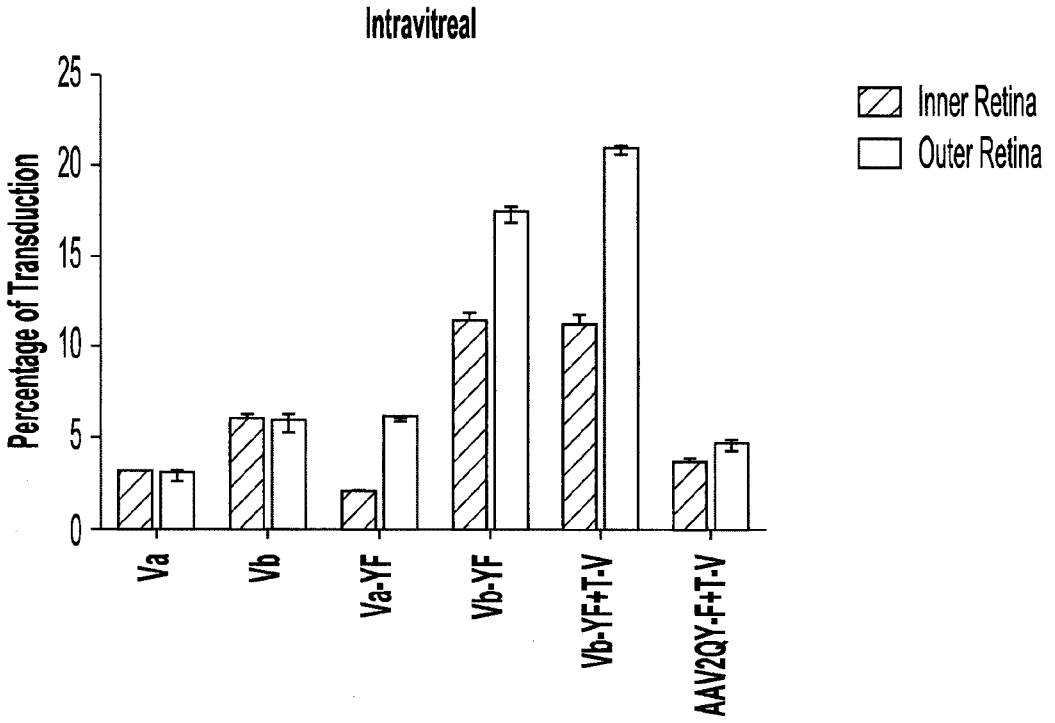


FIG. 13 continued



sTiter: 2E+12	Inner Retina	Outer Retina
Va	3%	2.9%
Vb	5.9%	5.8%
Va-YF	1.9%	6%
Vb-YF	11.3%	17.3%
Vb-YF+T-V	11.1%	20.8%
AAV2QY-F+T-V	3.6%	4.6%

FIG. 14

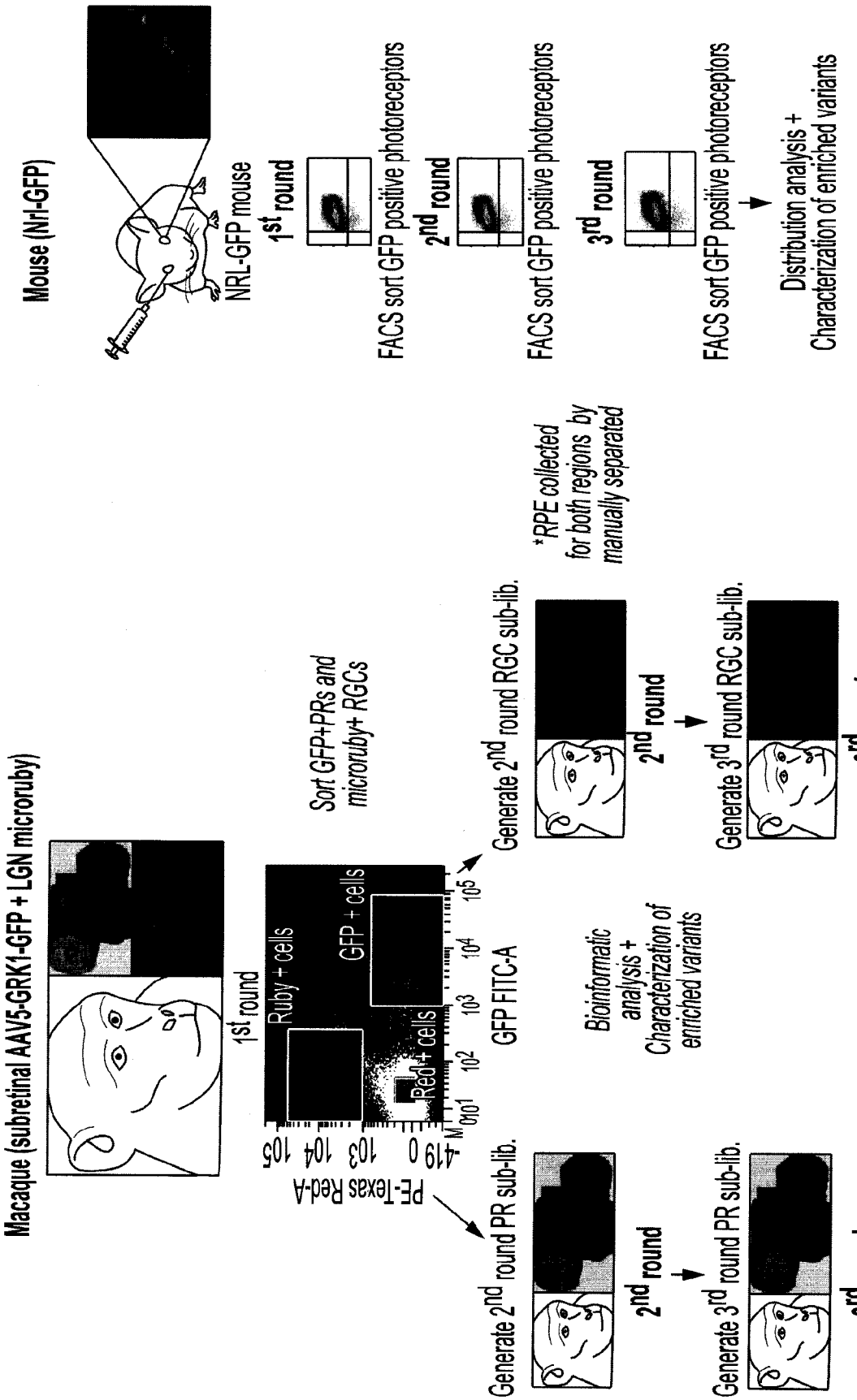

Mouse (Nrl-GFP)
NRL-GFP mouse
1st round
FACS sort GFP positive photoreceptors
2nd round
FACS sort GFP positive photoreceptors
3rd round
FACS sort GFP positive photoreceptors
Distribution analysis + Characterization of enriched variants
Macaque (subretinal AAV5-GRK1-GFP + LGN microruby)
1st round
Ruby + cells
GFP + cells
Red+ cells
PE-Texas Red-A
GFP FITC-A
Sort GFP+PRs and microruby+ RGCs
Generate 2nd round RGC sub-lib.
2nd round
*RPE collected for both regions by manually separated
Generate 3rd round RGC sub-lib.
3rd round
Bioinformatic analysis + Characterization of enriched variants
Generate 2nd round PR sub-lib.
2nd round
Generate 3rd round PR sub-lib.
3rd round

FIG. 15

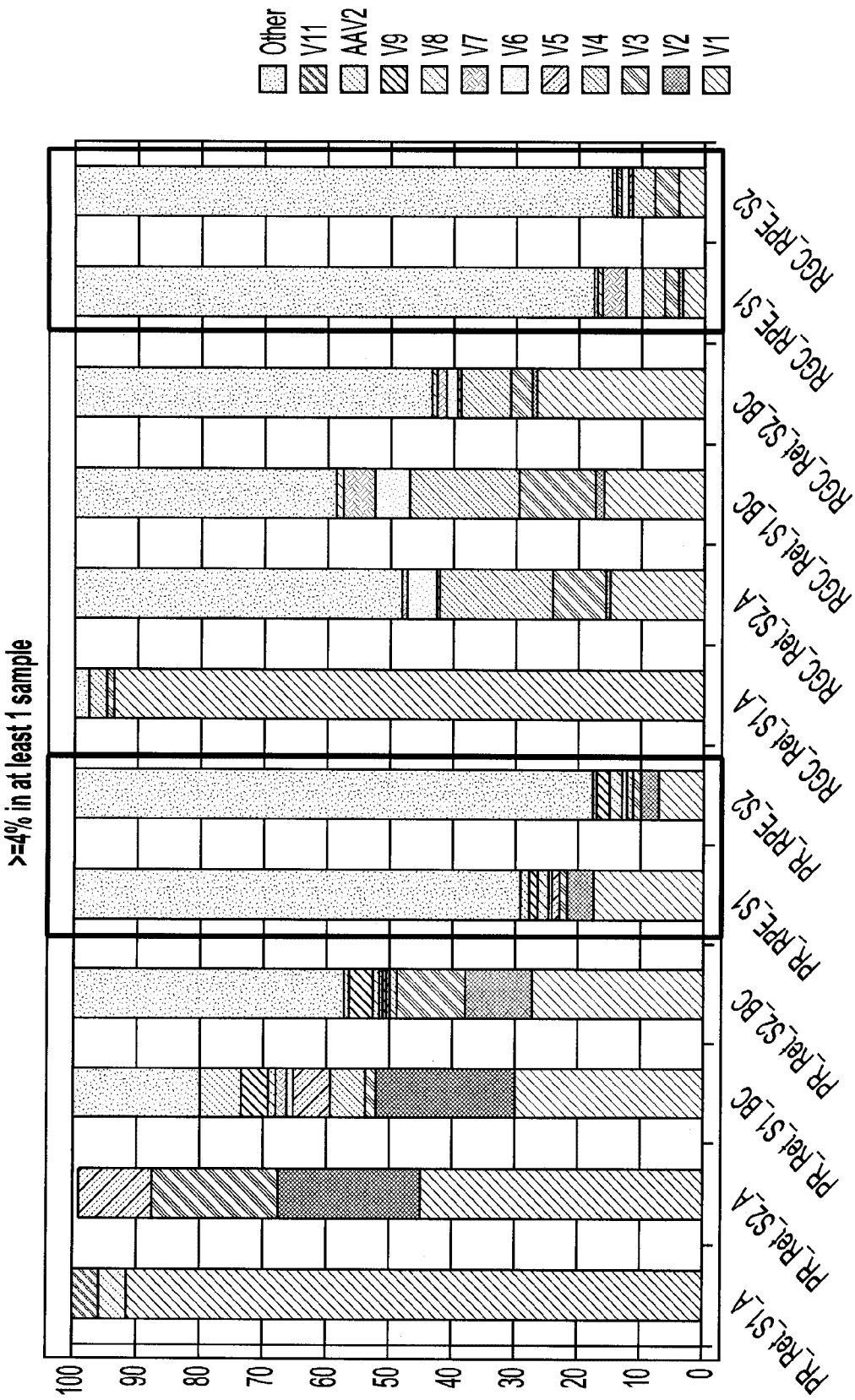

Other
V11
AAV2
V9
V8
V7
V6
V5
V4
V3
V2
V1
>=4% in at least 1 sample
100
90
80
70
60
50
40
30
20
10
0
PR_Rel_S1_A
PR_Rel_S2_A
PR_Rel_S1_BC
PR_Rel_S2_BC
PR_RPE_S1
PR_RPE_S2
RGC_Rel_S1_A
RGC_Rel_S2_A
RGC_Rel_S1_BC
RGC_Rel_S2_BC
RGC_RPE_S1
RGC_RPE_S2

FIG. 16

	Variable loop (position rel. AAV2)	VR-I (263-265)	VR-IV (450-466)	VR-V (490-503)	VR-VI (527-532)	VR-VII (545-556)	VR-VIII* (585-596)
Capsid Variants	Vb	2	--	5	--	9	--
	V2	1	--	4	1	--	--
	V3	2	5	--	3	--	--
	Va	--	--	5	--	9	--
	V5	--	--	--	1	--	--
	V6	--	--	5	--	--	--
	V7	2	--	2	--	8	--

FIG. 17

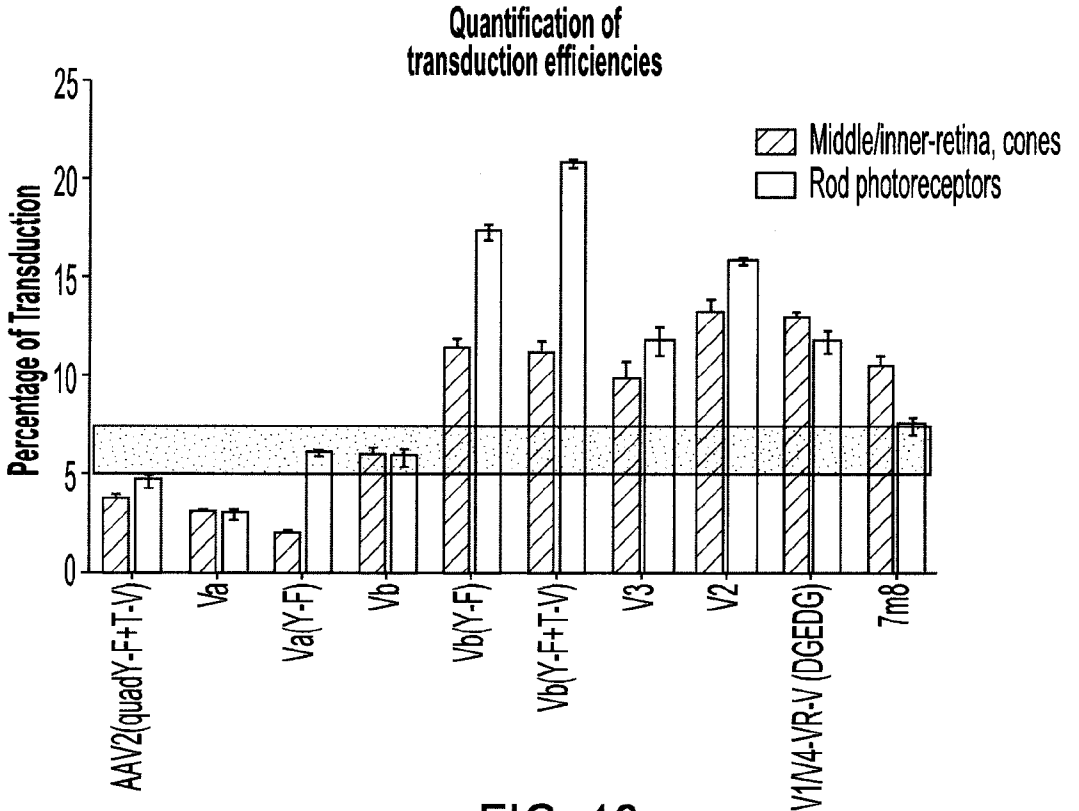


FIG. 18

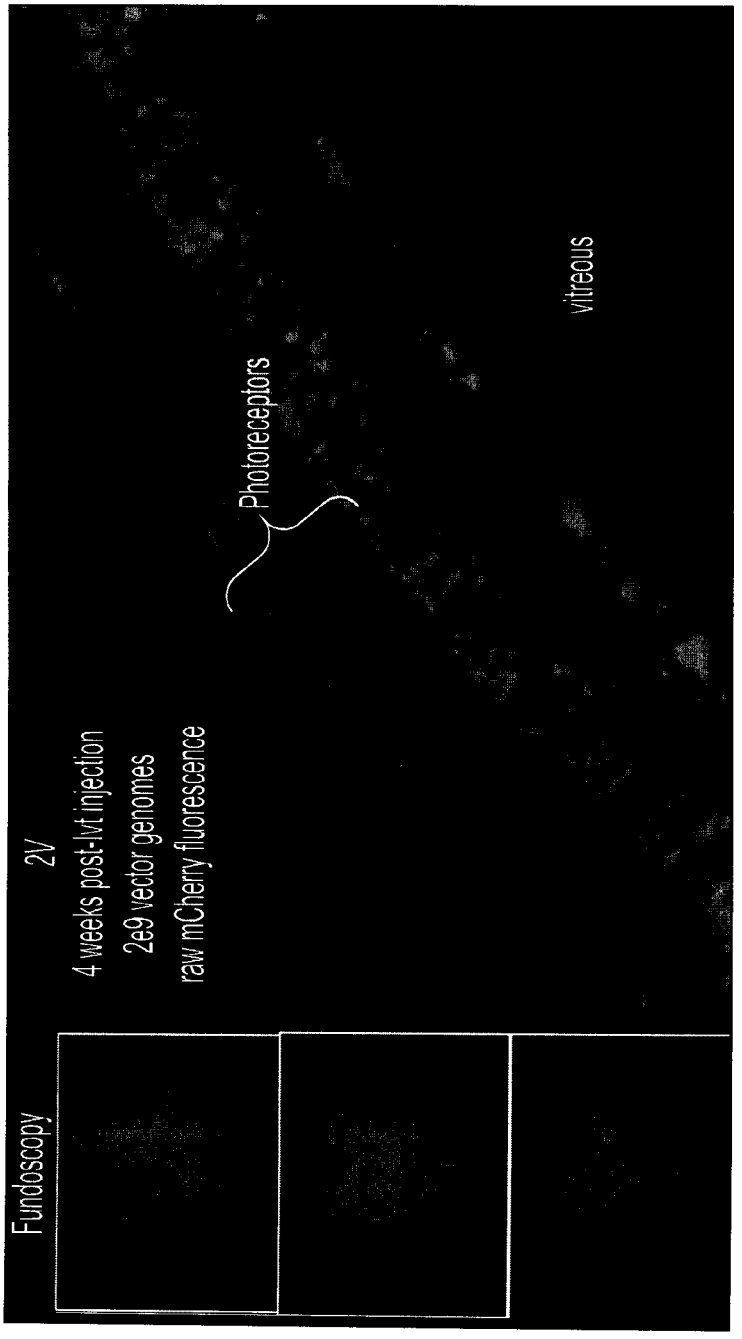

Fundoscopy
2V
4 weeks post-Ivt injection
2e9 vector genomes
raw mCherry fluorescence
Photoreceptors
vitreous

FIG. 19

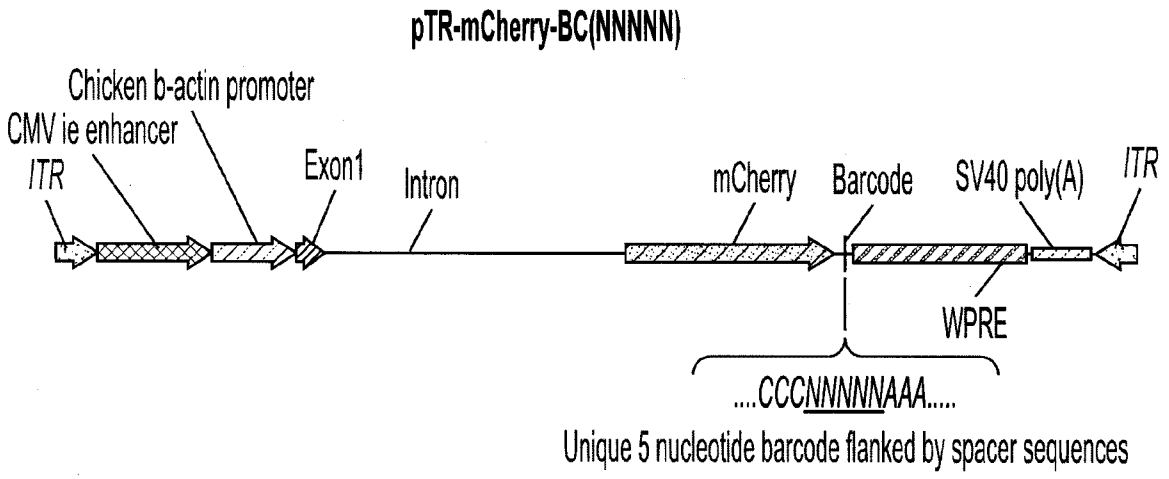

pTR-mCherry-BC(NNNNN)
Chicken b-actin promoter
CMV ie enhancer
ITR
Exon1
Intron
mCherry
Barcode
SV40 poly(A)
ITR
WPRE
....CCCNNNNNAAA.....
Unique 5 nucleotide barcode flanked by spacer sequences

FIG. 20

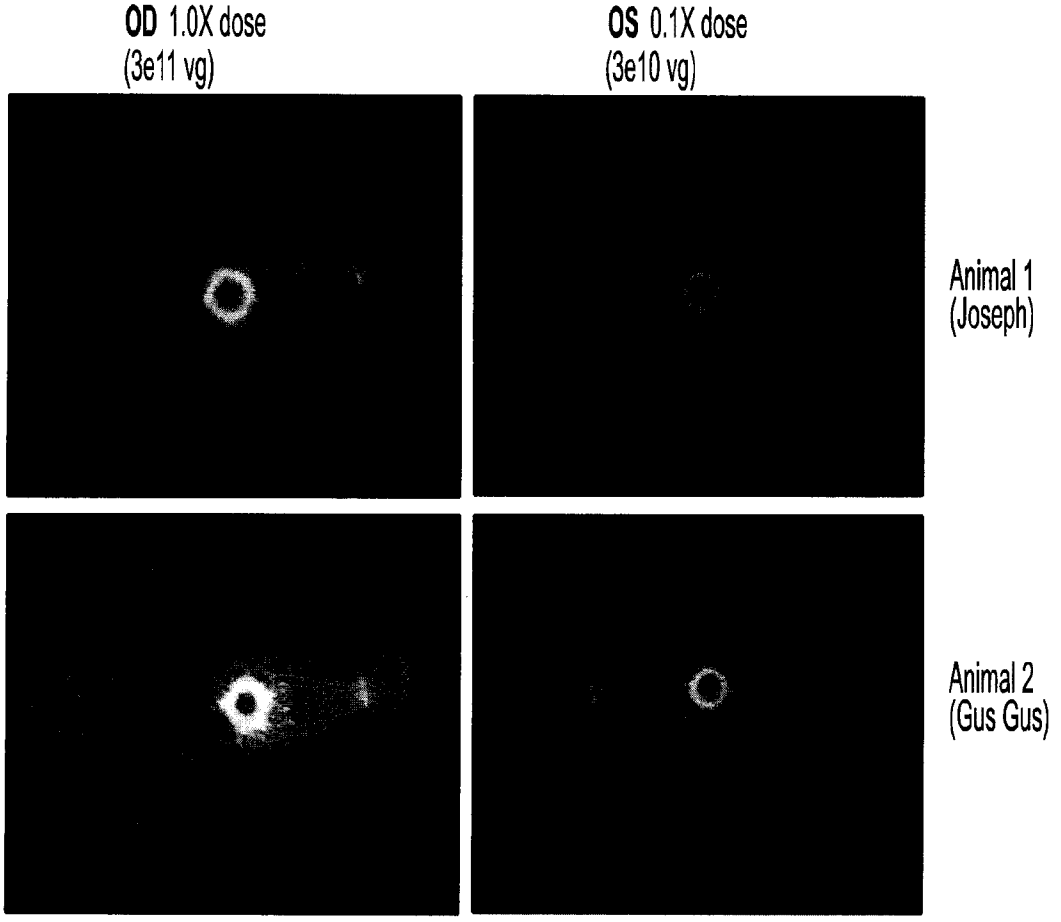

OD 1.0X dose
(3e11 vg)
OS 0.1X dose
(3e10 vg)
Animal 1
(Joseph)
Animal 2
(Gus Gus)

FIG. 21

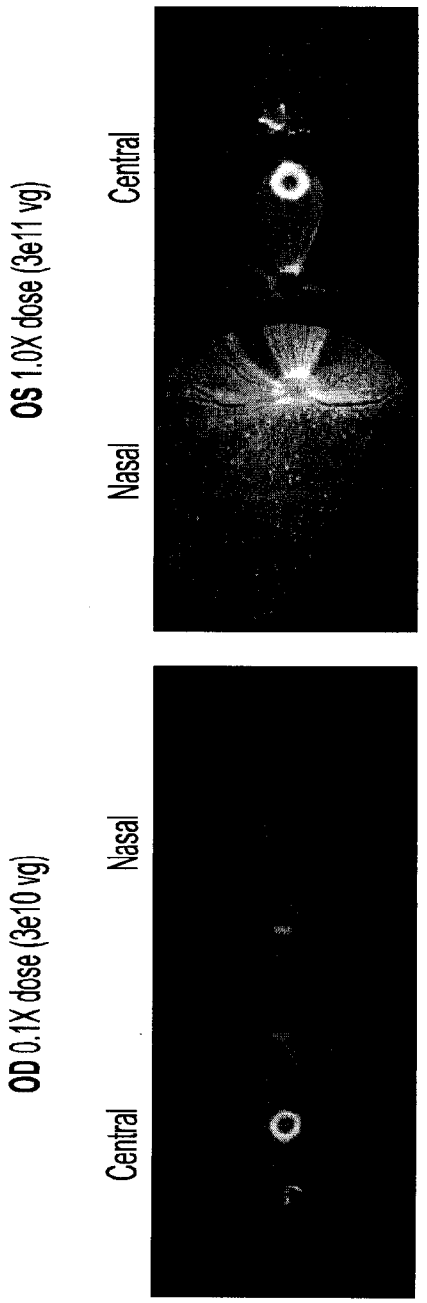

OS 1.0X dose (3e11 vg)
Nasal
Central
OD 0.1X dose (3e10 vg)
Central
Nasal

FIG. 22

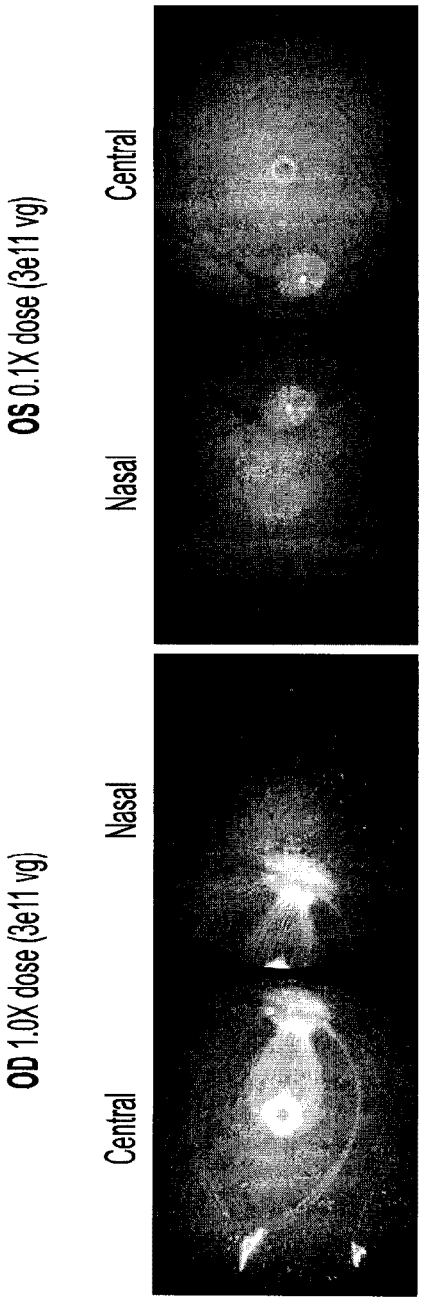

OD 1.0X dose (3e11 vg)
Central
Nasal
OS 0.1X dose (3e11 vg)
Nasal
Central

FIG. 23

263	444	490	527	545	585				
QSGAS	YLSRTNTPSGTTTQSRLQ	KTSADNNNSEYSWTGATK	KDDEEK	QGSEKTNVDIEK	RGNRQAATADVN	%PR3	%RG3	Names
XX...	....	.QD.E.....F..P....	......	....	....	0.7	13.9	P3-RGC1
XX...	F.....SD..ID...N.M	..A......DF.......	...DG.	....	....	0.0	2.2	P3-RGC2
XX..X	F.....D...S..M.T.R	.V........F.......	....D.	....	....	0.0	1.2	P3-RGC3
NA...	....	..DGE....DF.......	......	E...........	....	3.5	0.0	P3-PR1
DA..T	F.........A.M.K.H.	..YN......F......T	...D.R	....	....	2.0	0.0	P3-PR2
.....	F......GA.NM.T.A.R	T.P......DF.......	...D..	....	....	1.2	0.0	P3-PR3
XX...	....	.V........F.......	......	..AAADD.E.DG	....	3.5	1.0	-V7
XX...	....	....	...D..	..AGRADI...S	....	0.1	0.7	P3-RGC4
XX..X	....	.QD.E.....F..P....	......	.SAAGAD.A.DS	....	0.0	0.7	P3-RGC5

FIG. 24

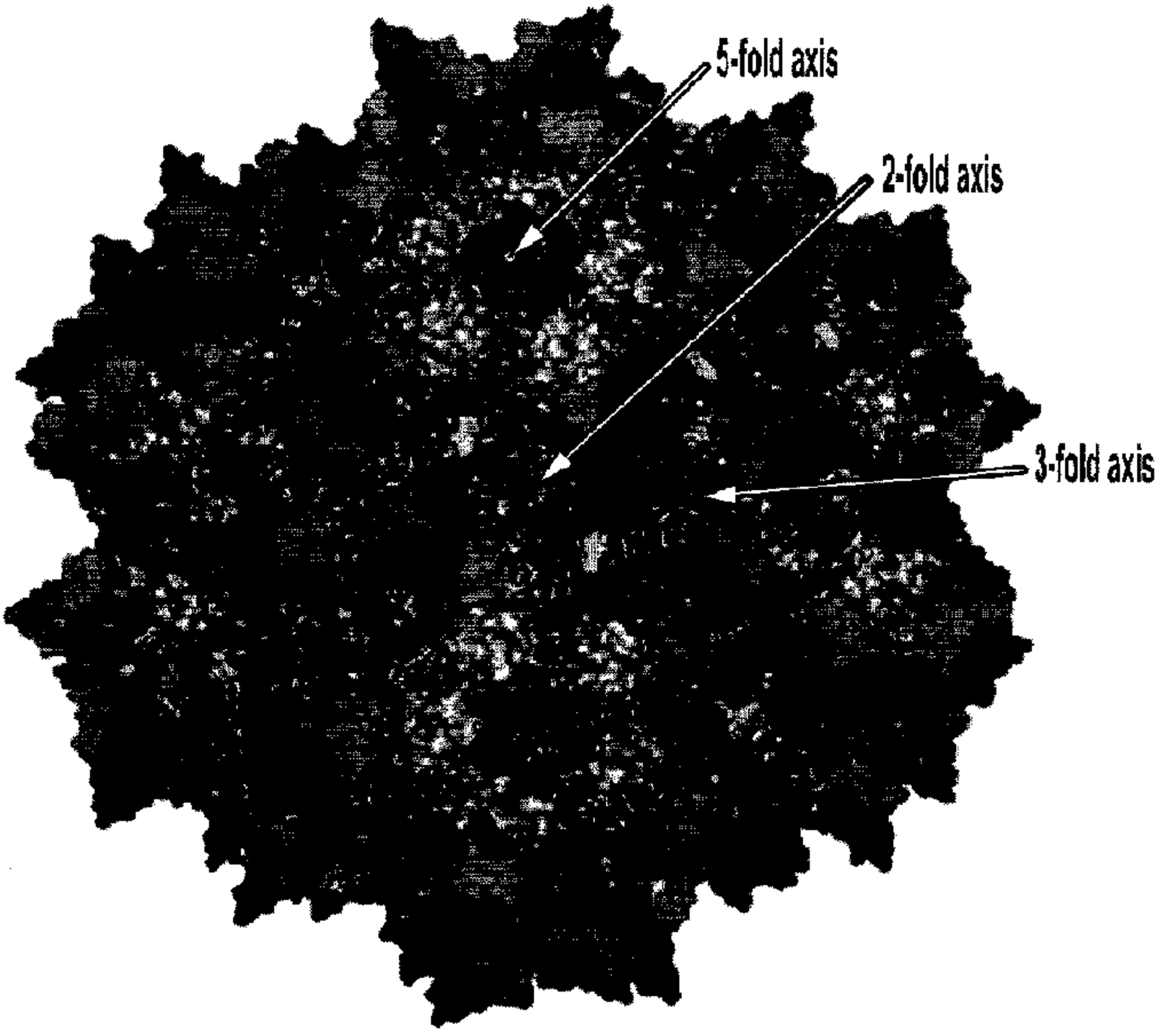
Round 3 NHP screen top variants

263	444	490	527	545	585	
QSGAS	YLSRTNTPSGTTTQSRLQ	KTSADNNNSEYSWTGATK	KDDEEK	QGSEKTNVDIEK	RGNRQAATADVN	Names
NA...	F.....A..NV...T..	.....	R..DD.	.....	.....	V3
NA...	.....	..DGE....DF.....	.....	.SAAGAD.A.DS	.....	V1, Vb
A....	.....	T.P.....DF.....	...D..	.....	.....	V2
.....	.....	.....	.....	E.....	.....	P3-1
.....	F.....A..NV...T..	.....	R..DD.	.....	.....	V3 variant
NA...	.....	.....F..P...T	.....	.....	.....	P3-2
A....	F.....A..NV...T..	.....	R..DD.	.....	.....	P3-3
.....	.....	..DGE....DF.....	.....	.SAAGAD.A.DS	.....	Vd
.....	.....	..DGE....DF.....	.....	EDATEN.I..DR	.....	V4, Va
NA...	.....	..DGE....DF.....	.....	.....	.....	P3-4
A....	.....	.....	...DD.	.....	.....	P3-5
A....	.....	..DGE....DF.....	.....	.SAAGAD.A.DS	.....	P3-6
NA...	.....	.....	.....	E.....	.....	P3-7
GT...	.....	.....F.....T	R..D.R	.....	.....	P3-8

Variants of interest based on differential enrichment between PRs and RGCs in round 3 NHP screen

263	444	490	527	545	585			
QSGAS	YLSRTNTPSGTTTQSRLQ	KTSADNNNSEYSWTGATK	KDDEEK	QGSEKTNVDIEK	RGNRQAATADVN	%PR3	%RG3	Names
XX...	.....	.QD.E.....F..P....	.....	.....	.....	0.7	13.9	P3-RGC1
XX...	.....	.....F.....T	R..D.R	.....	.....	4.9	4.7	P3-V14
NA...	.....	..DGE....DF.....	.....	E.....	.....	3.5	0.0	P3-PR1
XX...	F.....SD..ID...N.M	..A.....DF.....	...DG.	.....	.....	0.0	2.2	P3-RGC2
DA..T	F.....A.M.K.H	..YN.....F.....T	...D.R	.....	.....	2.0	0.0	P3-PR2
.....	F.....GA.NM.T.A.R	T.P.....DF.....	...D..	.....	.....	1.2	0.0	P3-PR3
XX..X	F.....D...S..M.T.R	.V.....F.....	....D.	.....	.....	0.0	1.2	P3-RGC3
XX...	.....	.....	...D..	..AGRADI...S	.....	0.1	0.7	P3-RGC4
XX..X	.....	.QD.E.....F..P....	.....	.SAAGAD.A.DS	.....	0.0	0.7	P3-RGC5

FIG. 25


5-fold axis
2-fold axis
3-fold axis