Article

Impact of Vital Dyes on Cell Viability and Transduction Efficiency of AAV Vectors Used in Retinal Gene Therapy Surgery: An In Vitro and In Vivo Analysis

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Purpose: Treatment of inherited retinal degenerations using adeno-associated viral (AAV) vectors involves delivery by subretinal injection. In the latter stages, alteration of normal anatomy may cause difficulty in visualizing the retinotomy, retinal detachment extension, and vector diffusion. Vital dyes may be useful surgical adjuncts, but their safety and impact on AAV transduction are largely unknown.

Methods: The effects of Sodium Fluorescein (SF), Membrane Blue (MB), and Membrane Blue Dual (DB) at a range of dilutions were assessed on human embryonic kidney cells in vitro using an AAV2-green fluorescent protein (GFP) reporter at different multiplicities of infection. Flow cytometry analysis was performed to assess both cell viability and transduction efficiency. The effect on quantitative (q)PCR titer was determined. Balanced salt solution (BSS) or dilute DB (1:5 in BSS) were delivered subretinally into left/right eyes of C57BL/6J mice ($n = 12$). Retinal structure and function were analyzed by optical coherence tomography, autofluorescence, dark- and light-adapted full-field electroretinography.

Results: DB and MB were not toxic at any concentration tested, SF only when undiluted. The presence of dyes did not adversely affect the genomic titer. DB even increased the values, due to presence of surfactant in the formulation. AAV2-GFP transduction efficiency was not reduced by the dyes. No structural and functional toxic effects were observed following subretinal delivery of DB.

Conclusions: Only undiluted SF affected cell viability. No effects on qPCR titer and transduction efficiency were observed. DB does not appear toxic when delivered subretinally and improves titer accuracy. DB may therefore be a safe and helpful adjunct during gene therapy surgery.

Translational Relevance: This paper might be of interest to the retinal gene therapy community: it is a "bench to bedside" research paper about the potential use of dyes as a surgical adjunct during the gene therapy surgery. We have tested the potential toxicity and impact on transduction efficiency in an in vitro and in vivo model.

Introduction

Gene therapy represents a promising approach for the treatment of inherited retinal dystrophies (IRD), a group of heterogeneous degenerative diseases affecting the retina.[1] IRDs are caused by mutations in single genes mainly expressed in the retinal pigmented epithelium (RPE) and photoreceptors. Monogenic recessive disorders might be amenable to treatment by gene augmentation therapy through the delivery of normal working copies of the defective gene carried by replication-deficient viral vectors.

The most widely used vector for retinal gene therapy is the adeno-associated virus (AAV), which transduces both the photoreceptors and RPE.[2] Early results of gene therapy clinical trials for IRDs have been encouraging, with proof of long-term treatment effects over many years in choroideremia and Leber congenital amaurosis-2, including successful second

eye treatments of the latter.[3,4] In order to achieve a maximal concentration of vector in proximity to the target cells (photoreceptors and RPE), the retina needs to be detached from the underlying RPE through a subretinal injection.[5] One of the key challenges for IRD gene therapy is ensuring that the vector is delivered in an appropriate distribution within the subretinal space. This is especially important in clinical trials for which visual acuity is an endpoint, as the fovea must necessarily be targeted. In very advanced IRDs, complete degeneration of the RPE layer may impede separation of the subretinal anatomical cleavage plane. Furthermore, the severe choroidal thinning typical of choroideremia means that inadvertent supra-choroidal delivery of the vector may go undetected due to a lack of clear color contrast. Vital dyes are routinely used in cataract and vitreoretinal surgery to aid in the visualization of a number of anatomical structures including the crystalline lens capsule, internal limiting membrane, epiretinal membranes, and the vitreous,[6] and may therefore have a role in guiding subretinal delivery of viral vector solutions.

The aim of this study was therefore to assess the suitability of vital dyes commonly used in vitreoretinal surgery as adjuncts for human AAV gene therapy surgery. We evaluated Sodium Fluorescein (SF; Martindale Pharma, Wooburn Green, Buckinghamshire, UK), Membrane Blue USA (MB; DORC, Zuidland, The Netherlands), and Membrane Blue Dual (DB; DORC) with regard to potential cell toxicity in vitro, along with the effects of these dyes on AAV titer and transduction efficiency. Lastly, an in vivo analysis was performed to assess any negative effects on retinal structure and function.

Methods

Cell Culture

Toxicity and transduction efficiency were assessed using HEK293 cells. The HEK cell line was chosen because Master Cell Banks exist that are approved by the US Food and Drug Administration (FDA) for Good Laboratory Practice (GLP) studies to support clinical trial applications.

HEK293 cells (ECACC #85120602) were cultured in Minimum Essential Media (MEM; Sigma-Aldrich Company Ltd) supplemented with L-glutamine (2 mM), penicillin (100 units/mL), streptomycin (100 μg/mL), nonessential amino acids (1%), and 10% fetal bovine serum. Cells were maintained at 37°C in a 5% CO$_2$ environment. For all cell culture experiments, five biological replicates were run and processed on different days to each other.

Flow Cytometry

ViViD, an amine reactive dye (LIVE/DEAD fixable violet dead cell stain; Thermo-Fisher Scientific, Loughborough, UK), was used to discriminate between live and dead cells. Stock solutions of LIVE/DEAD dye were prepared in dimethyl sulfoxide (DMSO; Sigma-Aldrich Company Ltd, Dorset, UK) according to the manufacturer's instructions. Cells were incubated with reconstituted LIVE/DEAD dye for 30 minutes in the dark. Media were removed, the cells resuspended in PBS, and then fixed with 4% paraformaldehyde for 15 minutes at room temperature. Fixative was replaced with 1 mL of PBS with 1% bovine serum albumin and the samples kept on ice. Samples were run on a LSRFortessa (BD Biosciences, Oxford, UK). Based on compensated green fluorescent protein (GFP)-fluorescence, gating settings were set to isolate GFP-positive, ViViD-positive, and negative cell fractions.

In Vitro Dye Toxicity Assessment

Three dyes were used in this study: SF, MB, and DB. SF is routinely used for fluorescein angiography and consists of 10% fluorescein in balanced salt solution (BSS; 566 mg sodium fluorescein in 5 mL). MB and DB are licensed for use in vitreoretinal surgery in the United States and Europe, respectively. DB is a combination of 0.15% trypan blue (TB) and 0.025% brilliant blue G (BBG) in a 4% polyethylene glycol carrier, while MB is composed of 0.15% TB without a carrier.

Cells were seeded in 6-well plates at a density of 7.5E+05 cells per well. One day post-seeding, the media was replaced with 200 μL of dye solution. A range of dye dilutions were tested: neat, 1:50, 1:5000, 1:50,000, and 1:500,000 for SF, and neat, 1:50, 1:125, 1:150, and 1:500 for DB and MB. Dye solutions were made up to a total volume of 200 μL with PBS (Fig. 1). After incubating the cells at 37°C for 1 hour, 1.8 mL of complete MEM was added to each well.

Acute and chronic toxicity was assessed by performing flow cytometry (FC) of dye-exposed cells after 1 hour and 3 days, respectively. Three days was selected as a time point as this is the time at which AAV transduction of HEK293 cells is typically maximal.[7]
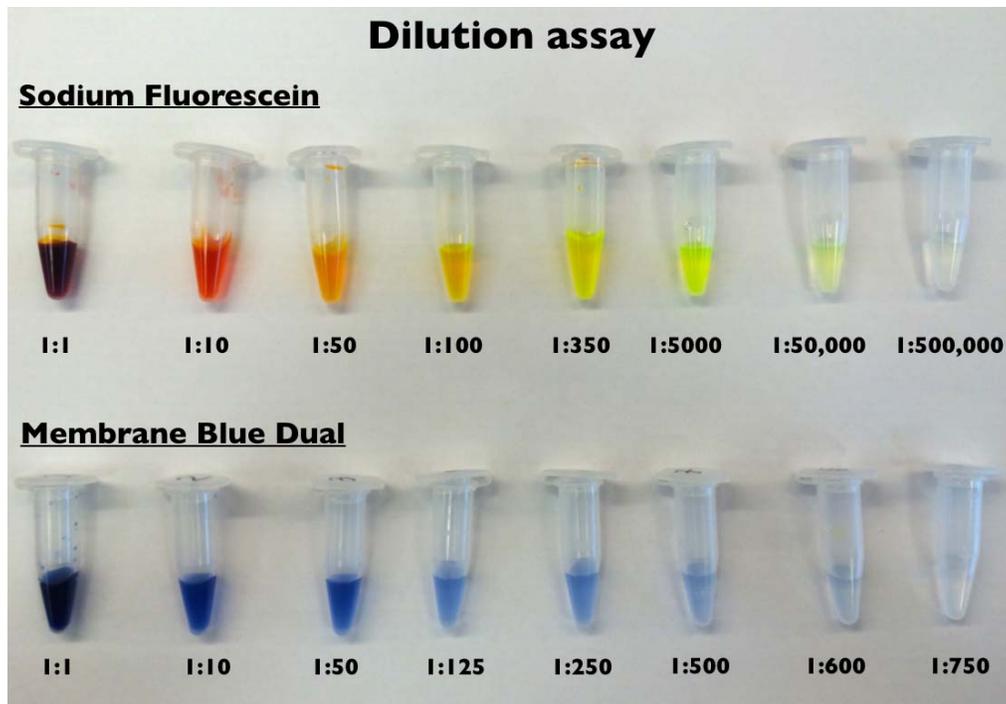
Figure 1. Dilution series assay. Different dye dilutions were tested for SF (neat, 1:50, 1:5000, 1:50,000, and 1:500,000) and for MB and DB (neat, 1:50, 1:125, 1:150 and 1:500, DB). Dye solutions were made up to a total volume of 200 μL with PBS.

Quantitative PCR

The AAV vector used in all experiments described here was AAV2/2-CAG-GFP-WPRE (referred herein as AAV2-GFP), cloned, and prepared as described elsewhere.[8] AAV samples were DNAase treated and heat denatured for use in quantitative PCR (qPCR) titration. One microliter of AAV solution was combined with 1 μL of DNase I, 1 μL of DNase I buffer (both New England Biolabs, Ipswich, MA), and 7 μL of molecular grade water in a nuclease-free microcentrifuge tube. The tube was incubated at 37°C for 1 hour. To this, 90 μL of molecular grade water was added and the mixture incubated at 90°C for 15 minutes. QPCR was performed using iTaq Universal SYBR Green Supermix kit (Bio-Rad, Hertfordshire, UK). A linearized vector plasmid of known concentration was used as a template standard and primers were designed to amplify a 72-bp fragment within the poly-A region. Reactions were run in triplicate in a CFX Connect machine (Bio-Rad). After a 3-minute activation step at 95°C, each reaction consisted of 40 cycles of 95°C for 10 seconds followed by 60°C for 30 seconds. The annealing temperature (60°C) was the temperature at which fluorescence readings were taken. Cycle threshold (Ct) values were determined using the default settings of the CFX Manager software (version 3.0; Bio-Rad). Data were exported to Excel (Microsoft, Redmond, WA). for analysis and technical replicates were averaged.

The effect of the dyes on viral titer was determined using the same viral preparation (AAV2-GFP at 5.96E+12 gc/mL). An aliquot was thawed on ice from −80°C and diluted 1 in 20 in PBS. One microliter of diluted AAV solution was added to 9 μL of either PBS (no dye), 1:125 diluted dye, 1:50 diluted dye, neat dye, TB (1:50 equivalent; Gibco, Waltham, MA), 4% polyethylene glycol (PEG; Sigma-Aldrich Company Ltd), 0.001% Pluronic F-68 (PF-68; Thermo-Fisher Scientific, Loughborough, UK), or 0.001% PF-68 in 1:50 DB in a microcentrifuge tube. Dyes (MB and DB) were diluted with PBS, unless otherwise specified. All AAV samples were incubated with dye solutions of different concentrations for 30 minutes at room temperature prior to DNase treatment and heat denaturation as described above. For data analysis, the titer values from samples with dye were normalized to the sample in each biological replicate that did not contain dye.

Transduction Efficiency Assessment

Transduction efficiency was assessed in vitro using AAV2-GFP at different multiplicities of infection

(MOI). HEK293 cells were seeded in 6-well plates (7.5E+05 cells per well) and 1-day post-seeding viral solutions at the appropriate MOI were prepared in a total volume of 300 µL of cell culture media to which 6 µL of neat dye was added. A 1:50 dilution of DB and MB dyes was chosen as, at this concentration, they both are still clearly visible in the surgical setting. Prior to application, the AAV/dye solution was incubated for 30 minutes (a reasonable duration that AAV might be expected to be exposed to dye prior to injection in the surgical setting). Cell media was then removed and replaced by AAV/dye solution and incubated for 1 hour at 37°C. After this, 2 mL of complete MEM was added to each well. FC was performed on day 3 according to the protocol detailed above.

Mice and Subretinal Injections

All animal experiments were performed in accordance with the United Kingdom's Animals (Scientific Procedures) Act of 1986, and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. C57BL/6J mice purchased from Charles River Laboratories (Harlow, UK) were housed in individually ventilated cages under a 12-hour light/dark cycle with food and water available ad libitum. Fifteen-week-old mice ($n = 12$) were anesthetized by intraperitoneal administration of ketamine (80 µg/g body weight, Vetalar; Bausch & Lomb, Kingston upon Thames, UK) and xylazine (10 µg/g body weight, Rompun; Bausch & Lomb) diluted in sterile 0.9% saline solution. Pupils were dilated using tropicamide 1% and phenylephrine hydrochloride 2.5% eye drops (both Bausch & Lomb, City, State/Country). Proxymetacaine hydrochloride 0.5% eye drops (Bausch & Lomb) were administered to provide topical anesthesia. Gel lubricant (Viscotears; Novartis, Camberley, UK) was applied to the corneal surface on to which a 6-mm cover glass was placed allowing direct visualisation of the fundus. Injections were performed with the aid of a surgical microscope (M620 F20; Leica Microsystems, Wetzlar, Germany) using a 35-G needle mounted on a 10-µL NanoFil syringe (WPI, Hitchin, UK). The superior rectus muscle was grasped with notched microsurgical forceps to stabilize the eye and the needle advanced into the subretinal space via a transscleral approach under direct visualization. Two microliters of DB diluted 1:5 in BSS was injected into the subretinal space of one eye while the other eye received 2-µL BSS delivered using identical methods as a surgical control. Half of the mice received dye injections in their right eye, while the other half received it in their left eye. Chloramphenicol 0.5% eye drops (Bausch & Lomb) were applied to the ocular surface following subretinal injection, and anesthetic reversal achieved by intraperitoneal injection of atipamezole (2 µg/g body weight in sterile 0.9% saline solution, Antisedan; Zoetis UK Ltd, Tadworth, UK).

Retinal Imaging

Eleven weeks after subretinal injection and under general anesthesia as described above, mice were subjected to wide-field spectral-domain optical coherence tomography (SD-OCT), and confocal scanning laser ophthalmoscopy (cSLO) including near infrared (IR) and blue autofluorescence (AF) imaging (Spectralis; Heidelberg Engineering, Heidelberg, Germany). AF images of both superior and inferior retina were acquired at a range of sensitivities (60, 70, 80 and 90). SD-OCT images were acquired using a high-speed modality (768 A scans per B scan). Mice were scanned with a volume protocol that included the superior and inferior hemiretina with 49 B scans per data set. All B scans were produced from a real-time average process (25 frames) to reduce speckle noise. Mean retinal thickness was calculated for SD-OCT images using two Early Treatment Diabetic Retinopathy Study (ETDRS) grids (provided by the Spectralis software) orientated such that their superior and inferior sectors were positioned over the optic nerve head. All sectors from both grids (except for the two including the optic nerve head) were averaged to provide a global measure of mean retinal thickness. Individual SD-OCT B-scans were manually segmented in cases where automatically determined reference planes were identified as being incorrect. Mean retinal thickness was compared between dye- and BSS-injected eyes by a paired t-test. We also assessed if there was any difference between the superior and inferior retinal thickness in each group (DB- versus BSS-injected eyes) to detect the potential impact of the dye in the detachment area. Due to the need for data matching, the number of mice included in this analysis was only nine: a few images were lost due to technical problems during the imaging acquisition or eyes excluded due to vitreous hemorrhage at the time of injection.

Qualitative analysis of AF and SD-OCT images was performed by two independent experienced observers (APS and HOO) in a masked fashion. The presence of RPE atrophy, photoreceptor layer defects and AF changes were determined.
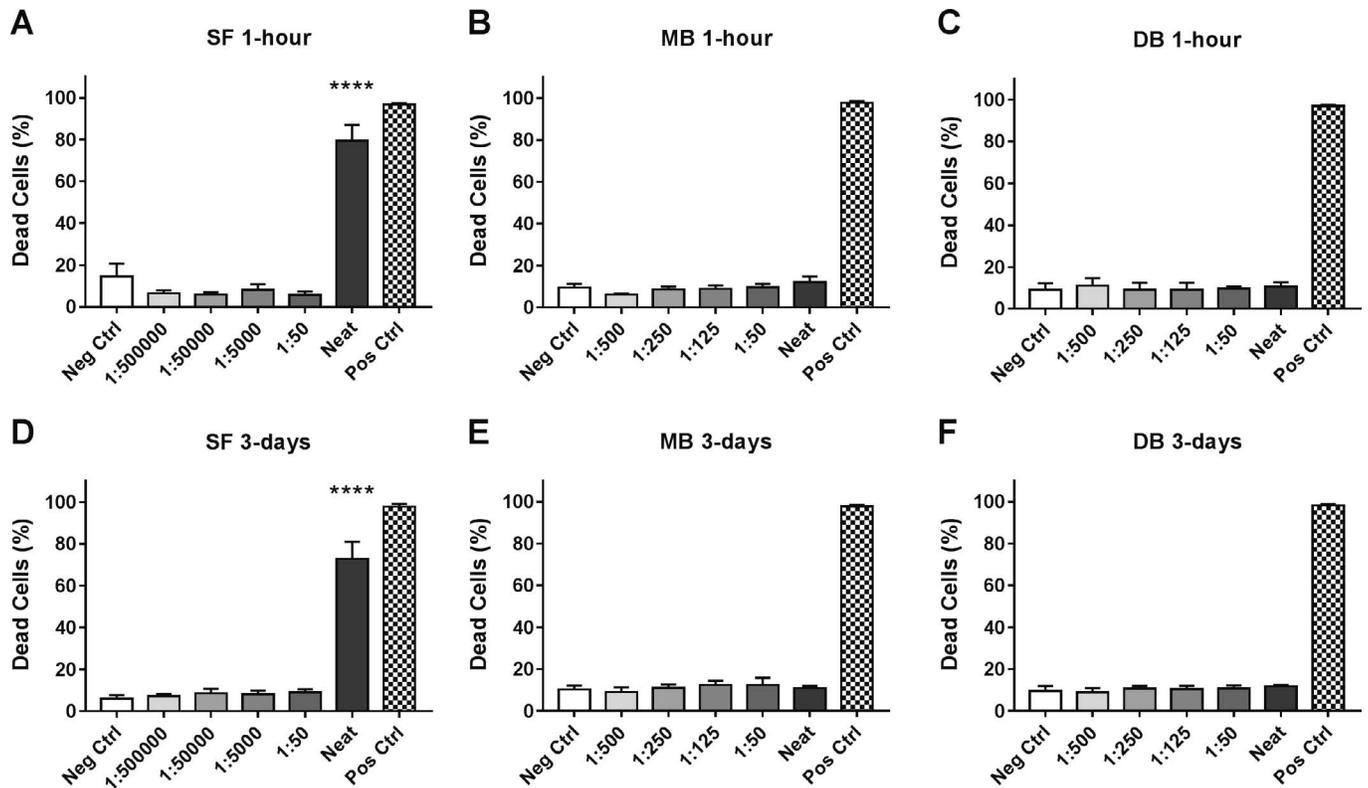
Figure 2. Potential toxicity of vital dyes was assessed in vitro. HEK293 cells were incubated with a range of dilutions of SF, MB, and DB for 1 hour. Toxicity was assessed immediately after (A–C) and 3 days later (D–F) by FC. Dye toxicity was compared with the negative control in each dataset (one-way ANOVA, with Dunnett's multiple comparison test). Values are mean of five replicates ± SEM. (A, D) show SF acute and chronic toxicity after 1-hour incubation and after 3 days, respectively, when applied undiluted (****$P < 0.0001$). Neither MB (B, E) nor DB (C, F) proved to be toxic at any of the dilutions tested.

Electroretinography

Dark- and light-adapted full-field electroretinography was performed 1 week after retinal imaging (12 weeks post-subretinal injection). Mice were dark-adapted overnight (>12 hours) prior to testing, anesthetized, and subjected to pupillary dilatation as described above. Mice were placed on a heated platform and silver thread Dawson, Trick, and Litzkow (DTL)-type electrodes placed on the eyes with custom contact lenses. Ground and reference electrodes were applied subcutaneously to the flank and between the eyes respectively. Dark-adapted responses to uniform white light flashes of increasing intensity ranging from −6 to 1.4 log cd.s/m^2 were recorded, and after a 10-minute period of light adaptation, photopic responses were recorded to flashes of −0.5 to 1.4 log cd.s/m^2. Recordings were acquired using the Espion E3 Ganzfeld system (Dyagnosis Limited, Aylesbury, UK) and responses analyzed offline. Dark- and light-adapted electroret-inogram (ERG) responses were compared between dye- and BSS-injected eyes using two-way ANOVA tests.

Statistical Analysis and Graphing

Statistical analysis and chart preparation was conducted using Prism software (GraphPad, San Diego, CA). When comparing one independent variable with more than two conditions and a single dependent variable, an ordinary one-way ANOVA test was applied. When comparing two independent variables, with at least two conditions and a single dependent variable, an ordinary two-way ANOVA was used. Post hoc tests were conducted with correction for multiple comparisons where appropriate. When available, sample groups were all compared with a single control group. When not available, all groups were compared with each other ($\alpha = 0.05$ for all tests). Graphical data is presented as mean ± 1 standard error of the mean (SEM) in all cases.

Results

The potential toxicity of SF, MB, and DB at a range of dilutions was compared with that of negative control samples (Fig. 2). For all data sets, the positive control sample (prepared by heating the cell suspension for 20 minutes at 60°C) showed a percentage of dead cells statistically higher than that of the negative control sample (one-way ANOVA with Bonferroni's multiple comparison test, $n = 5$, $P < 0.0001$ in all cases). After exposure to SF, the proportion of dead cells at both 1 hour and 3 days was found to be significantly greater than that of the negative control sample only when SF was used neat (one-way ANOVA with Dunnett's multiple comparison test, $n = 5$, $P < 0.0001$ both cases). Although this suggests that neat SF is toxic to the cells, it was not determined whether this was attributable to the fluorescein itself, or if it was an effect of the solution's excipients, pH, or osmolarity. No significant effects on the proportion of dead cells were found for any of the blue dye dilutions (MB and DB) at any given time point (one-way ANOVA with Dunnett's multiple comparison test, $n = 5$, $P > 0.9999$ in all cases). These data suggest that neither MB nor DB is toxic in vitro.

Having excluded fluorescein due to its potential toxicity at higher concentrations, MB and DB were applied in qPCR experiments designed to assess their potential impact on recorded AAV titer. After establishing that adding dyes did not have a significant impact on the qPCR method in general (see Supplementary Fig. S1), various dilutions of the two dyes were mixed with AAV solution and the AAV titer assessed by qPCR. An ordinary two-way ANOVA examining the effect of dyes (MB or DB) and dye dilutions on measured AAV titer showed statistically significant effects of dye type [$F_{(1, 30)} = 55.93$, $P < 0.0001$] and dye dilution on AAV titer [$F_{(3, 40)} = 21.71$, $P < 0.0001$] (Fig. 3). A Dunnett's multiple comparison test for the effect of the dilution within each dye tested showed that none of the MB dilutions affected the measured qPCR titer, when compared with no dye control ($n = 6$, neat dye, $P = 0.0835$; 1:50 dye, $P = 0.8011$; 1:125 dye, $P = 0.9999$). In the case of DB-treated samples, all these showed a significant increase of the reported titer (from 2.9- to 6-fold) when compared with no dye controls (neat dye and 1:50 dye, $P = 0.0001$; 1:125 dye, $P = 0.0067$). Moreover, the presence of the DB yielded a slightly higher AAV titer at every dilution tested when
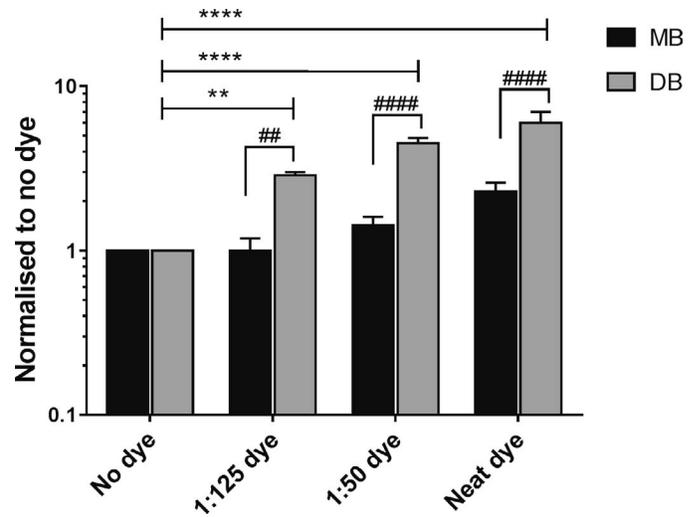
Figure 3. Impact of MB and DB on AAV titer assessed by quantitative PCR. Both dyes were used neat and in two dilutions (1:50 and 1:125) to prepare an AAV solution that was titered by qPCR and normalized to no dye sample. Values are mean of six replicates ± SEM. Only DB significantly increased the AAV titer at all dilutions tested (two-way ANOVA, with Dunnett's multiple comparison test; **$P < 0.01$, ****$P < 0.0001$). The comparison between the effect of the two dyes at every dilution showed that DB significantly increased measured AAV titer over MB (two-way ANOVA, with Sidak's multiple comparison test; ##$P < 0.01$, ####$P < 0.0001$).

compared with MB ($n = 6$, two-way ANOVA with Sidak's multiple comparison test: $P = 0.0093$ for 1:125, $P < 0.0001$ for 1:50 and neat; Fig. 3).

To investigate which component(s) of the dye solutions contributed to differing AAV titres, AAV solutions were exposed to dye dilutions as well as to isolated ingredients of the dye mixtures (Fig. 4). We again found that both neat and 1:50 dilution of DB significantly increased the measured AAV titer ($P < 0.005$ and $P < 0.0001$, respectively; one-way ANOVA with Dunnett's multiple comparisons test). Moreover, the addition on 0.0001% of PF68 to DB 1:50 also increase the measured genomic titer ($P < 0.0001$, one-way ANOVA with Dunnett's multiple comparisons test). TB (used in both MB and DB) and 4% PEG (used at this concentration in DB) were individually added to AAV samples. Of these two components, only PEG was found to significantly impact on the measured AAV titer relative to a no dye control ($P = 0.0403$, one-way ANOVA with Dunnett's multiple comparisons test). Hence, the 6-fold differences in AAV titer seen between MB and DB samples (Fig. 3) most likely reflects the use of a surfactant (PEG) in the latter, which is known to improve accuracy of AAV titers by reducing vector particle nonspecific
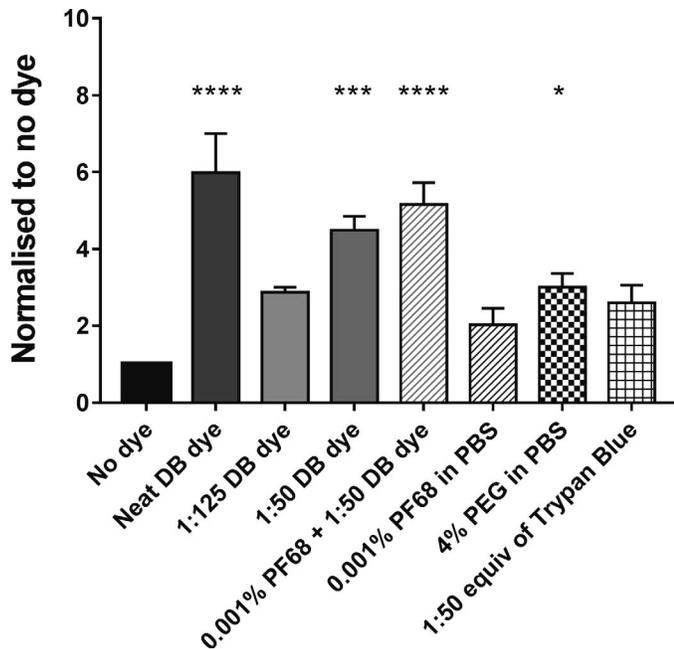
Figure 4. Effect of DB dilutions and of single dye components on AAV titer assessed by qPCR. DB was used neat and at two dilutions (1:50 and 1:125) to prepare an AAV solution that was titered by qPCR and normalized to no dye sample. The effects of three dye components (trypan blue, PEG, and PF-68) were tested in parallel; PF-68 was also tested in combination with DB diluted 1:50. Values are mean of six replicates ± SEM. Both neat and 1:50 diluted DB increased apparent AAV titer compared with no dye samples (one-way ANOVA, with Dunnett's multiple comparison test; ***$P < 0.001$; ****$P < 0.0001$). Of all three components tested, PEG was the only component that induced a significant increase in recorded qPCR titer (one-way ANOVA, with Dunnett's multiple comparison test; *$P = 0.0403$).

binding to plastic-ware during processing and incubation.[9]

Having established that the presence of DB was able to increase the titer that could be measured by qPCR, we wanted to test if this would also impact in the percentage of transduced cells in vitro. Therefore, we tested the transduction efficiency of AAV2-GFP at different MOI in the presence of DB at 1:50 dilution, the weakest dilution that is still visible to the human eye in a surgical setting (Fig. 1). MB was also included in this experiment to control for the effect of blue dyes in transduction efficiency. The amount of virus added to obtain a specific MOI was calculated identically for all conditions (i.e., no dye, MB, DB), using genome copies per milliliter values from the original qPCR of the stock virus (i.e., not correcting for improvements in measured titer gained by including DB). The percentage of GFP+ cells was assessed by FC at 3 days post transduction (Fig. 5A).

In all groups, there is a clear relationship between the MOI and the percentage of GFP+ cells, which was confirmed by a two-way ANOVA ($P < 0.0001$ for MOI as a source of variation). On first inspection, all treatment groups appear to have a very similar MOI-GFP+ cell percentage relationship. However, the presence of DB does appear to mildly improve transduction across the MOI range and we also observed the presence of dye to be a statically significant source of variation in the same analysis (two-way ANOVA, $P = 0.0203$ for dye). Although a Bonferroni's multiple comparisons test for the effect of the dye at any given MOI did not show any significant pairwise difference, we hypothesized that a better fit for the DB effect on the transduction efficiency could be achieved if the MOI was corrected based on the qPCR data for the specific condition, rather than using the original titer (Fig. 5B). Both original (dotted line) and corrected (dashed and solid lines) MOI of DB were plotted for direct comparison. Correcting for a 4.5-fold increase in AAV titer (obtained by qPCR for 1:50 DB, Fig. 3) plots a curve shifted too far to the right, indicating the effect on cell transduction is not directly proportional to the increase in qPCR titer found in our earlier experiments. Instead, we found that a theoretical 1.5-fold increase in the effective AAV titer results in calculated MOI values and a curve that directly overlies the no dye control. Overall, this suggests that addition of DB contributes to improved transduction efficiency in vitro, although the alteration is not as large as might be suggested by differences in the qPCR data. This discrepancy may result from the differences in the plastic surfaces and contact geometries in the qPCR and in vitro experiments.

Next, we assessed whether DB would have a toxic effect in vivo. The dye was diluted in BSS at a ratio of 1:5. This was 10 times the concentration used in the AAV assays (1:50) and was clearly visible underneath the retina following subretinal injection in mice (Fig. 6). Eleven weeks after subretinal injection, mean retinal thickness was not significantly different between eyes injected with 1:5 DB or those injected with BSS (252.5 ± 2.2 μm and 254.4 ± 2.9 μm for DB and BSS-injected eyes, respectively, $P = 0.799$, paired t-test, $n = 10$). A post-hoc power calculation based on this data suggested that the potential difference in mean retinal thickness of 5% or greater between the groups would have been detectable with P less than 0.05 and a power of 80%. In all cases, the superior retina (where the injection
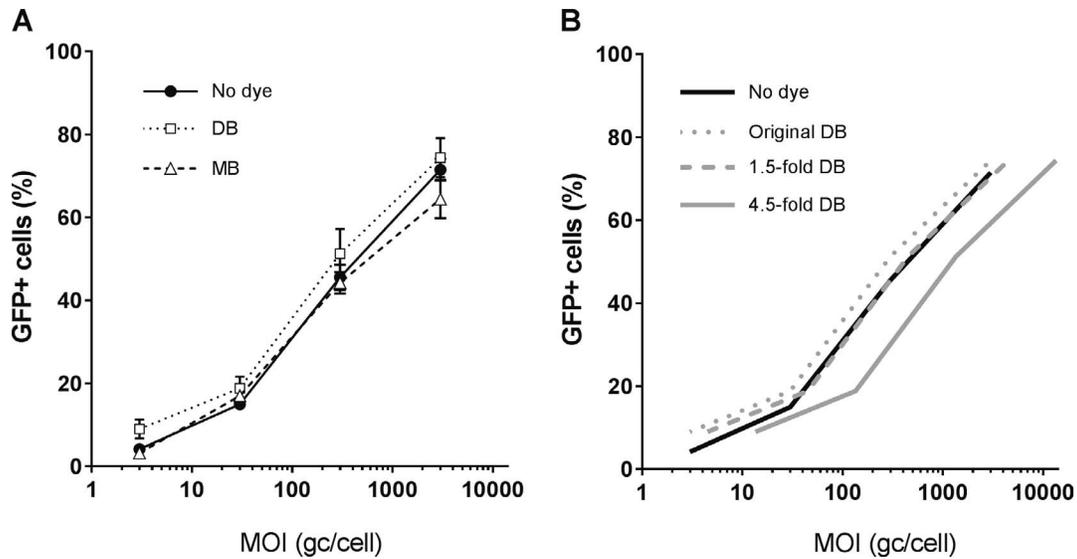
Figure 5. Effect of DB and MB on AAV2-GFP cell transduction. (A) HEK293 cells were incubated with increasing MOI of AAV2-GFP (3, 30, 300, and 3000) in 1:50 dilution of both MB and DB. Measuring GFP-positive (GFP+) cells by FC at 3 days post transduction assessed the effect of the dyes on cell transduction. Values are mean of replicates ± SEM (No dye, $n = 7$; MB, $n = 4$; DB, $n = 5$). Both MOI and dye contributed significantly to the source of variation (two-way ANOVA, $P < 0.0001$ for MOI, $P = 0.0203$ for dye). Bonferroni's multiple comparisons test for the effect of the dye at a given MOI did not show any significant pairwise difference. (B) MOI-transduction relationships for original titer (*dotted gray line*), qPCR corrected titer (*dashed gray line*), and theoretical titer (*solid gray line*) of DB were plotted for direct comparison to no dye (*solid black line*). While a 4.5-fold increase in AAV titer shifts the curve too far to the right, a theoretical fold increase of 1.5 seems to better explain the apparent increase transduction efficiency of DB in panel A.

bleb was formed) was found to be thinner compared with the inferior retina. This was however the case in both treatment groups (250.09 ± 2.89 μm vs. 258.28 ± 2.15 μm in the DB-injected eyes, and 252.73 ± 3.65 μm vs. $259.3.5 \pm 2.49$ μm in the BSS group, $n = 10$ F $(1, 8) = 26.35$, $P = 0.0009$ for effect of retinal location, two-way ANOVA), and no statistical interaction was detected between the factors of injected substance and retinal location [F $(1, 8) = 0.04486$, $P = 0.8376$, non-significant]. In this study, both eyes were treated of every mouse (one with DB, one with BSS). This matching design was used to reduce the inter-animal variability and improve the statistical power of our most important comparison (DB versus BSS treatment), but as a consequence of this, there are no data on uninjected eyes. We cannot therefore ascribe the observed differences between superior and inferior retinal thickness to retinal detachment with any confidence, or indeed exclude the possibility of a pre-existing superior-inferior retinal thickness gradient in these mice. The effect of induced retinal detachment alone has been investigated elsewhere[10] and is beyond the scope of the present study.

No differences between DB- and BSS-injected eyes both in terms of the presence of hyperfluorescent spots on AF images, or ellipsoid zone/RPE layer defects in the superior and inferior hemiretina of OCT scans were noted by two independent masked observers. RPE atrophy and clumping were noticed at the injection site in one case in each group, where significant subretinal hemorrhage had been documented at the time of injection. No RPE defects were seen elsewhere in any mice. Ellipsoid layer disruption was detected in four mice per group at the injection site, but was never widespread within the retina.

Electrophysiological testing was subsequently performed in order to exclude any adverse effects on retinal function, or selective involvement of cone photoreceptors, which contribute minimally to overall retinal thickness in the mouse. No differences were detected between the amplitudes of both dark-adapted a- and b-wave responses between the two groups based on their respective intensity-response relationships [F(1, 20) = 0.00299, $P = 0.957$ for a-wave amplitudes and F(1, 20) = 0.0331, $P = 0.857$ for b-wave amplitudes, two-way repeated measures ANOVA, $n = 11$]. Similarly, no difference in light-adapted b-wave response was detected between dye- and BSS-injected eyes [F(1, 20) = 0.1745, $P = 0.681$, two-way ANOVA, $n = 11$]. Hence, there was no obvious impairment in retinal function detected in either rod-
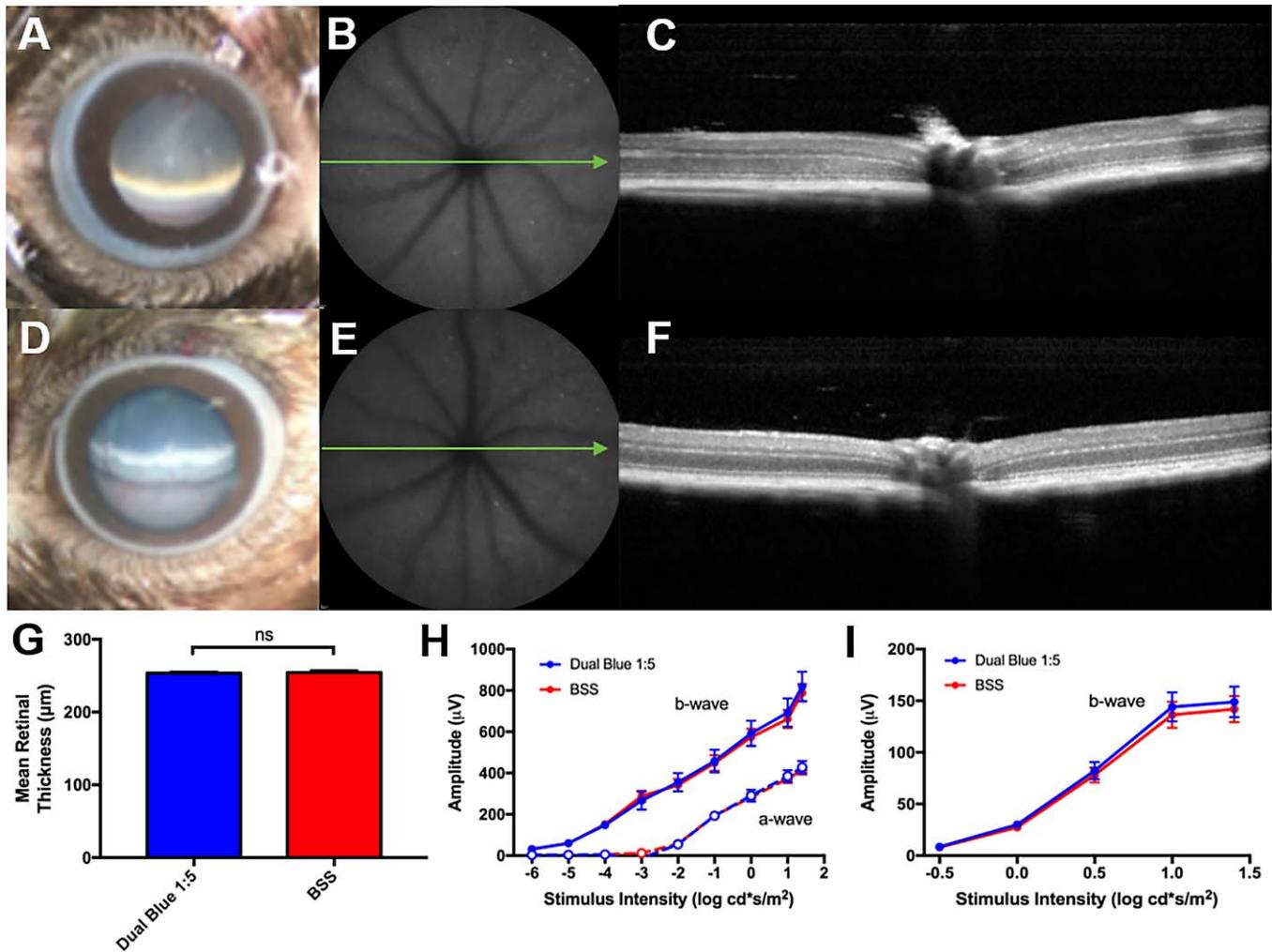
Figure 6. In vivo study. Morphological and functional analysis after subretinal injection of BSS and 1:5 DB. (A) Bleb created following subretinal injection of BSS and the corresponding AF and OCT images passing through the optic nerve head (B–C). (D) Bleb after subretinal injection of 1:5 dilution of Dual Blue. Note that the blue dye is clearly visible beneath the murine retina at this concentration. (E, F) Corresponding AF and OCT images. No defects in any retinal layers were visible on systematic review of all OCT sections. (B–E) Hyperfluorescent spots are visible and compatible with local macrophage activity. (G) No statistically significant difference in terms of retinal thickness was detected between eyes injected with DB or BSS. (H–I) Intensity response curves for dark- and light-adapted ERGs. No differences in a- or b-wave response amplitudes were apparent between dye and BSS-injected eyes.

or cone-pathways 12 weeks after subretinal injection with a 1:5 concentration of DB, equivalent to 0.03% TB, 0.005% BBG, and 0.8% PEG within the subretinal space. Furthermore, the magnitude of ERG responses was effectively identical when compared between dye injection and injection with BSS only.

Discussion

The results indicate that DB and MB, which are similar vital dyes approved for use in vitreoretinal surgery, can be combined with AAV without adverse-ly affecting either the genomic titer or the transduction efficiency. In the absence of PF-68 surfactant, the presence of PEG within DB may actually increase the observed measurement of genomic AAV titer, presumably by a similar mechanism of preventing particle adherence to plastic surfaces.[11] No toxicity was observed when neat concentrations of these dyes were applied to cells in vitro. Additionally, in vivo subretinal injection of a 1:5 dilution of DB appeared to be nontoxic to the retina and any potential impact on retinal structure or function was not significantly different when compared with an identical injection of BSS. Therefore, we propose that blue dyes may be a

useful adjunct to aid visualization in subretinal gene therapy surgery, without adversely impacting on either the efficiency of viral transduction or the potential surgical safety.

Subretinal dyes are routinely used to identify occult retinal breaks during retinal detachment repair.[11–14] Their use has been investigated through subretinal[12–14] and transscleral[15] dye injection, according to surgeon's preferences. Chorioretinal atrophy potentially caused by the accidental subretinal injection of TB was reported in three clinical cases.[16] It should however be noted that in all three cases, the accidental subretinal administration occurred when the dye was injected as a jet of fluid though the 23-G sclerostomy under air. The force of the fluid jet was sufficient to create a retinal hole and create a rapid mechanical retinal detachment. Because the effects of a fluid jet induced retinal detachment without dye has not been assessed, the authors commented that forceful mechanical disruption of the deep retinal structures as a result of the accidental subretinal administration may have contributed to the chorioretinal atrophy.[16]

A study of the effects of subretinal injection of 0.15% TB has been reported in eight rabbits and is worthy of further discussion because it claimed toxicity that was not observed after subretinal injection of BSS.[17] However, it should be noted that the study was descriptive and no significant changes were observed. At the latest 14-day follow-up, only two eyes with TB were compared with two eyes receiving BSS. Two of the TB-treated eyes, but only one of two BSS-treated eyes showed mild photoreceptor changes on semithin sections and electron microscopy. However, they were not detectable in any of the histological sections presented in the figures, all of which show good outer nuclear thickness and outer segments. Even if this was clinically significant, the observed changes could easily be accounted by variability in fixation with $n = 2$ eyes being compared. Finally, the RPE changes observed on angiography were focal and also seen to a lesser extent in the non-TB–treated eyes. They appear to be related to the subretinal needle rather than the wider area of retina exposed to TB. In our project we have used the OCT microscope, rather than retinal sections, to provide an overview of the retinal structure. SD-OCT allowed us to scan the whole retina with a 5- to 7-μm transversal resolution. This is more sensitive compared with histology considering the potential mechanical damage related to the procedure. The use of the ERG is also a well-established method for assessing photore-

ceptor health. While morphologically normal photoreceptors may have a functional loss on ERG, there are no reports of abnormal/damaged photoreceptors having a normal ERG. Hence, the combination of a sensitive functional test combined with an advanced imaging technique provides structural and functional confirmation that there was no detectable photoreceptor damage, beyond which would normally be expected following subretinal injection.

The effects of many dyes have been assessed on human RPE cells over the past 15 years, including indocyanine green (ICG), infracyanine green (IfCG), TB, bromophenol blue (BrB), patent blue (PB), and BBG.[18] We selected three dyes that are routinely used in clinical practice: SF, MD, and DB. SF was investigated as it is routinely used during clinical fluorescein angiography testing and it has been shown that diffusion within the retina is not toxic.[19] SF is also an effective and safe dye when used as a marker for subretinal transplantation of human fetal neural retina.[20]

Past data on dye toxicity are somewhat equivocal. Human fetal RPE cells incubated with TB at 0.5%, 0.1%, and 0.05% showed corresponding increasing apoptosis rates when analyzed with Annexin V-PE staining at 5 and 30 minute post exposure.[21] However, no toxicity in cultured human RPE cells was detected after 5-minutes incubation at the commercially available concentration of 0.15%.[22] At concentrations of 0.15 % and 0.25 %, TB is significantly toxic for ARPE-19 cells only after 30-minutes exposure and not at a shorter incubation time. The absence of toxicity at lower concentrations led us to investigate the use of dyes at higher, but still visible, dilutions for surgical application. It has also been shown that addition of 0.025 % BBG to 0.15% TB (the same dye concentrations present in the DORC Dual Blue solution) resulted in almost complete restoration of cell viability at both 5 and 30 minutes.[23] This might be explained by the fact that BBG acts as an antagonist on the P2X7 cell death purinergic receptor.[24] This receptor is physiologically activated by extracellular adenosine 5′-triphosphate. Additionally, a study of BBG in a rat model of subretinal injection demonstrated biocompatibility of the dye for surgical use.[25]

A prospective multicenter study has shown that the combination of BBG, TB, and PEG (as in the commercially available DB) both increases the viscosity and the density of the solution, and leads to improved staining of epiretinal and inner limiting membranes when compared with BBG alone.[26] This might be of benefit when administering retinal gene

therapy, where staining of the retinotomy may allow it to be identified and reused in instances where further detachment is required. In general, the presence of a small amount of dye in the viral stock may also permit visualization of diffusion of the AAV suspension beneath the macula, and would allow for the identification of any unwanted reflux of vector through the retinotomy in the vitreous.

The potential in vitro toxicity of dyes has been mainly investigated using the tetrazolium dyes assays by colorimetric assays using tetrazolium dyes,[27] which measure intra- and extracellular dehydrogenase activity. Although these assays deliver quantifiable data, we decided to use FC analysis to address this question, as it enabled simultaneous quantification of GFP expression and a cell viability marker. HEK293 cells have been used as a cell model, as they are a commercially available source of known cell lineage as per the requirements of regulatory authorities for preclinical investigations. Further, these cells are transduced efficiently and consistently by AAV, hence providing a sensitive substrate for assessing any reduction in efficacy. The use of neuronal cell lines, such as SH-SY5Y, might have some advantages considering the same neuro-ectodermal origin of the retina. However, the lack of consensus on many fundamental aspects that are associated with their use, such as the effects of culture media composition, variations in differentiation protocols, the lack of well-characterized 'Good Medical Practice' (GMP) cell lines, and of consistency in transduction, would result in a less sensitive assay not replicable for clinical approval by the regulatory authorities.[28]

Our data suggest that the TB itself and PEG in DB may be acting as a surfactant, similar to PF-68. Barnard at al.[9] previously demonstrated an approximate 81% loss of vector particles in the injection system in the absence of PF-68 surfactant. PF-68 0.01% was therefore included in the final vector suspension currently in use in a gene therapy for choroideremia. The data presented above suggest that if DB were to be added to the vector solution for retinal gene therapy, the presence of polyethylene glycol would not be detrimental, and could even act as an additional agent to maximize availability of AAV. Our in vitro data showing cell transduction in the presence of DB supports this statement.

In conclusion, DB and MB may be safe and useful adjuncts for use during gene therapy surgery to enable better visualization of the retinal detachment and to identify vector diffusion and/or reflux. Additionally, the higher genomic AAV titer observed in presence of the DB suggests that this dye may confer additional benefits to AAV solutions that are prepared without an endogenous surfactant in the vector formulation, in terms of reducing vector particles that are retained in the injection system.

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