A new dual dye for vitreoretinal surgery with increased transparency

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Supplemental Information

Contents

Synthesis of DDG

The synthesis of the new dye proceeded smoothly by reacting the green chlorophenyl dye **1**, described by Takahagi et al. [1], (Figure S1) with *p*-anisidine in refluxing butanol. Purification by repeated precipitations was not sufficient to remove all excess *p*-anisidine, and column chromatography on silica gel with a gradient of dichloromethane and methanol was essential. The dye was characterized by ^{1}H-NMR (Figure S2) and high-resolution mass spectrometry (Figure S3) and was found to conform to the structure given in Figure S1. HPLC on RP-18 with a gradient from phosphate buffer to acetonitrile showed absence of both p-anisidine and the chlorophenyl dye (Figure S4).

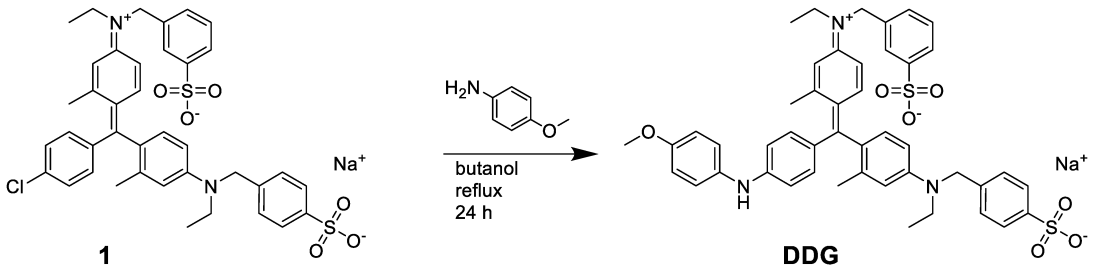

Figure S1. Synthesis of DDG.

The work-up consisted of adding Na_2CO_3 to the butanol solution, filtering off the solids after 30 min, precipitating the dye by pouring the butanol solution into ten times the volume of diethyl ether, centrifugation to recover the dye, and chromatography on silica gel with a gradient 100% dichloromethane to 60% dichloromethane 40% methanol. Chromatography was essential to remove all p-anisidine. Pure fractions, as determined by HPLC on an RP-18 column with a gradient from 0.02 M sodium phosphate buffer pH = 6.0 and acetonitrile, were pooled and evaporated.

^{1}H-NMR

The spectrum was taken in methanol-d4 on a JEOL spectrometer at 400 MHz. The spectrum is shown in Figure S2.

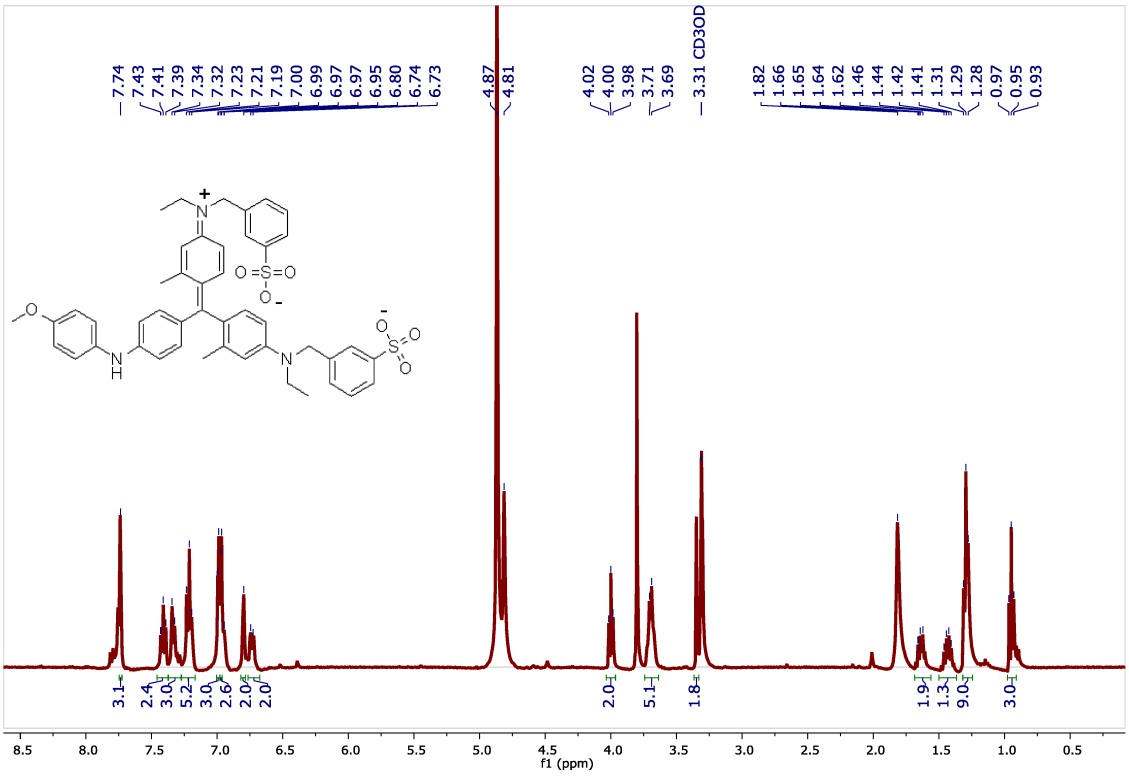

Figure S2. ^{1}H-NMR spectrum of DDG

High-resolution mass spectrometry

ESI-HR-MS: Data were acquired on an Impact II q-ToF mass spectrometer (Bruker Daltonics, Bremen) in negative mode using direct injection. The sample was dissolved in MeOH and injected via a syringe pump with a flow rate of 3 µL/min. I.on source

parameters: capillary voltage 4.5 kV, end plate offset 500V, nebulizer gas pressure 0.4 bar, dry gas flow 4 L/min, dry gas temperature 180°C. Data were acquired for 0.5 min and averaged; internal recalibration was performed with mass spectra obtained from a 10-4 M sodium acetate solution. Data processing and generation of molecular formulae was done with Bruker DataAnalysis 4.4.

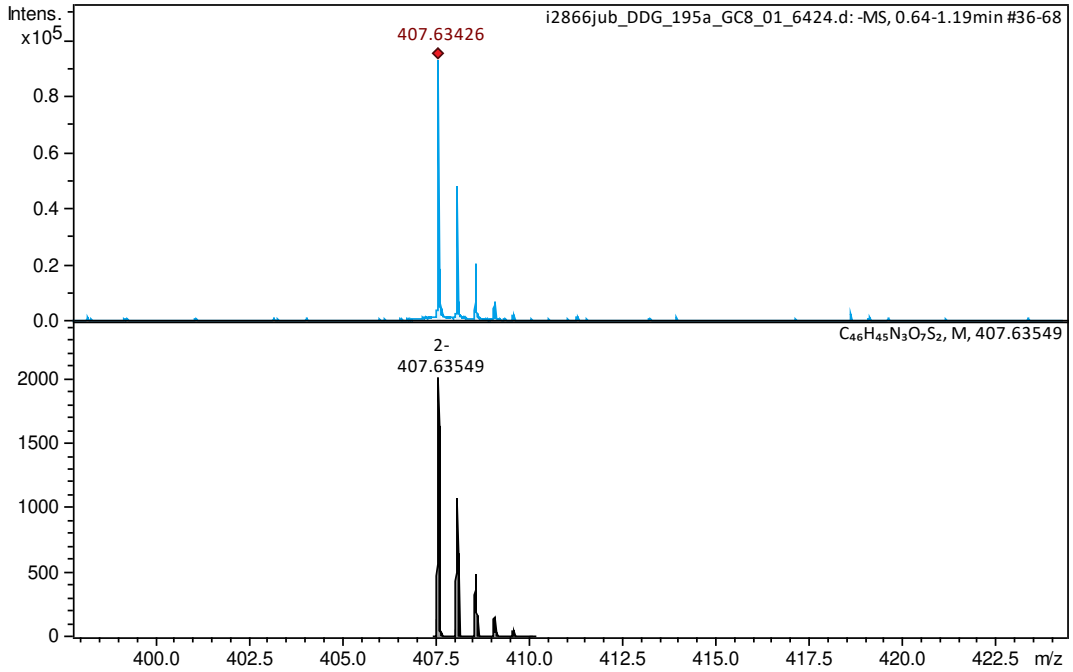

Figure S3. High-resolution mass spectrum of M-H(2-) (top) and the simulated isotope pattern (bottom.

HPLC

HPLC was carried out using RP-18 chromatography on a NUCLEODUR column 100-5, 5 µm, 50x4,6 mm, with a linear gradient from 100% 0.02 M phosphate buffer pH = 6.0 to 100% acetonitrile in 10 minutes. Detection was at 650 nm, in order to detect any remaining compound **1**.

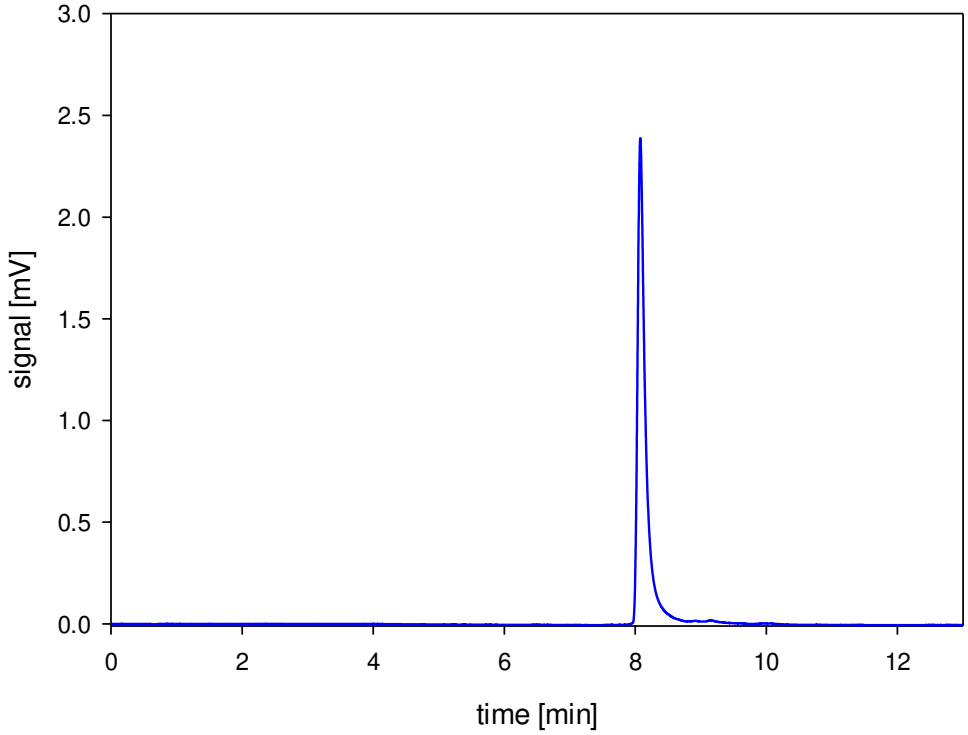

Figure S4. HPLC trace of DDG, with detection at 650 nm.

Dye solutions

Solutions with 0.02% DDG in PBS with diglycerol (Innovy, Brussels, Belgium) at 2.6%, either alone (single posterior dye, SPD) or in the presence of 0.09% TB (double posterior dye, DPD) were prepared under GLP conditions by an external company. TB was purified extensively from impurities.

Solutions of ILM Blue® (ILB) and MembraneBlue Dual® (MBD) were from DORC (Zuidland, The Netherlands).

For toxicity assays, BBG and DDG were dissolved in PBS at concentrations of 0.025% and 0.050%.

PEG3350 (polyethylene glycol MW 3350) was from Sigma (Germany), PEG with higher molecular weight were gifts from BASF (Ludwigshafen, Germany).

Staining models and their evaluation

The following models were used:

Filter and membrane: A 96-well filter plate (MSBVN1210 MultiScreen BV Filter Plate, Merck-Millipore, Darmstadt, Germany) (hydrophilic polyvinylidene difluoride (PVDF) was used as ILM model. A Collagen Cell Carrier membrane (CCC) (Viscofan, Weinheim, Germany) was used as model for the ERM. CCC consists of long collagen fibers of type I. Membrane punches (5 mm diameter) were fixed to the filter with a hypodermic needle and soaked in PBS for 30 minutes. PBS was removed by vacuum filtration, and 50 µL of the dye solution was pipetted onto the filter. After 30 s, the dye

was removed by vacuum filtration, and membrane and filter were washed three times with PBS. The membrane was removed and put into a 96-well plate for spectrum recording (Tecan Spark 20M (Tecan Group Ltd., Männedorf, Switzerland). Staining was recorded photographically. The intensity of staining was graded in four steps (0 to 3), as by Rodrigues et al. [5].

Proteins on plastic: A 96-well flat-bottom cell culture plate was coated with 3.2 µg/well collagen IV, 3.2 µg/well collagen I, 1.6 µg/well fibronectin, or 0.64 µg/well laminin and combinations of those. After incubation overnight, the solutions were removed and the wells were incubated with dyes for 30 s. After removing the dyes and washing with PBS, absorbance spectra were recorded as above. Results are shown in Figure S5.

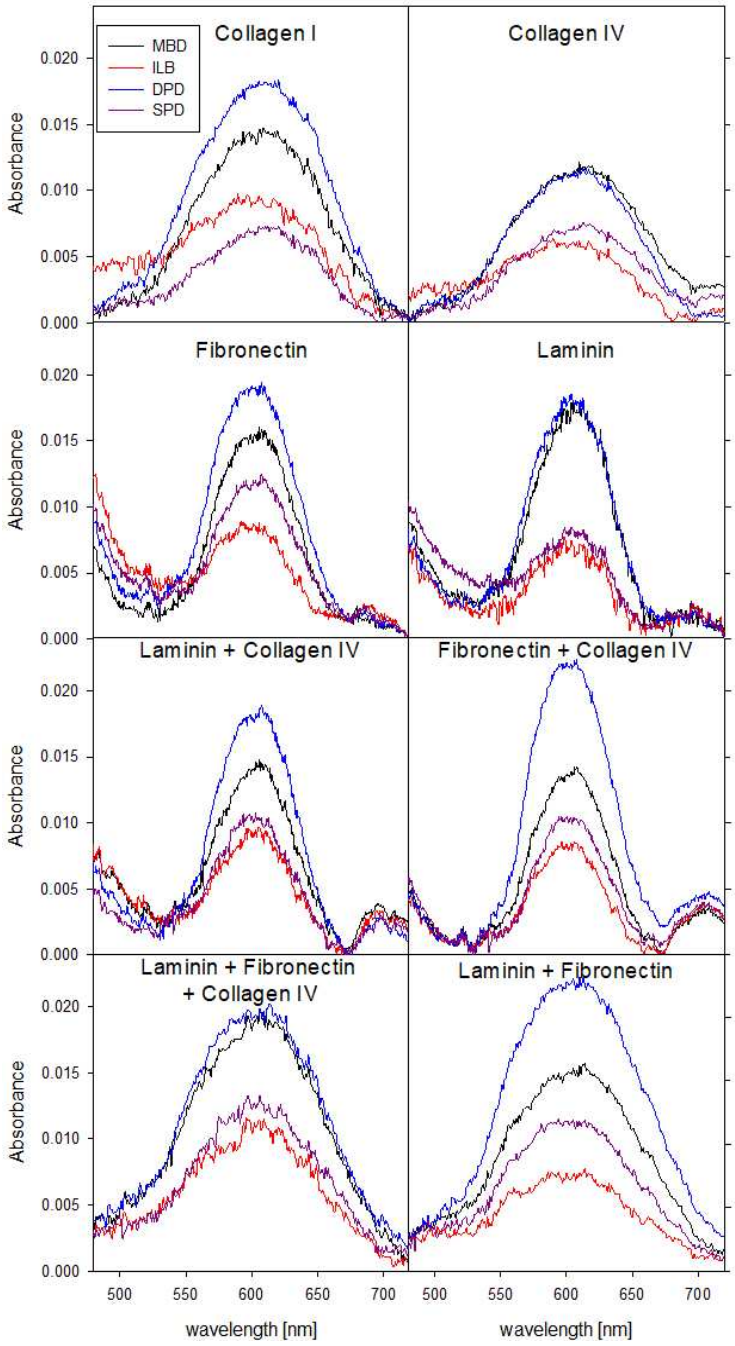

Figure S5. Staining of proteins on surfaces by dyes.

Evaluation of toxicity and phototoxicity

Toxicity and phototoxicity was evaluated as described before [2]:

Cell culture and toxicity

ARPE cells were from LGC, Wesel, Germany. Cells were used at passages between 30 and 50. Dulbecco's Modified Eagle's Medium/Ham's F-12 (DMEM/F-12) and MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) cell proliferation

reagent was from SigmaAldrich. The cells were grown in DMEM/F-12 supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37°C in 5% CO_2 humidified air.

Exposure to dyes and light

Cells were seeded at 10,000 cells/well in 96-well, flat-bottom tissue culture plates and grown for 48 h. The culture medium was removed, the cells were washed once with PBS and incubated with the dye solutions for 30 min, then washed three times with 200 µl PBS. Cells incubated with PBS were used as a control.

For phototoxicity, 100 µl PBS was added to each well after the removal of the dye solution, and the plate was illuminated for 15 min by the floodlight. Experiments with cells exposed only to the ambient light of the laboratory are called „in the dark“, although technically they were exposed to low levels of light (only 0.4% of the intensity seen by the cells on the floodpanel). Also, manipulation times were shorter (usually only 5-10 minutes) than the exposure to the light panel.

Toxicity assay

After exposure, cells were incubated under culture conditions either immediately with 10 µl MTT solution (5 mg/mL), or with cell culture medium for 24 h before adding the MTT solution. After 1 hour, the solution was removed and 100 µL DMSO was added to each well. Absorbance was measured at 570 nm in a plate reader (TECAN M1000, Männedorf, Switzerland). The intensity of developed color was taken as proportional to the number of viable cells.

Percentage of cell viability was calculated by relating the measured absorbance to that of the control, treated identical with the exception of the exposure to the dye.

All experiments were run in triplicate.

F-test and t-test data were computed using the proper Excel functions.

Evaluation of live-dead staining

Calcein-acetoxymethyl ester (living cells) and propidium iodide (dead cells) were used.

Cells were grown in 96-well plates. Growth medium was removed and a solution of 20% ethanol was added for 5 minutes in order to induce cell death in some cells. The ethanol solutions was removed, and the cells were stained with DDG (0.025%) in PBS for 15 minutes. After washing with PBS, the cells were exposed to a live-dead staining combination (Sigma-Aldrich) with calcein-acetoxymethyl ester (calcein-AM) in PBS for 15 minutes at room temperature according to the manufacturer’s instructions. After a wash with PBS, the cells were then exposed to propidium iodide (PI) for 5 minutes, and microphotographs were taken with a black-and-white camera, using a halogen lamp for illumination for the picture showing the stain, and with a xenon lamp and appropriate filter combinations for the two fluorescent dyes.

The pictures were overlaid as blue (black-and-white picture), red (PI) and green (calcein) using ImageJ.

Evaluation of transparency

An eye model (Fundus ADV, Bioniko, Miami FL, USA) with a photorealistic fundus (fovea, optic disc, blood vessels) was used in „open-sky“, i.e. the lens and iris part of the model was removed, and the globe was filled with PBS. Illumination was with the light guide (TotalView Endoillumination Probe) from standard surgical equipment

(EVA, DORC, Zuidland, The Netherlands). A standard operating microscope with attached camera was used for recording. Photos were taken with PBS only, with 100 µL DPD, and with 100 µL MBD.

In addition, a wedge-shaped cuvette (constructed from microscope slides) was filled with each of the two dye solutions (DPD and MBD), and photographed against a background. Camera settings and illumination were kept constant, and the two photos were combined into one image.

Correlation of staining

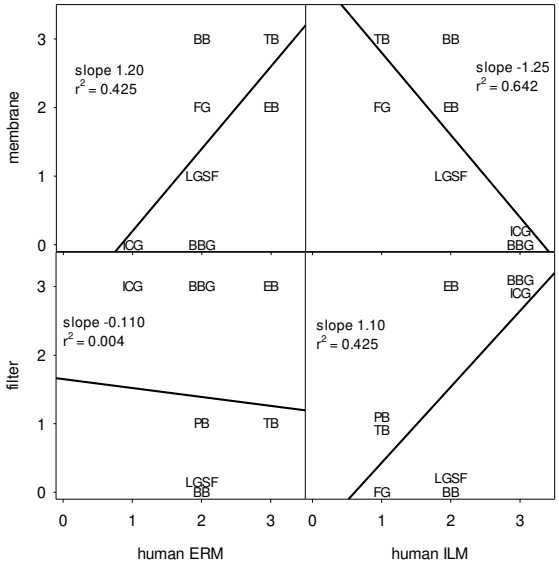

Figure S6. Correlation of staining between CCC membrane, PVDF filter, and biological tissues. Staining data for ILM and ERM are from [2].

Influence of PEG on the staining of the CCC membrane

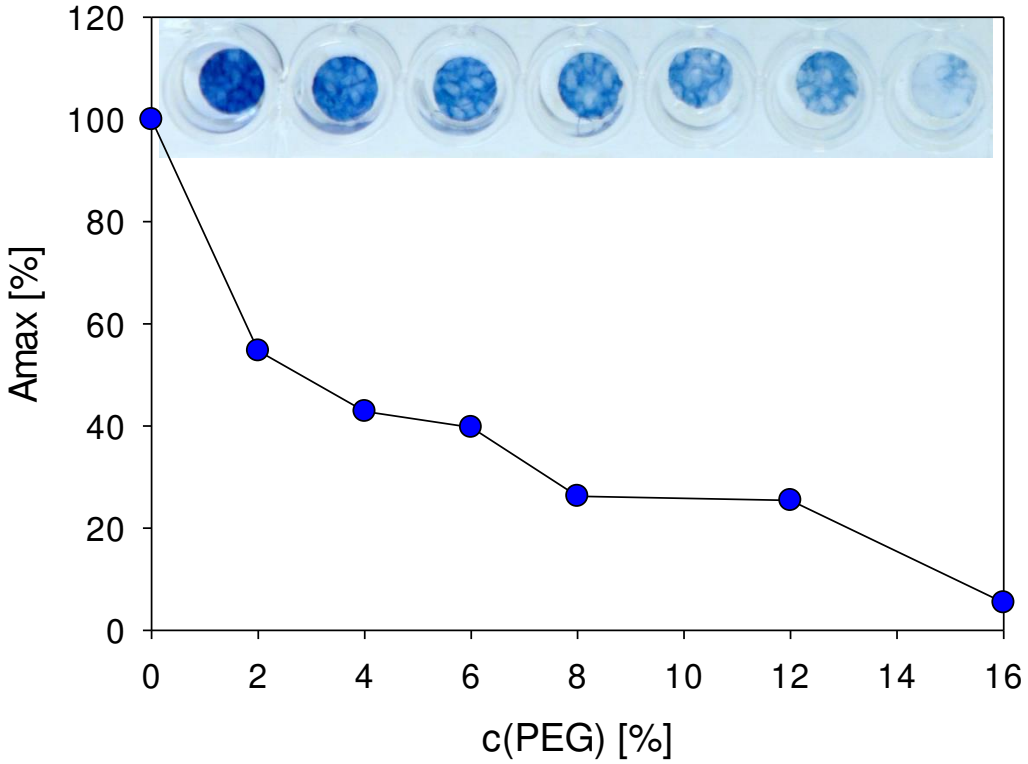

Figure S7. Staining of the CCC membrane with Trypan Blue in the presence of increasing concentrations of PEG3350. The maximum absorbance of the membrane is plotted, set to 100% in the absence of PEG3350. Insert: Photo of the membranes.

Staining of dead cells

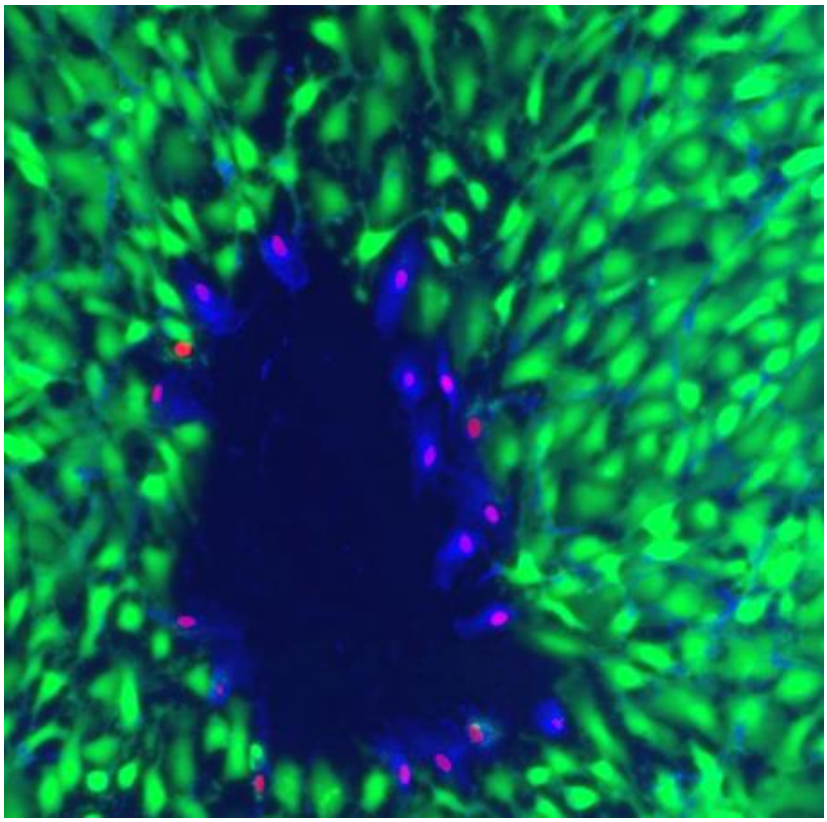

Figure S8. Live-dead staining of cells stained with SPD. Green: calcein (live cells); red: propidium iodide; blue: staining with DDG and phase contrast. Cells stained with DDG and PI simultaneously have the nucleus appear in pink, while cells not stained with DDG, but with PI, show up in red.

References

1. Takahagi M, Haga N, Atsushi I, Hirose T (2017) Triarylmethane composition, dye composition for ocular membrane dyeing. W02017/090349A1
2. Rodrigues EB, Penha FM, de Paula Fiod Costa E, Maia M, Dib E, Moraes MJ, Meyer CH, Magalhaes OJ, Melo GB, Stefano V, Dias AB, Farah ME (2010) Ability of new vital dyes to stain intraocular membranes and tissues in ocular surgery. Am J Ophthalmol 149: 265-277.