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# Safety testing of indocyanine green and trypan blue on retinal pigment epithelium cells

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# *Safety testing of indocyanine green and trypan blue on retinal pigment epithelium cells.*

## Abstract

***Background and aims:*** Indocyanine green (ICG) and Trypan blue (TB) are frequently used vital stains for ILM-staining during macular hole surgery. Lately, there are growing concerns in terms of safety to retinal tissues, especially to the retinal pigment epithelium (RPE). The aim of this experimental study was to examine potentially cytotoxic effects of ICG and TB on cultured human RPE cells.

***Methods:*** ARPE-19 cells were incubated with ICG (0.025–5.0 mg/ml) and with ICG-free solutions of corresponding osmolarities. TB was applied at 0.0375 mg/ml and 1.5 mg/ml. Incubation lasted 1–20 minutes with or without vitrectomy endolight illumination for 1–5 minutes. To mimic clinical practice, exposure time was set at 1 minute, followed by 5 minutes of illumination. Cell viability and morphology were examined after the follow-up times of 6, 24 and 72 hours.

***Results:*** ICG reduced cell viability at concentrations of 2.5 mg/ml and higher, when incubated for more than 5 minutes. Almost no cytotoxicity was observed for ICG at a concentration of 1.0 mg/ml and below, at any incubation time. Hypo-osmolar solutions below 270 mOsm/kg induced severe cytotoxicity independently of ICG, especially at exposure times of 10 minutes and more. At incubation times below 1 minute, osmolarity didn't play a major role. Incubation with ICG for 1 minute, and illumination for 5 minutes, did not cause damage at concentrations of up to 1.0 mg/ml. TB-related cell toxicity occurred at 1.5 mg/ml for incubation times above 5 minutes. No phototoxic effects for TB solutions were shown in any set-up with vitrectomy endolight illumination.

***Conclusions:*** For clinical use ICG concentration should not exceed 1.0 mg/ml, exposure and illumination time should remain below 5 minutes, the osmolarity being within physiological range. TB at short incubation times or low concentrations seems to be a safe alternative without phototoxicity.

**Key words:** ARPE-19, indocyanine green, trypan blue, dye concentration, osmolarity, vitrectomy endolight illumination, cell viability, cell morphology.

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# Abbreviations

| | |
|---|---|
| ARPE-19 | adult retinal pigment epithelial cell line |
| BSS | balanced salt solution |
| DAPI | 4’,6-diamidine-2’-phenylindole-dihydrochloride |
| D-MEM | Dulbecco’s modified Eagle medium |
| ERG | electroretinography |
| ERM | epiretinal membrane |
| ICG | indocyanine green |
| IFCG | infracyanine green |
| ILM | internal limiting membrane |
| MH | macular hole |
| MHS | macular hole surgery |
| OCT | optical coherence tomography |
| PFA | paraformaldehyde |
| PI | propidium iodide |
| PPV | pars plana vitrectomy |
| R28 | rat neurosensory retinal cell |
| RGC | retinal glial cell(s) |
| RPE | retinal pigment epithelium |
| SLO | scanning laser ophthalmoscope |
| TB | trypan blue |
| VA | visual acuity |

# 1. Introduction

## 1.1 Macular holes

Macular holes (*Figure* 1) were first described 1896 by Knapp in Germany and 1900 by Collins in Britain [23, 90]. Trauma, as well as cystic degeneration, were initially implied as reasons for macular hole development. However, the most frequently described type is the idiopathic macular hole. Idiopathic macular holes develop predominantly in older patients at an incidence ranging from 0.03 to 0.05%. The prevalence is three times greater among women than men [114]. Macular hole formation in the fellow eye was described at an incidence of 0 to 30% [1]. However, this initially incurable disease, leading to a prominent loss of visual acuity, can today be treated successfully by surgical intervention.

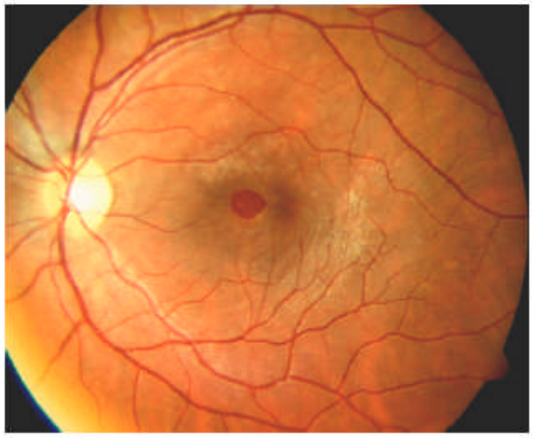


***Figure 1:*** Macular hole.

### 1.1.1 Pathogenesis

The pathogenesis of macular holes is still not completely understood. The original descriptions comprised trauma as being the most important predisposing factor, but myopia [129], intraocular inflammation [8], cataract surgery [49, 50], central vein occlusion [84], as well as diabetic maculopathy

have all subsequently been associated with this disease pattern. In 1955, Schepens noted the importance of the vitreous configuration in the development of macular holes [143]. Earlier in 1953, Irvine described aphakic macular oedema and suggested vitreomacular traction as contributing to its development [80]. This concept was reviewed again in the late sixties by several authors observing improvement of visual acuity following spontaneous relief of vitreomacular traction [136]. More recently, the concept of idiopathic macular holes has been established [1], showing a female predominance and an occurance in the sixties and seventies age groups. However, according to numerous publications over the past years, the role of the vitreoretinal traction in macular hole pathogenesis seems to be of predominant relevance [45, 51, 52]. Thus, the comprehensive and extensive description of the four stages of macular hole formation by Gass was an important step in understanding of the pathogenesis of macular holes.

### 1.1.2 Diagnosis and Stages

Recently, the diagnosis of macular holes includes functional analyses like the Watzke-Allen- or Amsler-Test, optical coherence tomography (OCT) as well as the confocal scanning laser ophthalmoscope (SLO).

OCT imaging is analogous to ultrasound, except that reflected light, instead of sound, is used. The analysis of the reflected light is processed with the technique of low-coherence interferometry. Therefore, it is a useful examination technique for diagnosis and follow-up examination of different macular diseases like diabetic retinopathy, cystic macular oedema, chorioretinal neo-vascularisation, vitreoretinal traction syndrome and epiretinal gliosis [41, 149]. With the use of a scanning laser ophthalmoscope (SLO), variable patterned stimuli can be projected onto the retina. During alternation of these patterns, visual evoked cortical potentials and pattern ERGs can be recorded. The configurations of the SLO-elicited potentials and peak latencies correspond to those evoked during conventional stimulation. During pattern stimulation the fundus and alternating pattern stimuli are observed simultaneously on a video

monitor. Consequently, the examiner always knows the exact location of the stimulus on the retina [155]. Static microperimetry using SLO examination is described to be an important tool to evaluate results of foveal retinal function in macular hole surgery [59, 70].

The stages of macular hole development (*Figure 2*) were described in two previous publications by Gass [51, 52].

**Stage 1:** Describes the foveal detachment. The biomicroscopy, shows in most of the patients a yellow spot of 100-200 µm in diameter (stage 1 A) or a ring of 200-350 µm in its diameter (stage 1 B), which is present in the foveal area. The foveal retina is elevated to the level of the surrounding retina (but not anterior). Most of the patients come to their first examination when they already show a yellow ring. A few fine radiating retinal striae often surround the yellow ring. As the ring enlarges, the rim becomes narrower and slightly serrated, and the center appears to be reddish. Patients present a mild decrease in visual acuity, or metamophopsia. The VA is typically in a range of 20/25 to 20/70. The vitreous is clear and attached.

**Stage 2:** Describes the early hole formation. The biomicroscopy initially shows a crescent- shaped macular hole, followed by a horseshoe- shaped hole, and finally a round shaped macular hole with an operculum attached to the posterior hyaloid surface. In some cases, hole formation began in two separate areas which then fused to form one single hole. As the hole enlarges, the yellow ring turns gray and appears to coincide with the area of retinal detachment surrounding the hole. In other cases, round yellowish deposits develop on the surface of the pigment epithelium within the area of the hole and surrounding detached retina. The patients experience a gradual loss of VA, as the hole increases, with reaching a maximum at the middle stages of hole development.

**Stage 3:** Describes a fully developed macular hole with vitreofoveal separation. The biomicroscopy shows a central round retinal defect of > 400 µm diameter, with an operculum suspended within the area of the hole. The operculum slightly oscillates with eye movements and is suspended on the surface of the posterior hyaloid membrane. Patients with fully developed stage 3 macular holes do not show a progression of visual loss or enlargement of the rim of retinal detachment. VA is usually in a range of 20/70 to 20/400. Most holes do not progress to stage 4.

**Stage 4:** Describes the macular hole with posterior vitreous separation from the optic disc and macula. The biomicroscopy shows a central round retinal defect of > 500 µm diameter, surrounded by a rim of elevated retina. In most cases an operculum could be located on the posterior surface of the anteriorly displaced mobile posterior hyaloid membrane.

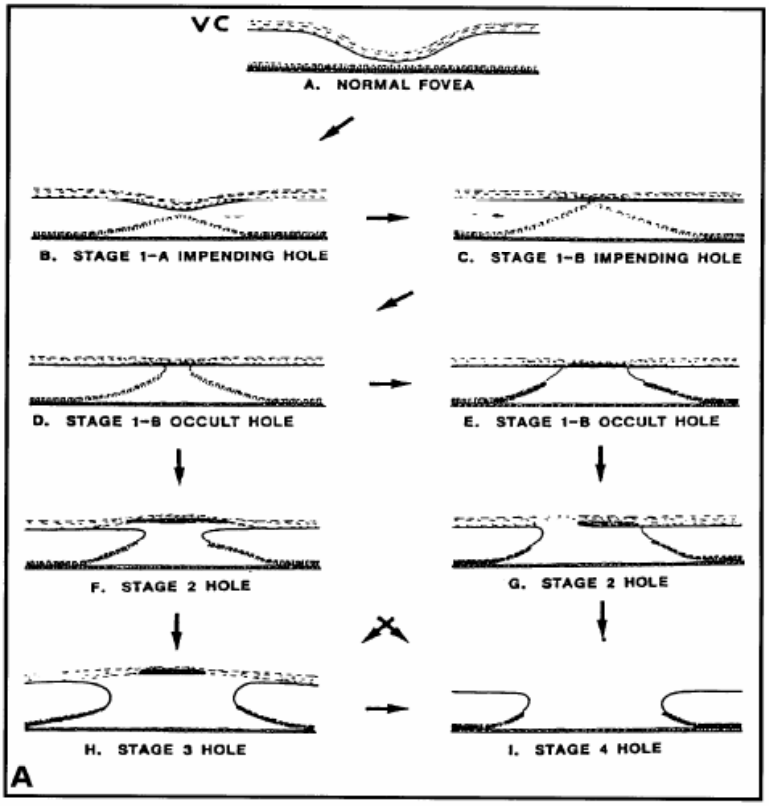



***Figure 2:*** Stages of development of an idiopathic senile macular hole.
(figure by Gass J.D., *Reappraisal of biomicroscopic classification of stages of development of a macular hole*. Am. J. Ophthalmol. 1995; 119(6): 752–759)

### 1.1.3 Macular hole treatment modalities

**Stage 1:** Without treatment 23 to 40% of stage 1 macular holes progress to full-thickness MH [69]. The study of Hikichi et al. [69] noticed for 30% of eyes with stage 1 macular holes a further decrease in visual acuity of 2 or more Snellen lines during a three year observation period. It was reported that intravitreal gas injection inhibited further progression in 91% of eyes with stage 1 macular holes [16]. However, it is generally recommended to monitor rather than treat the patient at this stage.

**Stage 2:** Hikichi et al. [69] noticed for 68% of eyes with stage 2 macular holes a further reduction of the visual acuity of 2 or more Snellen lines during the three year observation period. Because of the high risk of progression, surgical treatment is therefore recommended.

**Stage 3 and 4:** At these stages only few cases of spontaneous hole closure were observed [58]. In the study of Hikichi et al. [69], 29% of eyes with stage 3 and 13% of eyes with stage 4 macular holes showed a further decrease in the visual acuity of 2 or more Snellen lines during the three year observation period. The present standard treatment for stage 3 and 4 macular holes is pars plana vitrectomy, in combination with internal limiting membrane (ILM) peeling and epiretinal membrane removal, respectively.

## 1.2 Macular hole surgery with ILM-peeling

The developments in the past years clearly show that surgical treatment of stage 3 and 4 macular holes is superior to the former therapeutic treatment methods. However, the surgical treatment of stage 2 macular holes without epiretinal gliosis remains controversial. Today, the standard surgical procedure in macular hole surgery includes pars plana vitrectomy, removal of the vitreous cortex, removal of epiretinal membranes, gas tamponade and proper positioning of the patient [160].

Since Kelly and Wendel first reported on the successful closure of macular holes by pars plana vitrectomy (PPV) with gas-fluid exchange in a pilot study [89], several modifications and improvements of the surgical technique have been suggested [33, 111, 130, 159]. Kelly and Wendel reported about approximately 58% anatomical and 42% functional success rates. With the intention to improve the results of macular hole surgery, many surgeons started to use some type of adjuvant to create a permanent seal at the hole's edge, whereas others prefer to work without any adjuvant [29, 148]. Frequently used adjuvants include autologous serum [111], autologous platelet concentrate [53, 131] and transforming growth factor-beta 2 [54, 106]. In fact, the use of adjuvants was reported to further improve the macular hole closure rate, showing the best results using autologous platelet concentrate with 95% anatomical and 89% functional success rates [53].

However, postoperative complications including cataract, retinal breaks and detachment [140, 163], peripherial visual field loss [47, 115], increased intraocular pressure [157], as well as the alteration of the retinal physiology in the macular region [156], have been increasingly described in the literature. Thus, the role of ILM-peeling in macular hole surgery still remains a subject of debate and various investigators recommended to evaluate the surgical success by improvement of visual acuity, and not only by higher anatomical closure rate [6, 68, 94].

According to numerous clinical studies, the removal of the ILM has successfully shown both a visual and anatomical improvement [14, 59, 133]. The internal limiting membrane forms a physiological border between the retinal tissue and the vitreous interface (*Figure 3*). This membrane is derived mainly from the basement membrane elaborated by footplates of Müller cells [36].

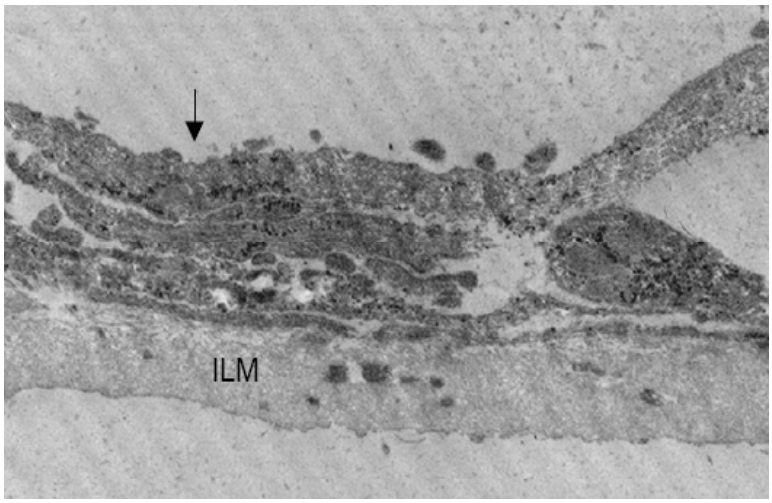



***Figure 3:*** EM-picture of the ILM, the arrow points on the retinal part of the ILM (EM-picture at 30210x magnification by Schumann R., *Epiretinale Membranen. Die Ultrastruktur nach Anwendung von Kryomethoden und chemischer Fixierung.* Department of Ophthalmology, University Eye Hospital Munich, Ludwig-Maximilians-University, Germany)

As a basement membrane, the ILM can act as a scaffold for cellular proliferation and may be involved in the pathophysiology of disorders affecting the vitreomacular surface, including epiretinal membranes (ERMs), vitreomacular traction and macular holes (MHs) [22, 57, 121, 146, 147]. The aim of the surgical intervention is the elimination of the anteroposterior and tangential traction by removing the posterior vitreous. As previously described, these traction forces are suspected to create, and later maintain, the macular hole. As a consequence, macular hole surgery including pars plana vitrectomy, ILM-peeling and ERM-removal has become an essential part of posterior segment surgery.

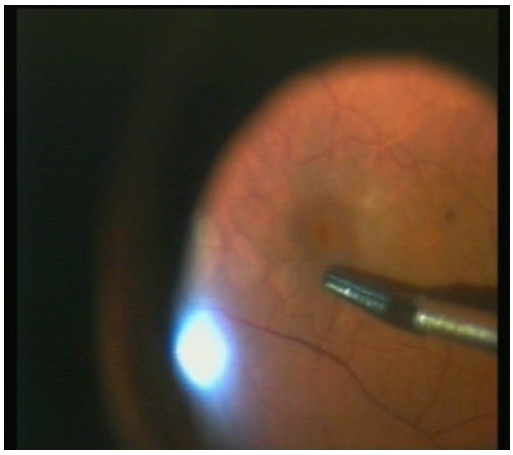


***Figure 4:*** intraoperative view on ILM-peeling in macular hole surgery without ILM-staining performed in the early nineties
(image by Prof. Dr. Kirchhof, University of Cologne)

Although the peeling of the nearly invisible ILM (*Figure 4*) is difficult to perform, its thorough and complete removal seems to be an important requirement for visual and anatomical success in macular hole surgery [14, 118]. Lately, several authors reported that vital stains, like indocyanine green or trypan blue, can be used to stain the ILM or ERMs selectively [34, 42, 85, 134, 135]. Staining the ILM or ERMs allows surgeons to work more quickly and precisely, thereby improving safety and anatomical outcomes in macular hole surgery.

## 1.3 Indocyanine green in ophthalmology

Indocyanine green (*Figure* 5) is an amphiphilic tricarbocyanine dye which was initially introduced in 1957. It soon became popular for measuring the cardiac output, liver function and for ophthalmic angiography [37, 93, 109, 166]. The main advantages were the confinement to the vascular compartment by binding to plasma proteins and rapid excretion into the bile. However, it has been shown that absorption properties of ICG show significant variations which depend on the solvent solution and on the concentration used [26, 62, 122]. ICG has little systemic toxicity [9, 46], although there is no information regarding overdosage in humans [75,170].

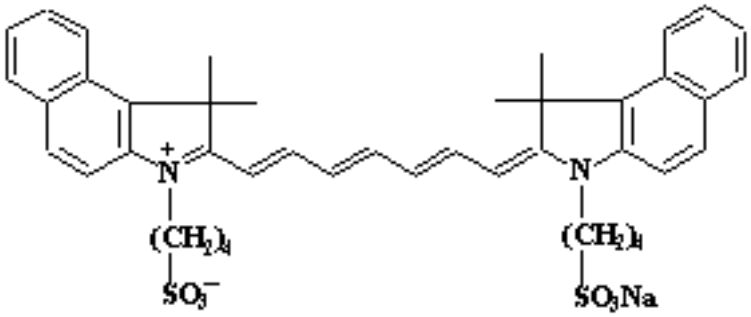



***Figure 5:*** Chemical formula of indocyanine green

ICG was initially introduced to ophthalmology in 1973 to study the choroidal circulation [37]. The tendency of the dye to bind to basement membranes was first recognised by cataract surgeons and used to improve visualisation of the anterior lens capsule for anterior capsulorrhexis in dense white cataract [76]. The main present use of ICG in posterior segment surgery is to facilitate the visualisation of the primary invisible internal limiting membrane (ILM) during macular hole surgery (steps of surgical procedure shown in *Figure 6*), allowing surgeons to peel more quickly and completely [85, 96].

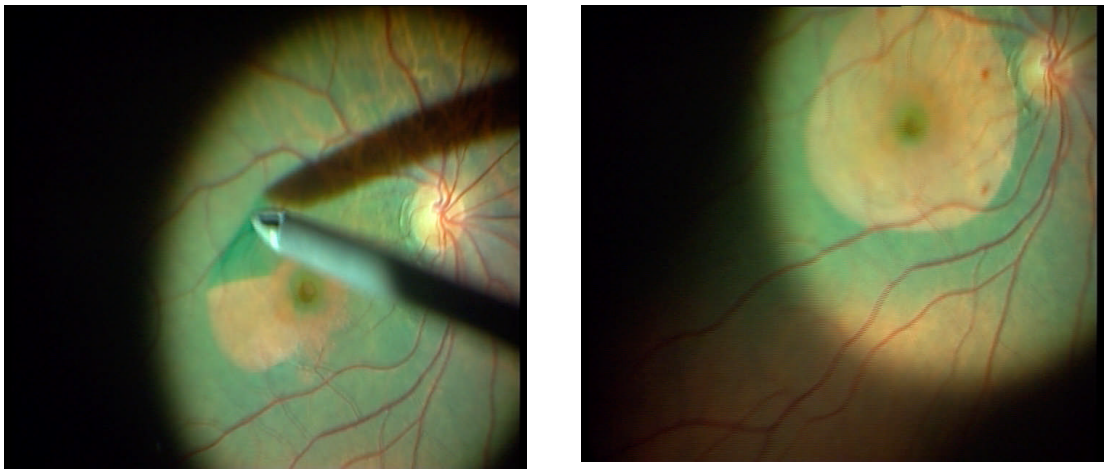


***Figure 6:*** intraoperative view on ICG-assisted ILM-peeling and peeled area in macular hole surgery (images by Prof. Dr. Grisanti, University of Tuebingen)

Since the first report by Grizzard and Tornambe and in numerous following publications, the application of ICG became an eminent tool in facilitating ILM-peeling [24, 85, 98–102] during macular hole surgery. The enthusiasm associated with this controlled and improved technique of ILM-peeling led to the widespread use of the dye. As a result of gaining experience, several studies using ICG have been conducted and results have been published during the past years. Although the majority of these reports exhibited positive functional and anatomical outcomes [24, 25, 63, 85, 95, 96, 98–102, 141, 152, 167], numerous authors added to the growing suspicion that intravitreal ICG may be toxic to retinal tissues [3, 19, 32, 43, 48, 59–61, 64, 71, 161].

So far there is no standardized protocol and the surgical techniques as well as volumes, doses and concentrations of ICG used were highly variable among the different series. In macular surgery, the concentration of ICG injected into the air- or fluid-filled vitreous ranged from 0.5 to 5.0 mg/ml, and the dye volumes used for ILM-staining ranged from 0.1 to 2.0 ml. However, the use of such wide ranges could not explain most of the negative outcomes, and results were highly contradictory as shown in two previous clinical studies [3, 24]. Ando et al. [3] used 0.1 to 0.2 ml of 5.0 mg/ml ICG (corresponding to doses of 0.5 to 1.0 mg) and reported unfavourable visual acuity after brief exposure (few seconds) to the dye. In contrast, Da Mata and associates [24] used 0.3 ml of 5.0 mg/ml ICG (corresponding to a dose of 1.5 mg) and observed no dye-related adverse effects even after an exposure time of 3 to 5 minutes. Several investigations similarly reported conflicting results on outcome with an intraocular persistence of ICG up to several months [5, 21, 71, 77, 124, 153, 164].

Another important criterium in ICG-assisted macular hole surgery is the use of vitrectomy endolights and the possible photosensitizing effects of ICG on retinal tissues. It is well known that the wavelenghts emitted by vitrectomy endolights range between 380 and 760 nm [62], and that the absorption maximum of ICG is approximately 700 nm [26, 62, 105, 122, 171]. The photosensitizing effects of ICG were described in several ophthalmologic [10, 12, 116, 127, 168, 169] and non-ophthalmologic studies [2, 35].

Regarding these conflicting reports and potential phototoxicity of ICG, various laboratory studies, including animal, *ex vivo* and *in vitro* experiments, were performed to verify outcomes [18, 31, 40, 44, 55, 72–74, 79, 81, 82, 88, 91, 107, 112, 113, 123, 137, 145, 150, 158]. According to these investigations, dye concentration, osmolarity of the solvent solution, incubation and illumination times, as well as the wavelengths of illumination were found to be responsible for dye related toxicity.

To summarise, there is still no standardised protocol for indocyanine green-assisted ILM- or ERM-staining during macular hole surgery, and the parameters responsible for ICG induced toxicity are still controversial. Thus, further investigations which consider most of the parameters that occur in clinical practice (appropriate dye concentrations and exposure times as well as vitrectomy endolight illumination) are required.

## 1.4 Trypan blue in ophthalmology

Indocyanine green was uncritically and widely used to stain the ILM and ERMs in macular hole surgery for a long time. However increasing reports on its possible ocular toxicity led to concerns against its intravitreal application. Consequently, these findings led to a demand for methods which prevent dye related toxicity, as well as the search for alternative dyes for intraoperative membrane staining.

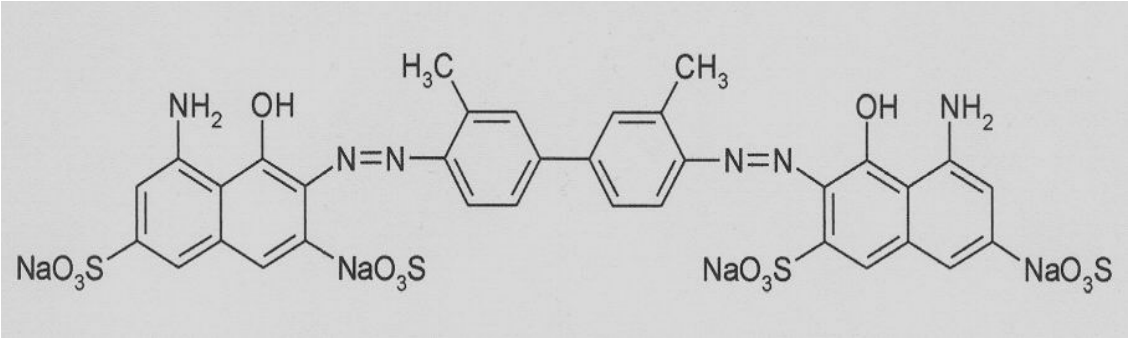



***Figure 7:*** Chemical formula of trypan blue

Trypan blue (TB) is a water-soluble acid dye (*Figure 7*) that has long been used in cell viability assays in the laboratory. It stains collagen and helps to distinguish living cells from dead cells, by being excluded from intact cellular membranes. More recently TB has been used as an intraoperative stain to facilitate anterior segment surgery [83, 117, 128]. Although no adverse effects of TB were noticed in clinics, the toxic potential of TB has already been described in several studies [20, 30]. In posterior segment surgery, TB directly stains ERMs and to a lesser degree the ILM, making it a useful tool for both macular hole and macular pucker surgery [110, 119, 139, 154]. In addition, it is well known that ICG selectively stains the ILM with a lower affinity to ERMs [38]. Hence, the staining attributes of TB resulted in it being an excellent complementary dye to ICG and IFCG, respectively, in already reported double vital staining during macular hole and macular pucker surgery [103, 152].

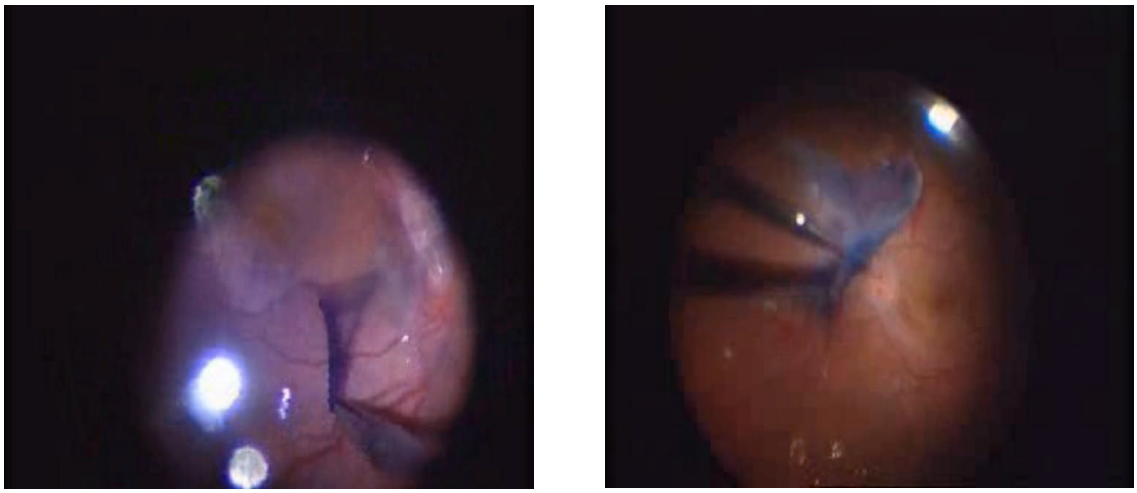


***Figure 8:*** intraoperative view on TB-assisted ILM-peeling (left picture) and ERM-removal (right picture) in macular hole surgery (figures by D.O.R.C. International, Zuidland, The Netherlands)

In current clinical studies, TB solutions of 0.6 and 1.5 mg/ml were used successfully to stain the ILM and ERMs (*Figure 8*) during macular hole surgery [7, 34, 65, 110, 134, 135]. However *in vitro* and animal studies showed contradictory results for different TB solutions. Short term exposure to TB with concentrations between 0.6 and 3.0 mg/ml did not have a toxic effect on cultured RPE cells under certain conditions [151]. Similar results were also shown in an experimental study with *post mortem* porcine eyes conducted

by our study group: TB of 1.5 mg/ml was demonstrated to cause no acute toxicity after an exposure time of 1 minute, as recommended by the manufacturer, followed by 10 minutes of illumination [56]. Unlike these findings, concentration-dependent toxicity was noticed in two past studies. One study used rabbit eyes, where TB at a concentration of 2.0 mg/ml was shown to be toxic compared to TB at 0.6 mg/ml after a period of 4 weeks [162], while the other study used cultured RPE cells, where TB at 4.0 mg/ml compared to TB at 0.6 and 0.06 mg/ml led to reduction of cell viability, as well as to upregulation of apoptosis-related and cell cycle arrest genes [104]. Several other clinical and in vitro studies were performed which compared outcomes after using ICG and TB [40, 82, 91, 108]. Gale et al. [40] indicated ICG to be more toxic than TB to the human RPE. The result was independent of any phototoxic potentiating effect of fiberoptic light or solvent toxicity. In addition, Jackson et al. [82] demonstrated no reduction in cell viability when RPE and glial cells were exposed from 1 to 30 minutes to TB at concentrations up to 2.0 mg/ml. However, the study showed damage to retinal glial cells when using ICG preparations at the same exposure times. Lee et al. [108] suggested TB to be less toxic than ICG when used in dye-assisted peeling of ILM during macular hole repair, as reflected by the better visual results. The *in vitro* study of Kodjikian [91] and associates compared the effects of ICG, IFCG and TB on cultured RPE cells. In contrast to previous findings, these authors noticed beside acute and chronic toxicity for ICG and IFCG, respectively, also a delayed toxicity for TB at all tested concentrations ranging from 0.5 to 5.0 mg/ml.

In conclusion TB at a concentration of 0.6 mg/ml appears to be safe to retinal tissues at exposure times of 1 minute or below, but according to the results of the last-mentioned studies, no clear statement on superiority of one of these dyes can be made yet.

## 1.5 Aim of the study

In recent, as well as in past clinical and experimental studies, several publications addressed both positive and negative effects of vital stains. It is indisputable that the initiation and use of ICG and TB, for ILM-peeling and ERM-removal in MHS, facilitated the work of numerous surgeons and led in most cases to more favourable functional and anatomical results. Contrary to these findings, the question of safety of vital stains to retinal tissues, especially to the RPE, has not been completely clarfied yet and this has led to growing controversial safety discussions in the past years [11, 87, 143].

The aim of our study was to undertake a safety investigation with ICG and TB on cultured ARPE-19 cells *in vitro*. We considered the parameters related to the clinical use of these dyes. Therefore, we tested the effects of different dye concentrations, incubation times, follow-up times, osmolarity and vitrectomy endolight illumination on the retinal pigment epithelium.

# 2. Materials and Methods

## 2.1 Cell culturing

For our *in vitro* experiments we used the human ARPE-19 cell line. It is a well-characterised line derived from primary human retinal pigment epithelium cell lines with normal karyology, which forms polarized epithelial monolayers. This cell line was established by selective trypsinisation of a primary retinal pigment epithelial culture, resulting in a uniform population of highly differentiated epithelial cells, which exhibit a strong growth potential [28]. The ARPE-19 cell line is therefore frequently and preferentially used for in vitro experiments.

Initially, we cultured ARPE-19 cells in cell culture flasks. For cell culture medium, we used fresh Dulbecco's modified Eagle medium (D-MEM) containing 15% foetal bovine serum (Gibco, Invitrogen Life Technologies GmbH, Karlsruhe, Germany), and the antibiotics, penicillin (10000 U/ml) and streptomycin (10000 µg/ml). ARPE-19 cells were grown in a humified atmosphere with 5.0% carbon dioxide at 37°C for approximately one week until reaching 80–100% confluence. During this time, culture medium was changed every second day. After cell cultures formed the typical monolayers, cells were trypsinised for a period of 5 minutes with a Trypsin/EDTA-solution containing 0.05% trypsin and 0.02% EDTA. Following trypsinisation, the cell suspension was removed and centrifuged at 1000 x g for 3 minutes. The supernatant was removed and the cells were resuspended in fresh culture medium. Afterwards, 300 µl of the cell suspension was applied to glass cover slips of 2.0 mm diameter placed in each well of a 12-well plate, leading to a density of approximately $1x10^5$ cells/well. These cells were grown until they formed monolayers of 80–100% confluence for our in vitro experiments. Confluent ARPE-19 cells growing in cell culture after one and after 4 days incubation are shown in figures 9 and 10. These previously described steps of cell culturing were conducted under sterile conditions to prevent possible damage and

infection to these sensitive cell cultures. Thus, culture medium changes, trypsinisation and application of the ARPE-19 cells to the wells were all performed under a sterile bench with laminar flow.

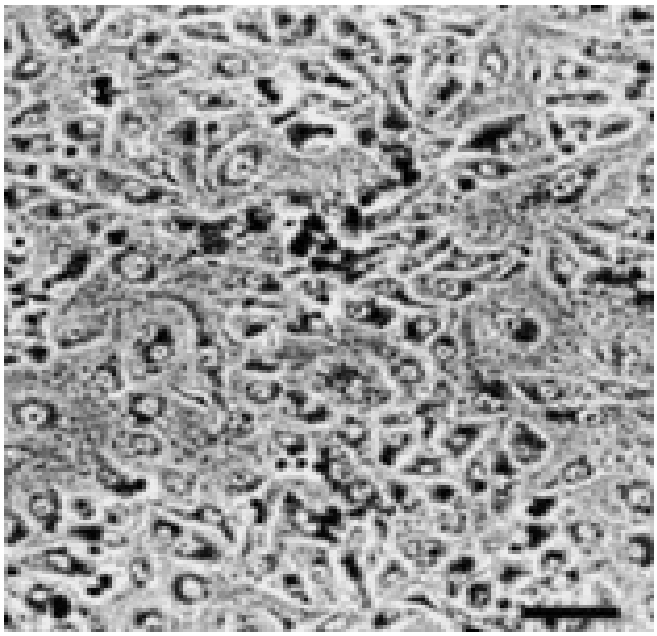


***Figure 9:*** ARPE-19 cell monolayer with 100% confluence but low cell density after 1 day of cell culturing (phase contrast microscopy image at 100x magnification)

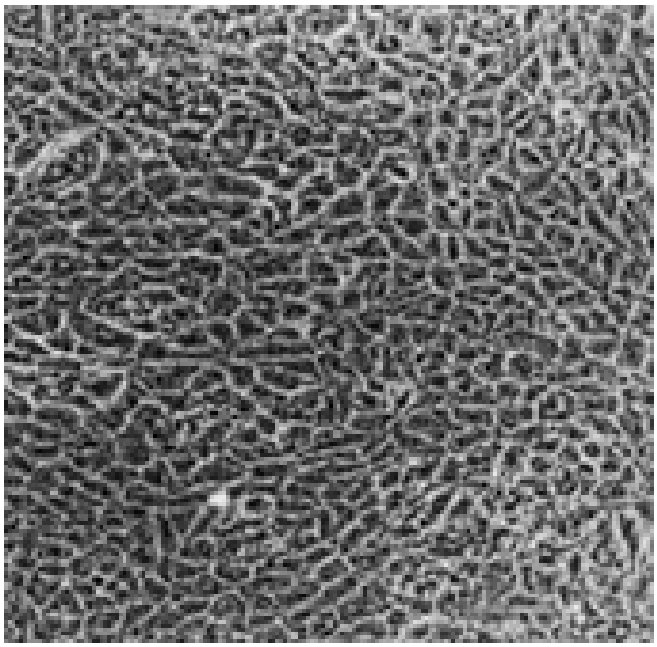


***Figure 10:*** ARPE-19 cell monolayer showing high cell density after 4 days of cell culturing (phase contrast microscopy image at 100x magnification)

## 2.2 Dye Preparation

We previously described the chemical characteristics of indocyanine green (ICG), as a consequence of its chemical nature this dye requires a special dilution scheme before its application. ICG is commercially available as a powder in combination with an ICG-Solvent solution, which is distilled water. We started with an indocyanine green stock solution at a concentration of 25 mg/ml. This solution was prepared by dissolving 25 mg of sterile ICG powder (Pulsion, Munich, Germany) in 1.0 ml sterile distilled water (ICG-Solvent, Pulsion, Munich, Germany). The solution was shaken for approximately 2 minutes to ensure that the ICG powder was completely dissolved and a homogeneous green solution was obtained. ICG solutions were aliquotted in Eppendorf tubes and covered with aluminium foil to prevent exposure to light. Keeping ICG solutions in the dark is important to avoid a degradation of dissolved ICG exposed to light [142].

In order to imitate the setting of an air- or gas-filled globe, the ICG stock solution was further diluted with appropriate amounts of balanced salt solution BSS (Pharmacia, Karlsruhe, Germany) to achieve concentrations of 5.0 mg/ml (0.50%), 2.5 mg/ml (0.25%) and 1.0 mg/ml (0.10%). To imitate the setting of a fluid-filled globe the aforementioned three ICG solutions of 5.0, 2.5 and 1.0 mg/ml were once again diluted with BSS to achieve final ICG concentrations of 0.125 mg/ml (0.0125%), 0.0625 mg/ml (0.0625%) and 0.025 mg/ml (0.0025%). Controls consisted of distilled water without ICG, which was diluted with BSS to achieve similar osmolarities to the tested ICG solutions (*Table 1).*

Commercially available trypan blue (TB) (Membrane Blue®, D.O.R.C. Int., Zuidland, The Netherlands) was supplied at a concentration of 1.5 mg/ml (0.15%). In order to mimic a fluid-filled globe, the TB solution was further diluted with BSS similar to the ICG solutions described above, to achieve a final trypan blue concentration of 0.0375 mg/ml (0.00375%).

***Table 1:*** Solutions of indocyanine green, trypan blue and BSS-Solvent control used in this study. Dilutions for experiments to mimic fluid-filled eyes were conducted based on the assumption that the vitreous cavity has a volume of approximately 4.0 ml.

| Solution | Dilution | Concentration |
|---|---|---|
| **ICG stock solution** | 25 mg ICG powder + 1.0 ml ICG-Solvent | **25.0 mg/ml** |
| **Solution # 1 (ICG)** | 1.0 ml stock solution + 4.0 ml BSS | **5.0 mg/ml** |
| **Solution # 1 (ICG-free Control)** | 1.0 ml ICG-Solvent + 4.0 ml BSS | **-** |
| **Solution # 2 (ICG)** | 1.0 ml stock solution + 9.0 ml BSS | **2.5 mg/ml** |
| **Solution # 2 (ICG-free Control)** | 1.0 ml ICG-Solvent + 9.0 ml BSS | **-** |
| **Solution # 3 (ICG)** | 1.0 ml stock solution + 24.0 ml BSS | **1.0 mg/ml** |
| **Solution # 3 (ICG-free Control)** | 1.0 ml ICG-Solvent + 24.0 ml BSS | **-** |
| **Solution # 4 (ICG)** | 0.1 ml solution # 1 (ICG) + 3.9 ml BSS | **0.125 mg/ml** |
| **Solution # 4 (ICG-free Control)** | 0.1 ml solution # 1 (ICG-free) + 3.9 ml BSS | **-** |
| **Solution # 5 (ICG)** | 0.1 ml solution # 2 (ICG) + 3.9 ml BSS | **0.0625 mg/ml** |
| **Solution # 5 (ICG-free Control)** | 0.1 ml solution # 2 (ICG-free) + 3.9 ml BSS | **-** |
| **Solution # 6 (ICG)** | 0.1 ml solution # 3 (ICG) + 3.9 ml BSS | **0.025 mg/ml** |
| **Solution # 6 (ICG-free Control)** | 0.1 ml solution # 3 (ICG-free) + 3.9 ml BSS | **-** |
| **Membrane blue®** | No dilution, commercially available! | **1.5 mg/ml** |
| **Membrane blue® (diluted)** | 0.1 ml Membrane blue + 3.9 ml BSS | **0.0375 mg/ml** |

## 2.3 Osmolarity and pH measurement

### 2.3.1 Osmolarity measurement

Osmolarity was measured with a freezing point osmometer (OSMOMAT 030, Gonotec, Berlin, Germany). According to this method, the total osmolarity of aqueous solutions is determined by comparative measurements of the freezing points of pure water and of the test solutions. Whereas water has a freezing point of 0°C, a solution with saline concentration of 1 osmol/kg has a freezing point of -1.858 °C (*Figure 11)*. The reproducibility of the osmometer is < ±0.5% or < ±2 digits for 50 µl and ±1.0% or <±4 digits for 30 µl. Measurements were performed with an amount of 50 µl.

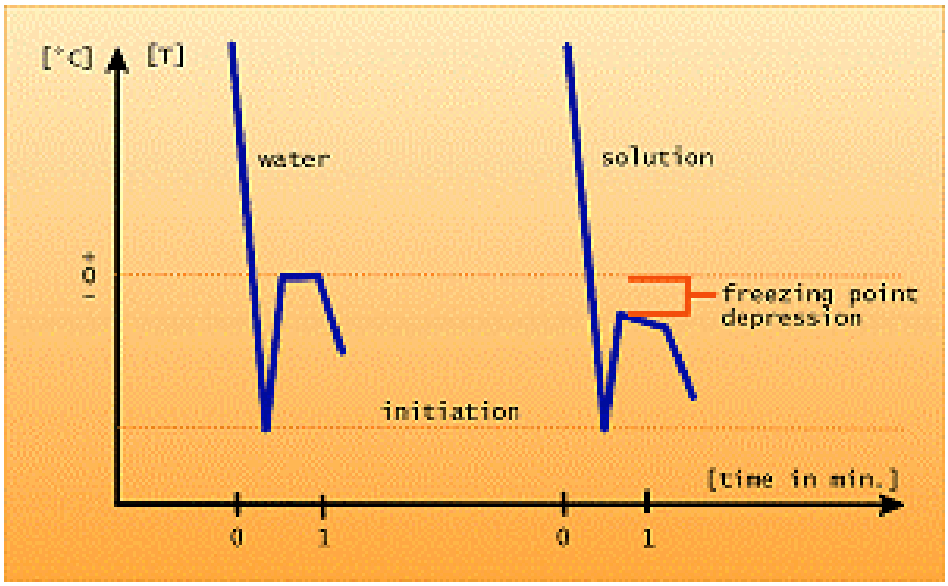



***Figure 11:*** Osmolarity determination by comparative measurement of the freezing points of pure water and experimental solutions (figure by Gonotec, www.gonotec.de)

### 2.3.2 pH measurement

pH-measurement (*Figure 12*) was performed using a microprocessor-based bench pH- and °C-meter (pH 211, Hanna Instruments, Kehl am Rhein, Germany). The accuracy of the pH-meter was ± 0.01 pH and ± 0.5°C at room temperature of 20°C, the average deviation amounted to ± 0.03 pH and ± 0.3°C. Before pH-measurement, we conducted a three point calibration using buffer solutions of pH = 4.01, 7.01 and 10.01. The pH-values in our experiments were measured at a solution temperature of 37°C.

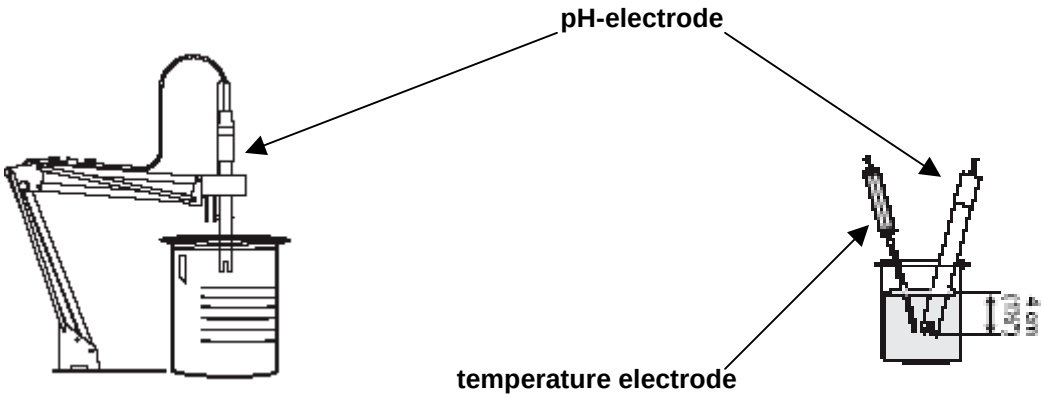



***Figure 12:*** pH- and C°-measurement (figures by Hannah instruments, www.hannainst.com)

## 2.4 Experimental set-up

### 2.4.1 Air- or gas-filled eyes

Intravitreal application of a vital dye can be performed either in an air- or a gas-filled eye, or in a fluid-filled globe. ICG was tested at a concentration of 5.0 (0.5%), 2.5 (0.25%) and 1.0 mg/ml (0.1%). In addition, to BSS the tested controls (ICG-free) were as described in Table 1. Within this setting, TB was tested at the commercially available concentration of 1.5 mg/ml (0.15%). A standard volume of 100 µl was applied to each well. Control experiments were conducted with 70%-ethanol as positive control and D-MEM culture medium as negative control (*Figures 13* and *14*). The loading consisted of triplets of the same solution, was repeated at least three times and was conducted with or without illumination under different conditions and in a clinical set-up.

**Plate 1 I, II and III:**

| | A | B | C | D |
|---|---|---|---|---|
| 1 | 70%-ethanol | D-MEM | Solution 1 (ICG) | Solution 1 (ICG free) |
| 2 | 70%-ethanol | D-MEM | Solution 1 (ICG) | Solution 1 (ICG free) |
| 3 | 70%-ethanol | D-MEM | Solution 1 (ICG) | Solution 1 (ICG free) |

**Plate 2 I, II and III:**

| | A | B | C | D |
|---|---|---|---|---|
| 1 | 70%-ethanol | D-MEM | Solution 2 (ICG) | Solution 2 (ICG free) |
| 2 | 70%-ethanol | D-MEM | Solution 2 (ICG) | Solution 2 (ICG free) |
| 3 | 70%-ethanol | D-MEM | Solution 2 (ICG) | Solution 2 (ICG free) |

**Plate 3 I, II and III:**

| | A | B | C | D |
|---|---|---|---|---|
| 1 | 70%-ethanol | D-MEM | Solution 3 (ICG) | Solution 3 (ICG free) |
| 2 | 70%-ethanol | D-MEM | Solution 3 (ICG) | Solution 3 (ICG free) |
| 3 | 70%-ethanol | D-MEM | Solution 3 (ICG) | Solution 3 (ICG free) |

**Plate 4 I, II and III:**

| | A | B | C | D |
|---|---|---|---|---|
| 1 | 70%-ethanol | D-MEM | Membrane blue® | BSS |
| 2 | 70%-ethanol | D-MEM | Membrane blue® | BSS |
| 3 | 70%-ethanol | D-MEM | Membrane blue® | BSS |

***Figure 13:*** Loading of 12-well plates used for experiments imitating air- or gas-filled eyes

### 2.4.2 Fluid-filled eyes

This set-up mimiced the situation in fluid-filled eyes. Thus we further diluted the solutions used in the setting for air/gas-filled eyes with BSS, taking into consideration that the vitreous cavity has an approximate volume of 4.0 ml [44]. ICG was tested at a concentration of 0.125 (0.0125%), 0.0625 (0.00625%), and 0.025 mg/ml (0.0025%). TB was diluted at a concentration of 0.0375 mg/ml (0.00375%). Similar to the previous set-up the same volume and controls were used.

**Plate 5 I, II and III:**

| | A | B | C | D |
|---|---|---|---|---|
| 1 | 70%-ethanol | D-MEM | Solution # 4 | Solution # 4 (ICG free) |
| 2 | 70%-ethanol | D-MEM | Solution # 4 | Solution # 4 (ICG free) |
| 3 | 70%-ethanol | D-MEM | Solution # 4 | Solution # 4 (ICG free) |

**Plate 6 I, II and III:**

| | A | B | C | D |
|---|---|---|---|---|
| 1 | 70%-ethanol | D-MEM | Solution # 5 | Solution # 5 (ICG free) |
| 2 | 70%-ethanol | D-MEM | Solution # 5 | Solution # 5 (ICG free) |
| 3 | 70%-ethanol | D-MEM | Solution # 5 | Solution # 5 (ICG free) |

**Plate 7 I, II and III:**

| | A | B | C | D |
|---|---|---|---|---|
| 1 | 70%-ethanol | D-MEM | Solution # 6 | Solution # 6 (ICG free) |
| 2 | 70%-ethanol | D-MEM | Solution # 6 | Solution # 6 (ICG free) |
| 3 | 70%-ethanol | D-MEM | Solution # 6 | Solution # 6 (ICG free) |

**Plate 8 I, II and III:**

| | A | B | C | D |
|---|---|---|---|---|
| 1 | 70%-ethanol | D-MEM | Diluted Membrane blue® | BSS |
| 2 | 70%-ethanol | D-MEM | Diluted Membrane blue® | BSS |
| 3 | 70%-ethanol | D-MEM | Diluted Membrane blue® | BSS |

***Legend :***

**I = set-up without illumination (1, 5, 10 and 20 minutes of dye exposure, no illumination)**
**II = set-up with illumination (1 and 5 minutes of dye exposure under illumination)**
**III = clinical set-up (1 minute dye exposition, 5 minutes total illumination)**

***Figure 14:*** Loading for 12-well plates used for experiments imitating fluid-filled eyes

### 2.4.3 The experimental set-up without illumination

The experimental set-up without illumination was performed to examine the effects of the two dyes, ICG and TB, and their solvent solutions on cell morphology and survival of cultured ARPE-19 cells. The amount of solution applied to each well was 100 µl as previously described in several clinical publications. The incubation times ranged from 1 to 20 minutes, and no endolight illumination was used. Afterwards the experimental solutions were removed by aspiration, and cells were washed twice with 300 µl of fresh sterile balanced salt solution. Finally, the ARPE-19 cells were again incubated with culture medium. Our control experiments were accomplished by using ethanol-70% as positive control, culture medium as negative control and BSS-solvent mixes of corresponding osmolarities as dye-free controls. The follow-up times for live-dead test evaluations were 6, 24 and 72 hours.

The main intention of this set-up was to examine the acute and postponed effect of the dyes without the influence of illumination after short and prolonged exposure times. We determined at which incubation time almost 0% cell survival and 100% morphologic change was achieved in previous pilot studies. For these experiments, we used 5.0 mg/ml ICG and 1.5 mg/ml TB. These experiments disclosed that for both dyes, an incubation time of 20 minutes or longer is required to reach the volitional effects. Consequently, our incubation times were set to 1, 5, 10 and 20 minutes. Furthermore, the effect of osmolarity was examined. Therefore, the effect on cell survival and morphologic change was compared using the solutions 1 to 6 (ICG) and their dye free control solutions 1 to 6 (ICG-free) with nearly similar osmolarities.

### 2.4.4 The experimental set-up with illumination

In this experimental set-up a standard amount of 100 µl of freshly prepared experimental solutions was applied to each well. Incubation and illumination time were the same, 1 and 5 minutes. Afterwards solutions were removed and cell cultures were washed twice with 300 µl fresh sterile BSS

solution. Finally the ARPE-19 cells were again incubated with culture medium. Evaluation was performed by fluorescent live-dead staining after 6, 24 and 72 hours. Illumination experiments were performed by using a standard light pipe (Spectra-Band Fiberoptic Endo-Illuminator, Altomed Ltd., Tyne and Wear, UK) powered by a fiberoptic light source (50 W, Pentalux, Ruck, Eschweiler, Germany). The light pipe was set to maximum illumination power and the distance between the endolight and the cell culture measured 8 mm, in accordance with previous studies [44, 55]. The illuminated area included almost the whole glass cover slip where ARPE-19 cells were grown on (*Figure 15*), thus most of the cells were exposed to illumination.

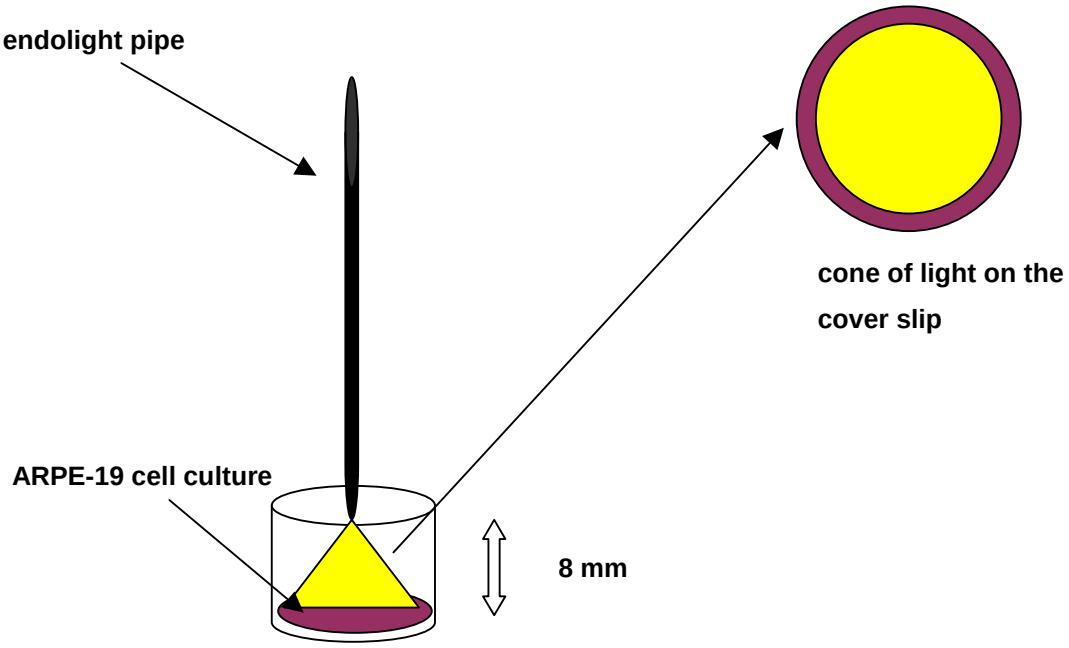



***Figure 15:*** Experimental set-up for our experiments with illumination.

### 2.4.5 The clinical experimental set-up

In this set-up we investigated the effects of both dyes, indocyanine green and trypan blue, on the retinal pigment epithelium under conditions mimicking clinical practise. Similar to the previous two set-ups, 100 µl of dye solution were used per well. The incubation time was set to 1 minute, which is recommended for TB but above the exposure time used for ICG. The total illumination time

was set to 5 minutes. For illumination, we used the aforementioned light pipe and light source at a distance of 8 mm. The experimental solutions were incubated for 1 minute under illumination, afterwards solutions were removed by aspiration and cells were washed twice with 300 µl fresh sterile BSS. The BSS solution was left in place for 4 minutes under illumination, resulting in a total illumination time of 5 minutes. Finally endolight illumination was turned off, BSS was removed and the ARPE-19 cells were again incubated with fresh culture medium. Evaluation was performed by fluorescent live-dead staining after 6, 24 and 72 hours.

This duration of dye incubation and total illumination was chosen to mimic the actual clinical situation during surgery. Previously, investigators conducting ILM-peeling with ICG or TB, reported dye exposure times ranging from few seconds [3, 4, 85] to 5 minutes [19, 24] under illumination. At present, however, it is generally accepted and sufficient to use ICG for less then one minute. In our clinical practise of macular hole surgery, ICG is usually applied for approximately 15 seconds. TB was used as recommended by the manufacturer for 1 minute. After removal of the dyes, the vitreous is usually irrigated with BSS. This surgical procedure of ILM-peeling is performed under vitrectomy endolight illumination, with halogen or xenon light sources at a distance of 5 to 8 mm, and usually takes around 3 minutes [66]. Thereafter, a fluid-air exchange is performed if required.

### 2.4.6 Acute and chronic toxicity testing

Several publications report about the safety and the harmfulness of ICG and TB at different incubation and follow-up times. In clinical studies, incubation times nowadays are usually below 1 minute. In contrast, experimental studies use longer incubation times (up to 1 month) [88, 162]. Longer incubation times may be tested for safety regarding persistence of the dye. ICG related fluorescence has been demonstrated to persist for weeks [164] and even for months [5, 21]. The amount of dye that may persist, however, is far below the concentrations applied. This situation is reflected also in the experimental set

up. After incubation with the dye, this is removed and the cultures are flushed with fresh medium. Though most of the dye is removed, minimal amounts will persist in the culture well. In order to detect the effects that may be caused by these remnants as well as postponed chronic effects of the initial solutions, the study included testing not only after 6 hours for acute toxicity testing but also after 24 and 72 hours. The period of 6 hours for acute toxicity testing, was chosen because of 0% cell survival after 1 minute incubation with ethanol-70%. In addition, first noticeable damage to RPE cells, incubated with ICG or TB, was similarly shown after this follow-up time. The longest follow-up time of 72 hours was chosen, because of our experience which compared pre experimental results with the highest dye concentrations, ICG 5.0 and TB 1.5 mg/ml. The outcomes of these cell survival experiments after 24 and 72 hours showed no prominent or clinically relevant difference. Thus, in our opinion this maximum follow-up time of 72 hours for our in vitro study was adequate.

## 2.5 Quantification of cell viability and morphologic change

### **2.5.1** DAPI / PI–staining

Cell viability/damage was evaluated with the live-dead staining technique involving the dye DAPI (4',6-diamidine-2'-phenylindole-dihydrochloride) and PI (propium iodide). DAPI binds to nuclear DNA, PI stains nuclear DNA and cytoplasmic RNA, but both dyes do not penetrate intact cellular membranes. By applying PI before permeabilizing cells, the dye will only stain damaged cells with defect membranes. By applying DAPI after permeabilization all nuclei (of dead and living cells) are stained. Live-dead staining was performed according to our staining protocol shown in *Table 2*.

DAPI is a fluorescent probe that selectively binds to DNA. It forms blue and highly fluorescent DNA-DAPI-complexes (*Figure 16*). DAPI is rapidly assimilated by cells and the highest fluorescence is mainly visible in the nucleus. The chemical formula is $C_{16}H_{15}N_5$ x 2HCl, its absorption maximum at λ = 340 nm and its emission maximum at λ = 488 nm in aqueous solutions. DAPI

remains stable for approximately 6 months stored at a temperature from 2 to 8°C in the dark.

In our experiments we used fresh sterile DAPI at a concentration of 6 µg/ml and incubated the cells with 300 µl DAPI/cover slip for 20 minutes in the dark in a humified atmosphere with 5% carbondioxide at 37°C.

The red propidium iodide (PI) dye binds to DNA by intercalating between the bases with little or no sequence preference, forming red PI-DNA complexes (*Figure 17*). Furthermore, PI also binds to cytoplasmatic RNA. The absorption maximum is at λ = 535 nm and its emission maximum is at λ = 617 nm in aqueous solutions. PI remains stable for at least 6 months protected from room light at a temperature from 2 to 6 °C.

In our live-dead assay we diluted the PI stock solution of 1 mg/ml with D-PBS at a ratio of 1:1500 and incubated the cells with 300 µl DAPI/cover slip for 15 minutes in the dark and a humified atmosphere with 5% carbondioxide at 37°C.

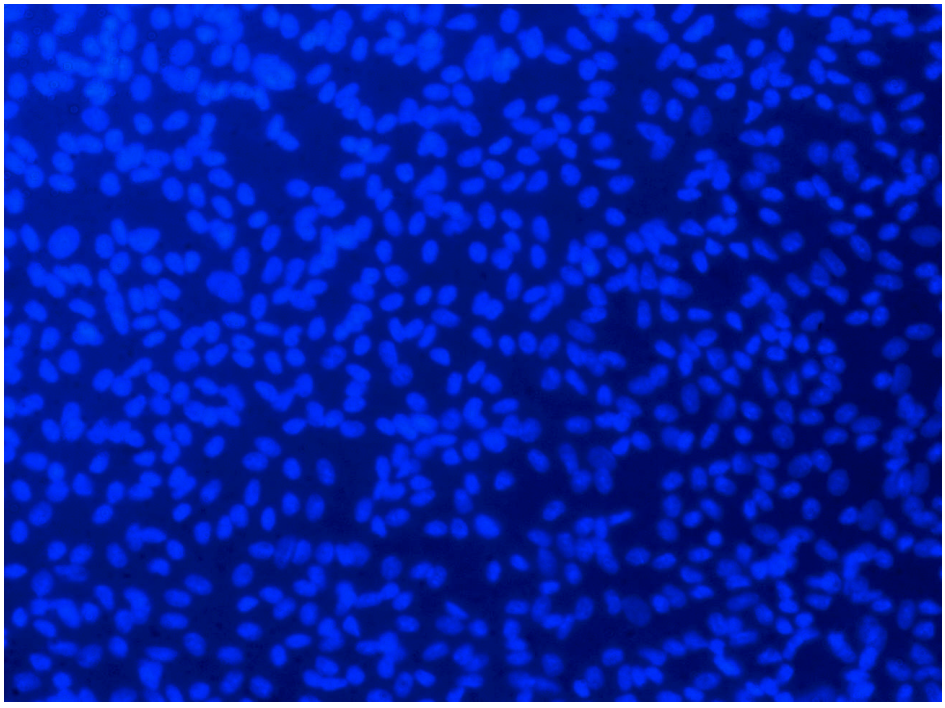


***Figure 16:*** Vital DAPI-stained ARPE-19 cells.
(Image by flourescence microscopy at 200x magnification)

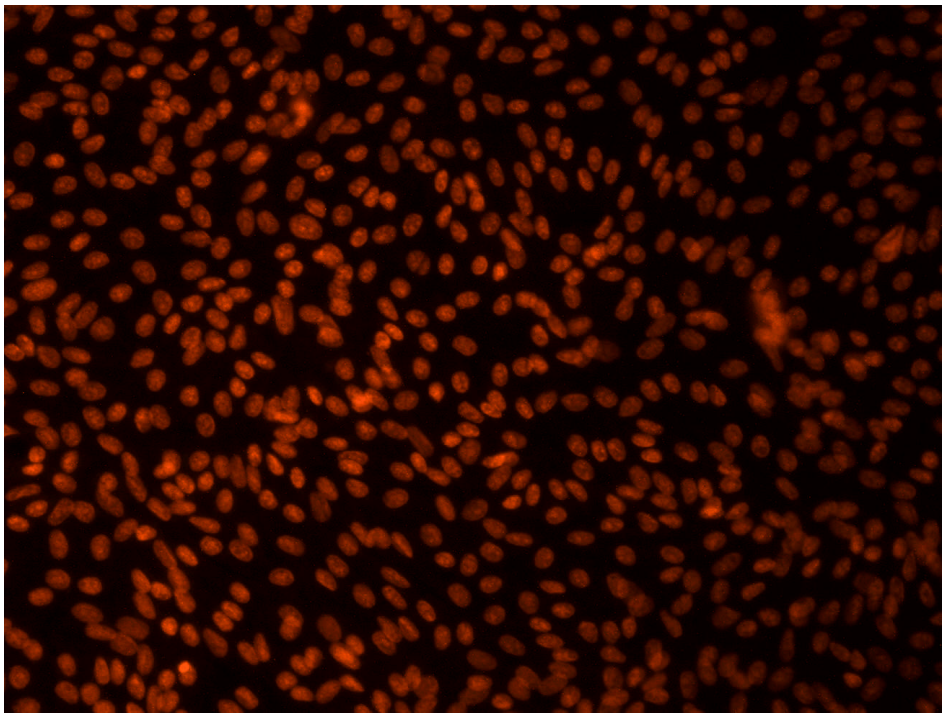


***Figure 17:*** Damaged PI-stained human ARPE-19 cells
(Image by flourescence microscopy at 200x magnification)

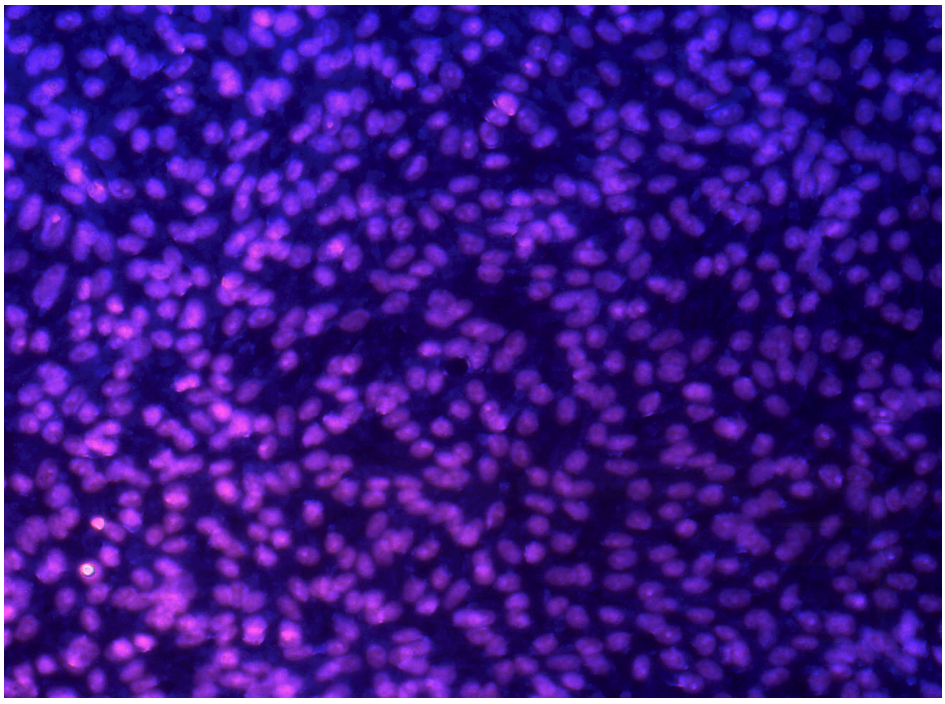


***Figure 18:*** Dead DAPI/PI-stained human ARPE-19 cells
(Image by flourescence microscopy at 200x magnification)

***Table 2:*** Protocol of live-dead staining with 4',6-diamidine-2'-phenylindole-dihydrochloride (DAPI) and propidium iodide (PI)

| Step | Action |
|---|---|
| **1** | Culturing cells to 80-100% confluence. |
| **2** | Conduction of cell experiments and staining after 6, 24 or 72 hours. |
| **3** | Remove the culture medium and wash the cells 3x with D-PBS. |
| **4** | **Apply 300 µl freshly made propidium iodide and incubate the specimen for 15 minutes in the dark at 37°C.** |
| **5** | Remove propidium iodide and wash the cells 3x with D-PBS. |
| **6** | **Apply 300 µl 4% PFA in PBS for fixation and incubate the specimen for 30 minutes in the dark at 37°C.** |
| **7** | Remove 4% PFA in PBS and wash the cells 3x with D-PBS. |
| **8** | **Apply 300 µl 0,1% Triton X-100 in PBS for permeabilisation and incubate the specimen for 10 minutes in the dark at 37°C.** |
| **9** | Remove 0,1% Triton X-100 in PBS and wash the cells 3x with D-PBS. |
| **10** | **Apply 300 µl DAPI and incubate the specimen for 20 minutes in the dark at 37°C.** |
| **11** | Remove DAPI and wash the cells 3x with D-PBS. |
| **12** | **Remove cover slips and view samples using a flourescence microscope with appropriate filters.** |

### 2.5.2 Fluorescence microscopy and cell counting

Flourescence microscopy (Axioplan 2 imaging, Zeiss, Jena, Germany) was performed to evaluate the cytotoxicity experiments. Images were made with at 200x magnification with appropriate filters, i.e., the DAPI-filter and the Rhodamine-filter. DAPI stains the nucleus of every cell on the cover slip, while PI selectively stains cells with damaged membranes. Therefore, only DAPI-stained cells are viable and double stained (DAPI/PI, *Figure 18*) cells are damaged. For cell counting, 8 images/cover slip were made, in total 24 images for every experimental solution (*Figure 21*). Cells were counted manually within the microscope images produced by the software Axiovision imaging (Zeiss, Jena, Germany) and digitally by a image analysis program (Analysis®, Soft Imaging System GmbH, Münster, Germany). Part of the evaluation also included assessment of the cell morphology (Figures 19–20) and the nucleus-cytoplasm relation. These cells were only counted manually, because the

counting this program is based on the identification of different colours only and does not take morphology into account.

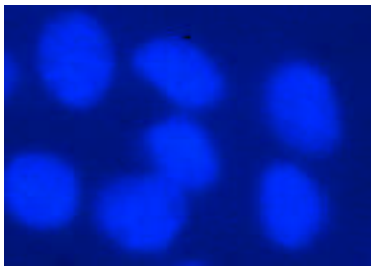


***Figure 19:*** Living ARPE-19 cell of normal morphology and nucleus-cytoplasm-relation (DAPI/PI-stained image at 400x magnification)

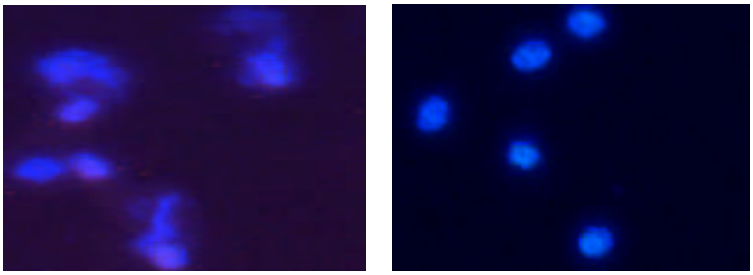


***Figure 20:*** Dead ARPE-19 cell of changed morphology and nucleus-cytoplasm-relation (DAPI/PI-stained images at 400x magnification)

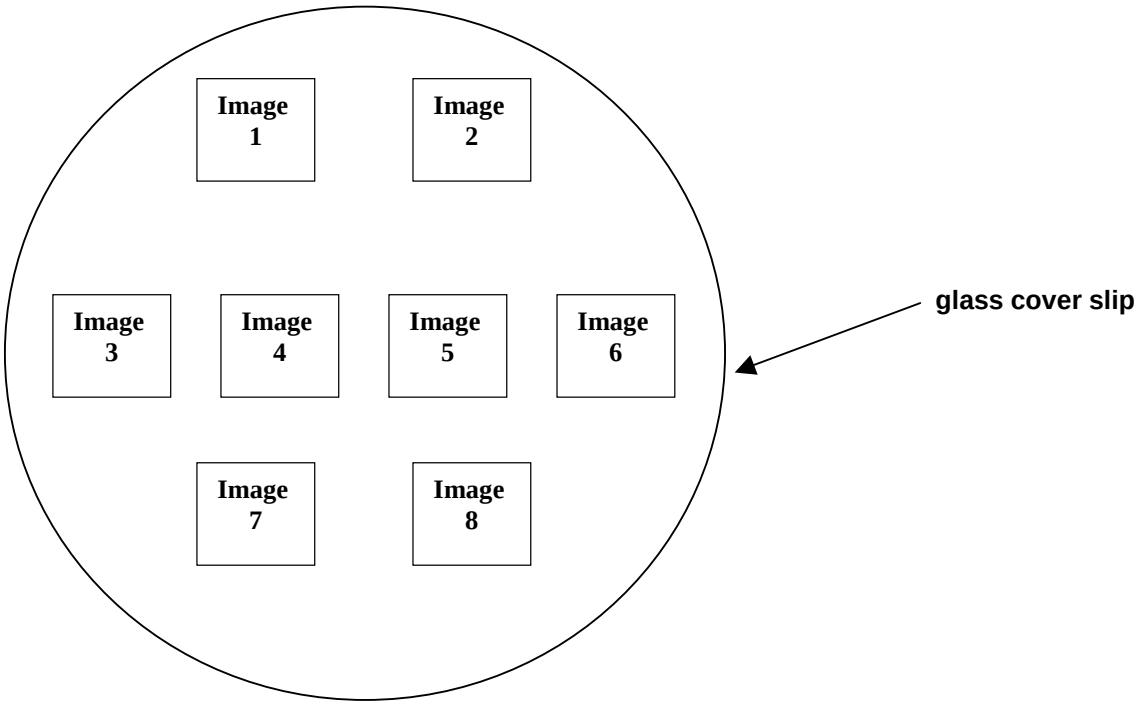
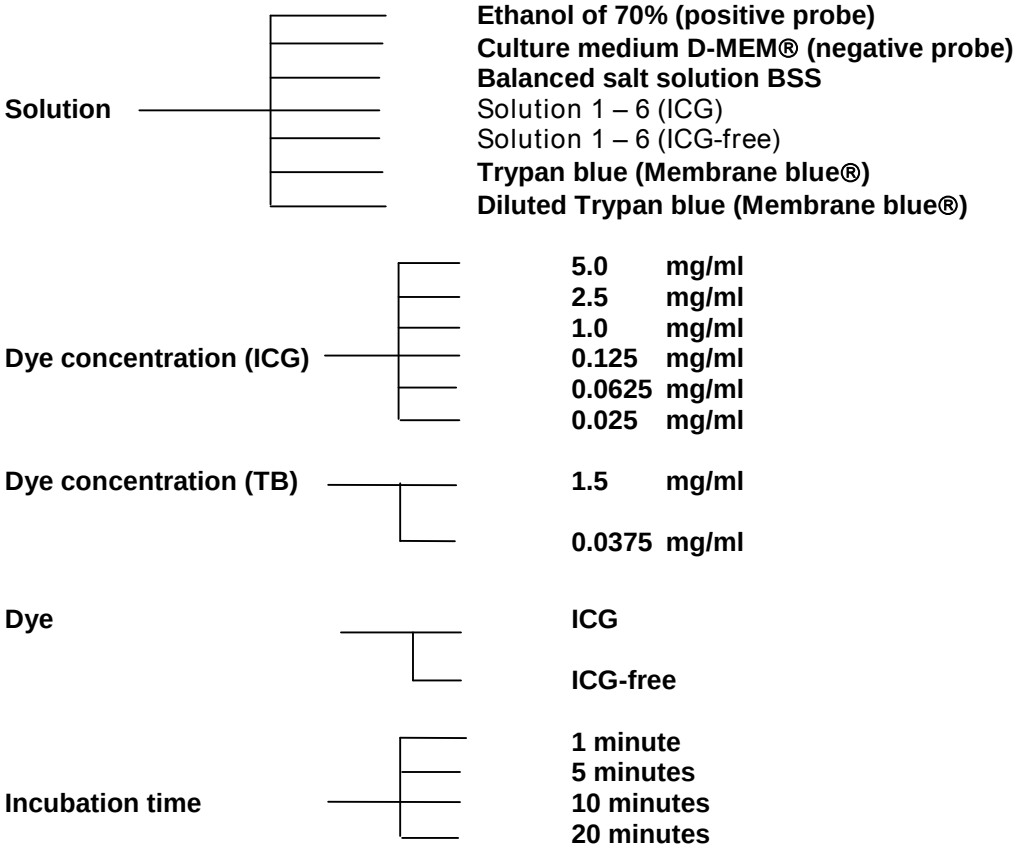



***Figure 21:*** Image making principle with 8 images / cover slip (4 images from the central region and 4 images from the peripheral region were counted)

## 2.6 Statistical method

Statistics were performed with a software program (JMP IN 5.1, SAS Inst. Inc., 2003, Cary, NC, USA). The variables were grouped in factors and observations. The factors were solution, dye, dye concentration, incubation time, illumination time and the follow-up time (*Figure 22*). Observations were the proportions of cell survival (living/total) and the proportions of morphologic change (morphologically changed/total). These proportions were transformed to arcsine of squareroot for analysis of variance to get homogenous variances. Goodness of fit was described by coefficient of determination $R^2$ and residual standard deviation *s*. Influences of the factors as well as their interactions on cell survival and morphology were tested. Expected values and 95% confidence limits were transformed back to the original scale to estimate medians.



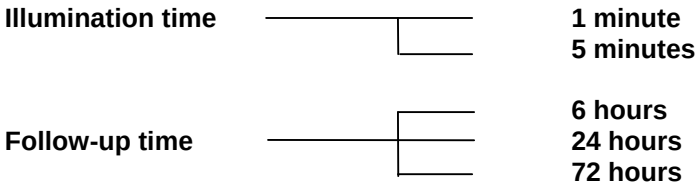



***Figure 22:*** Factors and their levels used for statistical analysis

### 2.6.1 Statistical analysis of the experimental set-up without illumination

In the set-up without illumination we conducted four different analyses of variance for four clinically relevant questions. For every question we had the follow-up times of 6, 24 and 72 hours.

The first question was: Is there a dose and incubation time dependent influence of indocyanine green on survival and morphology of cultured RPE cells? To answer this we compared the solutions 1 to 4 (ICG) with decreasing concentrations of 5.0 mg/ml to 0.125 mg/ml at different incubation times ranging from 1 to 20 minutes.

The second question was: Is there a dose and incubation time dependent influence of trypan blue on survival and morphology of cultured RPE cells? To determine this we compared trypan blue and diluted trypan blue with at concentrations of 1.5 mg/ml and 0.0375 mg/ml at different incubation times ranging from 1 to 20 minutes.

The third question was: If we notice toxicity, is this only related to ICG or is this also related to the hypotonic solvent solutions? Therefore, we compared the solutions 1 to 3 (ICG) with their dye-free control solutions 1 to 3 (ICG-free) at different incubation times ranging from 1 to 20 minutes.

The fourth question was: Is there a dye dependent difference between indocyanine green and trypan blue on survival and morphology of cultured ARPE-19 cells? Therefore, we compared the dyes with concentrations used for air/gas-filled eyes, the solutions 1 to 3 (ICG) at 5.0 down to 1.0 mg/ml and trypan blue at 1.5 mg/ml, as well as the dyes with concentrations used for fluid-filled eyes, the solution 4 (ICG) at 0.125 mg/ml and diluted trypan blue at 0.0375 mg/ml, at different incubation times ranging from 1 to 20 minutes.

### 2.6.2 Statistical analysis of the experimental set-up with illumination

In our set-up with illumination we conducted five different analyses of variance for five clinically relevant questions. For every question we similarly to above had the follow-up times of 6, 24 and 72 hours.

The questions one, two and four were the same as in the set-up without illumination, but here we had shorter incubation & illumination times of only 1 and 5 minutes. The new factor incubation & illumination time, was introduced only for this set-up, because the ARPE-19 cells were simultaneously incubated with the solutions and illuminated by the vitrectomy endolight. Due to this additional factor, we had to change the third question: If we notice possible phototoxicity, is this only related to light absorption by ICG or is this also related to light absorption by the solvent solutions? To answer this, we compared the solutions 1 to 3 (ICG) with the ICG-free control solutions 1 to 3 (ICG-free) at the different incubation and illumination times of 1 and 5 minutes.

A recent publication reported about the absorption of short wavelengths by ICG and the solvent solution BSS [86]. Therefore, we performed statistical analysis for a fifth question: is there a difference in survival and morphology of cultured ARPE-19 cells between an ICG solution of low concentration and balanced salt solution BSS under illumination? Therefore, we compared solution 4 (ICG) and BSS at the different incubation and illumination times of 1 and 5 minutes.

### 2.6.3 Statistical analysis of the clinical set-up

Finally in the third set-up, the clinical set-up, we tried to mimic the clinical situation, where dyes are used to stain the internal limiting membrane (ILM) or epiretinal membranes (ERM) in macular hole surgery. Although there is no standardised protocol of ILM-staining in clinical macular hole surgery, we standardised some factors in our study. The dye incubation time was 1 minute and the total illumination time was 5 minutes. Afterwards we conducted analyses of variance for three clinically relevant questions. The previously used follow-up times of 6, 24 and 72 hours were also adhered to in this set-up.

The first question was whether there is a dose dependent toxicity of ICG under a standardised incubation and illumination time. To determine this, we compared the solutions 1 to 4 (ICG).

The second question was whether there is a dose dependent toxicity of TB under a standardised incubation and illumination time. To answer this, we compared the trypan blue solutions of 1.5 and 0.0375 mg/ml.

The third question was which dye is safer for macular hole surgery under a standardised incubation and illumination time. Therefore, we compared the dyes with concentrations used for air/gas-filled eyes, the solutions 1 to 3 (ICG) of 5.0 down to 1.0 mg/ml and trypan blue of 1.5 mg/ml, as well as the dyes with concentrations used for fluid-filled eyes, the solution 4 (ICG) of 0.125 mg/ml and diluted trypan blue of 0.0375 mg/ml.

### 2.6.4 Exclusion from statistical analysis

We excluded some of our experimental solutions from statistical analysis. The main reason for the exclusion was the lack of statistical spread. The 70%-ethanol and the culture medium D-MEM solutions served as positive and negative controls for live-dead staining. As we expected, cell survival was approximately 0% for 70%-ethanol and approximately 100% for D-MEM in every set-up. Consequently, we excluded these solutions from statistical analysis, because of the lack of clinical relevance and the lack of statistical spread of the results of cell survival and morphologic change.

The solutions 5 and 6 (ICG) of 0.0625 and 0.025 mg/ml, respectively, as well as the solutions 5 and 6 (ICG-free) showed almost 100% cell survival and less than 1% morphologic change in every experimental set-up conducted. Thus, these solutions were also excluded because of the lack of statistical spread of the results.

# 3. Results

## 3.1 Results of osmolarity and pH measurement

The results of the osmolarity measurements showed possible toxic effects related to the hypoosmolarity of some indocyanine green (ICG) solutions used in our experimental set-ups. The ICG-solvent and the ICG stock solution of 25.0 mg/ml were highly hypotonic, but there is no clinical usage for these solutions. The osmolarities of the solutions 1 to 3 (ICG), used to mimic the situation in gas-filled eyes, with concentrations of 5.0, 2.5 and 1.0 mg/ml as well as the osmolarities of the solutions 1 to 3 (ICG-free), used as controls, were similarly below physiological limits. The solutions 4 to 6 (ICG) used to mimic the situation in fluid-filled eyes with concentrations of 0.125, 0.0625 and 0.025 mg/ml, the control solutions 4 to 6 (ICG-free), BSS as well as both trypan blue solutions were in the physiological limit of 295 – 315 mOsm/kg (data shown in *Table 3*).

***Table 3:*** Results of the osmolarity measurement. Hypotonic solutions are marked in **red**

| Solution | Dye concentration (mg/ml) | Osmolarity (mOsm/kg) |
|---|---|---|
| Ethanol of 70% (positive control) | - | Not measurable! |
| D-MEM (negative control) | - | 316 ± 0.3 |
| BSS | - | 302 ± 0.5 |
| D-PBS | - | 287 ± 0.3 |
| ICG-Solvent | - | 0 |
| ICG stock solution | 25.0 | 4 ± 0.4 |
| Solution # 1 (ICG) | 5.0 | 242 ± 0.2 |
| Solution # 1 (ICG free) | - | 246 ± 0.3 |
| Solution # 2 (ICG) | 2.5 | 277 ± 0.2 |
| Solution # 2 (ICG free) | - | 282 ± 0.5 |
| Solution # 3 (ICG) | 1.0 | 290 ± 0.6 |
| Solution # 3 (ICG free) | - | 293 ± 0.2 |
| Solution # 4 (ICG) | 0.125 | 297 ± 0.4 |
| Solution # 4 (ICG free) | - | 301 ± 0.5 |
| Solution # 5 (ICG) | 0.0625 | 303 ± 0.2 |
| Solution # 5 (ICG free) | - | 304 ± 0.3 |
| Solution # 6 (ICG) | 0.025 | 307 ± 0.6 |
| Solution # 6 (ICG free) | - | 305 ± 0.2 |
| Membrane blue® | 1.5 | 297 ± 0.3 |
| Diluted Membrane blue® | 0.0375 | 304 ± 0.5 |

The results of the pH measurement of our experimental solutions showed that pH-values were mainly in the physiological limit of 7.35 – 7.45. Only the ICG-solvent solution and the ICG stock solution with a concentration of 25.0 mg/ml had a far higher pH (data shown in *Table 4)*. Whether the higher pH-value of 7.53 of solution 1 (ICG) plays an important role for ICG dye related toxicity remains controversial, because no cellular damage related to higher or lower pH-values of ICG- or TB-solutions has been reported so far.

***Table 4:*** Results of the pH measurement at 37°C. Non-physiologic pH-values are marked in **red**

| Solution | Dye concentration (mg/ml) | pH-value |
|---|---|---|
| Ethanol of 70% (pos. control) | - | 7.6 ± 0.02 |
| D-MEM (neg. control) | - | 7.3 ± 0.06 |
| BSS | - | 7.3 ± 0.03 |
| D-PBS | - | 7.4 ± 0.03 |
| ICG-Solvent | - | 7.6 ± 0.01 |
| ICG stock solution | 25.0 | 7.7 ± 0.05 |
| Solution # 1 (ICG) | 5.0 | 7.5 ± 0.03 |
| Solution # 1 (ICG free) | - | 7.5 ± 0.02 |
| Solution # 2 (ICG) | 2.5 | 7.5 ± 0.01 |
| Solution # 2 (ICG free) | - | 7.4 ± 0.04 |
| Solution # 3 (ICG) | 1.0 | 7.5 ± 0.03 |
| Solution # 3 (ICG free) | - | 7.4 ± 0.04 |
| Solution # 4 (ICG) | 0.125 | 7.4 ± 0.01 |
| Solution # 4 (ICG free) | - | 7.4 ± 0.01 |
| Solution # 5 (ICG) | 0.0625 | 7.4 ± 0.03 |
| Solution # 5 (ICG free) | - | 7.4 ± 0.02 |
| Solution # 6 (ICG) | 0.025 | 7.4 ± 0.02 |
| Solution # 6 (ICG free) | - | 7.4 ± 0.01 |
| Membrane blue® | 1.5 | 7.4 ± 0.04 |
| Diluted Membrane blue® | 0.0375 | 7.4 ± 0.03 |

## 3.2 Results of the set-up without illumination

### 3.2.1 Results of statistical analysis of cell survival

For the first question we compared outcomes of cell survival, when cells were incubated for 1, 5, 10 and 20 minutes with the Solutions 1 to 4 (ICG) of 5.0, 2.5, 1.0 and 0.125 mg/ml. Thus we performed an analysis of variance with the factors solution (levels: Solution 1 to 4 (ICG)), incubation time (levels: 1, 5, 10 and 20 min.), follow-up time (levels: 6, 24 and 72 h) and their interactions.

We had $n$ = 288 observations. $R^2$ = 99.73% of variance was explained by the model, leaving a residual standard deviation of $s$ = 0.0243. We found a significant decrease in cell survival depending on the solution ($p$ < 0.0001), the incubation time ($p$ < 0.0001) and the follow-up time ($p$ < 0.0001). The interactions between the factors: solution and incubation time ($p$ < 0.0001), solution and follow-up time ($p$ < 0.0001), incubation time and follow-up time ($p$ = 0.0116) as well as the interaction between the three factors ($p$ < 0.0001, shown in Figures 23–25) were also significant (data shown under *6.5.1 Figures and tables, Table 6*). Most remarkable decreases in cell survival were noticed for the Solutions 1 and 2 (ICG). Median cell survival after the follow-up time of 6 hours decreased from 100% for both after 1 to 0% and 88.79% (CI: 87.52 to 89.99) after 20 minutes incubation time, respectively. After 72 hours, we noticed a decrease in median cell survival from 98.47% (CI: 97.95 to 98.91%) and 99.22% (CI: 98.84 to 99.53%) after 1 to 0% and 83.11% (CI: 81.62 to 84.55%) after 20 minutes incubation time, respectively (photographs shown under *6.2 Figures and tables, Figures 68 to 74*). Little changes were noticed for the solutions 3 and 4 (ICG) at all follow-up times (data shown under *6.5.1 Figures and tables, Table 7*).

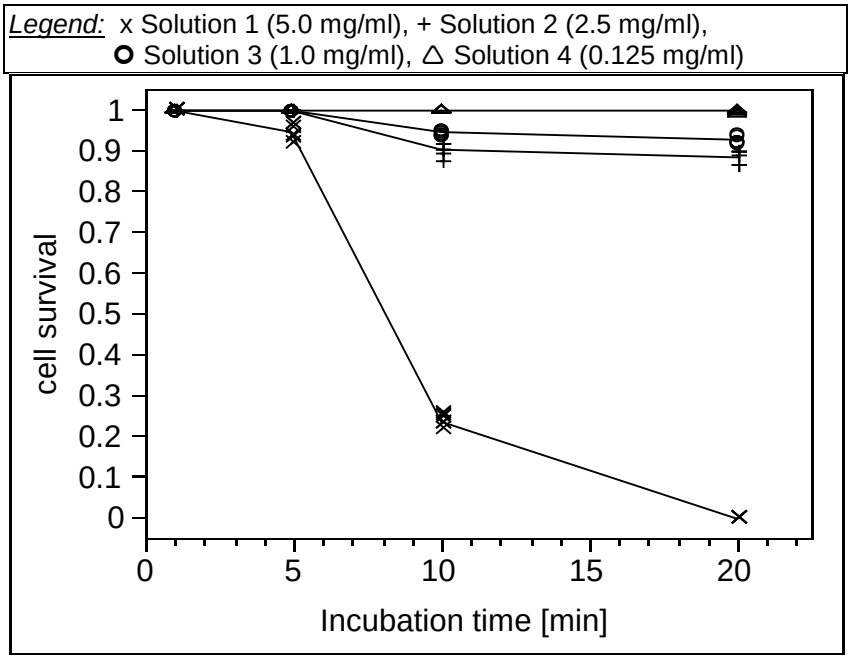



***Figure 23:*** Cell survival over incubation time when comparing the solutions 1 to 4 (ICG) after the follow-up time of **6 hours**, medians are connected.

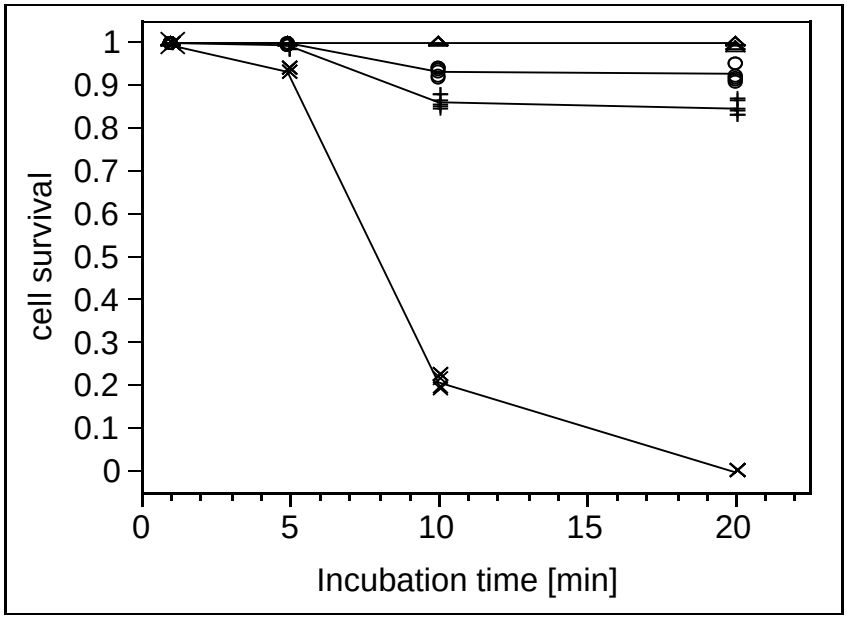



***Figure 24:*** Cell survival over incubation time when comparing the solutions 1 to 4 (ICG) after the follow-up time of **24 hours**, medians are connected.

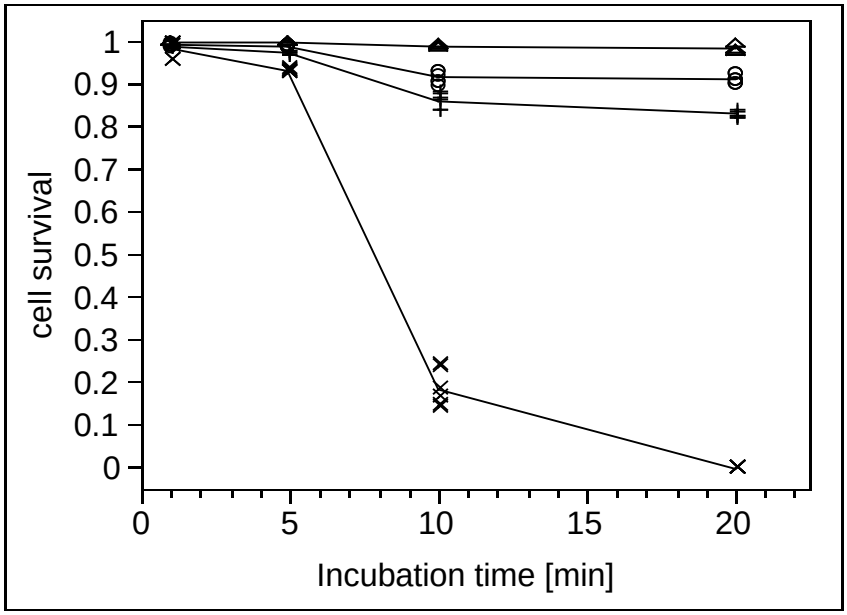



***Figure 25:*** Cell survival over incubation time when comparing the solutions 1 to 4 (ICG) after the follow-up time of **72 hours**, medians are connected.

For the second question we compared outcomes of cell survival, when cells were incubated for 1 to 20 minutes with trypan blue and diluted trypan blue of 1.5 and 0.0375 mg/ml respectively. For this, we performed an analysis of variance with the factors solution (levels: trypan blue, diluted trypan blue), incubation time (levels: 1, 5, 10 and 20 min.), follow-up time (levels: 6, 24

and 72 h) and their interactions. The number of observations was $n$ = 144. $R^2$ = 99.88% of variance was explained by the model, leaving a residual standard deviation of $s$ = 0.0201. We found a significant decrease in cell survival depending on the solution ($p < 0.0001$), the incubation time ($p < 0.0001$) and the follow-up time ($p < 0.0001$). The interactions between the factors: solution and incubation time ($p < 0.0001$), incubation time and follow-up time ($p = 0.0004$) as well as the interaction between the three factors ($p < 0.0001$, shown in *Figures* 26–28) were also significant (data shown under *6.5.2 Figures and tables, Table 9*). Regarding the interaction between the factors solution and follow-up time, we noticed no statistical significance ($p = 0.0511$).

Prominent decrease in cell survival was only noticed for TB 1.5 mg/ml. After the follow-up time of 6 hours, median cell survival decreased from 100% after 1 to 61.65% (CI: 60.06 to 63.22%) after 10 and even to 0% after 20 minutes incubation time. Almost similar results were noticed after the follow-up time of 72 hours, where median cell survival decreased from 99.71% (CI: 99.50 to 99.86) after 1 to 56.27% (CI: 54.65 to 57.88%) after 10 and 0% after 20 minutes incubation time (data shown under *6.5.2 Figures and tables, Table 10*, photographs shown under *6.2 Figures and tables, Figures 82 to 85*).

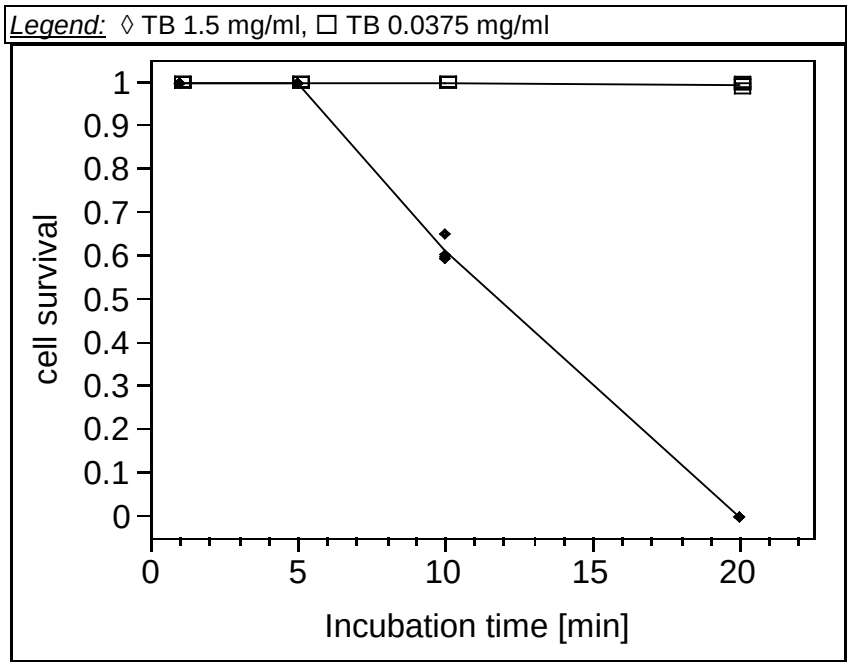



***Figure 26:*** Cell survival over incubation time when comparing TB with diluted TB after the follow-up time of **6 hours**, medians are connected.

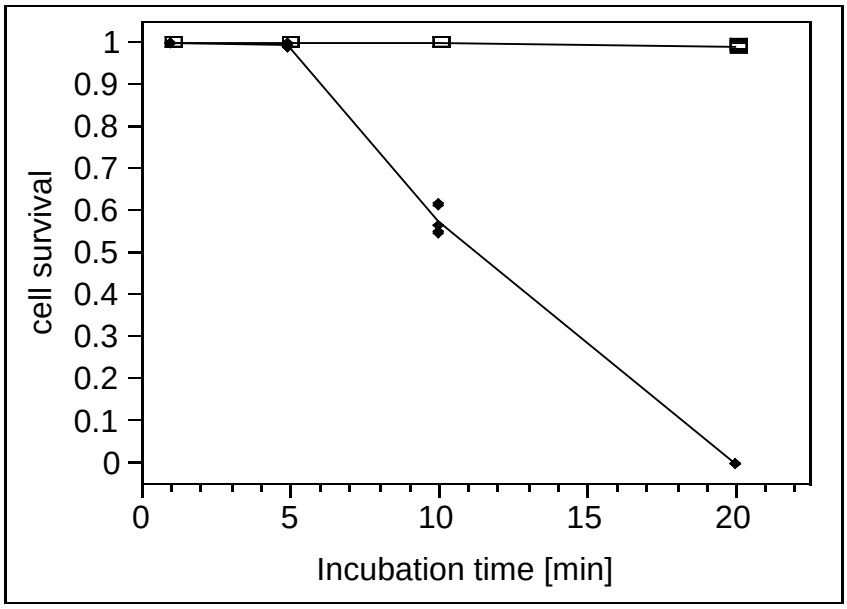



***Figure 27:*** Cell survival over incubation time when comparing TB with diluted TB after the follow-up time of **24 hours**, medians are connected.

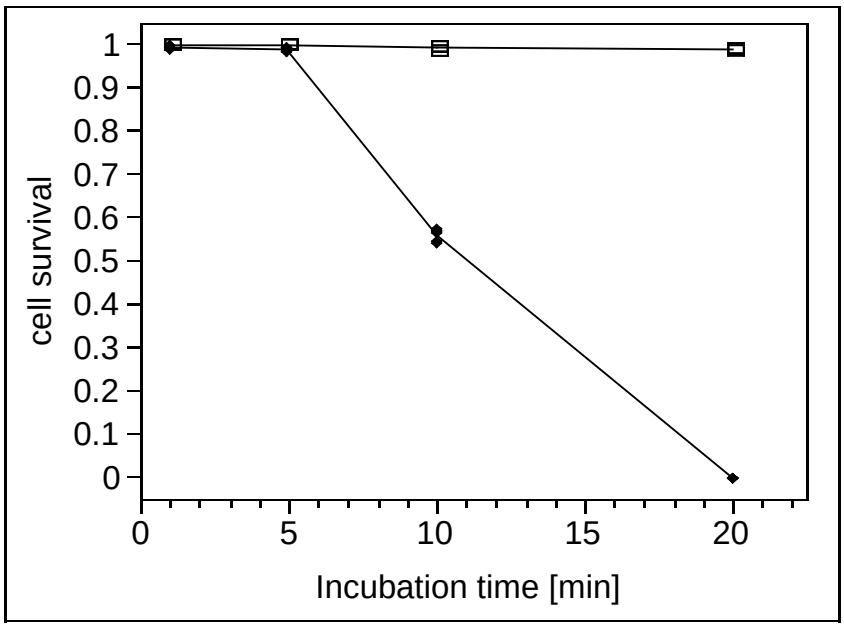



***Figure 28:*** Cell survival over incubation time when comparing TB with diluted TB after the follow-up time of **72 hours**, medians are connected.

The third question was mainly performed to verify the effects of osmolarity on the survival of RPE cells. Thus, we compared outcomes of cell survival, when cells were incubated for 1 to 20 minutes with the solutions 1 to 3 ICG) and the solutions 1 to 3 (ICG-free). Therefore we conducted an analysis of variance with the factors solution number (levels: Solution 1-3), dye (levels: ICG

and ICG-free), incubation time (levels: 1, 5, 10 and 20 min.), follow-up time (levels: 6, 24 and 72 h) and their interactions. The new factor dye was added to investigate the sole effect of ICG. We had $n$ = 432 observations. $R^2$ = 99.76% of variance was explained by the model, leaving residual a standard deviation of $s$ = 0.0243. We found significant decrease in cell survival depending on the solution number ($p$ = 0.0000), the dye ($p$ = 0.0057), the incubation time ($p$ = 0.0000) and follow-up time ($p$ < 0.0001). The two-fold interactions between the factors: solution number and dye ($p$ < 0.0001), solution number and incubation time ($p$ = 0.0000), solution number and follow-up time ($p$ < 0.0001) and incubation time and follow-up time ($p$ < 0.0001) were significant, too. In contrast no significant differences were noticed for the two-fold interactions dye and incubation time ($p$ = 0.7660), and dye and follow-up time ($p$ = 0.3244). The three-fold interactions between the factors: solution number, dye and follow-up time ($p$ = 0.0416); solution number, incubation time and follow-up time ($p$ < 0.0001); dye, incubation time and follow-up time ($p$ < 0.0001), as well as the fourfold interaction between all factors ($p$ = 0.0007, shown in Figures 23–25 and 29–31) were also significant (data shown under *6.5.3 Figures and tables, Table 12*). No significance was found for the threefold interaction between the factors solution number, dye and incubation time ($p$ = 0.0927).

The four-fold interaction showed significance, although there were no prominent differences in cell survival between the solutions regarding the aspect whether they contained ICG or not. Comparing the solutions 1 (ICG) and 1 (ICG-free) after the follow-up time of 6 hours, we noticed a decrease in median cell survival from 100% and 99.89% (CI: 99.72 to 99.98%) after 1 to 0% and 1.00% (CI: 0.65 to 1.43%) after 20 minutes, respectively. The differences between these two solutions became even smaller after our maximum follow-up time of 72 hours, where median cell survival decreased from 98.47% (CI: 97.95 to 98.91%) and 99.12% (CI: 98.72 to 99.45%) after 1 to 0% for both solutions after 20 minutes, respectively. For the solutions 3 (ICG) and 3 (ICG-free) after the follow-up time of 6 hours, we noticed a decrease in median cell survival from 100% for both solutions after 1 to 92.91% (CI: 91.88 to 93.88%) and 94.41% CI: 93.48 to 95.27%) after 20 minutes, respectively. The differences between these

two solutions after the follow-up time of 72 hours were also minimal (data shown under *6.5.3 Figures and tables, Table 13*, photographs shown under *6.2 Figures and tables, Figures 68 to 73 and 75 to 80*).

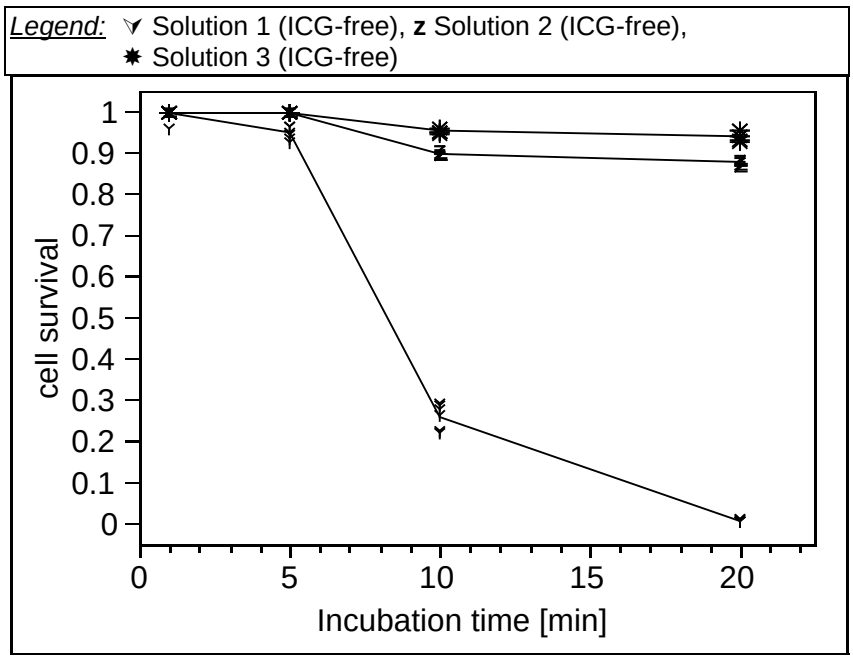



***Figure 29:*** Cell survival over incubation time when comparing the solutions 1 to 3 (ICG-free) after the follow-up time of **6 hours**, medians are connected.

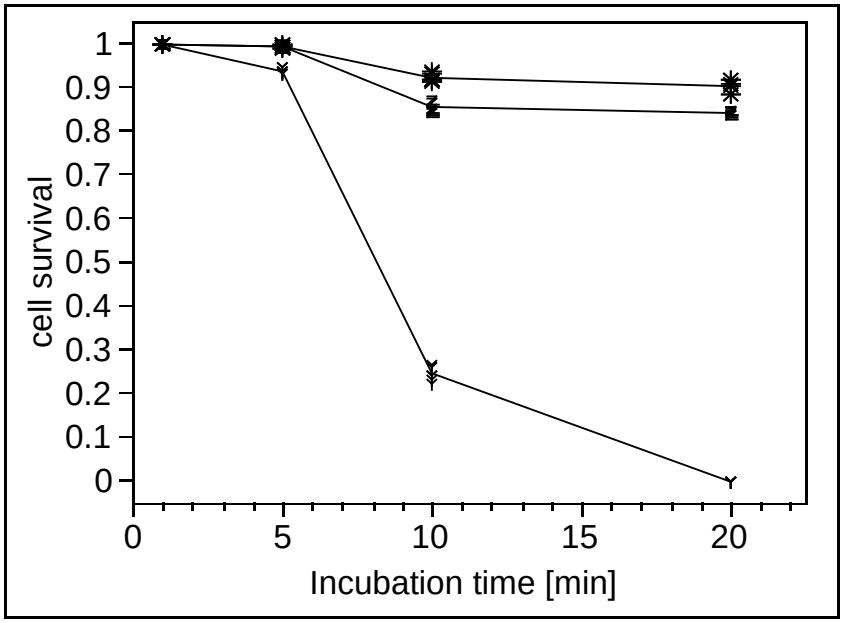



***Figure 30:*** Cell survival over incubation time when comparing the solutions 1 to 3 (ICG-free) after the follow-up time of **24 hours**, medians are connected.

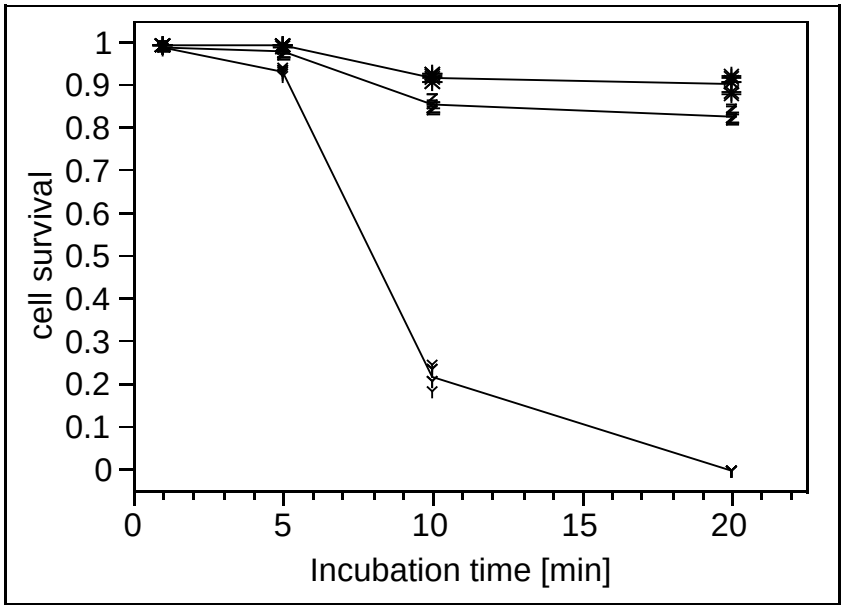



***Figure 31:*** Cell survival over incubation time when comparing the solutions 1 to 3 (ICG-free) after the follow-up time of **72 hours**, medians are connected.

For the first part of the fourth question we compared outcomes of cell survival, when cells were incubated for 1 to 20 minutes with the solutions 1 to 3 (ICG) and trypan blue 1.5 mg/ml. Therefore, we performed an analysis of variance with the factors solution (levels: solution 1 to 3 (ICG) and trypan blue), incubation time (levels: 1, 5, 10 and 20 min.), follow-up time (levels: 6, 24 and 72 h) and their interactions. We had $n$ = 288 observations. $R^2$ = 99.81% of variance was explained by the model, leaving a residual standard deviation of $s$ = 0.0244. We found a significant decrease in cell survival depending on the solution ($p < 0.0001$), the incubation time ($p < 0.0001$) and the follow-up time ($p < 0.0001$). Interactions between the factors: solution and incubation time ($p < 0.0001$), solution and follow-up time ($p < 0.0001$) and incubation time and follow-up time ($p < 0.0001$) as well as the interaction between all the factors ($p < 0.0001$, shown in Figures 23–25 and 26–28) were also significant (data shown under *6.5.4.1 Figures and tables, Table 15* ). A remarkable decrease in cell survival was seen for solution 1 (ICG) of 5.0 mg/ml and TB 1.5 mg/ml. Especially after the incubation time of 10 minutes, severe cell damage was noticed for both dyes (data shown under *6.5.4.1 Figures and tables, table 16*,

photographs shown under *6.2 Figures and tables, figures 68 to 73* and *82 to 84*).

For the second part of the fourth question we compared outcomes of cell survival, when cells were incubated for 1 to 20 minutes with solution 4 (ICG) of 0.125 mg/ml and trypan blue 0.0375 mg/ml. Therefore, we performed a second analysis of variance with the factors solution (levels: Solution 4 (ICG) and diluted trypan blue), incubation time (levels: 1, 5, 10 and 20 min.), follow-up time (levels: 6, 24 and 72 h) and their interactions. We had $n$ = 144 observations. $R^2$ = 82.93% of variance was explained by the model, leaving a residual standard deviation of $s$ = 0.0200. We found a significant decrease in cell survival depending on the incubation time ($p$ < 0.0001) and the follow-up time ($p$ < 0.0001). Interactions between the factors: solution and follow-up time ($p$ = 0.0293) and incubation time and follow-up time ($p$ < 0.0001) as well as the interaction between all the factors ($p$ = 0.0461, shown in Figures 23–25 and 26–*28*) were also significant (data shown under *6.5.4.2 Figures and tables, Table 18*). No significance was shown for the factor solution ($p$ = 0.4934) and the interaction between the factors solution and incubation time ($p$ = 0.1591). Thus, minimal changes were noticed for both dyes over the whole range of incubation and follow-up times (data shown under *6.5.4.2 Figures and tables, Table 19*, photographs shown under *6.2 Figures and tables, Figures 74 and 85*).

### 3.2.2 Results of statistical analysis of morphologic change

To answer the first question, whether there is also a change in morphology of cultured ARPE-19 cells, we compared the outcomes of morphologic change when RPE cells were incubated for 1 to 20 minutes with the solutions 1 to 4 (ICG). Thus, we performed an analysis of variance with the same factors and their interactions as already used for the outcomes of cell survival. We had $n$ = 144 observations. $R^2$ = 99.81% of variance was explained by the model, leaving a residual standard deviation of $s$ = 0.0220. We found a significant increase morphologic change depending on the solution ($p$ < 0.0001), incubation time ($p$ < 0.0001) and follow-up time ($p$ < 0.0001). The

interactions between the factors: solution and incubation time ($p < 0.0001$), solution and follow-up time ($p = 0.0056$), incubation time and follow-up time ($p < 0.0001$) as well as the three-fold interaction ($p < 0.0001$, shown in Figures 32–*34*) were also significant (data shown under *6.5.5 Figures and tables, Table 21*). Similar to the results of cell survival, most remarkable changes were noticed for the solutions 1 and 2 (ICG). Regarding these solutions after the first follow-up time of 6 hours, median morphologic change increased from 2.27 % (CI: 1.58 to 3.09%) and 0.67% (CI: 0.32 to 1.15%) after 1 to 100% and 11.83% (CI: 10.25 to 13.51%) after 20 minutes incubation time, respectively. After the maximum follow-up time of 72 hours median morphologic change increased from 2.75% (CI: 1.98 to 3.64%) and 2.48% (CI: 1.76 to 3.33%) after 1 to 100% and 17.27% (CI: 15.40 to 19.22%) after 20 minutes incubation time, respectively. Negligable to little changes were shown for the other solutions 3 and 4 (ICG) after the whole range of incubation and follow-up times (data shown under *6.5.5 Figures and tables, Table 22*, photographs shown under *6.2 Figures and tables, Figures 68 to 74*).

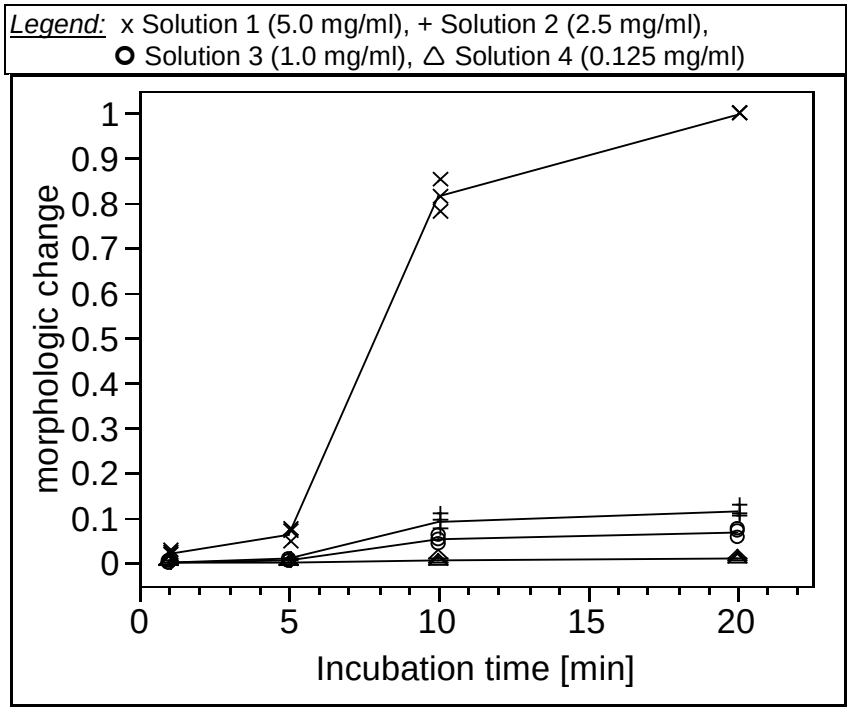



***Figure 32:*** Morphologic change over incubation time when comparing the solutions 1 to 4 (ICG) after the follow-up time of **6 hours**, medians are connected.

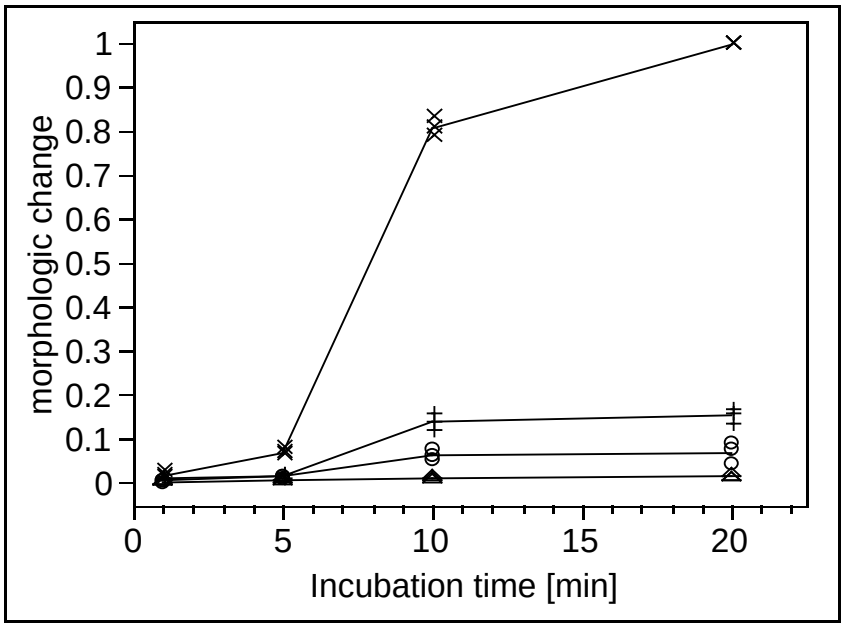



***Figure 33:*** Morphologic change over incubation time when comparing the Solutions 1 to 4 (ICG) after the follow-up time of **24 hours**, medians are connected.

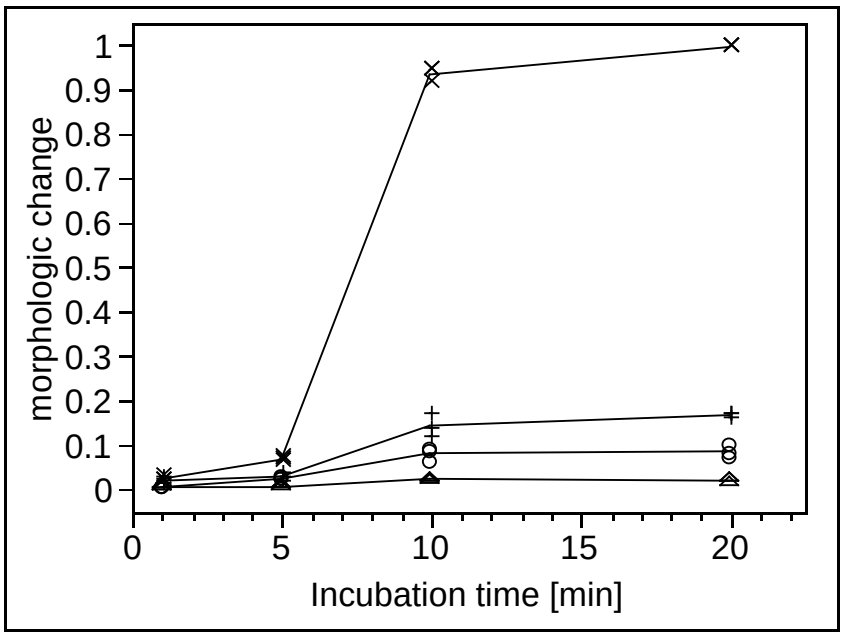



***Figure 34:*** Morphologic change over incubation time when comparing the solutions 1 to 4 (ICG) after the follow-up time of **72 hours**, medians are connected.

For the second question we compared the outcomes of morphological change, when cells were incubated for 1 to 20 minutes with trypan blue and diluted trypan blue at 1.5 and 0.0375 mg/ml. Therefore, we performed an analysis of variance with the factors solution (levels: trypan blue, diluted trypan

blue), incubation time (levels: 1, 5, 10 and 20 min.), follow-up time (levels: 6, 24 and 72 h) and their interactions. We had $n$ = 72 observations. $R^2$ = 99.92% of variance was explained by the model, leaving a residual standard deviation of $s$ = 0.0173. The statistical analysis showed a significant increase in morphologic change depending on the solution ($p < 0.0001$), incubation time ($p < 0.0001$) and follow-up time ($p < 0.0001$). The interactions between the factors: solution and incubation time ($p < 0.0001$), incubation time and follow-up time ($p = 0.0137$) as well as the interaction between all factors ($p = 0.0035$, shown in Figures 35–37) also showed significance (data shown under *6.5.6 Figures and tables, Table 24*). In contrast, no significance was noticed for the interaction solution and follow-up time ($p = 0.6304$).

According to the results of the statistical analysis of cell survival, a prominent increase in morphologic change was only noticed for the undiluted trypan blue solution of 1.5 mg/ml after incubation times of 10 minutes or longer at every follow-up time (data shown under *6.5.6 Figures and tables, Table 25*, photographs shown under *6.2 Figures and tables, Figures 82 to 85*).

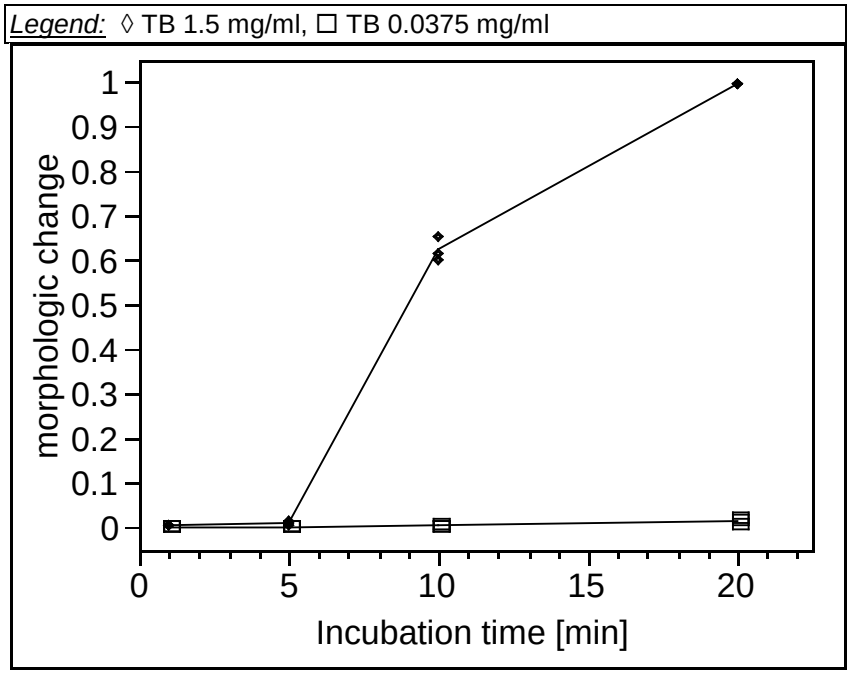



***Figure 35:*** Morphologic change over incubation time when comparing TB with diluted TB after the follow-up time of **6 hours**, medians are connected.

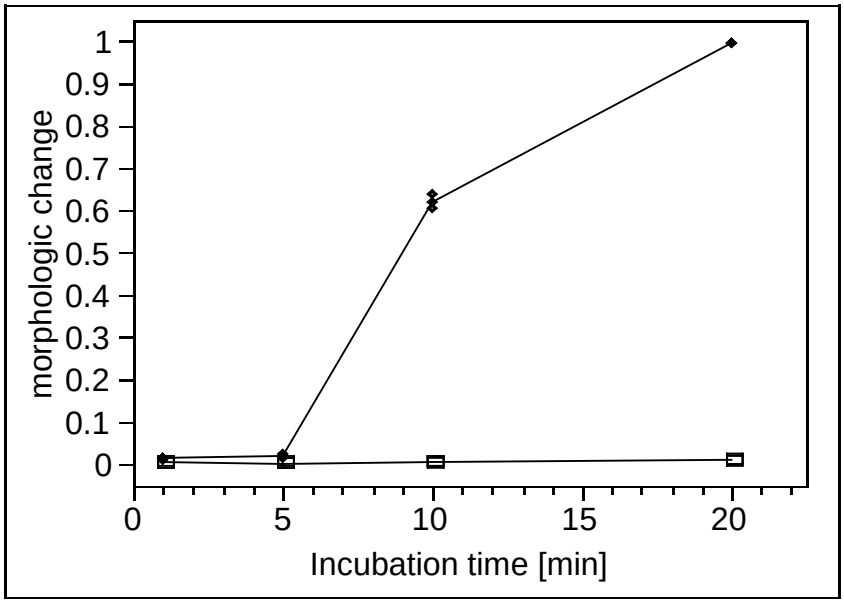



***Figure 36:*** Morphologic change over incubation time when compariing TB with diluted TB after the follow-up time of **24 hours**, medians are connected.

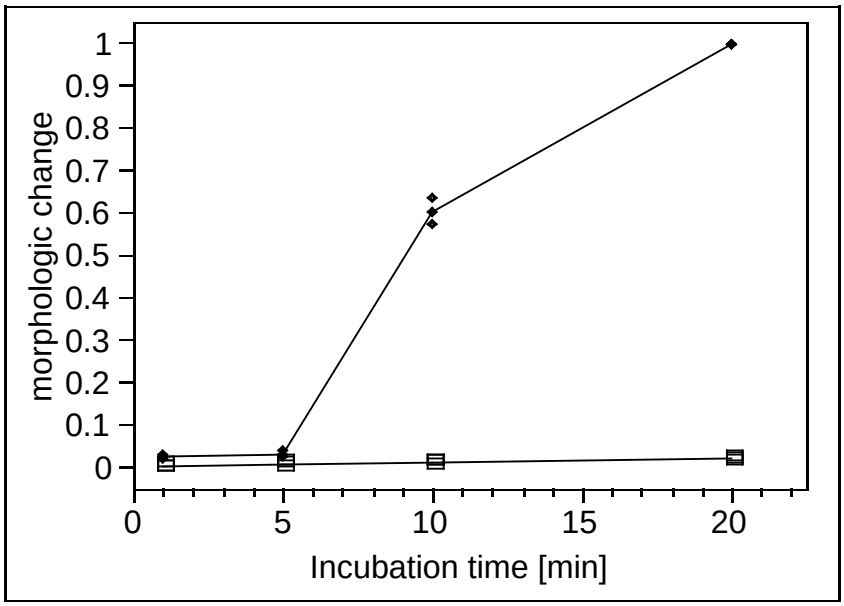



***Figure 37:*** Morphologic change over incubation time when comparing TB with diluted TB after the follow-up time of **72 hours**, medians are connected.

As previously described, our third question was performed to verify the effects of osmolarity not only on the survival, but also on the morphology of cultured ARPE-19 cells. Consequently, we compared outcomes of morphologic change, when cells were incubated for 1 to 20 minutes with the solutions 1 to 3 (ICG) and the solutions 1 to 3 (ICG-free). Thus, we performed an analysis of

variance with the factors solution number (levels: Solution 1-3), dye (levels: ICG and ICG-free), incubation time (levels: 1, 5, 10 and 20 min.), follow-up time (levels: 6, 24 and 72 h) and their interactions. The number of observations amounted to $n$ = 216. $R^2$ = 99.78% of variance was explained by the model, leaving a residual standard deviation of $s$ = 0.0254. We found a significant increase in morphological change depending on the solution number ($p$ < 0.0001), the dye ($p$ = 0.0014), the incubation time ($p$ < 0.0001) and the follow-up time ($p$ < 0.0001). The two-fold interactions: solution number and dye ($p$ < 0.0001), solution number and incubation time ($p$ < 0.0001), solution number and follow-up time ($p$ < 0.0001), incubation time and follow-up time ($p$ < 0.0001) as well as dye and follow-up time ($p$ = 0.0085) were also significant. No significance could be determined for the interaction between the factors dye and incubation time ($p$ = 0.3507).

The three-fold interaction between the factors solution number, incubation time and follow-up time ($p$ < 0.0001) also showed significance. In contrast, we could not determine a statistical significance for the three-fold interactions between the factors: solution number, dye and incubation time ($p$ = 0.0628); solution number, dye and follow-up time ($p$ = 0.6174), as well as for the interaction dye, incubation time and follow-up time ($p$ = 0.5648). The four-fold interaction between all factors ($p$ = 0.5950, shown in Figures 32–34 and 38–40) likewise was also not significant (data shown under *6.5.7 Figures and tables, Table 27*).

Regarding the solutions 1 (ICG) and (ICG-free) after the maximum follow-up time of 72 hours, our results showed only minimal differences in the outcome for morphological change. Median morphological change increased from 2.75% (CI: 1.88 to 3.78%) and 2.54% (CI: 1.71 to 3.53%) after 1 to 100% for both solutions after 20 minutes incubation time, respectively (data shown under *6.5.7 Figures and tables, Table 28*). After 10 minutes, we noticed the greatest difference in morphological change between these two solutions, showing a median morphological change of 94.04% (CI: 92.60 to 95.34%) for solution 1 (ICG) and 88.38% (CI: 86.46 to 90.18%) for Solution 1 (ICG-free). Almost similar results where found when comparing the other two ICG solutions at 2.5

and 1.0 mg/ml respectively with their dye-free controls (photographs shown under *6.2 Figures and tables, Figures 68 to 73* and *75 to 80*).

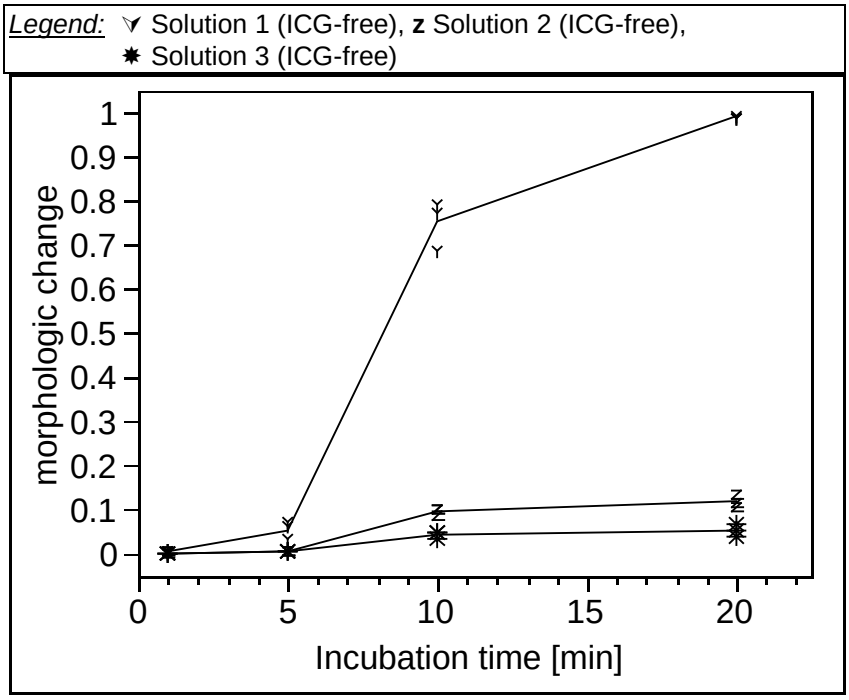



***Figure 38:*** Morphologic change over incubation time when comparing the solutions 1 to 3 (ICG-free) after the follow-up time of **6 hours**, medians are connected.

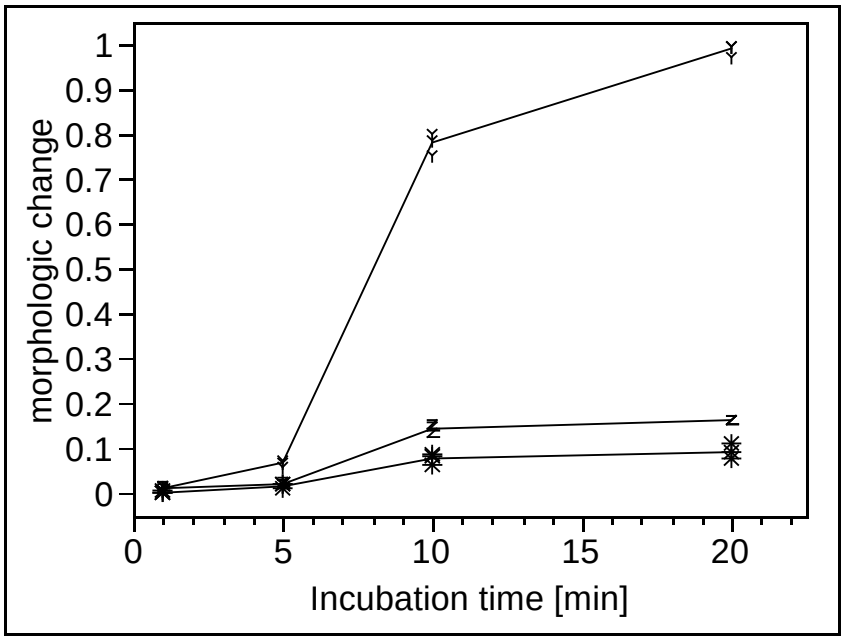



***Figure 39:*** Morphologic change over incubation time when comparing the solutions 1 to 3 (ICG-free) after the follow-up time of **24 hours**, medians are connected.

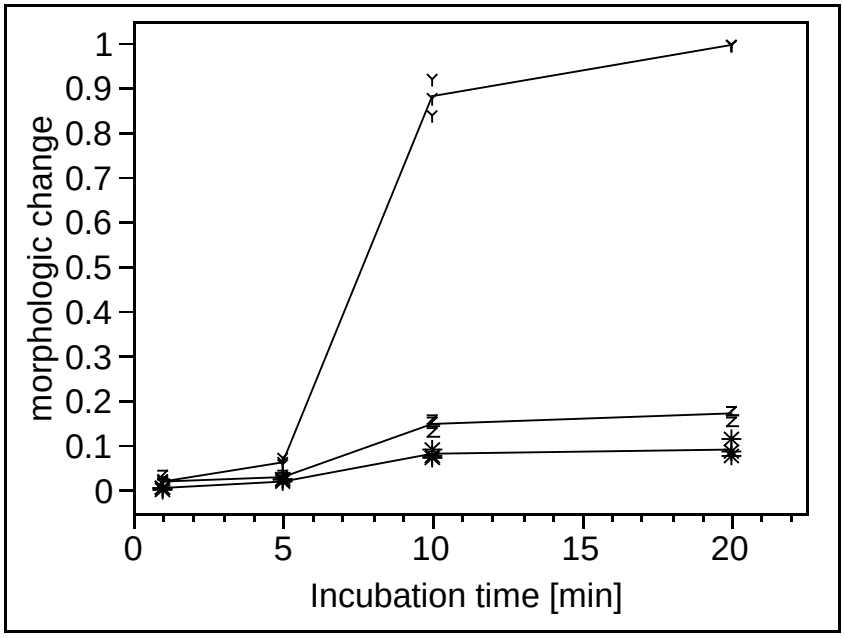



***Figure 40:*** Morphologic change over incubation time when comparing the solutions 1 to 3 (ICG-free) after the follow-up time of **72 hours**, medians are connected.

For the first part of the fourth question we compared outcomes of morphological change, when cells were incubated for 1 to 20 minutes with the solutions 1 to 3 (ICG) and trypan blue at 1.5 mg/ml. Therefore we performed an analysis of variance with the factors solution (levels: solution 1 to 3 (ICG) and trypan blue), incubation time (levels: 1, 5, 10 and 20 min.), follow-up time (levels: 6, 24 and 72 h) and their interactions. We had $n$ = 144 observations. $R^2$ = 99.88% of variance was explained by the model, leaving a residual standard deviation of $s$ = 0.0215. We found significant increase in morphologic change depending on the solution ($p < 0.0001$), the incubation time ($p < 0.0001$) and the follow-up time ($p < 0.0001$). Interactions between the factors: solution and incubation time ($p < 0.0001$), solution and follow-up time ($p = 0.0003$) and incubation time and follow-up time ($p = 0.0063$) as well as the interaction between all the factors ($p < 0.0001$, shown in Figures 32–34 and 35–37) were also significant (data shown under *6.5.8.1 Figures and tables, Table 30*). According to the results of the cell survival observation, a prominent increase in morphological change was seen for solution 1 (ICG) at 5.0 mg/ml and trypan blue at 1.5 mg/ml (data shown under *6.5.8.1 Figures and tables, Table 31*).

For the second part of the fourth question we compared outcomes of morphologic change, when cells were incubated for 1, 5, 10 and 20 minutes with the solution 4 (ICG) at 0.125 mg/ml and diluted trypan blue at 0.0375 mg/ml. Therefore we performed a second analysis of variance with the factors solution (levels: solution 4 (ICG) and diluted trypan blue), incubation time (levels: 1, 5, 10 and 20 min.), follow-up time (levels: 6, 24 and 72 h) and their interactions. We had $n$ = 72 observations. $R^2$ = 79.37% of variance was explained by the model, leaving a residual standard deviation of $s$ = 0.0185. We found a significant increase in morphological change depending on the solution ($p$ = 0.0301), the incubation time ($p$ < 0.0001) and the follow-up time ($p$ < 0.0001). Interactions between the factors: solution and incubation time ($p$ = 0.2874), solution and follow-up time ($p$ = 0.2757), incubation time and follow-up time ($p$ = 0.4926) as well as the interaction between all the factors ($p$ = 0.6484, shown in Figures 32–34 and 35–37) were not significant (data shown under *6.5.8.2 Figures and tables, Table 33*). Consequently, minimal changes were noticed for both dyes over the whole range of incubation and follow-up times (data shown under *6.5.8.2 Figures and tables, table 34*, photographs shown under *6.2 Figures and tables, Figures 74 to 85*).

## 3.3 Results of the set-up with illumination

### 3.3.1 Results of statistical analysis of cell survival

In the first question of this set-up we compared outcomes of cell survival using the ICG solutions 1 to 4 under illumination with a vitrectomy endolight. Thus, we had the factors solution (levels: solution 1-4 (ICG)), incubation & illumination time (levels: 1 and 5 minutes), follow-up time (levels: 6, 24 and 72 hours) and the interactions between these factors. We had $n$ = 144 observations. $R^2$ = 97.54% of variance was explained by the model, leaving a residual standard deviation of $s$ = 0.0302. There was a statistically significant decrease in cell survival depending on the factors solution ($p$ < 0.0001), incubation & illumination time ($p$ < 0.0001) and follow-up time ($p$ < 0.0001). The interactions between the

factors: solution and incubation & illumination time ($p < 0.0001$), solution and follow-up time ($p < 0.0001$), incubation & illumination time and follow-up time ($p = 0.0007$), as well as the interaction between all the factors ($p = 0.0001$, shown in Figures 41–43), were also significant (data shown under *6.6.1 Figures and tables, Table 36*).

The most remarkable decrease in cell survival was shown for solution 1 (ICG) after the follow-up time of 72 hours, where median cell survival decreased from 84.83% (CI: 83.04 to 86.54%) after 1 to 66.15% (CI: 63.82 to 68.44%) after 5 minutes of incubation & illumination time. Only minimal changes were noticed for the solutions 3 and 4 (ICG), where median cell survival after the follow-up time of 72 hours, decreased from 97.95% (CI: 97.20 to 98.59%) and 100% after 1 to 96.34% (CI: 95.38 to 97.21%) and 99.29% (CI: 98.82 to 99.64%) after 5 minutes of incubation & illumination time, respectively (data shown under *6.6.1 Figures and tables,Table 37*, photographs shown under *6.3 Figures and tables, Figures 86 to 91*).

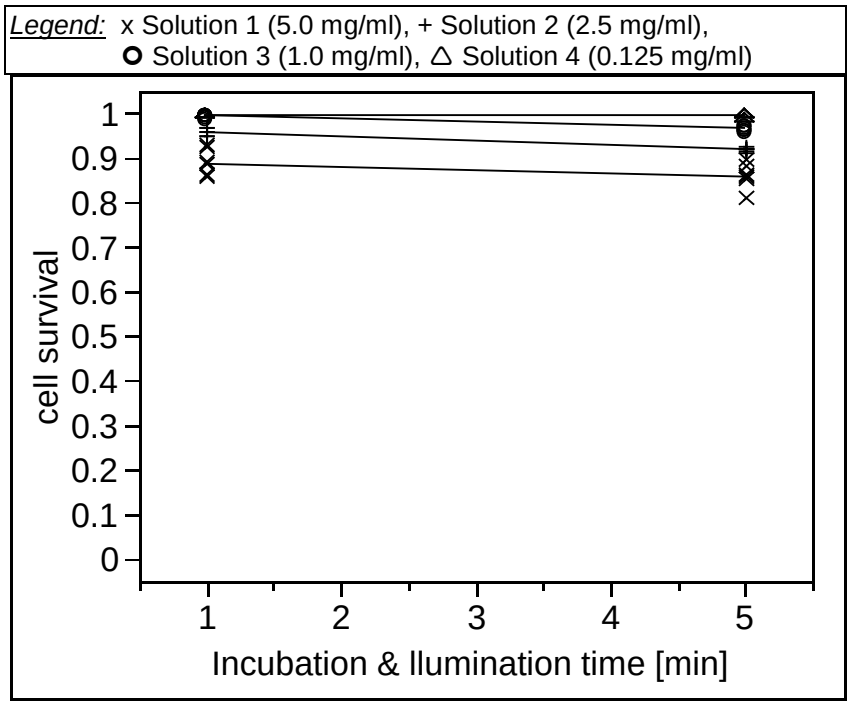



***Figure 41:*** Cell survival over incubation & illumination time when comparing the solutions 1 to 4 (ICG) after the follow-up time of **6 hours**, medians are connected.

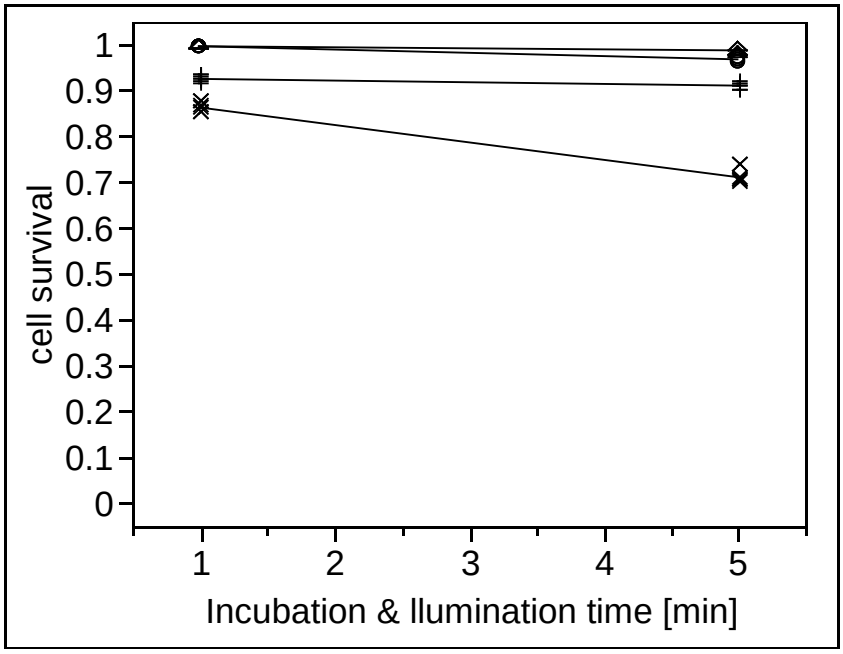



***Figure 42:*** Cell survival over incubation & illumination time when comparing the solutions 1 to 4 (ICG) after the follow-up time of **24 hours**, medians are connected.

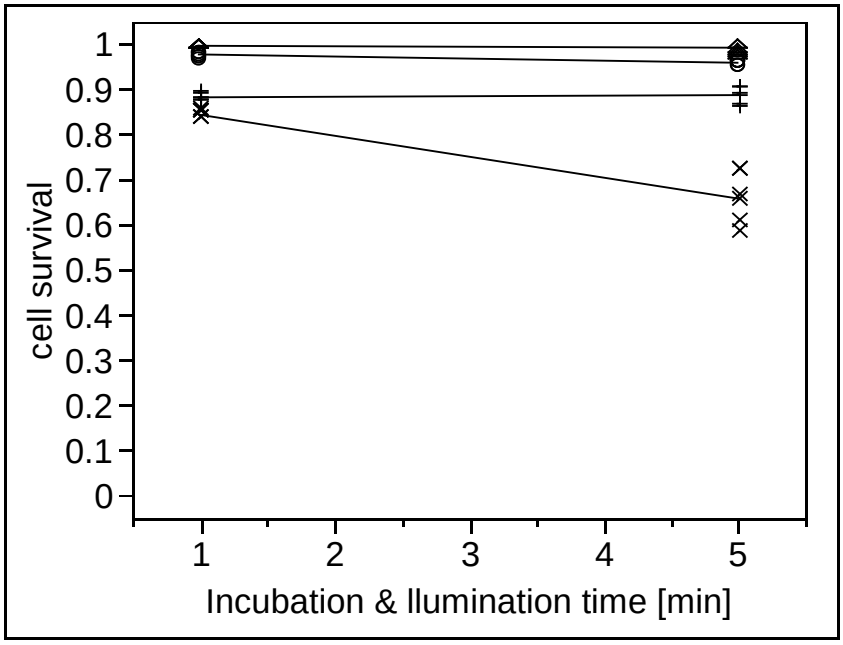



***Figure 43:*** Cell survival over incubation & illumination time when comparing the solutions 1 to 4 (ICG) after the follow-up time of **72 hours**, medians are connected.

The second question served to detect the effects of both trypan blue solutions on cell survival of cultured RPE-cells under illumination. The factors were solution (levels: trypan blue and diluted trypan blue), incubation & illumination time (levels: 1 and 5 minutes), follow-up time (levels: 6, 24 and 72

hours) and their interactions. We had $n$ = 72 observations. $R^2$ = 26.25% of variance was explained by the model, leaving a residual standard deviation of $s$ = 0.0658. A statistically significant decrease in cell survival was only shown for the factor follow-up time ($p$ = 0.0079). No significance was shown for the factors solution ($p$ = 0.1530) and incubation & illumination time ($p$ = 0.1250). The interactions between the factors: solution and incubation & illumination time ($p$ = 0.8145), solution and follow-up time ($p$ = 0.5921), incubation & illumination time and follow-up time ($p$ = 0.0849), as well as the three-fold interaction ($p$ = 0.9644, shown in Figures 44–46) similarly did not yield a statistically significant result (data shown under *6.6.2 Figures and tables, Table 39*).

Consequently, there were only minimal changes for both trypan blue dyes, showing an almost similar median cell survival over the whole range of incubation & illumination and follow-up times (data shown under *6.6.2 Figures and tables, Table 40*, photograph for TB 1.5 mg/ml shown under *6.3 Figures and tables, Figure 94*).

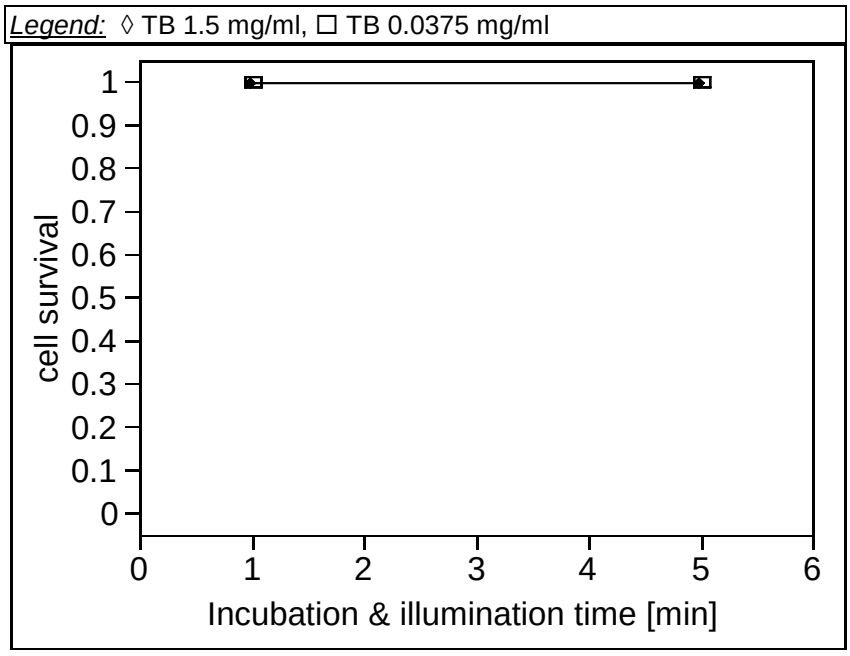



***Figure 44:*** Cell survival over incubation & illumination time when comparing TB with diluted TB after the follow-up time of **6 hours**, medians are connected.

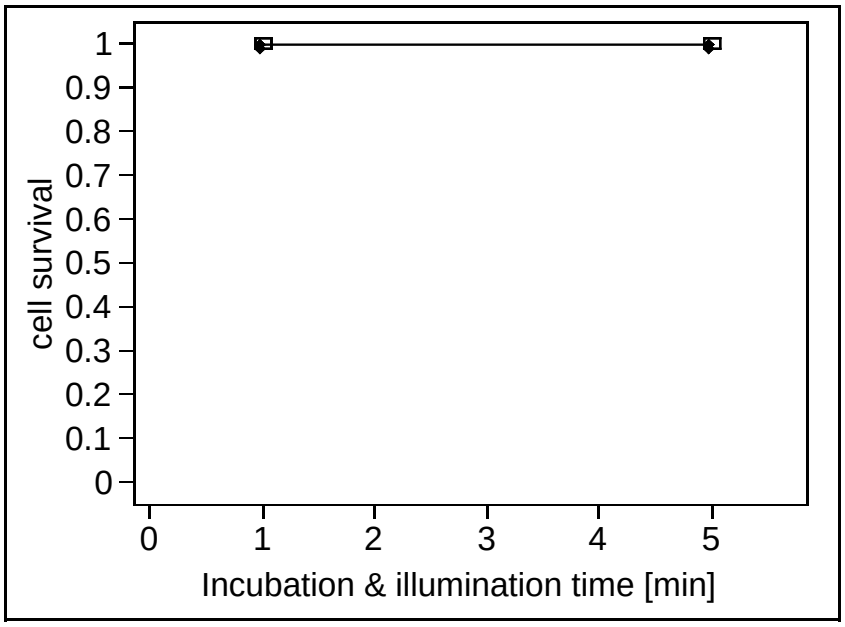



***Figure 45:*** Cell survival over incubation & illumination time when comparing TB with diluted TB after the follow-up time of **24 hours**, medians are connected.

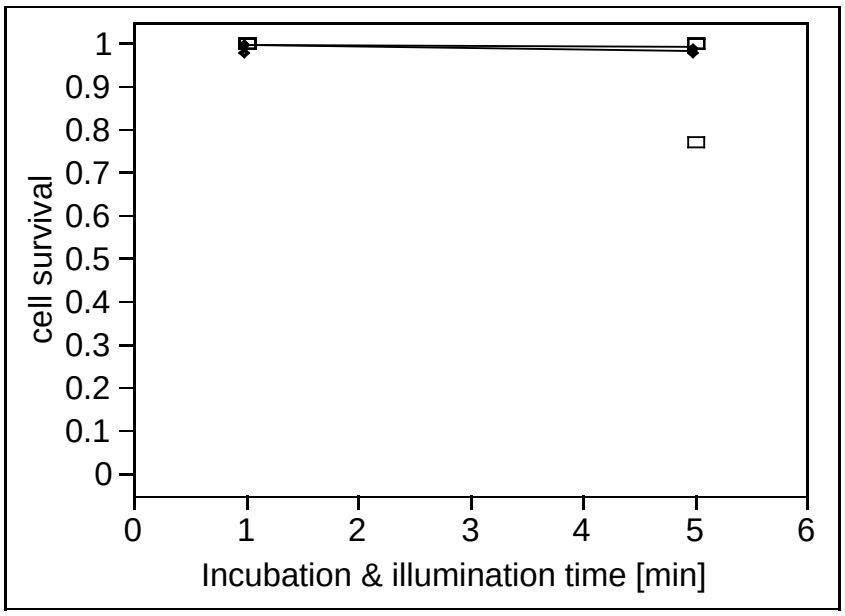



***Figure 46:*** Cell survival over incubation & illumination time when comparing TB with diluted TB after the follow-up time of **72 hours**, medians are connected.

Our third question compared the effects of the solutions 1 to 3 (ICG) and 1 to 3 (ICG-free) on the survival of ARPE-19 cells under illumination. The factors were solution number (levels: solutions 1-3), dye (levels: ICG and ICG-free), incubation & illumination time (levels: 1 and 5 minutes), follow-up time (levels: 6, 24 and 72 hours) and their interactions. The number of observations

was $n$ = 216. $R^2$ = 97.58% of variance was explained by the model, leaving a residual standard deviation of $s$ = 0.0281. We noticed a significant decrease in cell survival depending on the solution number ($p$ < 0.0001), the dye ($p$ < 0.0001), the incubation & illumination time ($p$ < 0.0001) and the follow-up time ($p$ < 0.0001).

The two-fold interactions between the factors: solution number and dye ($p$ < 0.0001), solution number and incubation & illumination time ($p$ < 0.0001), solution number and follow-up time ($p$ < 0.0001), dye and incubation & illumination time ($p$ = 0.0002), dye and follow-up time ($p$ < 0.0001), were also significant. No significance was shown for the interaction between the factors illumination & incubation time and follow-up time ($p$ = 0.4328). All three-fold interactions between the factors: solution number, dye and incubation & illumination time ($p$ < 0.0001); solution number, dye and follow-up time ($p$ < 0.0001); solution number, incubation & illumination time and follow-up time ($p$ = 0.0002); dye, incubation & illumination time and follow-up time ($p$ < 0.0001) showed significance. The four-fold interaction ($p$ < 0.0001, shown in *Figures 41-43* and 47–49) likewise showed significance, and there were considerable differences in cell survival comparing the solutions 1 to 3 (ICG) with their ICG-free controls (data shown under *6.6.3 Figures and tables, Table 42*). The median cell survival after the incubation & illumination time of 5 minutes serves as an example. Using the solutions 1 and 2 (ICG), median cell survival decreased from 85.99% (CI: 84.38 to 87.52%) and 92.33% (CI: 91.08 to 93.49%) after 6 to 66.15% (CI: 63.99 to 68.27%) and 89.03% (CI: 87.58 to 90.41%) after 72 hours, respectively. In contrast, using the control solutions 1 and 2 (ICG-free), median cell survival only decreased from 98.22% (CI: 97.58 to 98.77%) and 98.49% (CI: 97.89 to 98.99%) after 6 to 98.10% (CI: 97.44 to 98.67%) and 97.38% (CI: 96.61 to 98.06%) after 72 hours, respectively (data shown under *6.6.3 Figures and tables, Table 43*, photographs shown under *6.3 Figures and tables, Figures 86 to 90* and *92 to 93*).

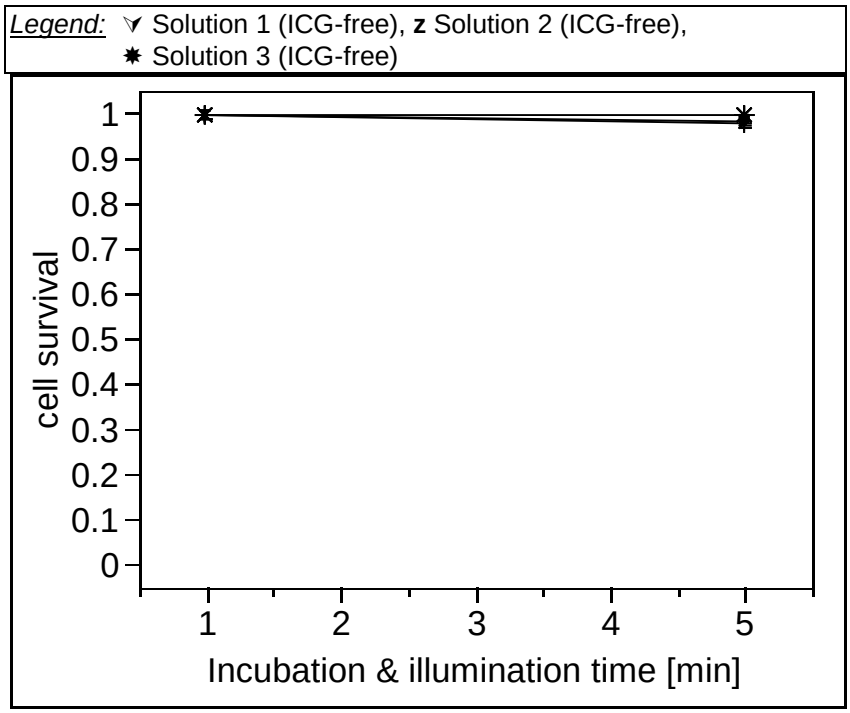



***Figure 47:*** Cell survival over incubation time when comparing the solutions 1 to 3 (ICG-free) after the follow-up time of **6 hours**, medians are connected.

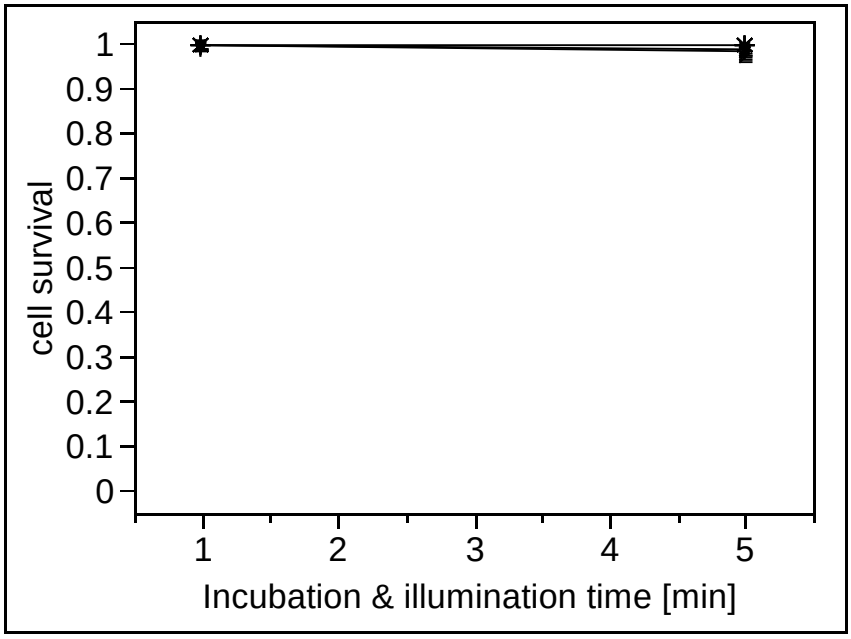



***Figure 48:*** Cell survival over incubation time when comparing the solutions 1 to 3 (ICG-free) after the follow-up time of **24 hours**, medians are connected.

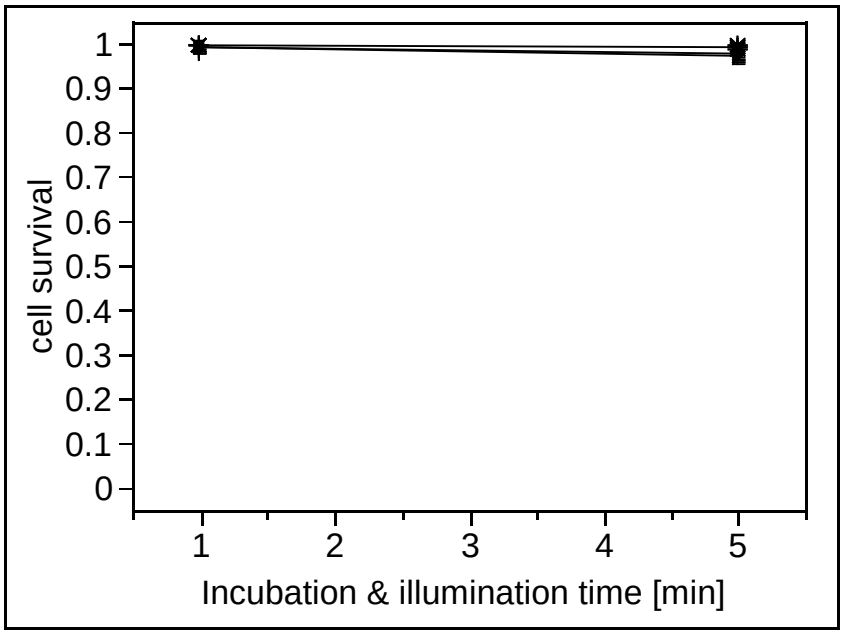



***Figure 49:*** Cell survival over incubation time when comparing the solutions 1 to 3 (ICG-free) after the follow-up time of **72 hours**, medians are connected.

For the first part of the fourth question we had the factors solution (levels: solutions 1 to 3 (ICG) and trypan blue), incubation & illumination time (levels: 1 and 5 minutes), follow-up time (levels: 6, 24 and 72 hours) and their interactions. The number of observations amounted to $n = 144$. $R^2 = 97.33\%$ of variance was explained by the model, leaving a residual standard deviation of $s = 0.0315$. We found a significant decrease in cell survival depending on the solution ($p < 0.0001$), the incubation & illumination time ($p < 0.0001$) and the follow-up time ($p < 0.0001$). The interactions between the factors: solution and incubation & illumination time ($p < 0.0001$), solution and follow-up time ($p < 0.0001$) as well as the three-fold interaction ($p < 0.0001$, shown in Figures 41–*43* and 44–46) were also significant (data shown under *6.6.4.1 Figures and tables, Table 45*). No significance was found for the interaction between the factors incubation & illumination time and follow-up time ($p = 0.1128$).

A remarkable decrease in cell survival was seen for the solutions 1 and 2 (ICG) at 5.0 and 2.5 mg/ml, while a minimal decrease only was shown for solution 3 (ICG) at 1.0 mg/ml and TB at 1.5 mg/ml (data shown under *6.6.4.1 Figures and tables, Table 46*, photographs shown under *6.3 Figures and tables, Figures 86 to 90* and *94*).

For the second part of the fourth question we had the factors solution (levels: solution 4 (ICG) and diluted trypan blue), incubation & illumination time (levels: 1 and 5 minutes), follow-up time (levels: 6, 24 and 72 hours) and their interactions. The number of observations amounted to $n$ = 72. $R^2$ = 30.81% of variance was explained by the model, leaving a residual standard deviation of $s$ = 0.0646. We only found a significant decrease in cell survival depending on the factor incubation & illumination time ($p$ = 0.0013). No significance was shown for the factors solution ($p$ = 0.1278), follow-up time ($p$ = 0.2076). The interactions between the factors: solution and incubation & illumination time ($p$ = 0.1278), solution and follow-up time ($p$ = 0.3607), incubation & illumination time and follow-up time ($p$ = 0.2076) as well as the threefold interaction ($p$ = 0.3607, shown in Figures 41–43 and 44–46) were similarly not significant (data shown under *6.6.4.2 Figures and tables, Table 48*).
A minimal decrease in cell survival with almost similar results were seen for both diluted solutions, solution 4 (ICG) at 0.125 mg/ml and trypan blue at 0.0375 mg/ml (data shown under *6.6.4.2 Figures and tables, Table 49*).

The fifth question served to elucidate differences in cell survival between a low concentrated ICG solution and the balanced salt solution (BSS), when using illumination. Thus, we had the factors solution (levels: Solution 4 (ICG) and BSS), incubation & illumination time (levels: 1 and 5 minutes), follow-up time (levels: 6, 24 and 72 hours) and the interactions between these factors. We had $n$ = 72 observations. $R^2$ = 74.82% of variance was explained by the model, leaving a residual standard deviation of $s$ = 0.0274. We noticed a significant decrease in cell survival depending on the factors incubation & illumination time ($p < 0.0001$), follow-up time ($p < 0.0001$) and their interaction ($p < 0.0001$). No significance was shown for the factor solution ($p$ = 0.2618), the interaction between the factors solution and incubation & illumination time ($p$ = 0.2618), solution and follow-up time ($p$ = 0.2692) as well as the threefold interaction ($p$ = 0.2692, shown in Figures 50–52 and under *6.6.5 Figures and tables, Table 51*). Median cell survival even after the follow-up time of 72 hours, barely decreased for both solutions (data shown under *6.6.5 Figures and tables, Table 52*).

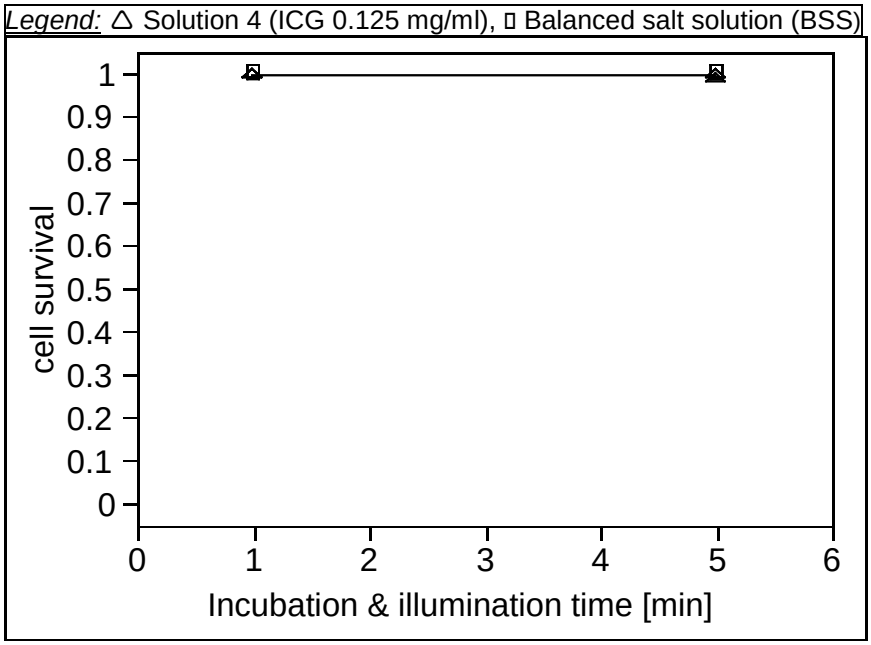



***Figure 50:*** Cell survival over incubation & illumination time when comparing solution 4 (ICG) with BSS after the follow-up time of **6 hours**, medians are connected.

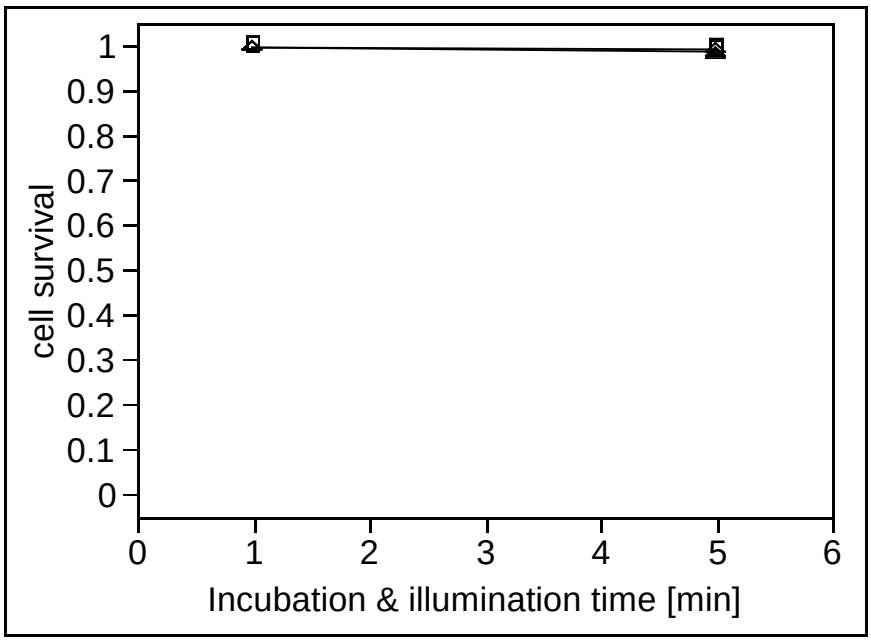



***Figure 51:*** Cell survival over incubation & illumination time when comparing solution 4 (ICG) with BSS after the follow-up time of **24 hours**, medians are connected.

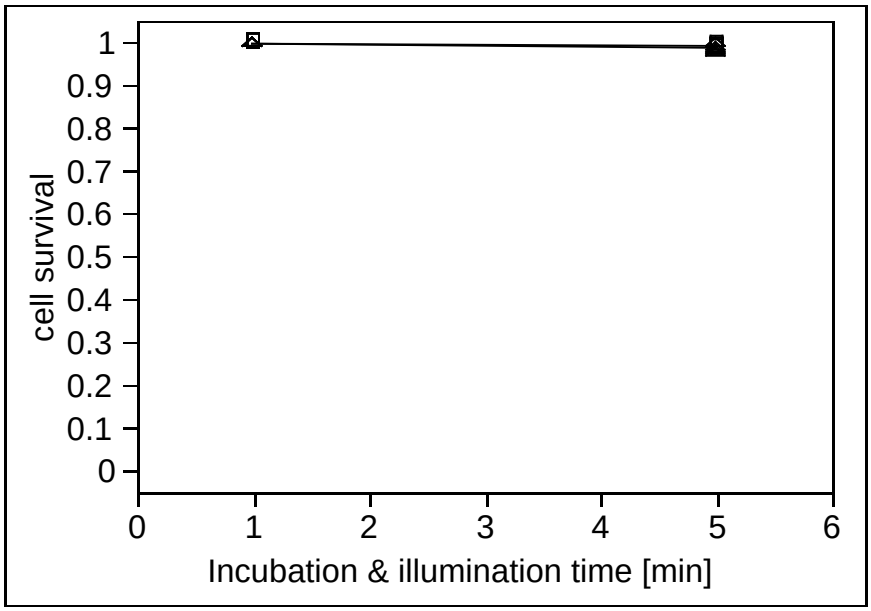



***Figure 52:*** Cell survival over incubation & illumination time when comparing solution 4 (ICG) with BSS after the follow-up time of **72 hours**, medians are connected.

### 3.3.2 Results of statistical analysis of morphologic change

The factors comparing the outcomes of morphological change using illumination and the solutions 1 to 4 (ICG) were the same as for the first question regarding the outcomes of cell survival. We had $n$ = 72 observations. $R^2$ = 98.59% of variance was explained by the model, leaving a residual standard deviation of $s$ = 0.0230. We noticed a significant increase of morphological change depending on the solution ($p < 0.0001$), the incubation & illumination time ($p < 0.0001$) and the follow-up time ($p < 0.0001$). The interactions: solution and incubation & illumination time ($p < 0.0001$), solution and follow-up time ($p < 0.0001$), incubation & illumination time and follow-up time ($p = 0.0330$), as well as the three-fold interaction ($p < 0.0001$, shown in Figures 53–55) were also significant (data shown under *6.6.6 Figures and tables, Table 54*).

Most remarkable differences in morphological change were noticed for the solutions 1 and 2 (ICG). Median morphological changes after 5 minutes incubation & illumination time raised from 15.33% (CI: 13.44 to 17.31%) and 7.93% (CI: 6.54 to 9.44%) after 6 to 41.09% (CI: 38.46 to 43.75%) and 11.50% (CI: 9.84 to 13.27%) after 72 hours follow-up time, respectively (data shown

under *6.6.6 Figures and tables, Table 55*, photographs shown under *6.3 Figures and tables, Figures 86 to 91*).

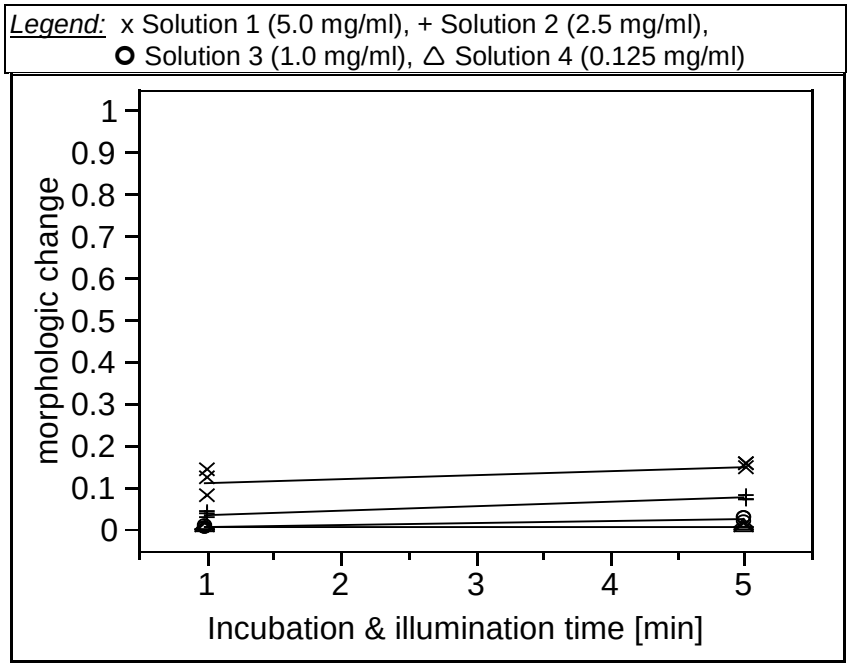



***Figure 53:*** Morphologic change over incubation & illumination time for the solutions 1 to 4 (ICG) after the follow-up time of **6 hours**, medians are connected.

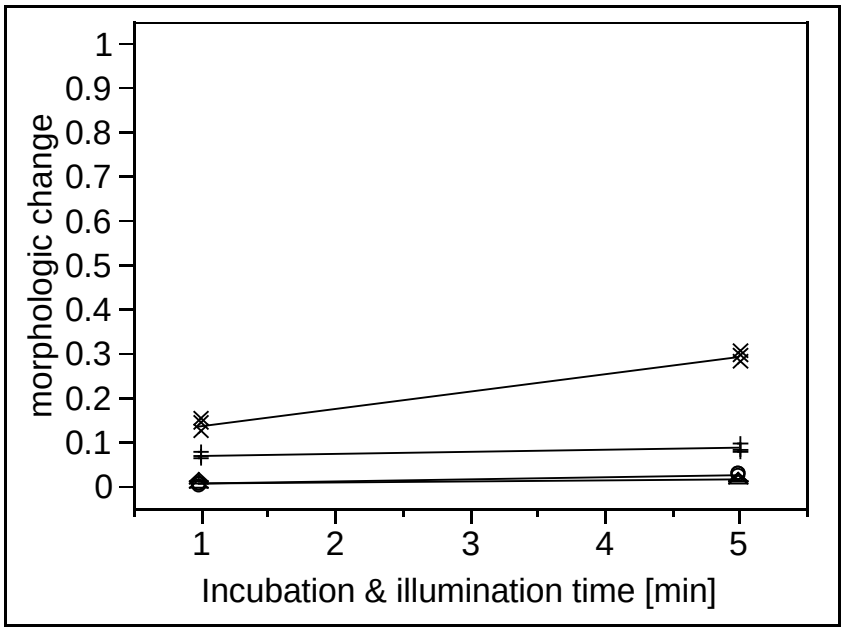



***Figure 54:*** Morphologic change over incubation & illumination time for the solutions 1 to 4 (ICG) after the follow-up time of **24 hours**, medians are connected.

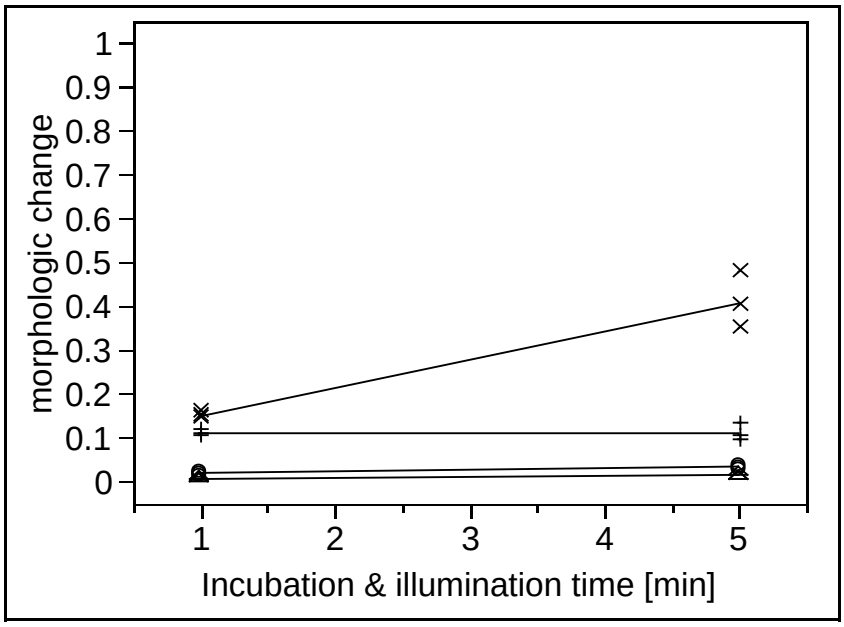



***Figure 55:*** Morphologic change over incubation & illumination time for the solutions 1 to 4 (ICG) after the follow-up time of **72 hours**, medians are connected.

For the second question, we also focused on the outcomes of morphological change for both trypan blue solutions. The factors for analysis of variance were solution (levels: trypan blue and diluted Trypan blue), incubation & illumination time (levels: 1 and 5 minutes), follow-up time (levels: 6, 24 and 72 hours) and their interactions. We had $n$ = 36 observations. $R^2$ = 59.12% of variance was explained by the model, leaving a residual standard deviation of $s$ = 0.0163. There was a statistical significance depending on the solution ($p$ = 0.0115), follow-up time ($p$ = 0.0096) and the three-fold interaction ($p$ = 0.0216, shown in Figures 56–58). In contrast, there was no significant difference in morphological change depending on the incubation & illumination time ($p$ = 0.4871). The interactions between the factors solution and incubation & illumination time ($p$ = 0.2114), solution and follow-up time ($p$ = 0.1808), as well as incubation & illumination time and follow-up time ($p$ = 0.6132), similarly were not significant (data shown under *6.6.7 Figures and tables, Table 57*). Consequently, the observed morphologic change was minimal for both trypan blue dyes. Median morphological change after 5 minutes incubation & illumination time for trypan blue and diluted trypan blue increased from 0.50% (CI: 0.19 to 0.95%) and 0.56% (CI: 0.31 to 0.88%) after 6 to 1.47 (CI: 0.89 to

2.19%) and 0.71 (CI: 0.28 to 1.31%) after 72 hours follow-up time, respectively (data shown under *6.6.7 Figures and tables, Table 58*, photograph for TB 1.5 mg/ml shown under *6.3 Figures and tables, Figure 94*).

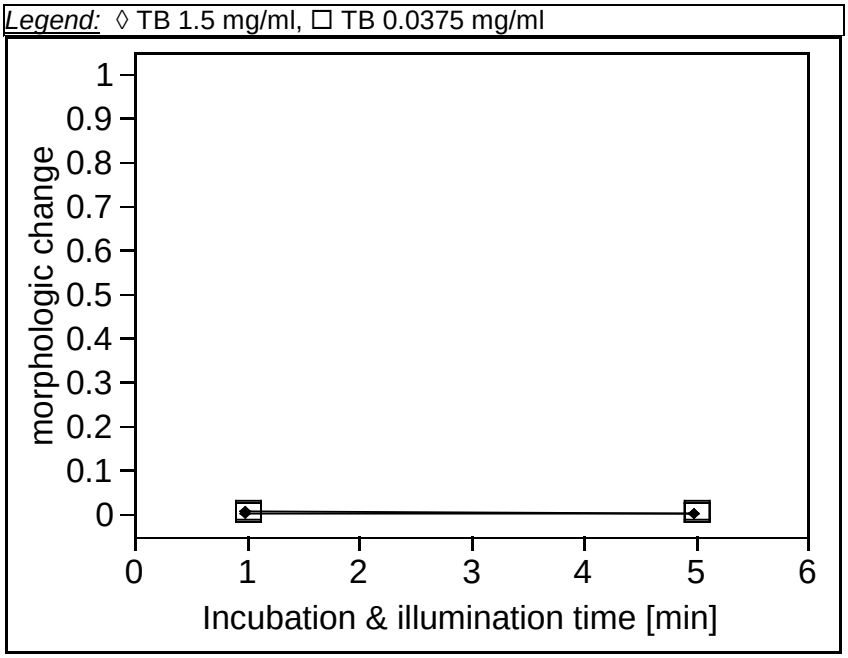



***Figure 56:*** Morphologic change over incubation & illumination time when comparing TB and diluted TB after the follow-up time of **6 hours**, medians are connected.

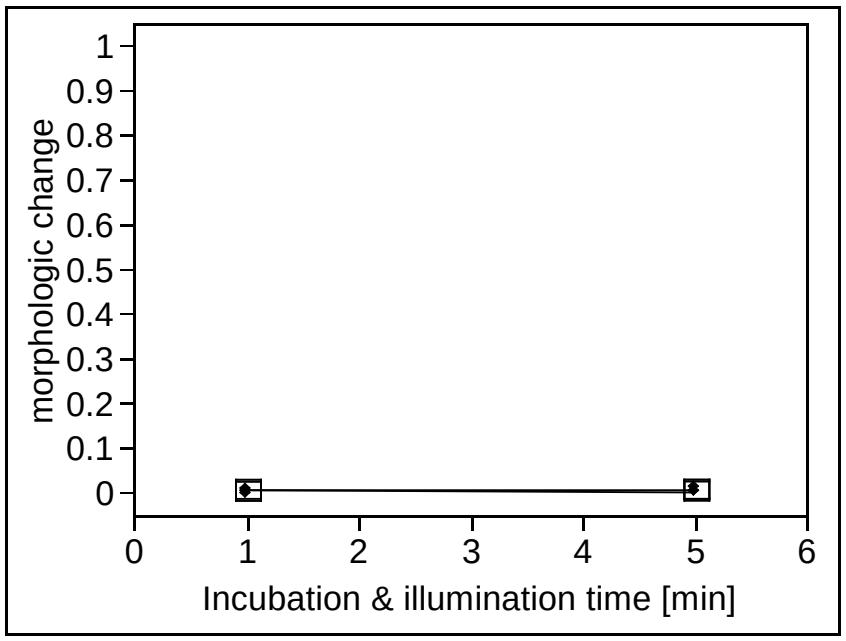



***Figure 57:*** Morphologic change over incubation & illumination time when comparing TB with diluted TB after the follow-up time of **24 hours**, medians are connected.

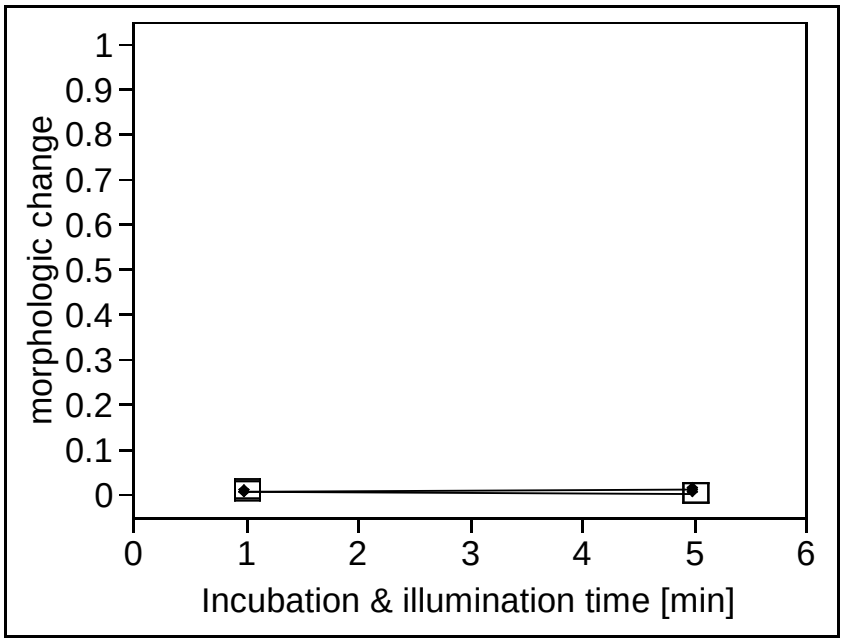



***Figure 58:*** Morphologic change over incubation & illumination time when comparing TB with diluted TB after the follow-up time of **72 hours**, medians are connected.

The third question investigated beside the outcomes of cell survival also the outcomes of morphologic change, using the solutions 1 to 3 (ICG) and the ICG-free control solutions. The factors were the same as in the statistical analysis of cell survival. The number of observations was $n$ = 108. $R^2$ = 98.54% of variance was explained by the model, leaving a residual standard deviation of $s$ = 0.0216. We found a significant increase in morphological change depending on the solution number ($p$ < 0.0001), the dye ($p$ < 0.0001), the incubation & illumination time ($p$ < 0.0001) and the follow-up time ($p$ < 0.0001).

The two-fold interactions: solution number and dye ($p$ < 0.0001), solution number and incubation & illumination time ($p$ < 0.0001), solution number and follow-up time ($p$ < 0.0001), dye and incubation & illumination time ($p$ < 0.0001), dye and follow-up time ($p$ < 0.0001) were also significant. No significance was noticed for the interaction between the factors incubation & illumination time and follow-up time ($p$ = 0.5327). The three-fold interactions: solution number, dye and incubation & illumination time ($p$ < 0.0001); solution number, dye and follow-up time ($p$ < 0.0001); solution number, incubation & illumination time and follow-up time; dye, incubation & illumination time and follow-up time ($p$ = 0.0285) as well as the fourfold interaction ($p$ < 0.0001) were also significant

(data shown under *6.6.8 Figures and tables, Table 60*). The fourfold interaction for these question obviously showed the effect of the ICG dye on morphologic change in comparison with the ICG-free solutions (shown in Figures 53–55 and 59–61).

Median morphologic change after an incubation & illumination time of 5 minutes, for the solution 1 (ICG) increased from 15.33% (CI: 13.58 to 17.16%) after 6 to 41.09% (CI: 38.66 to 43.56%) after 72 hours follow-up time. In absolute contrast to these findings median morphologic change for the solution 1 (ICG-free) only raised from 2.04% (CI: 1.40 to 2.81%) after 6 to 2.11% (CI: 1.46 to 2.89%) after 72 hours follow-up time. Little differences were noticed using the Solutions 3 (ICG) and 3 (ICG-free). Median morphologic change after the incubation & illumination time of 5 minutes, only increased from 2.97% (CI: 2.18 to 3.87%) and 0.90% (CI: 0.49 to 1.43%) after 6 to 3.75% (CI: 2.86 to 4.75%) and 1.07% (CI: 0.62 to 1.64%) after 72 hours follow-up time, respectively (data shown under *6.6.8 Figures and tables, Table 61*, photographs shown under *6.3 Figures and tables, Figures 86 to 90* and *92 to 93*).

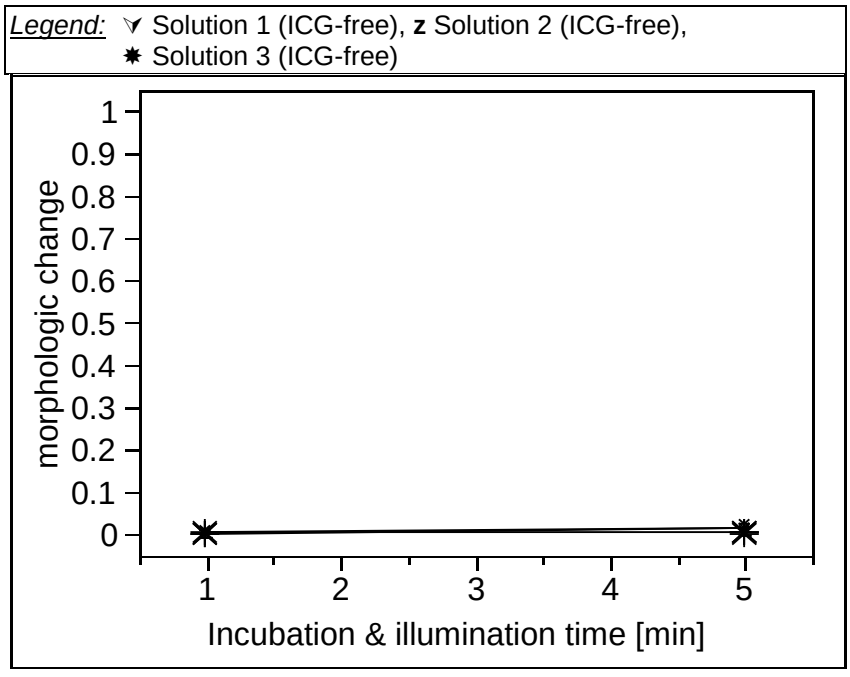



***Figure 59:*** Morphologic change over incubation time when comparing the solutions 1 to 3 (ICG-free) after the follow-up time of **6 hours**, medians are connected.

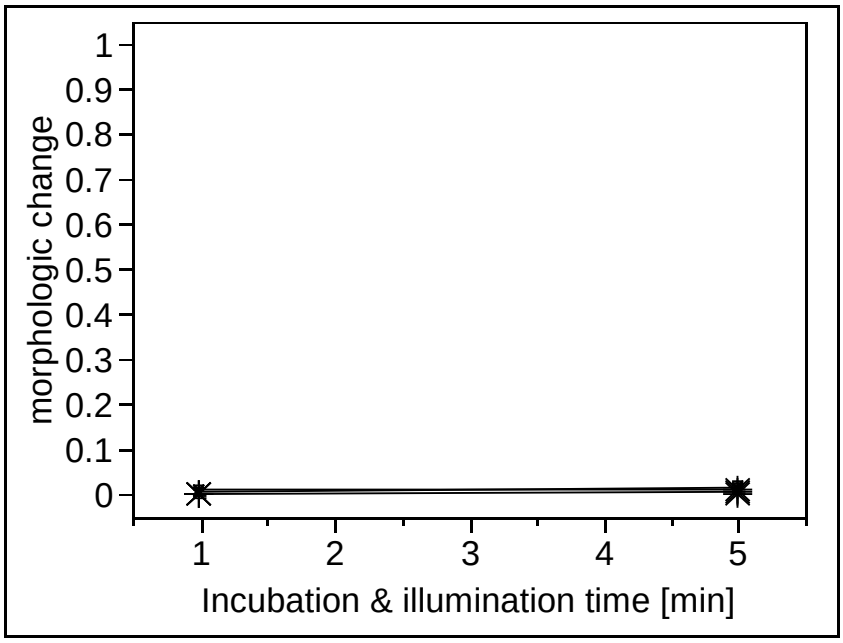



***Figure 60:*** Morphologic change over incubation time when comparing the solutions 1 to 3 (ICG-free) after the follow-up time of **24 hours**, medians are connected.

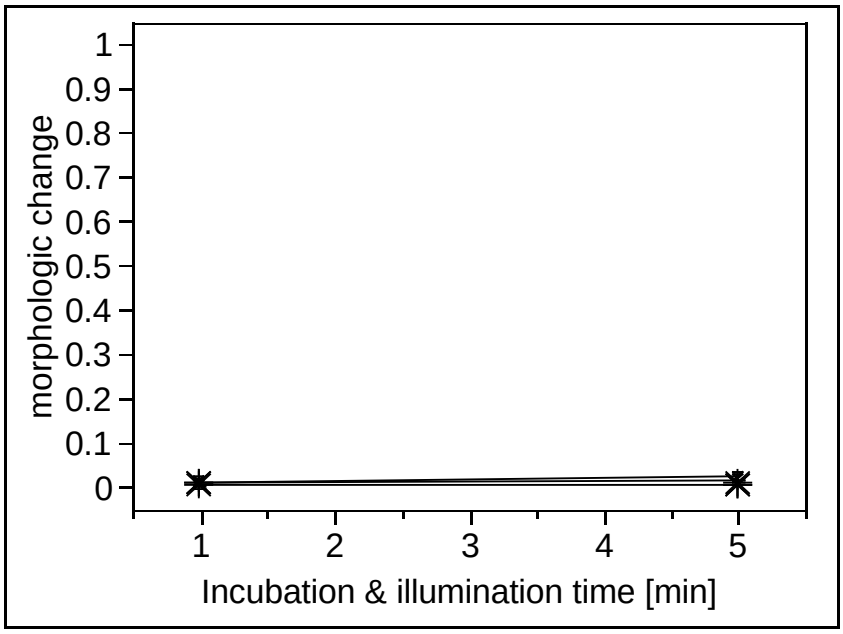



***Figure 61:*** Morphologic change over incubation time when comparing the solutions 1 to 3 (ICG-free) after the follow-up time of **72 hours**, medians are connected.

In the first part of the fourth question we compared the outcomes of morphological change, when cells were incubated for 1 and 5 minutes with the solutions 1 to 3 (ICG) and trypan blue under illumination. Therefore, we performed an analysis of variance with the factors solution (levels: solution 1 to 3 (ICG) and trypan blue), incubation & illumination time (levels: 1 and 5 minutes), follow-up time (levels: 6, 24 and 72 h) and their interactions. The number of observations was $n = 72$. $R^2 = 98.62\%$ of variance was explained by the model, leaving a residual standard deviation of $s = 0.2316$.

We found a significant increase in morphological change depending on the solution ($p < 0.0001$), the incubation & illumination time ($p < 0.0001$) and the follow-up time ($p < 0.0001$). The interactions between the factors: solution and incubation & illumination time ($p < 0.0001$), solution and follow-up time ($p < 0.0001$), incubation & illumination time and follow-up time ($p = 0.0209$), as well as the threefold interaction ($p < 0.0001$, shown in Figures 53–55 and 56–58) was also significant (data shown under *6.6.9.1 Figures and tables, Table 63*). An incubation & illumination time-dependent increase in morphological change was especially noticed for the solutions 1 and 2 (ICG). Little changes were observed for solution 3 (ICG) and minimal changes for trypan blue. Median morphological change after 5 minutes incubation & illumination time for solution 3 (ICG) and trypan blue was 2.97% (CI: 2.12 to 3.95%) and 0.50 (CI: 0.19 to 0.95%) after 6 to 3.75% (CI: 2.79 to 4.83%) and 1.47% (CI: 0.89 to 2.19%) after 72 hours follow-up time, respectively (data shown under *6.6.9.1 Figures and tables, Table 64*, photographs shown under *6.3 Figures and tables, Figures 86 to 90* and *94*).

In the second part of the fourth question we compared the outcomes of morphological change, when cells were incubated for 1 and 5 minutes with solution 4 (ICG) at 0.125 and diluted trypan blue at 0.0375 mg/ml under illumination. Therefore, we performed an analysis of variance with the factors solution (levels: Solution 4 (ICG) and diluted trypan blue), incubation & illumination time (levels: 1 and 5 minutes), follow-up time (levels: 6, 24 and 72 h) and their interactions. We had $n = 36$ observations. $R^2 = 68.78\%$ of variance was explained by the model, leaving a residual standard deviation of $s =$

0.0159. We found a significant increase in morphological change depending on the solution ($p < 0.0001$), the follow-up time ($p = 0.0292$), the interaction between the factors solution and incubation & illumination time ($p = 0.0096$) and the threefold interaction between all factors ($p = 0.0354$, shown in Figures 53–*55* and 56–58). No significance was found regarding the factor incubation & illumination time ($p = 0.4452$), the interaction between the factors solution and follow-up time ($p = 0.2264$) and incubation & illumination time and follow-up time ($p = 0.6785$, data shown under *6.6.9.2 Figures and tables, Table 66*). Although the increase in morphological change was significant for the factor solution, differences between these two solutions were minimal. Median morphological change after 5 minutes incubation & illumination time for solution 4 (ICG) and diluted trypan blue, increased from 0.92% (CI: 0.59 to 1.31%) and 0.71% (CI: 0.43 to 1.06%) after 6 to 1.76% (CI: 1.30 to 2.30%) and 1.71% (CI: 0.79 to 1.61%) after 72 hours follow-up time, respectively (data shown under *6.6.9.2 Figures and tables, Table 67*).

The fifth question investigated whether there were significant differences in morphological change, using solution 4 (ICG) at 0.125 mg/ml and the balanced salt solution BSS. The number of observations amounted to $n = 36$. $R^2 = 64.87\%$ of variance was explained by the model, leaving a residual standard deviation of $s = 0.0149$. Significant changes were noticed for the factors solution ($p = 0.0018$), incubation & illumination time ($p = 0.0304$) and the follow-up time ($p = 0.0006$). The interactions between the factors: solution and incubation & illumination time ($p = 0.1399$), solution and follow-up time ($p = 0.8833$), incubation & illumination time and follow-up time ($p = 0.6615$), as well as the threefold interaction ($p = 0.3066$) were not significant (data shown under *6.6.10 Figures and tables, Table 69 and 70*).

## 3.4 Results of the clinical set-up

### 3.4.1 Results of statistical analysis of cell survival

In contrast, to the previous two set-ups, incubation and illumination times where standardised in this set-up. Therefore, the observed effects were caused by the factors solution (levels: solution 1-4 (ICG)), follow-up time (levels: 6, 24 and 72 hours) and their interaction. We had $n$ = 72 observations. $R^2$ = 95.79% of variance was explained by the model, leaving a residual standard deviation of $s$ = 0.0437. We found a significant decrease in cell survival depending on the factors solution ($p$ < 0.0001), follow-up time ($p$ < 0.0001) and the interaction between these factors ($p$ <0.0001, shown in *Figure 62,* data shown under *6.7.1 Figures and tables, Table 72*). The decrease in cell survival was remarkable for the solutions 1 and 2 (ICG). Median cell survival decreased from 89.87% (CI: 87.62 to 91.92%) and 95.92% (CI: 94.40 to 97.22%) after 6 to 70.22% (CI: 66.91 to 73.43%) and 91.63% (CI: 89.55 to 93.50%) after 72 hours follow-up time, respectively (data shown under *6.7.1 Figures and tables, Table 73*, photographs shown under *6.4 Figures and tables, Figures 95 to 97*).

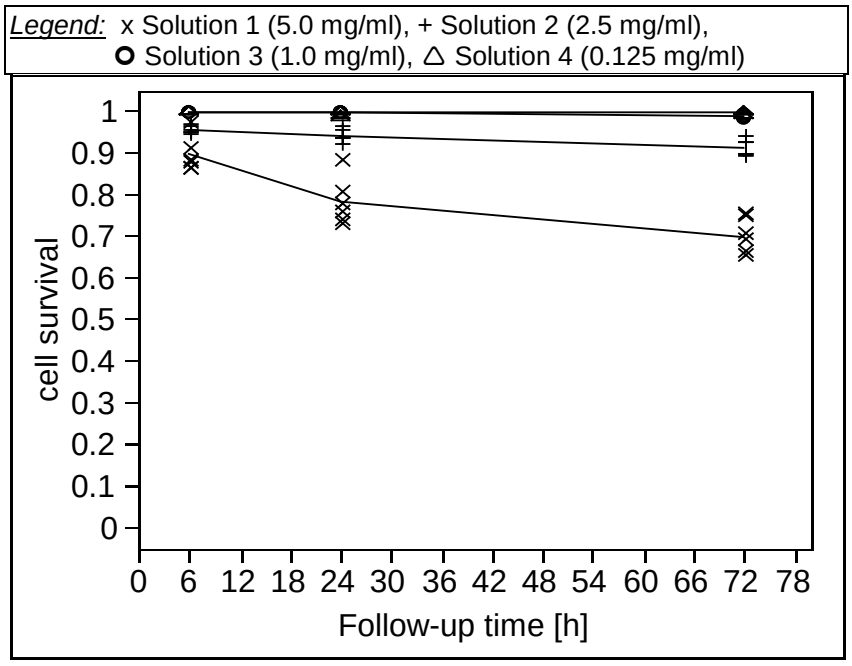



***Figure 62:*** Cell survival over follow-up time when comparing the solutions 1 to 4 (ICG) medians are connected.

The second question investigated the effects of trypan blue and diluted trypan blue on the survival of ARPE-19 cells. The factors for analysis of variance were solution (levels: trypan blue and diluted trypan blue), the follow-up time (levels: 6, 24 and 72 hours) and their interaction. The number of observations amounted to $n$ = 36. $R^2$ = 55.76% of variance was explained by the model, leaving a residual standard deviation of $s$ = 0.0267. We only found a significant decrease in cell survival depending on the solution ($p < 0.0001$). No significant differences in cell survival were shown for the follow-up time ($p$ = 0.2638) as well as for the interaction between the factors solution and the follow-up time ($p$ = 0.3854, shown in *Figure 63*, data shown under *6.7.2 Figures and tables, Table 75*). Follow-up time dependent decrease in cell survival was minimal for both trypan blue dyes. Median cell survival using TB and diluted TB decreased from 99.53% (CI: 99.18 to 99.79%) and 99.96% (CI: 99.83 to 100%) after 6 to 99.51% (CI: 99.15 to 99.77%) and 99.89% (CI: 99.69 to 99.99%) after 72 hours follow-up time, respectively (data shown under *6.7.2 Figures and tables, Table 76*, photograph for TB 1.5 mg/ml shown under *6.4 Figures and tables, Figure 95*).

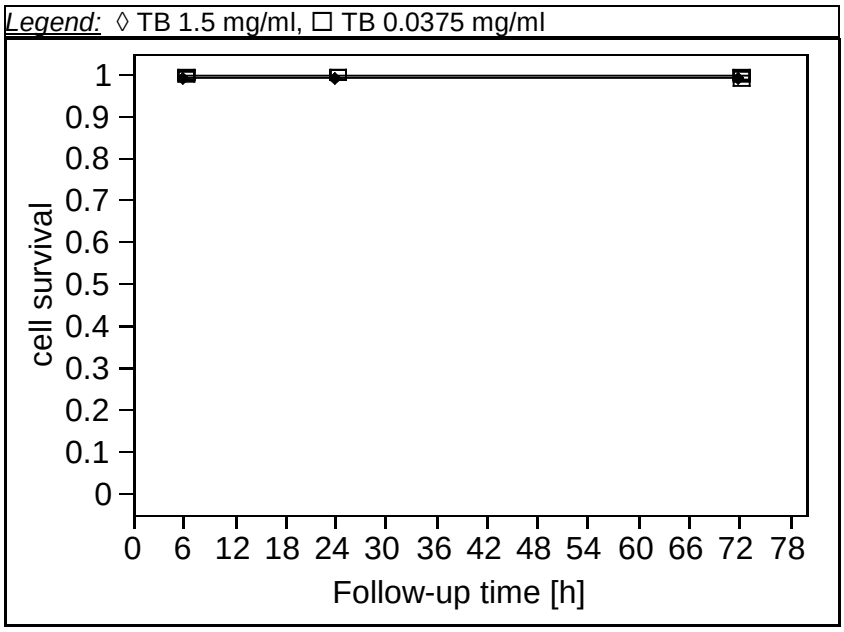



***Figure 63:*** Cell survival over follow-up time when comparing TB and diluted TB, medians are connected.

The third question examined which dye is safer for cultured ARPE-19 cells. Therefore we compared the solutions 1 to 3 (ICG) at 5.0 down to 1.0 mg/ml with TB at 1.5 mg/ml in a first analysis of variance and solution 4 (ICG) at 0.125 mg/ml with diluted TB at 0.0375 mg/ml in a second analysis.

Thus our factors for the first analysis of variance were solution (levels: solution 1 to 3 (ICG) and trypan blue), follow-up time (levels: 6, 24 and 72 hours) and the interaction between these factors. The number of observations was $n$ = 72. $R^2$ = 96.29% of variance was explained by the model, leaving a residual standard deviation of $s$ = 0.0388. The results showed a significant decrease in cell survival depending on the solution ($p$ < 0.0001), the follow-up time ($p$ < 0.0001) and the interaction between these factors ($p$ <0.0001, shown in *Figures 62* and *63*, data shown under *6.7.3.1 Figures and tables, Table 78*). The follow-up time dependent decrease in cell survival was especially shown for the solutions 1 and 2 (ICG). Only minimal changes were noticed for Solution 3 (ICG) and trypan blue, where cell survival decreased from 100% and 99.53% (CI: 99.00 to 99.87%) after 6 to 99.10% (CI: 98.40 to 99.60%) and 99.51% (CI: 98.96 to 99.85%) after 72 hours follow-up time, respectively (data shown under *6.7.3.1 Figures and tables, Table 79*, photographs shown under *6.4 Figures and tables, Figures 95 to 98*).

The factors for our second analysis of variance were solution (levels: Solution 4 (ICG) and diluted trypan blue), follow-up time (levels: 6, 24 and 72 hours) and the interaction between the factors. The number of observations was $n$ = 36. $R^2$ = 15.60% of variance was explained by the model, leaving a residual standard deviation of $s$ = 0.0388. The results showed no significant decrease in cell survival depending on the solution ($p$ = 0.6792), the follow-up time ($p$ = 0.3260) and the interaction between these factors ($p$ = 0.2348, shown in *Figures 62* and *63*, data shown under *6.7.3.2 Figures and tables, Table 81*). Thus, only little changes were noticed for solution 4 (ICG) and diluted TB, where cell survival decreased from 100% and 99.96% (CI: 99.73 to 99.98%) after 6 to 99.89% (CI: 99.57 to 100%) and 99.89% (CI: 99.57 to 100%) after 72 hours follow-up time, respectively (data shown under *6.7.3.2 Figures and tables, Table 82*).

### 3.4.2 Results of statistical analysis of morphologic change

In addition, to our first question about outcomes of cell survival under standardised conditions, we compared the outcomes of morphologic change using the solutions 1 to 4 (ICG). We performed an analysis of variance with the factors solution (levels: solution 1 to 4 (ICG)), follow-up time (levels: 6, 24 and 72 hours) and their interaction. We had $n$ = 36 observations. $R^2$ = 98.48% of variance was explained by the model, leaving a residual standard deviation of $s$ = 0.0270. We found statistical significance in morphological change depending on the solution ($p < 0.0001$), the follow-up time ($p < 0.0001$) and the interaction between the factors ($p < 0.0001$, shown in *Figure 64*, data shown under *6.7.4 Figures and tables, Table 84*). Follow-up time dependent increase in morphological change was especially noticed for solution 1 (ICG), where median morphologic change increased from 12.27% (CI: 10.23 to 14.46%) after 6 to 28.18% (CI: 25.33 to 31.12%) after 72 hours. Minor effects of follow-up time were shown for the solutions 3 and 4 (ICG) (data shown under *6.7.4 Figures and tables, Table 85,* photographs shown under *6.4 Figures and tables, Figures 95 to 97*).

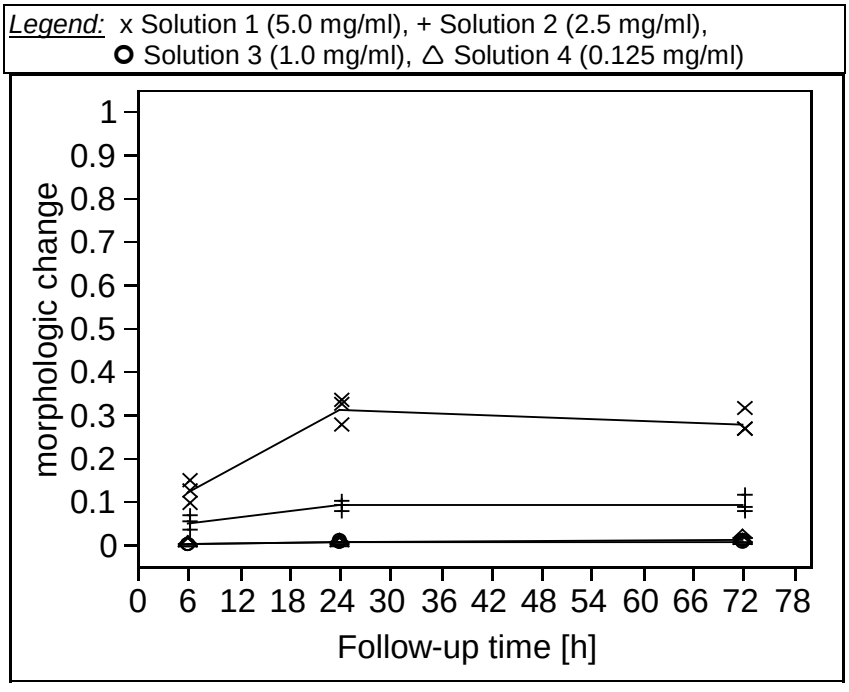



***Figure 64:*** Morphologic change over follow-up time when comparing the solutions 1 to 4 (ICG), medians are connected.

In the same way as for ICG-solutions, we compared the outcomes of morphological change for both TB-solutions. Consequently, we conducted an analysis of variance with the factors solution (levels: trypan blue and diluted trypan blue), follow-up time (levels: 6, 24 and 72 hours) and their interaction. The number of observations was $n$ = 18. $R^2$ = 47.80% of variance was explained by the model, leaving a residual standard deviation of $s$ = 0.0139. We only found a significant difference in morphological change depending on the follow-up time ($p$ = 0.0298). No significance was shown for the factor solution ($p$ = 0.3071) and the interaction of the factors ($p$ = 0.8635, data shown under *6.7.5 Figures and tables, Table 87*). As already shown in the statistical analysis for the observation cell survival, the differences between these solutions were minimal. The follow-up time dependent median morphological change increased from 0.57% (CI: 0.34 to 0.87%) after 6 to 1.04% (CI: 0.71 to 1.42%) after 72 hours for trypan blue and from 0.46% (CI: 0.25 to 0.73%) after 6 to 0.83% (CI: 0.54 to 1.17%) after 72 hours follow-up time for diluted trypan blue, respectively (data shown under *6.7.5 Figures and tables, Table 88*).

Similarly to the third question for cell survival, we assessed which of the dyes, ICG or TB, led to more morphological change to cultured ARPE-19 cells. We performed two analyses of variance, one with solutions used to mimic gas-filled eyes and the other with solutions used to mimic fluid-filled eyes. Thus, we first compared the outcomes of morphologic change using the factors solution (levels: solution 1 to 3 (ICG) and trypan blue), follow-up time (levels: 6, 24 and 72 hours) and their interaction. The number of observations was $n$ = 36. $R^2$ = 98.73% of variance was explained by the model, leaving a residual standard deviation of $s$ = 0.0249. There were significant differences depending on the solution ($p$ < 0.0001), the follow-up time ($p$ < 0.0001) as well as the interaction between the factors ($p$ < 0.0001, data shown under *6.7.6.1 Figures and tables, Table 90*). We found a remarkable increase in morphologic change using solution 1 and 2 (ICG), as well as a minimal increase for solution 3 (ICG) and trypan blue (data shown under *6.7.6.1 Figures and tables, Table 91*, photographs shown under *6.4 Figures and tables, Figures 95 to 98*).

In the second analysis we compared the outcomes of morphological change using the factors solution (levels: solution 4 (ICG) and diluted trypan blue), follow-up time (levels: 6, 24 and 72 hours) and their interaction. We had $n$ = 18 observations. $R^2$ = 48.59% of variance was explained by the model, leaving a residual standard deviation of $s$ = 0.0203. We found no significant differences in morphological change depending on the solution ($p$ = 0.1075), the follow-up time ($p$ = 0.0531) and their interaction ($p$ = 0.6968, data shown under *6.7.6.2 Figures and tables, Table 93*). Consequently, little changes were observed for solution 4 (ICG) and diluted trypan blue, where median morphological change increased from 0.61% (CI: 0.28 to 1.07%) and 0.46% (CI: 0.18 to 0.87%) after 6 to 1.42% (CI: 0.88 to 2.09%) and 0.83% (CI: 0.43 to 1.35%) after 72 hours follow-up time, respectively (data shown under *6.7.6.2 Figures and tables, Table 94*).

# 4. Discussion

## 4.1 Indocyanine green

Since the use of indocyanine green (ICG) was introduced in macular hole surgery for peeling the internal limiting membrane its usefullness for staining of the otherwise barely visible membrane has been widely accepted [24, 42, 63, 85, 96, 98, 167]. However, since reports of possible cytotoxicity appeared, [32, 43, 47, 59–61, 78, 125, 156, 161] studies focusing on the role of ICG in observed histological and clinical alterations after ICG-assisted ILM peeling receiced much attention. The concentration of the dye, retinal exposure time, osmolarity of the ICG solution, as well as the illumination time and the wavelength of the vitrectomy endolight were identified as possibly influencing factors. Though several publications already explored some of the parameters, conflicting results, as well as experimental set-ups that did not comply with the clinical practice, stimulated the present work.

### 4.1.1 Acute effects

Reported ICG concentrations range from 1.0-5.0 mg/ml when ILM-peeling is performed in air- or gas-filled eyes [3, 24, 32, 98 –102,141,167]. Lower concentrations are achieved, if ICG-staining is performed in fluid-filled eyes under balanced salt solution (BSS). In present clinical practice volumes of 0.1 to 0.5 ml are usually applied into the vitreous cavity [3, 24, 98–102] and left in place for less than 30 seconds [3, 4, 98] to up to 1 minute [48, 60].

Da Mata et al. [24] reported about 0.3 ml of ICG at 5.0 mg/ml (corresponding to a dose of 1.5 mg) as a safe and useful adjunct for ILM-peeling in macular hole surgery. The dye was left in the vitreous cavity for 3 to 5 minutes and removed thereafter by active suction. This technique was based on a preliminary study with human cadaver eyes by the same study group [15]. Sakamoto et al. [141] showed that 0.5 ml of ICG at 5.0 mg/ml (corresponding to a dose of 2.5 mg)

was similarly useful and without any evidence for toxicity. Contrary to these findings, Ando and associates [3] using 0.1 to 0.2 ml of ICG at 5.0 mg/ml (corresponding to doses of 0.5 to 1.0 mg), reported on less favourable outcomes and even irreversible peripherial visual field loss when results of ICG-assisted membrane peeling were compared to those without ICG. In contrast the same study group, using ICG at 0.5 mg/ml (corresponding doses of 0.05 to 0.1 mg) for ILM-staining in 16 patients, observed no dye-related adverse effects [4]. In both studies there was only brief exposure to the dye (around 10 seconds) and the same volume was applied. Thus, according to this study (a) ICG at 5.0 mg/ml appeared to be toxic and (b) ICG-related toxicity appeared to be concentration dependent even at exposure times below 1 minute. The results of Ando's studies therefore are consistent with those of our study. In the clinical set-up of our study we tried to mimic the situation that occurs in clinical practice. We used 0.1 ml of ICG at 5.0 mg/ml (corresponding to a dose of 0.5 mg) for 1 minute coupled with 5 minutes of illumination using a standard vitrectomy endolight pipe. We noticed a significant decrease in RPE cell viability (90% cell survival after 6 and 70% after 72 hours follow-up) and an increase in morphologic change (12% morphologically altered cells after 6 and 28% after 72 hours follow-up) compared to lower concentrated ICG solutions and the dye-free controls.

Regarding clinical reports about the effects of lower concentrated ICG solutions, Kwok et al. [98–102] reported in several clinical investigations about the safety of ICG-assisted ILM-peeling in macular hole surgery. The authors conducted ILM-staining with 0.2 ml of ICG at 0.25 to 1.25 mg/ml (corresponding to doses of 0.05 to 0.25 mg) and left the dye in place for approximately 30 seconds. Improved visual and anatomic outcomes as well as no dye-related toxicity were observed in all conducted studies. In contrast, Engelbrecht et al. [32] working with ICG at 1.0 mg/ml, reported on retinal pigment epithelial changes in 10 of 22 eyes after ICG-assisted ILM-peeling. Although the comparison of these studies is strongly limited, this was the first report about adverse effects for ICG at 1.0 mg/ml. Limitations of the study were that (a) 1 to 2 ml of ICG at 1.0 mg/ml (corresponding doses of 1 to 2 mg) were applied, (b) the ICG solution was

highly hypoosmotic (250 mOsm/kg) and (c) the dye exposure time was 30 to 150 seconds. In the clinical set-up of our study, using 0.1 ml of ICG at 2.5 and 1.0 mg/ml (corresponding to doses of 0.25 and 0.1 mg) we observed remarkably better results for RPE cell survival and morphological change. For ICG at 2.5 and 1.0 mg/ml, we found 96% and 100% cell survival after the 6 and 92% and 99% after the 72 hours follow-up, respectively. Thus, we agree with the findings of Kwok and associates [98–102] and suggest ICG doses up to 0.25 mg as being comparably safe to the retinal pigment epithelium at clinical relevant exposure and illumination times.

In addition, to clinical investigations in air- or gas-filled eyes, adverse effects were similarly noticed for ICG solutions injected into fluid-filled eyes. In fluid-filled eyes, ICG is further diluted by BSS, thus concentrations of the dye are lower than in air- or gas-filled eyes. Uemura et al. [161] noticed visual field defects in 4 of 7 eyes undergoing ICG-assisted ILM-peeling. In this study, 0.6 to 0.8 ml of ICG at 5.0 mg/ml was injected in the fluid-filled eye (corresponding to concentrations of approximately 0.75 to 1.0 mg/ml), and left in place for at least 3 minutes. Predominantly nasal visual field defects were observed when ICG-assisted peeling was performed, while no such damage was seen when ILM-peeling was conducted without ICG. Gandorfer et al. [43] reported that 0.2 to 1.0 ml of ICG at 5.0 mg/ml (corresponding to doses of 1 to 5 mg) injected into fluid-filled eyes may cause retinal damage even at brief exposure. Assuming a vitreous with a volume of around 4.0 ml, the dose of 1.0 to 5.0 mg ICG could have resulted in a concentration of 0.25 to 1.25 mg/ml in this study. The ultrastructural analyses of removed tissues indicated a cleavage plane not at the inner undulating aspect of the ILM, but within innermost retinal layers. No such defects were found when ILM-peeling was performed without ICG. Although no functional consequences were observed, the authors concluded that ICG may be responsible for the observed alterations. These observations were supported by several other studies, where possible alteration of the cleavage plane during ICG-assisted ILM-peeling was similarly noticed [60, 97, 125]. Nakamura et al. [125] described adverse effects when ILM removal assisted by 0.3 ml of ICG at 5.0 mg/ml (corresponding to a dose of 0.15 mg)

was performed in an animal model with primate eyes. The authors found fragments of glial tissues on excised ILM and a damaged vitreoretinal interface, which did not completely recover within 12 months. Kwok et al. [97] using ICG at 0.25 mg/ml also reported adhering cellular elements on the retinal surface of the ILM after ICG-assisted peeling in 10 patients. Although, visual and functional outcomes were less favourable compared to previous studies [24], they were still satisfying. In Haritoglou's study [60], 20 patients underwent ICG-assisted macular hole surgery with far lower ICG concentrations. Volumes of 0.2 to 0.5 ml of ICG at 0.5 mg/ml were injected into fluid-filled eyes (corresponding to 0.025 to 0.0625 mg/ml) and left in place for 1 minute. In seven patients postoperative visual defects occurred. ICG-stained specimens revealed cellular elements resembling the plasma membrane of Müller cells and other undetermined retinal structures adherent to the retinal side of the ILM in most of the 20 cases. Similar negative effects with poor visual and functional outcomes were again reported by the same study group some time later [48, 61]. As a consequence, the intraocular ICG application was stopped at their institution. These concerns against ICG were confirmed by Horio et al. [78]. In their study, 0.2 ml of ICG at 1.25 mg/ml was applied into fluid filled eyes (corresponding to 0.05 to 0.06 mg/ml) for 10 to 30 seconds. Their results indicated that even this brief exposure time affected the visual acuity. These results are contradicted by other clinical studies and our results of cell survival and morphological change in the clinical set-up. We tested diluted ICG solutions at 0.025 to 0.125 mg/ml, which would occure when 0.1 ml of ICG at 1.0 to 5.0 mg/ml is injected into a fluid-filled globe. In the clinical set-up, there was neither a decrease in cell viability nor an alteration of morphology of cultured RPE cells for every tested solution over the whole range of follow-up times.

Because the clinical reports were contradictory, concerns about retinal and especially RPE toxicity led to numerous laboratory studies [40, 72, 91, 123, 137, 158]. These experiments focussed on acute effects of high ICG concentrations and of exposure times far beyond clinical practice.

Rezai et al. [137] used ICG at 1.0, 5.0 and 20.0 mg/ml and incubated cultured retinal pigment epithelial cells for 30 minutes with the dye. All concentrations

induced a significant amount of apoptosis in RPE cells already after 24 hours follow-up. Additionally, Ho et al. [72] incubated cultured retinal pigment epithelial cells with 0.1 ml pf ICG at 0.001 to 5.0 mg/ml (corresponding to doses of 0.0001 to 0.5 mg) for 5 minutes up to 3 hours. The authors reported about cytotoxicity of indocyanine green in a dose- and time-dependent manner. Morphological changes, as well as reduction of mitchondrial dehydrogenase activity were found for ICG at 5.0 mg/ml after 10 minutes, for ICG at 1.0 mg/ml after 20 minutes and for ICG at 0.01 mg/ml after 3 hours. No adverse effects were noticed for the dye-free controls of corresponding osmolarities. The possible mechanism leading to ICG-related toxicity was similarly described by this study group some time later [73, 74]. Ho et al. [73, 74] postulated a $Na^+$-dependent ICG-uptake into retinal pigment epithelial cells for being responsible for cytotoxicity and increased photosensitising effects. Removal of sodium in both studies reduced the negative effects of ICG. Thus, according to these studies, ICG toxicity seems to be concentration and incubation time dependent; however, such incubation times of beyond 5 minutes are not clinically relevant and therefore outcomes cannot be fully transferred to clinical pratice. Some laboratory studies investigated the effect of different ICG concentrations at exposure times up to 5 minutes [91, 123, 158], which is closer to clinically-relevant dye incubation times. Kodjikian and associates [91] noticed reduced cell viability for ICG at 5.0 mg/ml, when RPE cell cultures were incubated for 5 minutes with this dye. No acute toxic effects were found for ICG at 0.5 mg/ml and below. These effects were observed even at a 3 minutes exposure in two other in vitro studies, one with cultured retinal glial cells [123] and the second similarly with cultured retinal pigment epithelial cells [40]. In the first study, ICG at 5.0 mg/ml caused increased expression of the apoptosis related gene *bcl-2,* as well as increased change in morphology in a concentration-dependent manner. Little adverse effects were shown for ICG at 0.5 mg/ml at this exposure time [123]. In the second study, cell viability decreased when ICG concentration was above 0.5 mg/ml [40]. In addition, Tokuda et al. [158] demonstrated retinal toxicity of 0.1 ml ICG 5.0 mg/ml (corresponding to dose of 0.5 mg) even at the exposure time of 1 minute in an *in vitro* model with isolated rat retinas. In this

study, severe structural damage in every retinal layer and a significantly higher relaese of LDH were observed when compared to the use of balanced salt solution.

In our study, using the solutions 1 to 3 (ICG) at concentrations decreasing from 5.0 to 1.0 mg/ml (corresponding to doses of 0.5, 0.25 and 0.1 mg), i.e., solutions which mimic the situation in air or gas-filled eyes, RPE damage was found only at incubation times beyond 5 minutes in the set-up without illumination. This damage was severe for ICG at 5.0 mg/ml, less severe for ICG at 2.5 mg/ml and little for ICG at 1.0 mg/ml. In this experimental setting, osmolarity of the solutions also seemed to play an important role for observed toxicity while dye-free controls showed similar rates of cell survival and morphologic change (data shown under chapter *6.5 Tables of the set-up without illumination*). The solutions 4 to 6 (ICG) up to a concentration of 0.125 mg/ml, mimicking the situation in fluid-filled eyes, showed no relevant adverse effects in this set-up. One reason might be the by far lower dye concentration of the solutions, another that osmolarity was in the physiological range of 295 – 315 mOsm/kg. No changes in cell viability or morphology were observed at all using the solutions 5 and 6 (ICG) at 0.0625 and 0.025 mg/ml, as well as their dye-free controls of corresponding osmolarities. Median cell survival was arround 100% and median morphological change never exceeded 1% in every set-up conducted (data not shown). For this reason, these four solutions were excluded from statistical analyses.

Recapitulating the results of our study, brief exposure of 1 minute or shorter to ICG at 1.0 mg/ml and below seems to cause no acute adverse effects to the retinal pigment epithelium even when illumination is present in clinically relevant limits (< 5 minutes). In contrast, care should be taken using higher concentrated hypoosmotic ICG solutions of 2.5 mg/ml and more at incubation times beyond 5 minutes.

### 4.1.2 Chronic effects

Recently, several clinical trials described persistence of ICG in the retina [5, 21, 71, 77, 124, 153, 164] and in other structures of the visual pathway [92,132]. In these studies, about 0.1 to 0.3 ml of ICG at 1.25 to 5.0 mg/ml was applied into the vitreous and left in place for time periods ranging from some seconds to a maximum of 5 minutes. Both, satisfying visual and functional outcomes as well as severe adverse effects were observed [19, 71, 124].

Weinberger et al. [164] noticed ICG persistence for 6 weeks after macular hole surgery. In their study, a small amount of ICG at 5.0 mg/ml was applied into the vitreous and left in place for 1 minute. No evidence for dye related toxicity could be determined in this study, as well as in a follow-up study with a mean of 8 months conducted by the same group [165]. The authors suggested that the persistent flourescence signal was due to the low metabolisation of ICG in the bradytrophic environment of the remaining adherent vitreous and internal limiting membrane. Horiguchi et al. [77] similarly reported persisting ICG flourescence for a mean of 2.7 months after macular hole surgery in 14 patients. In this study, ILM-staining was performed with 0.1 to 0.2 ml of ICG at 1.25 mg/ml, the dye was left in place for approximately 10 to 30 seconds and similarly no adverse effects due to ICG were observed. The investigators suggested ICG penetration and/or diffusion into the retina as being responsible for such long dye persistence. Two case reports by Ashikari et al. [5] showed ICG persistence at the fundus for even longer than 6 months. ICG at 5.0 mg/ml was used, left in place for only a few seconds, and no complications during surgical procedure or complications due to the dye were observed. Ciardella et al. [21] when using ICG at 2.5 mg/ml, demonstrated subfoveal flourescence persistence up to 8 months after uneventful macular hole surgery in 4 case reports. The authors' theory was an ICG uptake by the subfoveal retinal pigment epithelium, and this theory was also supported by two studies of Chang et al. [17, 18]. Another clinical investigation by Tadayoni et al. [153] reported beside flourescence persistence also on dye accumulation in the RPE and the optic nerve. The authors used infracyanine green (IFCG) at a concentration of

2.5 mg/ml and incubated the dye for 3 minutes. No adverse effects were noticed, but the investigators were concerned about the long-term safety of the dye [153]. These concerns were consistent to those of Kroemer et al. [92]. This study group worked with almost the same protocol (indocyanine green 2.5 mg/ml for 3 minutes), and similarly noticed dye accumulation in the area of the former macular hole, retinal axons and around the optic disc. According to the previous results, no functional implications and visual field defects were also observed in this study.

Da Mata et al. [25] recently published a follow-up study of their original clinical investigation with 114 patients receiving ICG-assisted macular hole surgery. In their original protocol from 1999, 0.2 to 0.4 ml of ICG at 5.0 mg/ml was used and was left in place for 3 to 5 minutes. Shortly thereafter, they showed that adaquate ILM-staining could also be achieved with 0.05 to 0.1 ml of ICG at 5.0 mg/ml incubated for 30 seconds. The results of the study with a mean follow-up of 26 months showed excellent anatomic and visual results, without the evidence of dye-related toxicity. To our knowledge, this is the only clinical study with such long follow-up times reporting about no adverse effects of ICG. However, these favourable outcomes may be due to the change of the staining technique at an early stage, using lower ICG doses and shorter incubation times.

In contrast to all these studies noticing dye persistence without dye-related toxicity, Cheng et al. [19] showed chronic toxic effects of residual ICG after macular hole surgery in case reports of 6 patients. In this study, 1.0 to 1.5 ml of ICG at 2.5 mg/ml (corresponding to doses of 2.5 to 3.75 mg) were instilled in the eyes and left in place for 1 to 5 minutes. All eyes had residual ICG left behind at the end of surgery, independent of exposure time. Patients were followed up to 1 year. Circular foveal RPE atrophy larger than the area of the macular hole and surrounding cuff was noted in 4 of 5 cases with preoperative macular hole. The first retinal damage was already seen 1 month after surgery [19]. These results are certainly concerning, but the clinical relevance of this study is limited because the number of patients was small and such high volumes (usually 0.1 to 0.2 ml) as well as such long exposure times (usually 10

to 30 seconds) usually are not used in present clinical work with ICG. More closer to present macular hole surgery were two other clinical studies reporting on delayed toxicity of ICG [71, 126]. Nakamura et al. [126] reported on ICG persistence for 7.3 months in average and the occurance of peripheral visual field defects in 2 of 34 eyes which underwent ICG-assisted macular hole surgery. In this study, 0.3 ml of ICG at 5.0 mg/ml (corresponding to a dose of 1.5 mg) were applied into the vitreous and removed immediately by aspiration. In the eyes with visual field defects, ICG persisted at the bottom of the former macular hole and led to retinal pigment epithelial (RPE) atrophy. These findings match with the case report of Hirata et al. [71], where accidental subretinal migration of ICG leading to RPE atrophy was described. In Hirata's study, the same volume of solution and the same concentration of ICG were used. Accidental subretinal migration is a dreaded complication during macular hole surgery [13]. During these situations, ICG has direct access to subretinal structures such as the retinal pigment epithelium. Consequently, RPE damage in context with ICG persistence demanded the further investigation of delayed toxicity of ICG solutions, and this has been conducted in numerous experimental studies [31, 40, 88, 91, 107, 113] as well as by our study group.

In our study with cultured ARPE-19 cells, we had follow-up observations at 6, 24 and 72 hours after ICG treatment. Although all these above mentioned clinical trials didn't show toxic effects of ICG at the follow-up time of 3 days, there were several animal and *in vitro* studies reporting about negative effects even after some days and even for low concentrated ICG solutions [31,79]. A concentration-dependent delayed ICG toxicity was shown by Kawaji et al. [88] in an animal model. These investigators injected 0.05 ml of ICG at concentrations of 25.0, 5.0 and 0.5 mg/ml (corresponding to doses of 1.25, 0.25 and 0.025 mg), as well as BSS into the subretinal space of rabbit eyes. Histological evaluation was performed up to 28 days after injection of ICG at 5.0 mg/ml, as well as at 14 days after injection of the other solutions. Severe RPE damage was shown for ICG 25.0 mg/ml after 14 days. In eyes with ICG 5.0 mg/ml the photoreceptors began disappearing within 3 days after the injection and over time showed the development of retinal atrophy. In contrast, no

damage to retinal layers was shown for ICG at 0.5 mg/ml and BSS 14 days after injection. Similar results were observed in another animal study by Maia et al. [113], where 0.02 ml of ICG at 5.0 mg/ml (corresponding to a dose of 0.1 mg) was injected into the subretinal space and was followed by 7 minutes of endolight illumination at maximum intensity or without light exposure. Animals were followed up to 14 days. First damage to retinal layers was already noticed on the first day after surgery, showing altered photoreceptor segments and degeneration of the outer nuclear layer. Until day 7, light exposure seemed to enforce the damaging potential. On day 14, all retinal layers were severely altered independent of light exposure. In contrast, no such effects were noticed, when 0.3 ml of ICG at 5.0 mg/ml (corresponding to a dose of 1.5 mg) were applied onto the retinal surface, left in place for 1 minute and was followed by 7 minutes of illumination. Thus, delayed toxicity of commonly used ICG solutions was demonstrated by both studies, however, exposure times of up to 28 days are not clinically relevant. More closer to the exposure times in clinical practice, Lee at al. [107] found ICG to have toxic effects at concentrations of 1.25 mg/ml or higher when injected into the subretinal space of rabbit eyes. In this study, 0.2 to 0.3 ml of ICG up to 5.0 mg/ml (corresponding to doses up to 1.5 mg) was used and left in place for only 1 minute. Eyes were followed up to 4 weeks. After 3 days significant degenerative changes were found in the retinal pigment epithelial cells, the photoreceptors and the outer nuclear layer when ICG 1.25 mg/ml (dose 0.25 mg) or higher was injected. In the course of time, the level of cellular damage got even higher, leading to focal RPE loss and complete destruction of the outer sensory retina. No significant changes were found for ICG 0.6 mg/ml (dose 0.12 mg) at all follow-up times.

The results of the aforementioned studies showed that damage to the retinal pigment epithelium was present already in the first 3 days after surgery, when using ICG 1.25 mg/ml or higher. The results of these studies are in accordance to ours, since prolonged exposure of the dye at certain concentrations clearly induces damage. However, these situations do not mimic clinical practice and can be therefore questioned for their clinical relevance. Nevertheless, an important message that can be derived from these reports is that the dye needs

to be removed after application. In our experimental set-ups mimicking air- or gas-filled eyes, 0.1 ml of ICG at 1.0, 2.5 and 5.0 mg/ml (corresponding to doses of 0.01, 0.25 and 0.5 mg) was applied to the cells. We similarly noticed delayed damage to our RPE cell cultures when using similar doses of 0.5 and 0.25 mg ICG. Although, the main damage already occured after 6 hours, there was a tendency towards decreasing cell survival and increasing change in morphology with prolongation of the follow-up time in every of the conducted set-ups (data shown under chapter *6. Figures and tables*). We similarly found delayed toxicity to a little degree for ICG at 1.0 mg/ml (dose 0.1 mg), which is contradictory to the results of Lee et al [107].

Additional to our findings, delayed toxicity of ICG even at lower concentrations has also been reported [31, 79]. Hsu et al. [79] demonstrated in their HRPE cell culture study that ICG at 0.1 mg/ml still significantly inhibited cell growth at an incubation time of 72 hours. Enaida et al. [31] even noticed functional damage to the retina without any apparent morphological change for ICG 0.025 mg/ml. In their study with rat eyes, 0.05 ml of ICG at 0.025 to 25.0 mg/ml (corresponding to doses of 0.00125 to 1.25 mg) were injected into the vitreous. Retinal toxicity was histologically assesed by light microscopy on day 10, and retinal function was evaluated by electroretinography (ERG) after 10 days, as well as after 2 months. For the higher concentrated ICG solutions, severe retinal damage with histologically detectable alterations of retinal tissues could be determined. For the lower concentrated ICG solutions at 0.25 and 0.025 mg/ml, no morphological damage, but decreased amplitudes of dark-adapted a- and b-waves in ERGs were occured 10 days, and there was no recovery within 2 months. However, there are some limitations of these studies, as (a) no irrigation was performed and (b) no clinical relevant incubation times were adhered to.

In contrast to these reports when using ICG solutions at 0.025 to 0.125 mg/ml (corresponding to doses of 0.0025 to 0.0125 mg) no chronic toxic effects were found in every set-up and at all follow-up times conducted in our study. To summarise, changes in cell survival and morphology were more prominent for ICG 1.0 mg/ml and above when comparing the 6 hour time point to the 24 hour

time point, than thereafter. This could be explained by the finding that the apoptosis-related gene *bax* and the cell cycle arrest protein P21 have peak values at 16-24 hours after ICG incubation [169]. In conclusion, the results of our study suggest that there is foremost an acute toxic effect of ICG at concentrations above 1.0 mg/ml rather than a chronic toxicity of ICG remnants after dye removal.

## 4.2 Trypan blue

Trypan blue (TB) is a vital stain that has been used as an intraoperative stain to facilitate anterior segment surgery [117, 128], particularly in the staining of the anterior capsule in white mature cataract [83, 117]. Recently, clinical trials have been published introducing trypan blue as a useful tool to visualise the internal limiting membrane (ILM) as well as epiretinal membranes (ERMs) in macular hole surgeries [7, 34, 65, 103, 110, 152]. One clinical study even reported about favourable visual results using trypan blue, when outcomes of ICG-assisted peeling of the internal litimiting membrane were compared to those with TB [108].

### 4.2.1 Acute effects

In clinical practice, 0.5 ml TB of 0.6 mg/ml (corresponding to a dose of 0.3 mg) is commonly used and left in place for 1-2 minutes. Favourable anatomical and functional results were reported in the majority of these studies [7, 34, 65, 108, 110]. Teba et al. [154] previously reported about successful ILM-staining by even higher concentrated TB of 2.0 mg/ml without any observed dye-related toxicity. Thus, TB seems to be a safe alternative to ICG and consequently has the potential to replace it in the latter clinical practice.

This initial enthusiasm, using trypan blue, was encouraged by several experimental studies verifying the safety of the dye [56, 82, 151]. Jackson and associates [82] demonstrated the safety of 0.1 ml of TB 0.125 to 2.0 mg/ml (corresponding to doses of 0.0125 to 0.2 mg) on the retinal pigment epithelium

at exposure times up to 30 minutes in a cell culture study. In contrast, Rezai et al. [138] noticed TB-induced apoptosis in cultured human RPE cells at all concentrations (0.5 to 5.0 mg/ml) when the dyes were applied to the cells for up to 30 minutes. However, such long incubation times are not used in clinical practice. Closer to clinical relevant exposure times, Stalmans et al. [151] demonstrated the safety of trypan blue by noticing no increased cell death, when cultured human RPE cells were exposed for 5 minutes to TB of 0.6 and even 3.0 mg/ml. A *post mortem* study with porcine eyes by our study group similarly found no adverse effects when 0.5 ml of TB 1.5 mg/ml (corresponding to a dose of 0.75 mg) was applied and left in place for 1 minute followed by 10 minutes of endolight illumination [56].

In contrast to these publications Kwok et al. [104] reported about the alteration of cell viability and gene expression in an *in vitro* model with cultured human ARPE-19 cells when using TB at concentrations of 0.6 mg/ml and higher. In addition, a recently published study by Lüke et al. [112] raised our interest, in which the authors reported about the loss of the b-wave in ERG after exposure of the bovine retina to TB 1.5 mg/ml for 15 seconds or longer. This effect was only partly reversible within the expected recovery time. To our knowledge, this was the first report about functional implications of the retina after brief exposure to TB. Due to strongly controversial outcomes further investigation was required.

Our in vitro study with cultured ARPE-19 cells is in agreement with studies reporting about TB-related toxicity, while acute toxic effects of trypan blue occured in a dose and time dependent manner when incubation time exceeded 10 minutes. In our set-up without illumination we applied 0.1 ml of TB at 1.5 and 0.0375 mg/ml (corresponding to doses of 0.15 and 0.00375 mg) to our cell cultures for 1 to 20 minutes. Median cell survival for TB at 1.5 mg/ml after the 6 hours follow-up, was 100% after 1 minute, similarly 100% after 5 minutes, 62% after 10 minutes and 0% after 20 minutes incubation time, respectively. Median morphological change for TB at 1.5 mg/ml after the 6 hours follow-up, was 1% after 1 minute, 1.5% after 5 minutes, 63% after 10 minutes and 100% after 20 minutes incubation time, respectively. No relevant acute toxic effects were

found for TB at 1.5 mg/ml when incubated up to 5 minutes with or without illumination, as well as for TB at 0.0375 mg/ml in every set-up conducted. To summarise, although our study has some limitations (cell culture study and long exposure times) we could demonstrate that trypan blue in clinical relevant doses can cause acute damage to the retinal pigment epithelium under certain conditions. Thus, we recommend exposure times below 5 minutes for TB at 1.5 mg/ml or the application of TB into fluid-filled eyes.

### 4.2.2 Chronic effects

Trypan blue (TB) has been used with a long history of safety in anterior segment surgery [128]. However, the toxic potential of TB is well known [20, 30]. Although some experimental studies already reported about possible chronic toxicity of different TB solutions to retinal tissues [91,162], only few clinical trials on the long-term safety of TB have yet been published [34, 65, 110].

In the study of Feron et al. [34] 10 patients underwent TB-assisted ERM-peeling during surgery for proliferative vitreoretinopathy. TB at 0.6 mg/ml was used and left in place for 1 minute. No adverse reactions related to the use of the dye were observed for up to 3 months after surgery. These results are consistent to the findings of Li et al. [110] and Haritoglou et al. [65], where ILM- as well as macular pucker removal were performed and the average follow-up times were 4.4 and 5.8 months, respectively. Both studies used TB at 0.6 mg/ml, in Li's study dye exposure time was 2 minutes and in that of Haritoglou's 1 minute. No toxic effects of TB were noticed in both clinical trials. Both authors reported about the facilitated removal of the membranes and a satisfying improvement of visual acuity. Another remarkable aspect of these studies was that outcomes of TB-assisted peeling were not significantly different from that without the use of the dye. Thus according to the authors, the main necessity of TB may be the facilitation of the surgeons work, allowing a quicker and safer removal of epiretinal membranes without any evidence of dye toxicity. As shown in these clinical investigations the clinical practice of using TB at 0.6 mg/ml seems to

safe even at follow-up times of up to around 6 months. The long-term safety of TB 0.6 mg/ml was similarly shown in Veckeneer's rabbit model [162]. Veckeneer et al. evaluated in their study the safety of TB at 0.6 and 2.0 mg/ml on retinal tissues up to 1 month. While marked damage was found for TB at 2.0 mg/ml, no abnormalities were noticed for TB at 0.6 mg/ml. However, some limitations of this study were that (a) the injected volume of 0.1 ml was much lower and therefore not consistent with the volume (0.5 ml) applied in clinical practice, (b) the dyes were injected into gas-filled eyes and (c) no irrigation of the dye was performed. Additionally, Kodjikian et al. [91] tested the chronic toxic effects of various TB at 0.5 to 5.0 mg/ml in a cell culture model with retinal pigment epithelial cells. In this study, cells were incubated for 6 days with TB solutions. Chronic cytotoxic effects of TB were observed at all TB concentrations, as morphological changes and decrease in cell viability occurred. Thus, according to this study, care should be taken even when TB solutions of low concentration are applied to the retina. However, the limitation of this study was that TB solutions were left in place for 6 days without irrigation. As already noted for Veckeneer's study, this aspect doesn't match with clinical practice where dye removal and irrigation is performed within 1 minute after application. Thus remnant TB doses in clinics are by far lower than in these studies. Closer to clinical practice was the experimental procedure performed in the cell culture study of Gale et al. [40], who found no evidence for delayed TB toxicity, when TB 0.003 mg/ml (1:500 dilution of the TB 1.5 mg/ml stock solution) was incubated for 2, 24 and 72 hours.

Regarding the results of our chronic toxicity testing using TB at 1.5 and 0.0375 mg/ml, there was rather an acute than a chronic toxic effect for TB at 1.5 mg/ml applied to the cultured RPE cells. Moreover, acute as well as chronic adverse effects were only present when TB at 1.5 mg/ml was incubated for 10 minutes or longer without illumination. Significant decrease in cell viability and alteration of morphology depending on the follow-up time ($p < 0.0001$ for both observations) were noticed for TB 1.5 mg/ml when compared to TB 0.0375 mg/ml in our set-up without illumination. Neither for TB at 1.5 mg/ml applied for 5 minutes in combination with endolight illumination, nor for the brief exposure

to this dye followed by 5 minutes illumination (clinical set-up) were chronic toxic effects observed at follow-up times up to 72 hours. For TB at 0.0375 mg/ml no chronic toxicity was noticed in every conducted set-up and at the whole range of follow-up times. Thus, in our opinion brief exposure to TB up to 1.5 mg/ml followed by illumination in clinical relevant limits seems to be safe to the retinal pigment epithelium at a short follow-up. Nevertheless, further long-term clinical studies are required in this context.

## 4.3 Influence of illumination

The technique of ILM-peeling and ERM-removal in macular hole surgery requires both staining with vital stains and illumination with vitrectomy endolights for fast and safe accomplishment. However, poor outcome of dye-assisted membrane removal in macular hole surgery reported by several authors and well-known photosensitising effects of vital stains, especially of ICG, have led to growing concerns. As responsible parameters, dye properties of ICG [26, 122], dye concentration [62, 105, 171], distance of the endolight pipe and duration of light exposure [39, 120], wavelength spectra emitted by vitrectomy endolights [39, 62, 120], as well as the type of the light source [66] were previously reported. These concerns consequently required further investigation of potential phototoxic effects of vital stains used for ILM-peeling and ERM-removal in macular hole surgery.

### 4.3.1 Indocyanine green and illumination

The indocyanine green (ICG) dye has a complex molecular structure with both hydrophilic and lipophilic properties [26, 122]. Depending on its concentration and the nature of the solvent, ICG tends to form monomers at lower concentrations and aggregates at higher concentrations [171]. Dissolution in physiologic saline solution also favours aggregation, although dissolution to a low concentration may favour monomers [105]. The maximum absorption spectrum is 785 nm for monomers and 690 nm for aggregates [26, 105, 122].

Similar results were noticed recently by Haritoglou et al. [62] in a study investigating light-absorbing properties of different ICG solutions. The authors also found two absorption maxima, one at approximately 700 nm and a second one at 780 nm. Thus in clinical practice, there is an overlap between the absorption maxima of ICG and the emission curve of the light source (380 – 760 nm), resulting in a possible photosensitising effect, especially at higher ICG concentrations. In addition, to this study, the effects of short (around 400 nm) as well as of longer wavelengths (beyond 760 nm) in combination with ICG were demonstrated in some studies [10, 12, 86]. As shown in the investigation by Kadonosono and associates [86] for the short wavelenghts (400 – 450 nm) emitted by the light source, the absorption coefficients of ICG were not greater than those of balanced salt solution alone, indicating that there is no additional phototoxicity by short wavelength light using ICG. In contrast, two other studies noticed increased diode laser uptake (absorption maximum at 810 nm) of retinal tissues after ICG-assisted internal limiting membrane removal in macular hole surgery [10, 12]. The authors concluded that protein binding of residual ICG led to decreased formation of polymers and shifted absorption beyond 785 nm toward a maximum of 810 nm. Similar observations were previously reported in other studies [26, 105, 122].

Because interaction of ICG and illumination is obvious, numerous experimental studies in this context were conducted, including *post mortem* studies [44, 55, 64] and cell culture studies [40, 72, 81, 82, 127, 145, 169].

Gandorfer et al. [44] demonstrated in their *ex vivo* model with 10 human donor eyes (eyes enucleated 16 to 30 hours after death), that exposure of the ICG-stained ILM to wavelengths beyond 620 nm resulted in severe damage to the inner retina, including loss of ILM, cellular disorganization and fragmentation of the cytoplasm. In this study, 0.05 ml of ICG 0.5 mg/ml (corresponding to a dose of 0.025 mg) was applied for 1 minute followed by 3 minutes of illumination with wavelengths of 380 to 760 nm. ICG in combination with wavelengths of 380 to 620 nm disclosed rupture of Müller cells with detachment of the ILM, but no other cellular disorganization. Eyes subjected to illumination only showed no such abnormalities. These results are consistent to findings of Haritoglou et al.

[64] using ICG at 0.5 mg/ml diluted with glucose 5% in combination with endolight illumination (380 – 760 nm) in a *post mortem* study one year later. The investigators reported about disorganisation of the inner retina and complete loss of ILM after application of the dye and illumination. No abnormalities were found without illumination and in unstained control specimens. Contrary to these outcomes, another *post mortem* study with porcine eyes (eyes processed within 5 hours after death) conducting similar experiments noticed no alteration of retinal structures even at higher ICG concentrations [55]. In this study, 0.5 ml of ICG at 0.1 to 2.0 mg/ml (corresponding to doses of 0.05 to 1.0 mg) were applied and left in place for either 30 or 60 seconds. After irrigation, the posterior pole was irradiated at maximum power for 3 minutes by a standard light pipe. Although differences between the species may contribute to these contradictory results, according to the authors it was conceivable that the postmortem time and the vitality of the tissue influenced the outcome in this *ex vivo* system.

In numerous cell culture studies testing the effects of ICG with illumination, mainly performed with cultured retinal pigment epithelial cells, phototoxic effects have been noticed [72, 81, 127, 145, 169].

Sippy et al. [145] reported about negative effects of ICG treatment combined with illumination. In this study, cultured human RPE cells were exposed for 20 minutes to ICG at 1.0 mg/ml followed by 10 minutes of endolight illumination. One observed effect was decreased mitochondrial enzyme activity, compared to cells exposed only to BSS and illumination. Paradoxically, no alterations of cellular morphology or ultrastructure were seen. In Ho's study [72], cultured retinal pigment epithelial cells were exposed to ICG at 2.5 mg/ml either dissolved in BSS or in sodium free BSS for 2 minutes. Afterwards, the cells were irradiated with a light beam for 40 minutes. The authors found photoreactive changes in RPE cells. These changes included cell shrinkage, cell death, pyknotic nuclei, reduced viability as well as reduced mitochondrial dehydrogenase activity. These effects were less severe when ICG was dissolved in sodium-free BSS. In another study by the same group, $Na^+$-dependent ICG uptake in RPE cells was reported to be responsible for such

observations [74]. In the same context of photoreactive changes in the RPE, Yam and associates [169] reported about concentration-dependent toxicity of ICG solutions in combination with acute endolight illumination on cultured retinal pigment epithelial cells. In this study, ICG at 0.25 and 2.5 mg/ml were applied to cultured ARPE-19 cells for 1 minute. After isotonic rinsing, the cells were irradiated with a light beam (400 – 800 nm) at a distance of 10 mm for 15 minutes. Cell viability decreased to 40% for ICG 2.5 and to 80% for ICG 0.25 mg/ml, respectively. The authors similarly noticed an upregulation of the apoptosis related genes *p63* and *bax,* as well as the gene for the cell cycle arrest protein P21. Contrary to these findings, Iriyama et al. [81] noticed no affection of cell viability of cultured retinal glial cells (RGC) when using a similar protocol. This aspect is remarkable, while another *in vitro* study using both cell lines suggested cultured RPE cells to be more resistant to light exposure after brief incubation to ICG than cultured RGC cells [82]. The different sensitivity of retinal cells was similarly shown by the study of Narayanan et al. [127], comparing ICG effects accompanied by 10 minutes illumination on viability of cultured human RPE cells and rat neurosensory retinal cells (R28). In this study, ICG caused a significant decrease in mitochondrial dehydrogenase activity in R28 and ARPE-19 cells. ICG without light exposure did not decrease mitochondrial dehydrogenase activity. In both cell lines, [H]thymidine incorporation was increased when treated with ICG with or without light indicating increased DNA synthesis. Surprisingly, R28 cells did not show any significant decrease in cell viability. Closer to clinically relevant illumination times, Gale et al. [40] tested the efffects of 0.75 ml ICG at 0.5 and 2.5 mg/ml (corresponding to doses of 0.375 and 1.875 mg) in combination with illumination on cultured RPE cells. Each solution was applied to the cells for 5 minutes coupled with 1 minute of intense fiberoptic illumination. Although there was a reduction of cell viability for both dyes, no significant differences were noticed when results were compared to those without the use of illumination. Therefore, according to this study, there seems to be a toxic effect of ICG independent of additional light exposure.

Regarding these contradictory clinical and experimental results, we deciced to test the effects of ICG combined with illumination on the retinal pigment epithelium in our *in vitro* study. In the set-up with illumination, cultured ARPE-19 cells were exposed to ICG at 0.025 to 5.0 mg/ml as well as their dye-free controls of corresponding osmolarities for either 1 or 5 minutes coupled with illumination by a standard halogen vitrectomy endolight pipe (380 – 760 nm) from a distance of 8 mm [44, 55]. Phototoxicity was not present with the dye-free controls, as well as with the diluted ICG solutions at 0.125 mg/ml and below used to mimic the situation that occurs in fluid-filled eyes. Severely decreased cell viability and increase of morphological change were found for ICG at 5.0 mg/ml at both incubation & illumination times. After the follow-up time of 72 hours, we noticed median cell survival of 85% after 1 and 66% after 5 minutes of incubation & illumination as well as median morphologic change of 15% after 1 and 41% after 5 minutes. Similarly, there was decreased median cell survival and median morphological change with growing incubation & illumination times to a lesser degree for ICG at 2.5 mg/ml (cell survival > 89%, morphologic change < 12%) and to a small degree for ICG at 1.0 mg/ml (cell survival > 98%, morphologic change < 3%).

To summarise, phototoxicity of ICG is concentration and illumination time dependent, when ICG is used in concentrations above 1.0 mg/ml, mimicking air- or gas-filled eyes at illumination times up to 5 minutes. ICG below 1.0 mg/ml coupled with illumination of 1 minute or shorter appears to be safe in our *in vitro* model. Concerning the type of illumination used during ICG-assisted macular hole surgery, one study with *post mortem* eyes noticed more favourable outcomes for the xenon light source compared to the halogen light source [66]. Thus, in our opinion this paramater requires further investigation.

### 4.3.2 Trypan blue and illumination

After phototoxicity of ICG was demonstrated in numerous studies [72, 81, 82, 169], concerns against the use of vital stains were growing once again. Consequently, many investigators also tested potential phototoxic effects of

different trypan blue dyes [40, 56, 82, 116, 126], although no clinical evidence of adverse effects of trypan blue (TB) in combination with vitrectomy endolight illumination were reported yet.

The cell culture study by Gale et al. [40] tried to mimic the lighting conditions within the globe during vitreoretinal surgery in one of the conducted experiments. Therefore, the investigators incubated TB at 1.5 mg/ml for 5 minutes coupled by 1 minute of intense fiberoptic illumination in a distance of 3 to 4 mm from the cells. The authors reported no significant alteration of cell viability independent of light exposure. Similar results were found in our experiments using illumination, although some parameters differed from that of Gale's study. In our set-up with illumination, we incubated TB at 1.5 mg/ml for 1 and 5 minutes in combination with vitrectomy endolight illumination at maximum intensity at a distance of 8 mm. Although the light pipe distance was double, our maximum illumination time was five times longer than in Gale's study. Cell viability in our experiments was not significantly altered, median cell survival rates were allways near 100% independent of the length of illumination. In contrast, Narayanan et al. [126] demonstrated potential phototoxic effects of TB at 1.0 mg/ml on rat neurosensory retinal cells. TB at 1.0 mg/ml incubated for 2 minutes and followed by light exposure of 5 and 10 minutes respectively led to significant reduction of mitochondrial dehydrogenase activity. No such effects were noticed for TB at 0.125 to 0.5 mg/ml. In this study, similar experiments were accomplished with ARPE-19 and no negative effects of the TB dyes were observed. The results regarding effects on ARPE-19 cells are in agreement with outcomes of our study and the study of Grisanti et al. [56]. In the clinical set-up of our study, where TB incubation time was 1 minute followed by 5 minutes of illumination, we also didn't notice any adverse effects to our RPE cell cultures. Median cell curvival was minimally below 100% and median morphologic change near 1%. Our results were confirmed by the study of Grisanti et al. with porcine eyes [56]. The authors similarly used TB at 1.5 mg/ml, incubated the dye for 1 minute, followed by even 10 minutes of illumination at maximum power by the same light pipe and in the same distance. No morphologically detectable acute adverse effects were noticed.

To summarise the results of previous studies and the results obtained in our study, TB at 1.5 mg/ml at incubation times of 5 minutes or below followed by endolight illumination up to even 10 minutes appears to be safe to cultured RPE cells. No adverse effects at all were noticed for our diluted TB solution at 0.0375 mg/ml in both set-ups where illumination occurred. However, the effects of TB and endolight illumination on other retinal tissues should be further investigated.

## 4.4 Influence of osmolarity

The influence of hypoosmotic solvent solutions for indocyanine green (ICG) is a controversially discussed topic when using ICG preparations [40, 62, 150]. ICG powder is primarily not soluble in balanced salt solution (BSS), but only in distilled aqueous solution (ICG solvent) at very low osmolarity. Thus, ICG solutions frequently used in macular hole surgery for air- or gas-filled eyes, ICG 1.0 to 5.0 mg/ml, are often hypoosmotic. No such problems occur if ICG is further diluted by BSS when applied into fluid-filled eyes. Osmolarities of these ICG dyes, as well as of the common used TB dyes (0.6 to 1.5 mg/ml) are within physiological limits (295 to 315 mOsm/kg). Furthermore, it is important to notice that there is still no standardised dilution protocol for ICG solutions and that there are remarkable differences in osmolarity of similar concentrated ICG solutions depending on the proportions of solvent solution and BSS in the final ICG preparation. For example, the osmolarity of ICG at 1.0 mg/ml ranged from around 240 [40, 151] to 299 mOsm/kg [72] in different investigations. Stalmans et al. [150] demonstrated the adverse effects of hypoosmotic ICG and solvent solutions in a study with RPE cells. The outcomes of cell suvival using ICG at 1.0 mg/ml of 248 mOsm/kg as well as the dye-free control solution of 247 mOsm/kg, were compared to outcomes when using BSS (311 mOsm/kg) and other isoosmotic solutions. The investigators noticed a significantly decreased cell viability for ICG and its dye-free control solution compared to the other solutions after an exposure time of 5 minutes. No statistically significant difference was found comparing this two hypoosmotic solutions ($p$ = 0.78). In contrast to these findings, Gale et al. [40] reported significant differences in the

outcomes of cell survival, when the hypoosmotic ICG at 1.0 mg/ml (240 mOsm/kg) was compared to the similarly hypoosmotic dye-free control (242 mOsm/kg) at the incubation time of 3 minutes. They reported about 103.7% cell survival for the dye-free solution compared to 89.9% for ICG at 1.0 mg/ml. These differences became even more prominent when ICG at 2.5 and 5.0 mg/ml were compared to their dye-free controls.

In our study, osmolarities were 290, 277 and 242 mOsm/kg for ICG at 1.0, 2.5 and 5.0 mg/ml, respectively. For the diluted ICG at 0.025, 0.0625 and 0.125 mg/ml we measured 307, 303 and 297 mOsm/kg, respectively. Similarly, the osmolarities of both TB at 0.0375 and 1.5 mg/ml (304 and 297 mOsm/kg) were found to lie within physiologic limits. To test the effects of osmolarity without interfering parameters such as illumination, we performed our *in vitro* experiments with ICG solutions and BSS/solvent mixes of corresponding osmolarities at incubation times up to 20 minutes in the dark. Although we noticed statistically significant differences in cell survival ($p$ = 0.0057) and morphologic change ($p$ = 0.0014) if the solutions contained ICG or not, the differences between the outcomes were small and clinically not relevant. Median cell survival for ICG at 5.0 mg/ml, after the follow-up time of 72 hours, were 93% after 5, 18% after 10 and even 0% after 20 minutes incubation time. For the dye-free control, after the same follow-up time, 93% after 5, 22% after 10 and similarly 0% after 20 minutes were noticed. The differences became even smaller when outcomes of ICG at 2.5 and 1.0 mg/ml were compared to their dye-free controls. As expected, there were no adverse effects for isoosmotic ICG solutions in our study, even after the maximum incubation time of 20 minutes.

To summarise, there is obviously an effect of osmolarity in higher concentrated ICG solutions on survival and morphology of cultured RPE cells. Isoosmotic ICG solutions below 1.0 mg/ml appeared to be safe at incubation times up to 20 minutes without the use of illumination. Hypoosmotic ICG solutions, as used in air- or gas-filled eyes, only seemed to be safe when incubation times were kept below 5 minutes and no illumination was used. In our opinion further investigations and a standardised dilution protocol are required.

# 5. Summary and conclusions

## 5.1 Summary

In the set-up without illumination, concentration, osmolarity and exposure time dependent toxicity for indocyanine green (ICG) solutions were apparent. Especially the hypo-osmotic ICG solutions from 1.0 to 5.0 mg/ml as well as their dye-free controls showed prominent cell damage at exposure times of 10 minutes and longer. Trypan blue (TB) of 1.5 mg/ml incubated for at least 10 minutes similarly showed unfavourable results as no viable cells were observed after 20 minutes. The iso-osmotic ICG solutions below 1.0 mg/ml, their dye-free controls as well as TB of 0.0375 mg/ml showed no toxic effects to cultured ARPE-19 cells even at the maximum incubation time of 20 minutes.

In the second set-up using illumination, concentration and incubation & illumination time dependent toxicity for ICG solutions were demonstrated. Especially, ICG solutions of 2.5 and 5.0 mg/ml showed prominent damage to the RPE cells with growing incubation & illumination time respectively. Negligible to minimal influence of vitrectomy endolight illumination was noticed for ICG solutions below 1.0 mg/ml, the ICG-free controls and both TB solutions.

In the clinical-set-up, where the exposure and the illumination time were standardised, only ICG concentration dependent cell damage was noticed. ICG solutions of 1.0 mg/ml or below as well as both TB dyes seemed to be safe to the cultured RPE cells at the exposure time of 1 minute and the total illumination time of 5 minutes, as used in this set-up.

Regarding the question of acute and chronic toxicity of both dyes, the most prominent decrease in cell viability and increase of morphological change were already noticed after the follow-up time of 6 hours. Indeed, a further damage after the follow-up time of 24 hours was seen but the differences to the results of the maximum follow-up time of 72 hours were less prominent and clinically not relevant.

## 5.2 Conclusions and recommendations

Our conclusions are, that indocyanine green and trypan blue are without any doubt important and useful dyes for macular hole surgery. However, surgeons working with these vital stains should note the following aspects for safe accomplishment of ILM-peeling or ERM-removal (shown in *figure 65*).

The toxic effects of indocyanine green to the retinal pigment epithelium are widespread and complex. The results of our experimental study showed that ICG toxicity to the retinal pigment epithelium is dependent on the dye concentration, the osmolarity of the solvent solutions, as well as on the lengths of dye exposure time and of vitrectomy endolight illumination time. For this reason, we recommend the use of isoosmolar ICG solutions (osmolarity ≥ 290 mOsm/kg) with a concentration of 1.0 mg/ml or less. In addition, we recommend to keep the exposure and the illumination times as short as possible, and to make sure that the dye is removed as thorough as possible by irrigation or aspiration, respectively. An incubation time of 1 minute or below followed by an illumination time of 5 minutes or less appeared to be safe in our *in vitro* study, when ICG at concentrations 1.0 mg/ml or less was used.

The toxic effect of trypan blue to the retinal pigment epithelium was, according to our experiments, only dependent on the dye concentration when the incubation time was prolonged. Harmful effects of TB at 1.5 mg/ml to our cell cultures were only found when the incubation time exceeded 10 minutes. In contrast, no toxicity at all could be demonstrated for TB at 0.0375 mg/ml. However, such long incubation does not have clinical relevance. As expected, no osmolarity dependent toxicity was found for both isoosmotic TB solutions. In addition, no harmful effects of trypan blue were found in every set-up including the use of vitrectomy endolight illumination. However, it is important to note that our maximum illumination time was 5 minutes and therefore no clear statement on TB related toxicity beyond these limits could be made in our study. Therefore, we conclude that TB at 1.5 mg/ml applied for 1 minute or less is a safe vital stain to the retinal pigment epithelium without phototoxicity at short illumination times in our *in vitro* investigation.

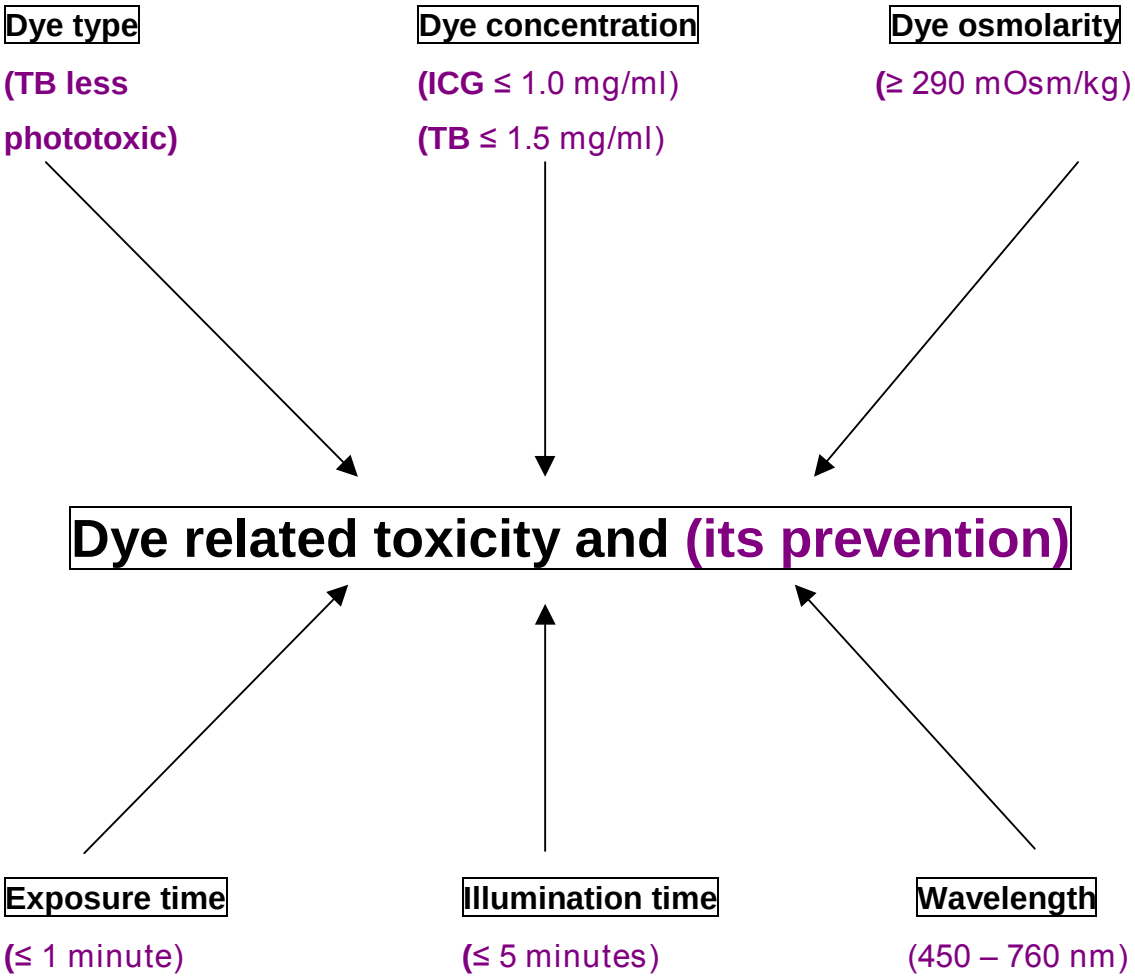



***Figure 65:*** Aspects leading to dye related toxicity and its **prevention** in macular hole surgery

## 5.3 Strengths and weaknesses of the study

The main strength of our study is its diversity of tested influences on the retinal pigment epithelium, when using the dyes ICG and TB. We tested the influences of different dyes, concentrations, osmolarities, exposure times, follow-up times and endolight illumination on survival and morphology of cultured ARPE-19 cells. The influences were tested either singly, for example our set-up without illumination with prolonged incubation times, or in combination in three different set-ups. Moreover, in our clinical set-up, we tried to develop a standardised protocol for ILM-staining in macular hole surgery based on our own experiences and on those published in clinical studies. Other

advantages of cell cultures studies are the speed of obtaining results, the low costs, no risk to human health and no ethical clearance for animal or human studies.

One weakness of the study is that these effects were only tested on cultured RPE cells and not also on other ocular tissues that also could be damaged by these dyes. One reason is the widespread availability of RPE cell lines, the other that several clinical reports suggested that mainly the retinal pigment epithelium is damaged by ICG and TB. In contrast, there are also reports about negative effects of ICG on retinal glial cells in vitro [81, 82, 123] as well as on spinal root axons [27]. Thus the toxic potential of vital stains to different retinal tissues should not be underestimated [67].

Another weakness of our cell culture study is that it cannot fully replicate the situation that occurs clinically in humans. Experimental studies with ARPE-19 cell cultures are a useful complementary tool to investigate potential toxic effects before clinical macular hole surgery. However, these studies alone cannot be used to reach a conclusion on the clinical safety of vital stains.

# 6. Figures and tables

## 6.1 Figures using the negative and positive controls

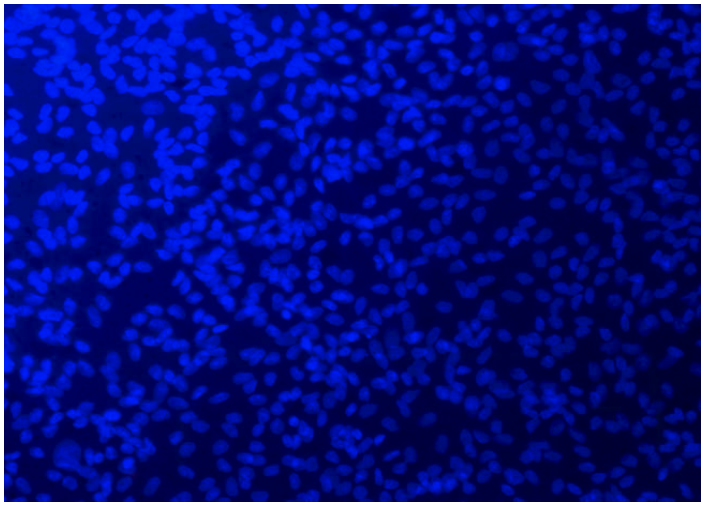


***Figure 66:*** Effect of the culture medium D-MEM, used as a negative control, on ARPE-19 cells. (DAPI/PI-stained living cells at 200x magnification)

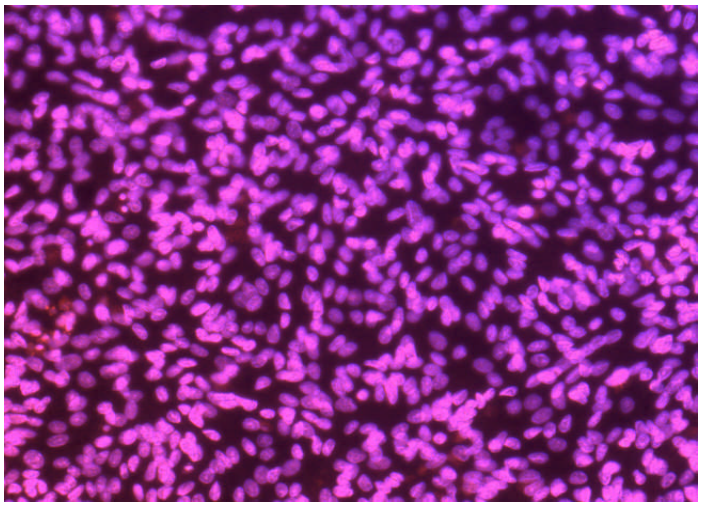


***Figure 67:*** Effect of 70% ethanol, used as positive control, on ARPE-19 cells. (DAPI/PI-stained dead cells at 200x magnification)

## 6.2 Figures of the set-up without illumination

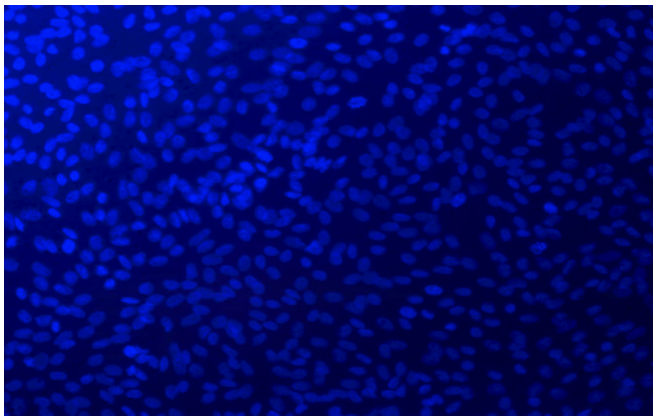


***Figure 68:*** Effect of **solution 1 (ICG 5.0 mg/ml)** on ARPE-19 cells after an incubation time of **5 minutes without illumination** at a follow-up time of 72 hours (DAPI/PI-stained cells at 200x magnification)

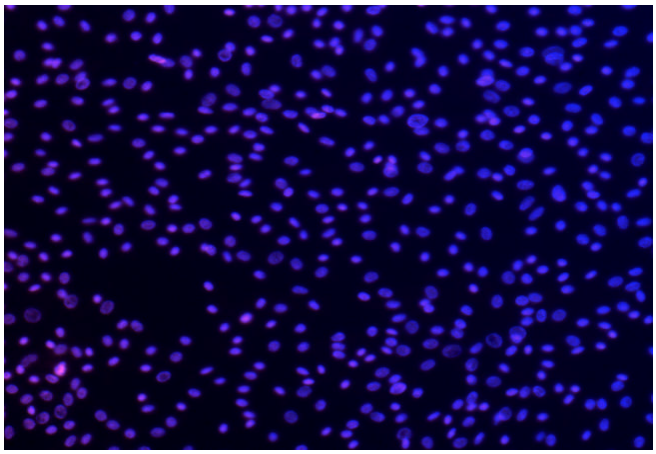


***Figure 69:*** Effect of **solution 1 (ICG 5.0 mg/ml)** on ARPE-19 cells after an incubation time of **10 minutes without illumination** at a follow-up time of 72 hours (DAPI/PI-stained cells at 200x magnification)

***Figure 70:*** Effect of **solution 1 (ICG 5.0 mg/ml)** on ARPE-19 cells after an incubation time of **20 minutes without illumination** at a follow-up time of 72 hours (DAPI/PI-stained cells showing prominent cell loss at 200x magnification)

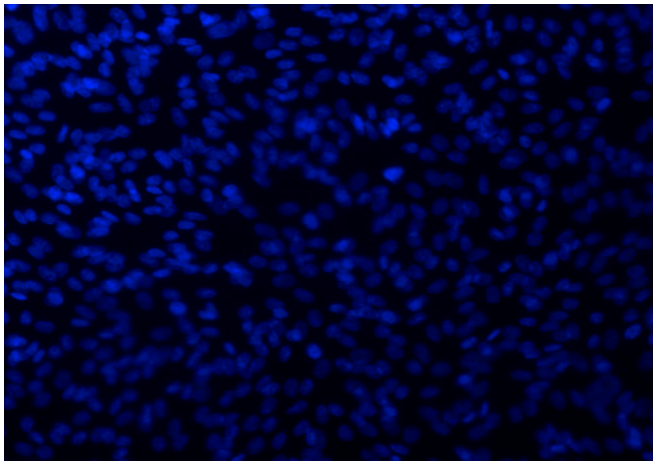


***Figure 71:*** Effect of **solution 2 (ICG 2.5 mg/ml)** on ARPE-19 cells after an incubation time of **10 minutes without illumination** at a follow-up time of 72 hours (DAPI/PI-stained cells with at 200x magnification)

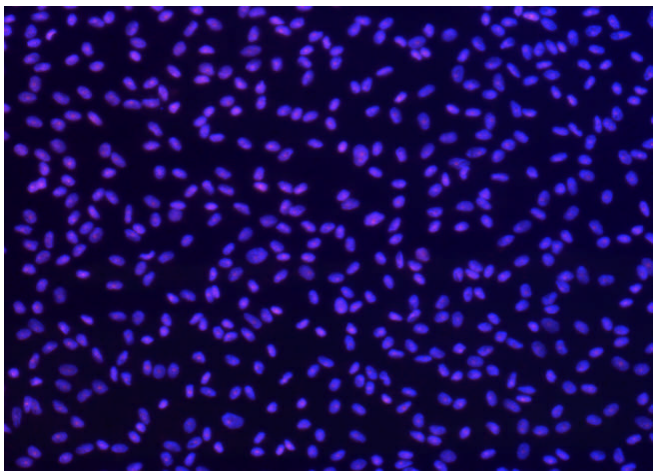


***Figure 72:*** Effect of **solution 2 (ICG 2.5 mg/ml)** on ARPE-19 cells after an incubation time of **20 minutes without illumination** at a follow-up time of 72 hours (DAPI/PI-stained cells with at 200x magnification)

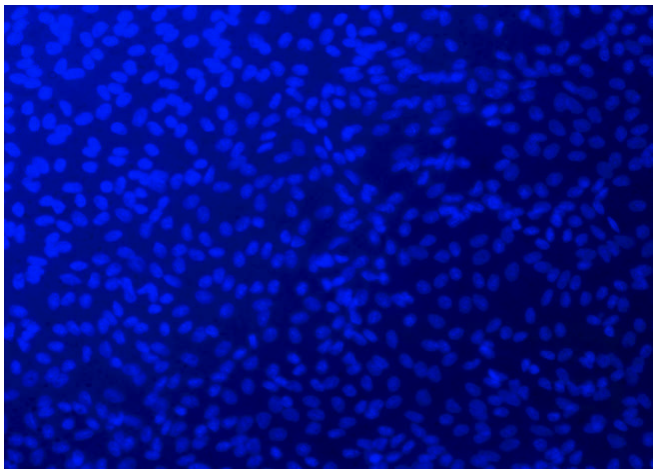


***Figure 73:*** Effect of **solution 3 (ICG 1.0 mg/ml)** on ARPE-19 cells after an incubation time of **20 minutes without illumination** at a follow-up time of 72 hours (DAPI/PI-stained cells at 200x magnification)

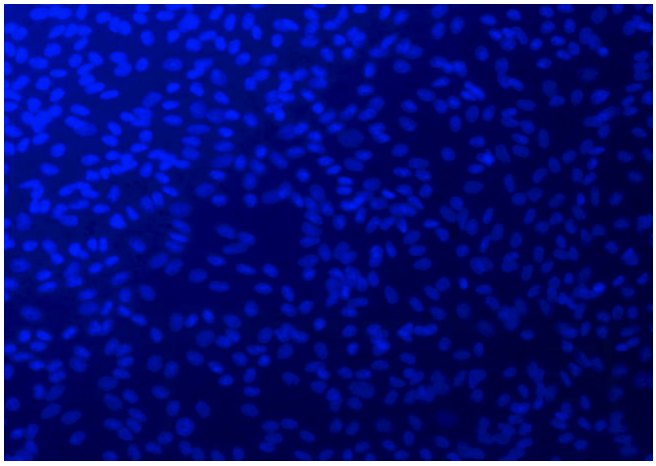


***Figure 74:*** Effect of **solution 4 (ICG 0.125 mg/ml)** on ARPE-19 cells after an incubation time of **20 minutes without illumination** at a follow-up time of 72 hours (DAPI/PI-stained cells at 200x magnification)

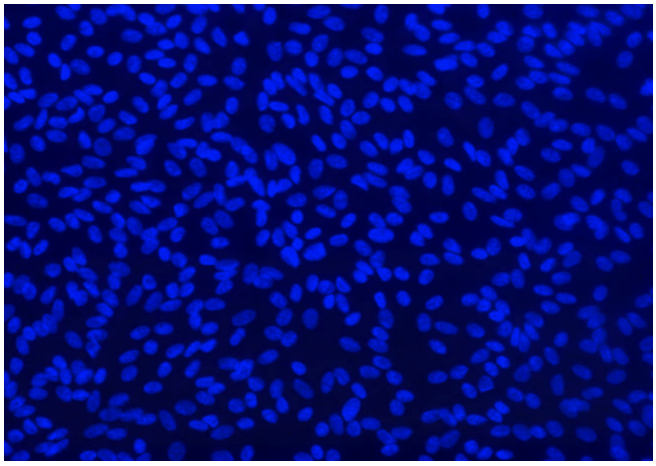


***Figure 75:*** Effect of **solution 1 (ICG-free)** on ARPE-19 cells after an incubation time of **5 minutes without illumination** at a follow-up time of 72 hours (DAPI/PI-stained cells at 200x magnification)

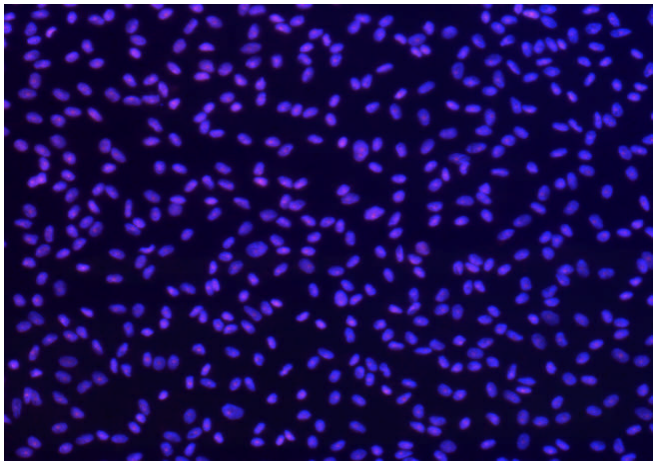


***Figure 76:*** Effect of **solution 1 (ICG-free)** on ARPE-19 cells after an incubation time of **10 minutes without illumination** at a follow-up time of 72 hours (DAPI/PI-stained living and dead cells at 200x magnification)

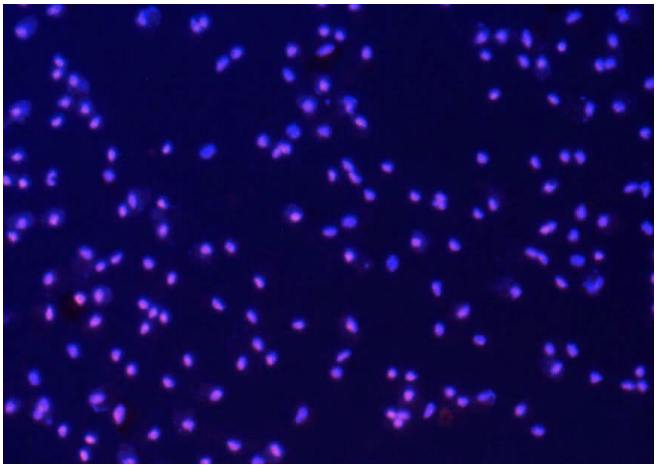


***Figure 77:*** Effect of **solution 1 (ICG-free)** on ARPE-19 cells after an incubation time of **20 minutes without illumination** at a follow-up time of 72 hours
(DAPI/PI-stained dead cells at 200x magnification)

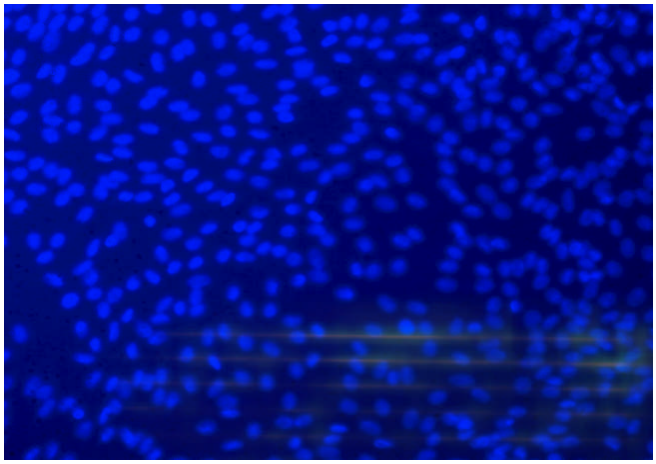


***Figure 78:*** Effect of **solution 2 (ICG-free)** on ARPE-19 cells after an incubation time of **10 minutes without illumination** at a follow-up time of 72 hours
(DAPI/PI-stained cells at 200x magnification)

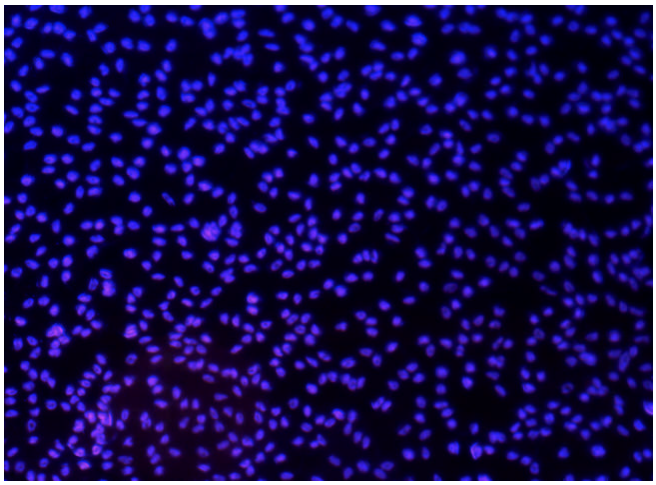


***Figure 79:*** Effect of **solution 2 (ICG-free)** on ARPE-19 cells after an incubation time of **20 minutes without illumination** at a follow-up time of 72 hours
(DAPI/PI-stained cells at 200x magnification)

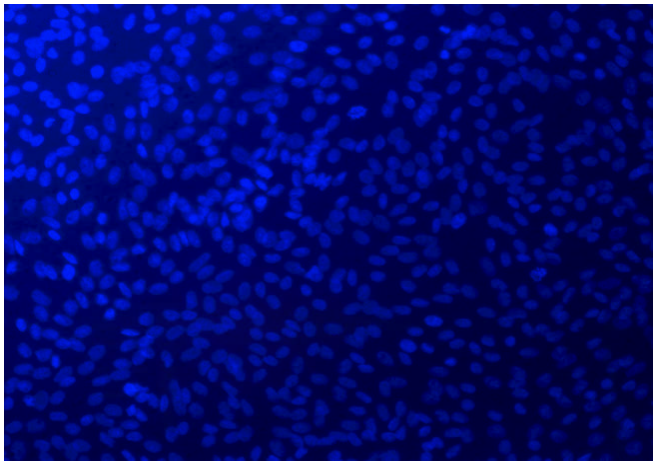


***Figure 80:*** Effect of **solution 3 (ICG-free)** on ARPE-19 cells after an incubation time of **20 minutes without illumination** at a follow-up time of 72 hours (DAPI/PI-stained cells at 200x magnification)

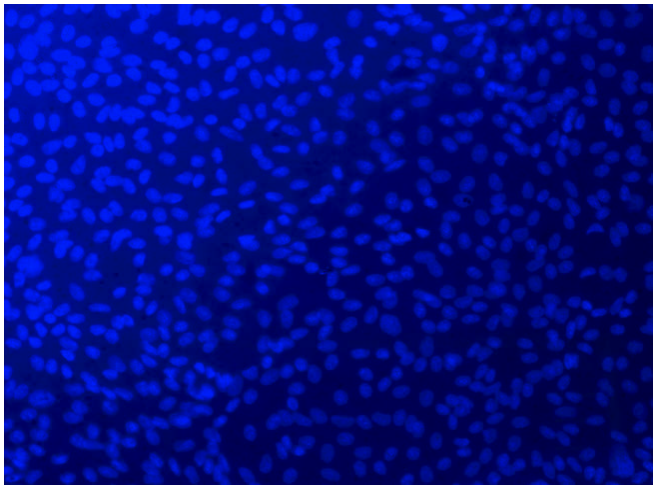


***Figure 81:*** Effect of **solution 4 (ICG-free)** on ARPE-19 cells after an incubation time of **20 minutes without illumination** at a follow-up time of 72 hours (DAPI/PI-stained cells at 200x magnification)

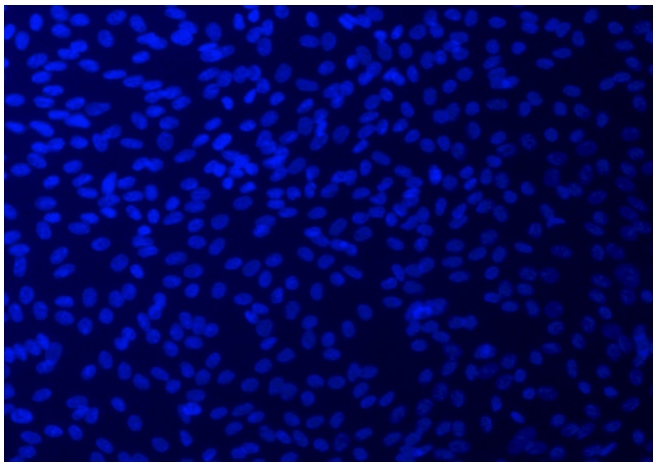


***Figure 82:*** Effect of **trypan blue 1.5 mg/ml** on ARPE-19 cells after an incubation time of **5 minutes without illumination** at a follow-up time of 72 hours (DAPI/PI-stained cells at 200x magnification)

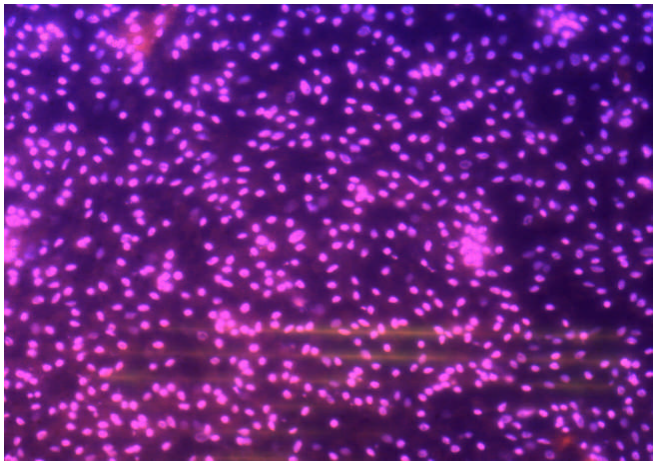


***Figure 83:*** Effect of **trypan blue 1.5 mg/m**l on ARPE-19 cells after an incubation time of **10 minutes without illumination** at a follow-up time of 72 hours (DAPI/PI-stained dead cells at 200x magnification)

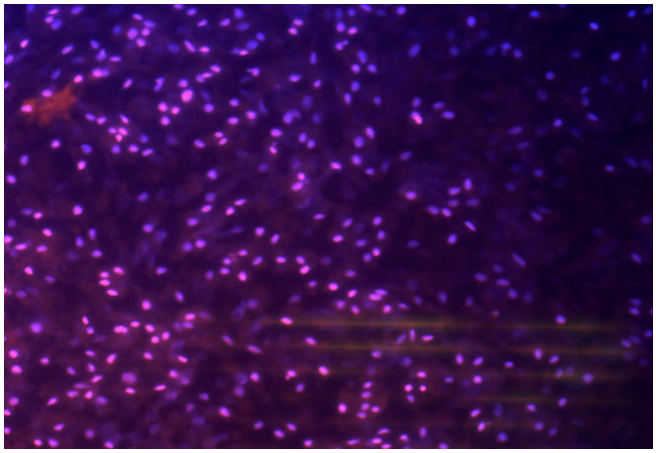


***Figure 84:*** Effect of **trypan blue 1.5 mg/ml** on ARPE-19 cells after an incubation time of **20 minute without illumination** at a follow-up time of 72 hours (DAPI/PI-stained dead cells at 200x magnification)

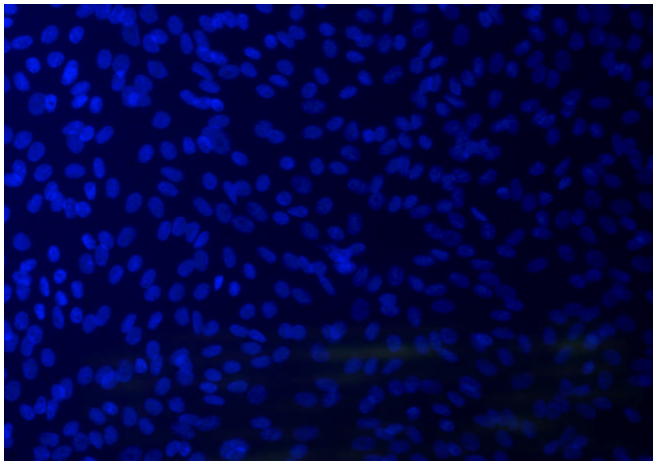


***Figure 85:*** Effect of **trypan blue 0.0375 mg/ml** on ARPE-19 cells after an incubation time of **20 minutes without illumination** at a follow-up time of 72 hours (DAPI/PI-stained cells at 200x magnification)

## 6.3 Figures of the set-up with illumination

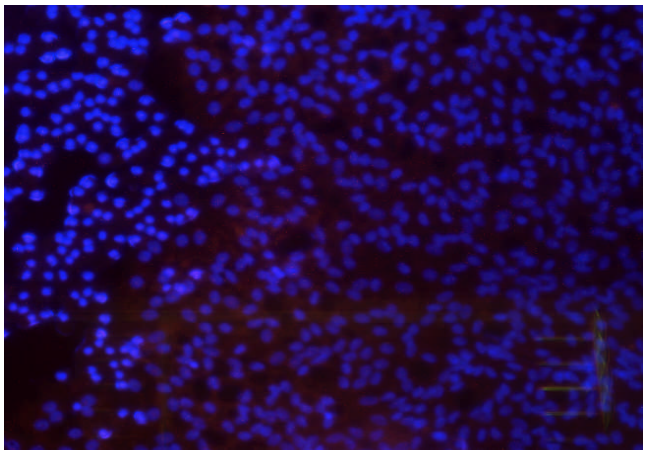


***Figure 86:*** Effect of **solution 1 (ICG 5.0 mg/ml)** on ARPE-19 cells after an incubation time of **1 minute under illumination** at a follow-up time of 72 hours (DAPI/PI-stained living and dead cells at 200x magnification)

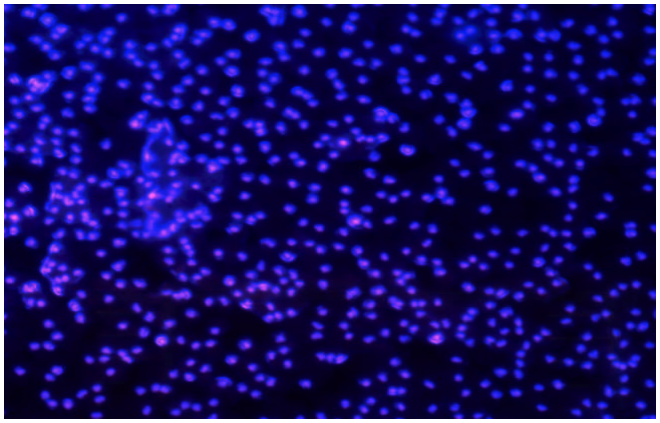


***Figure 87:*** Effect of **solution 1 (ICG 5.0 mg/ml**) on ARPE-19 cells after an incubation time of **5 minute under illumination** at a follow-up time of 72 hours (DAPI/PI-stained dead cells at 200x magnification)

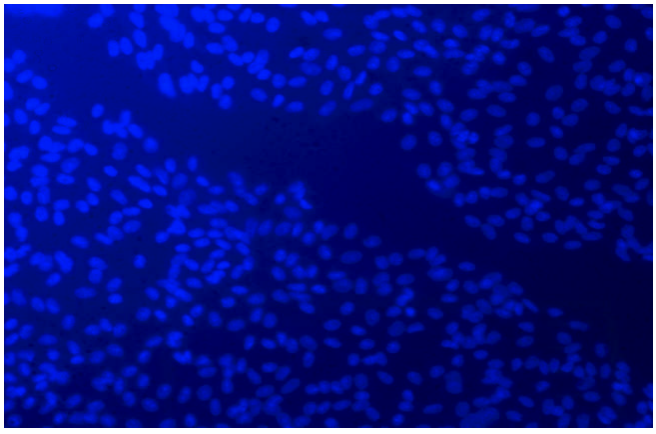


***Figure 88:*** Effect of **solution 2 (ICG 2.5 mg/ml)** on ARPE-19 cells after an incubation time of **1 minute under illumination** at a follow-up time of 72 hours (DAPI/PI-stained cells at 200x magnification)

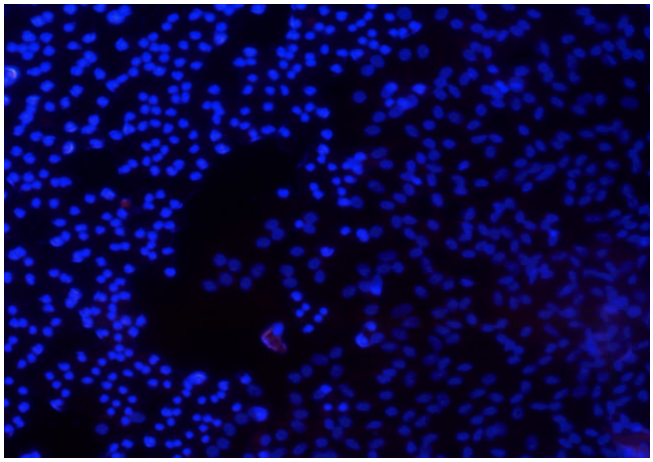


***Figure 89:*** Effect of **solution 2 (ICG 2.5 mg/ml)** on ARPE-19 cells after an incubation time of **5 minutes under illumination** at a follow-up time of 72 hours (DAPI/PI-stained living and dead cells at 200x magnification)

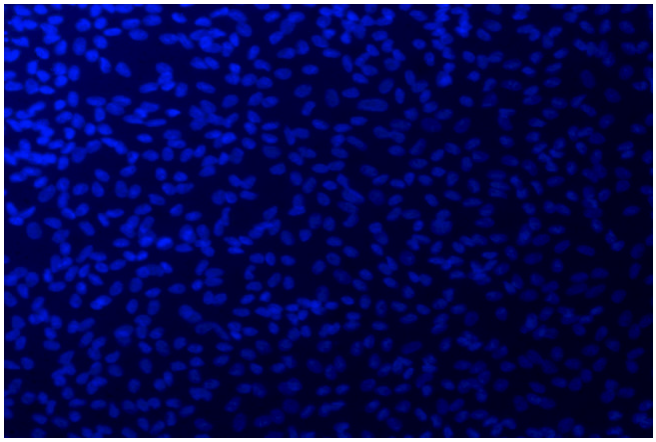


***Figure 90:*** Effect of **solution 3 (ICG 1.0 mg/ml)** on ARPE-19 cells after an incubation time of **5 minutes under illumination** at a follow-up time of 72 hours (DAPI/PI-stained cells at 200x magnification)

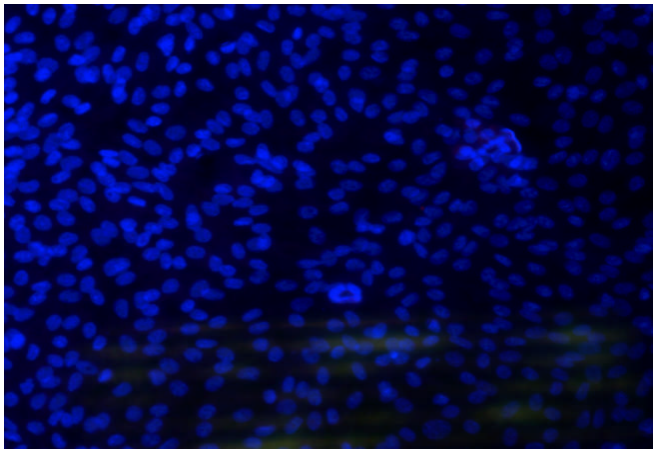


***Figure 91:*** Effect of **solution 4 (ICG 0.125 mg/ml)** on ARPE-19 cells after an incubation time of **5 minutes under illumination** at a follow-up time of 72 hours (DAPI/PI-stained cells at 200x magnification)

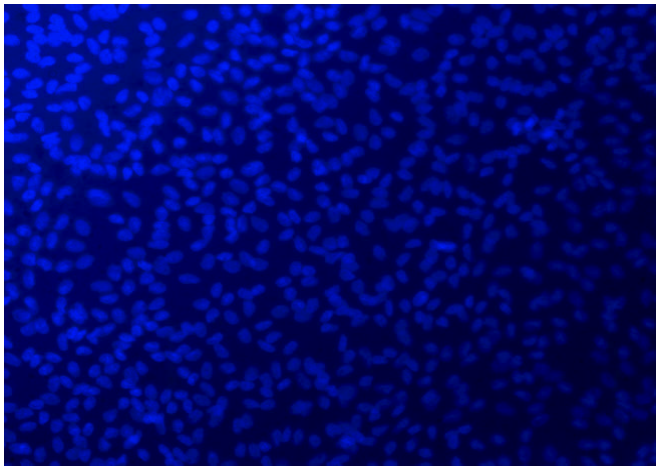


***Figure 92:*** Effect of **solution 1 (ICG free)** on ARPE-19 cells after an incubation time of **5 minutes under illumination** at a follow-up time of 72 hours (DAPI/PI-stained cells at 200x magnification)

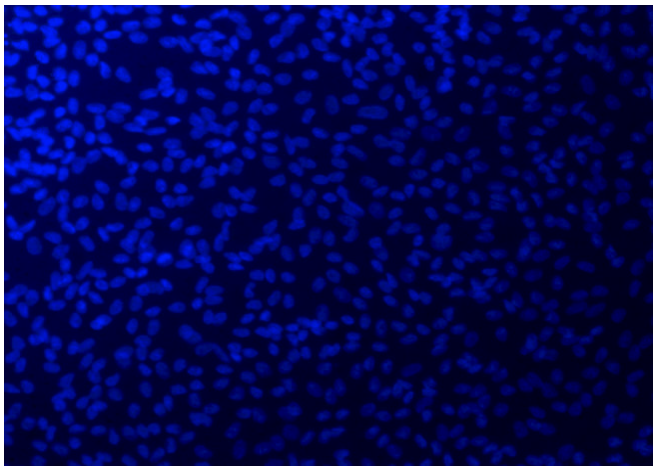


***Figure 93:*** Effect of **solution 2 (ICG free)** on ARPE-19 cells after an incubation time of **5 minutes under illumination** at a follow-up time of 72 hours (DAPI/PI-stained cells at 200x magnification)

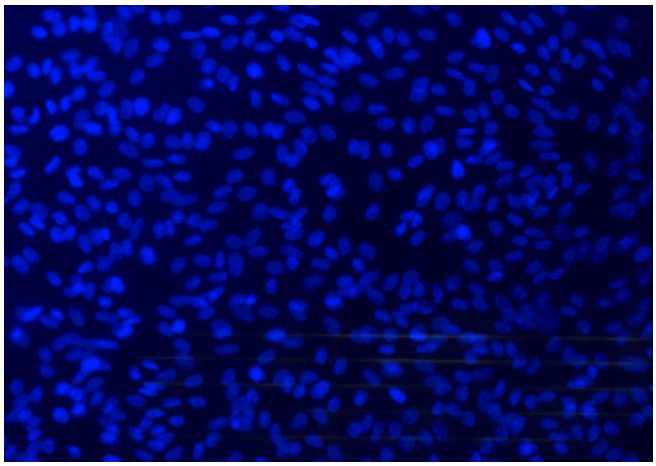


***Figure 94:*** Effect of **trypan blue 1.5 mg/ml** on ARPE-19 cells after an incubation time of **5 minutes under illumination** at a follow-up time of 72 hours (DAPI/PI-stained cells at 200x magnification)

## 6.4 Figures of the clinical set-up

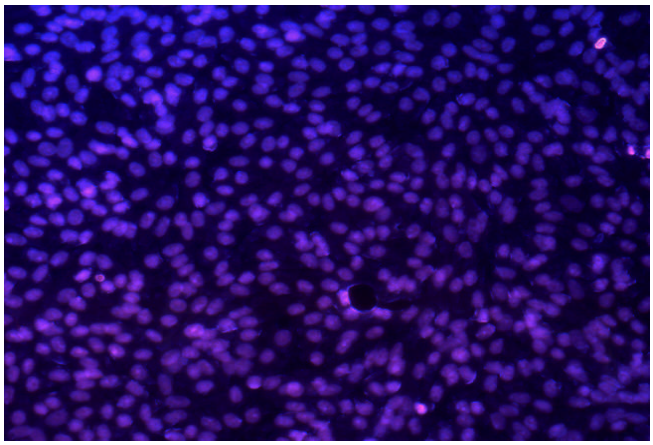


***Figure 95:*** Effect of **solution 1 (ICG 5.0 mg/ml)** on ARPE-19 cells after an incubation time of 1 minute and a total illumination time of 5 minutes at a follow-up time of 72 h (DAPI/PI-stained living and dead cells at 200x magnification)

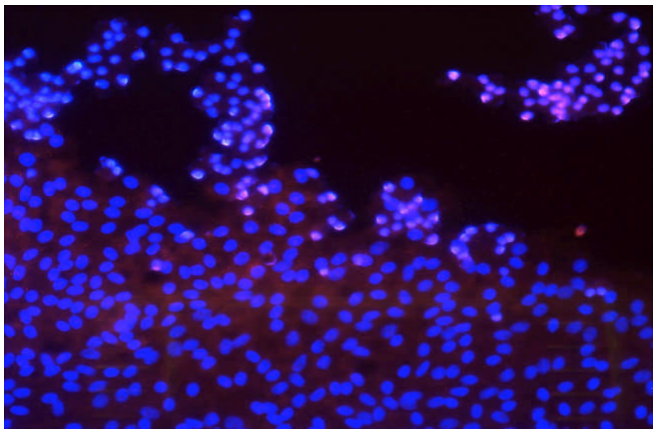


***Figure 96:*** Effect of **solution 2 (ICG 2.5 mg/ml)** on ARPE-19 cells after an incubation time of 1 minute and a total illumination time of 5 minutes at a follow-up time of 72 h (DAPI/PI-stained living and dead cells at 200x magnification)

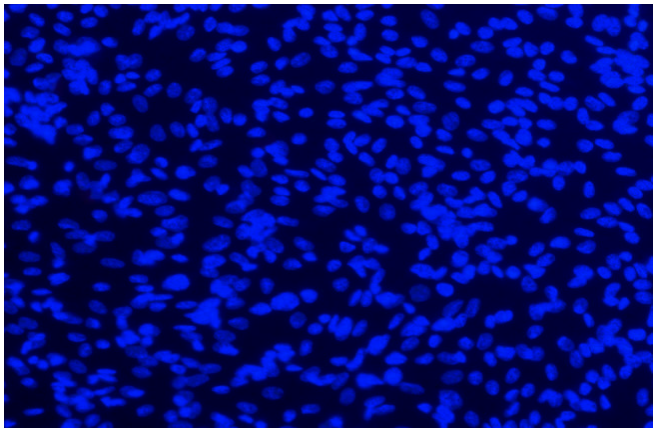


***Figure 97:*** Effect of **solution 3 (ICG 1.0 mg/ml)** on ARPE-19 cells after an incubation time of 1 minute and a total illumination time of 5 minutes at a follow-up time of 72 h (DAPI/PI-stained living cells at 200x magnification)

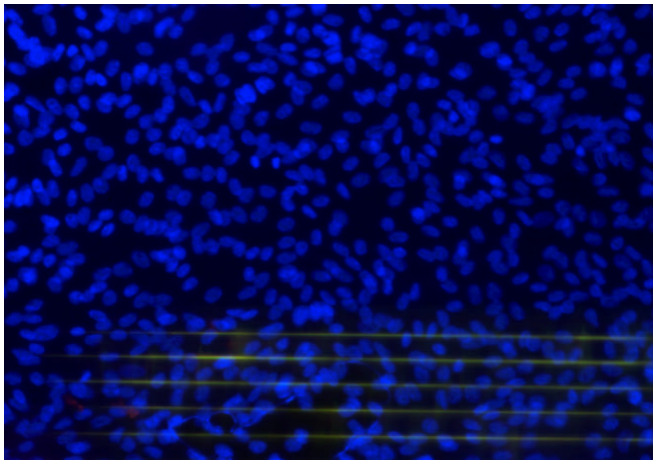


***Figure 98:*** Effect of **trypan blue 1.5 mg/ml** on ARPE-19 cells after an incubation time of 1 minute and a total illumination time of 5 minutes at a follow-up time of 72 h (DAPI/PI-stained living cells at 200x magnification)

## 6.5 Tables of the set-up without illumination

### 6.5.1 Question one – cell survival

***Table 5:*** Summary of fit for the first question–cell survival of the set-up without illumination

| | |
|---|---|
| $R^2$ | 99.73% |
| $R^2$ Adj | 99.68% |
| *s* | 0.0243 |
| Mean of Response | 1.2874 |
| *n* | 288 |

***Table 6:*** Effect tests for the first question–cell survival of the set-up without illumination

| Factors and Interactions | Nparm | DF | Sum of Squares | F Ratio | *P-value* |
|---|---|---|---|---|---|
| Solution | 3 | 3 | 21.7335 | 12242.08 | <.0001 |
| Incubation time [min] | 3 | 3 | 15.0983 | 8504.624 | <.0001 |
| Solution * Incubation time [min] | 9 | 9 | 15.0236 | 2820.84 | <.0001 |
| Follow-up time [h] | 2 | 2 | 0.1979 | 167.2211 | <.0001 |
| Solution * Follow-up time [h] | 6 | 6 | 0.0265 | 7.4752 | <.0001 |
| Incubation time [min] * Follow-up time [h] | 6 | 6 | 0.0100 | 2.8125 | 0.0116 |
| Solution * Incubation time [min] * Follow-up time [h] | 18 | 18 | 0.0829 | 7.7837 | <.0001 |

***Table 7:*** Median cell survival, lower and upper CL for the interaction between the factors solution, incubation time and follow-up time

| Solution | mg/ml | Incubation time [min] | Follow-up time [h] | Median cell survival [%] | Lower CL [%] | Upper CL [%] |
|---|---|---|---|---|---|---|
| Solution 1 (ICG) | 5.0 | 1 | 6 | 100.00 | 100.00 | 100.00 |
| Solution 1 (ICG) | 5.0 | 5 | 6 | 94.92 | 94.03 | 95.74 |
| Solution 1 (ICG) | 5.0 | 10 | 6 | 24.02 | 22.37 | 25.71 |
| Solution 1 (ICG) | 5.0 | 20 | 6 | 0.00 | 0.00 | 0.00 |
| Solution 2 (ICG) | 2.5 | 1 | 6 | 100.00 | 100.00 | 100.00 |
| Solution 2 (ICG) | 2.5 | 5 | 6 | 100.00 | 100.00 | 100.00 |
| Solution 2 (ICG) | 2.5 | 10 | 6 | 90.37 | 89.18 | 91.49 |
| Solution 2 (ICG) | 2.5 | 20 | 6 | 88.79 | 87.52 | 89.99 |
| Solution 3 (ICG) | 1.0 | 1 | 6 | 100.00 | 100.00 | 100.00 |
| Solution 3 (ICG) | 1.0 | 5 | 6 | 100.00 | 100.00 | 100.00 |
| Solution 3 (ICG) | 1.0 | 10 | 6 | 94.58 | 93.66 | 95.43 |
| Solution 3 (ICG) | 1.0 | 20 | 6 | 92.91 | 91.88 | 93.88 |
| Solution 4 (ICG) | 0.125 | 1 | 6 | 100.00 | 100.00 | 100.00 |
| Solution 4 (ICG) | 0.125 | 5 | 6 | 100.00 | 100.00 | 100.00 |
| Solution 4 (ICG) | 0.125 | 10 | 6 | 100.00 | 100.00 | 100.00 |
| Solution 4 (ICG) | 0.125 | 20 | 6 | 99.79 | 99.58 | 99.93 |
| Solution 1 (ICG) | 5.0 | 1 | 24 | 99.54 | 99.23 | 99.77 |
| Solution 1 (ICG) | 5.0 | 5 | 24 | 93.35 | 92.34 | 94.29 |
| Solution 1 (ICG) | 5.0 | 10 | 24 | 21.16 | 19.58 | 22.78 |
| Solution 1 (ICG) | 5.0 | 20 | 24 | 0.00 | 0.00 | 0.00 |
| Solution 2 (ICG) | 2.5 | 1 | 24 | 100.00 | 100.00 | 100.00 |
| Solution 2 (ICG) | 2.5 | 5 | 24 | 99.42 | 99.09 | 99.68 |
| Solution 2 (ICG) | 2.5 | 10 | 24 | 86.38 | 85.01 | 87.70 |
| Solution 2 (ICG) | 2.5 | 20 | 24 | 84.96 | 83.54 | 86.33 |
| Solution 3 (ICG) | 1.0 | 1 | 24 | 100.00 | 100.00 | 100.00 |
| Solution 3 (ICG) | 1.0 | 5 | 24 | 99.77 | 99.55 | 99.92 |
| Solution 3 (ICG) | 1.0 | 10 | 24 | 93.38 | 92.37 | 94.32 |

| Solution | mg/ml | Incubation time [min] | Follow-up time [h] | Median cell survival [%] | Lower CL [%] | Upper CL [%] |
|---|---|---|---|---|---|---|
| Solution 3 (ICG) | 1.0 | 20 | 24 | 93.07 | 92.04 | 94.03 |
| Solution 4 (ICG) | 0.125 | 1 | 24 | 100.00 | 100.00 | 100.00 |
| Solution 4 (ICG) | 0.125 | 5 | 24 | 100.00 | 100.00 | 100.00 |
| Solution 4 (ICG) | 0.125 | 10 | 24 | 100.00 | 100.00 | 100.00 |
| Solution 4 (ICG) | 0.125 | 20 | 24 | 99.86 | 99.68 | 99.97 |
| Solution 1 (ICG) | 5.0 | 1 | 72 | 98.47 | 97.95 | 98.91 |
| Solution 1 (ICG) | 5.0 | 5 | 72 | 93.18 | 92.16 | 94.13 |
| Solution 1 (ICG) | 5.0 | 10 | 72 | 18.49 | 16.99 | 20.03 |
| Solution 1 (ICG) | 5.0 | 20 | 72 | 0.00 | 0.00 | 0.00 |
| Solution 2 (ICG) | 2.5 | 1 | 72 | 99.22 | 98.84 | 99.53 |
| Solution 2 (ICG) | 2.5 | 5 | 72 | 97.75 | 97.13 | 98.29 |
| Solution 2 (ICG) | 2.5 | 10 | 72 | 86.41 | 85.04 | 87.72 |
| Solution 2 (ICG) | 2.5 | 20 | 72 | 83.11 | 81.62 | 84.55 |
| Solution 3 (ICG) | 1.0 | 1 | 72 | 99.57 | 99.27 | 99.79 |
| Solution 3 (ICG) | 1.0 | 5 | 72 | 99.20 | 98.81 | 99.51 |
| Solution 3 (ICG) | 1.0 | 10 | 72 | 91.81 | 90.70 | 92.85 |
| Solution 3 (ICG) | 1.0 | 20 | 72 | 91.61 | 90.50 | 92.66 |
| Solution 4 (ICG) | 0.125 | 1 | 72 | 100.00 | 100.00 | 100.00 |
| Solution 4 (ICG) | 0.125 | 5 | 72 | 100.00 | 100.00 | 100.00 |
| Solution 4 (ICG) | 0.125 | 10 | 72 | 99.04 | 98.62 | 99.38 |
| Solution 4 (ICG) | 0.125 | 20 | 72 | 98.59 | 98.10 | 99.02 |

## **6.5.2** Question two – cell survival

***Table 8:*** Summary of fit for the second question–cell survival of the set-up without illumination

| | |
|---|---|
| $R^2$ | 99.88% |
| $R^2$ Adj | 99.86% |
| *s* | 0.0201 |
| Mean of Response | 1.2648 |
| *n* | 144 |

***Table 9:*** Effect tests for the second question–cell survival of the set-up without illumination

| Factors and Interactions | Nparm | DF | Sum of Squares | F Ratio | *P-value* |
|---|---|---|---|---|---|
| Solution | 1 | 1 | 11.1861 | 27640.87 | <.0001 |
| Incubation time [min] | 3 | 3 | 15.8788 | 13078.85 | <.0001 |
| Solution * Incubation time [min] | 3 | 3 | 12.8551 | 10588.28 | <.0001 |
| Follow-up time [h] | 2 | 2 | 0.04057 | 50.1223 | <.0001 |
| Solution * Follow-up time [h] | 2 | 2 | 0.00247 | 3.0496 | 0.0511 |
| Incubation time [min] * Follow-up time [h] | 6 | 6 | 0.01095 | 4.5095 | 0.0004 |
| Solution * Incubation time [min] * Follow-up time [h] | 6 | 6 | 0.02645 | 10.8928 | <.0001 |

***Table 10:*** Median cell survival, lower and upper CL for the interaction between the factors solution, incubation time and follow-up time

| Solution | mg/ml | Incubation time [min] | Follow-up time [h] | Median cell survival [%] | Lower CL [%] | Upper CL [%] |
|---|---|---|---|---|---|---|
| Trypan blue | 1.5 | 1 | 6 | 100.00 | 100.00 | 100.00 |
| Trypan blue | 1.5 | 5 | 6 | 100.00 | 100.00 | 100.00 |
| Trypan blue | 1.5 | 10 | 6 | 61.65 | 60.06 | 63.22 |
| Trypan blue | 1.5 | 20 | 6 | 0.00 | 0.00 | 0.00 |
| diluted TB | 0.0375 | 1 | 6 | 100.00 | 100.00 | 100.00 |

| Solution | mg/ml | Incubation time [min] | Follow-up time [h] | Median cell survival [%] | Lower CL [%] | Upper CL [%] |
|---|---|---|---|---|---|---|
| diluted TB | 0.0375 | 5 | 6 | 100.00 | 100.00 | 100.00 |
| diluted TB | 0.0375 | 10 | 6 | 100.00 | 100.00 | 100.00 |
| diluted TB | 0.0375 | 20 | 6 | 99.69 | 99.49 | 99.85 |
| Trypan blue | 1.5 | 1 | 24 | 100.00 | 100.00 | 100.00 |
| Trypan blue | 1.5 | 5 | 24 | 99.64 | 99.42 | 99.81 |
| Trypan blue | 1.5 | 10 | 24 | 57.75 | 56.14 | 59.35 |
| Trypan blue | 1.5 | 20 | 24 | 0.00 | 0.00 | 0.00 |
| diluted TB | 0.0375 | 1 | 24 | 100.00 | 100.00 | 100.00 |
| diluted TB | 0.0375 | 5 | 24 | 100.00 | 100.00 | 100.00 |
| diluted TB | 0.0375 | 10 | 24 | 100.00 | 100.00 | 100.00 |
| diluted TB | 0.0375 | 20 | 24 | 99.20 | 98.88 | 99.46 |
| Trypan blue | 1.5 | 1 | 72 | 99.71 | 99.50 | 99.86 |
| Trypan blue | 1.5 | 5 | 72 | 99.16 | 98.84 | 99.43 |
| Trypan blue | 1.5 | 10 | 72 | 56.27 | 54.65 | 57.88 |
| Trypan blue | 1.5 | 20 | 72 | 0.00 | 0.00 | 0.00 |
| diluted TB | 0.0375 | 1 | 72 | 100.00 | 100.00 | 100.00 |
| diluted TB | 0.0375 | 5 | 72 | 100.00 | 100.00 | 100.00 |
| diluted TB | 0.0375 | 10 | 72 | 99.34 | 99.05 | 99.58 |
| diluted TB | 0.0375 | 20 | 72 | 98.98 | 98.63 | 99.29 |

## 6.5.3 Question three – cell survival

***Table 11:*** Summary of fit for the third question–cell survival of the set-up without illumination

| | |
|---|---|
| $R^2$ | 99.76% |
| $R^2$ Adj | 99.72% |
| *s* | 0.0243 |
| Mean of Response | 1.2045 |
| *n* | 432 |

***Table 12:*** Effect tests for the third question–cell survival of the set-up without illumination

| Factors and Interactions | Nparm | DF | Sum of Squares | F Ratio | *P-value* |
|---|---|---|---|---|---|
| Solution number | 2 | 2 | 29.1646 | 24685.57 | 0.0000 |
| Dye | 1 | 1 | 0.0046 | 7.7417 | 0.0057 |
| Incubation time [min] | 3 | 3 | 37.9211 | 21398.18 | 0.0000 |
| Follow-up time [h] | 2 | 2 | 0.3865 | 327.1213 | <.0001 |
| Solution number * Dye | 2 | 2 | 0.0186 | 15.7422 | <.0001 |
| Solution number * Incubation time [min] | 6 | 6 | 21.6000 | 6094.232 | 0.0000 |
| Solution number * Follow-up time [h] | 4 | 4 | 0.0164 | 6.9552 | <.0001 |
| Dye * Incubation time [min] | 3 | 3 | 0.0007 | 0.3820 | 0.7660 |
| Dye * Follow-up time [h] | 2 | 2 | 0.0013 | 1.1294 | 0.3244 |
| Incubation time [min] * Follow-up time [h] | 6 | 6 | 0.0559 | 15.7618 | <.0001 |
| Solution number * Dye * Incubation time [min] | 6 | 6 | 0.0065 | 1.8279 | 0.0927 |
| Solution number * Dye * Follow-up time [h] | 4 | 4 | 0.0059 | 2.5107 | 0.0416 |
| Solution number * Incubation time [min] * Follow-up time [h] | 12 | 12 | 0.0339 | 4.7760 | <.0001 |

| Factors and Interactions | Nparm | DF | Sum of Squares | F Ratio | *P-value* |
|---|---|---|---|---|---|
| Dye *<br>Incubation time [min] *<br>Follow-up time [h] | 6 | 6 | 0.0219 | 6.1702 | <.0001 |
| Solution number *<br>Dye *<br>Incubation time [min] *<br>Follow-up time [h] | 12 | 12 | 0.0206 | 2.9057 | 0.0007 |

***Table 13:*** Median cell survival, lower and upper CL for the interaction between the factors solution number, dye, incubation & illumination time and follow-up time

| Solution number (dye) | mg/ml | Incub. & illum. time [h] | Follow-up time [h] | Median cell survival [%] | Lower CL [%] | Upper CL [%] |
|---|---|---|---|---|---|---|
| Solution 1 (ICG) | 5.0 | 1 | 6 | 100.00 | 100.00 | 100.00 |
| Solution 1 (ICG) | 5.0 | 5 | 6 | 94.92 | 94.03 | 95.74 |
| Solution 1 (ICG) | 5.0 | 10 | 6 | 24.02 | 22.37 | 25.71 |
| Solution 1 (ICG) | 5.0 | 20 | 6 | 0.00 | 0.00 | 0.00 |
| Solution 1 (ICG-free) | 0.0 | 1 | 6 | 99.89 | 99.73 | 99.98 |
| Solution 1 (ICG-free) | 0.0 | 5 | 6 | 95.14 | 94.27 | 95.95 |
| Solution 1 (ICG-free) | 0.0 | 10 | 6 | 26.35 | 24.65 | 28.09 |
| Solution 1 (ICG-free) | 0.0 | 20 | 6 | 1.00 | 0.65 | 1.43 |
| Solution 2 (ICG) | 2.5 | 1 | 6 | 100.00 | 100.00 | 100.00 |
| Solution 2 (ICG) | 2.5 | 5 | 6 | 100.00 | 100.00 | 100.00 |
| Solution 2 (ICG) | 2.5 | 10 | 6 | 90.37 | 89.18 | 91.49 |
| Solution 2 (ICG) | 2.5 | 20 | 6 | 88.79 | 87.52 | 89.99 |
| Solution 2 (ICG-free) | 0.0 | 1 | 6 | 100.00 | 100.00 | 100.00 |
| Solution 2 (ICG-free) | 0.0 | 5 | 6 | 100.00 | 100.00 | 100.00 |
| Solution 2 (ICG-free) | 0.0 | 10 | 6 | 90.13 | 88.94 | 91.26 |
| Solution 2 (ICG-free) | 0.0 | 20 | 6 | 87.91 | 86.60 | 89.15 |
| Solution 3 (ICG) | 1.0 | 1 | 6 | 100.00 | 100.00 | 100.00 |
| Solution 3 (ICG) | 1.0 | 5 | 6 | 100.00 | 100.00 | 100.00 |
| Solution 3 (ICG) | 1.0 | 10 | 6 | 94.58 | 93.66 | 95.43 |
| Solution 3 (ICG) | 1.0 | 20 | 6 | 92.91 | 91.88 | 93.88 |
| Solution 3 (ICG-free) | 0.0 | 1 | 6 | 100.00 | 100.00 | 100.00 |
| Solution 3 (ICG-free) | 0.0 | 5 | 6 | 100.00 | 100.00 | 100.00 |
| Solution 3 (ICG-free) | 0.0 | 10 | 6 | 95.50 | 94.66 | 96.27 |
| Solution 3 (ICG-free) | 0.0 | 20 | 6 | 94.41 | 93.48 | 95.27 |
| Solution 1 (ICG) | 5.0 | 1 | 24 | 99.54 | 99.23 | 99.77 |
| Solution 1 (ICG) | 5.0 | 5 | 24 | 93.35 | 92.34 | 94.29 |
| Solution 1 (ICG) | 5.0 | 10 | 24 | 21.16 | 19.58 | 22.78 |
| Solution 1 (ICG) | 5.0 | 20 | 24 | 0.00 | 0.00 | 0.00 |
| Solution 1 (ICG-free) | 0.0 | 1 | 24 | 100.00 | 100.00 | 100.00 |
| Solution 1 (ICG-free) | 0.0 | 5 | 24 | 94.03 | 93.08 | 94.92 |
| Solution 1 (ICG-free) | 0.0 | 10 | 24 | 24.55 | 22.89 | 26.25 |
| Solution 1 (ICG-free) | 0.0 | 20 | 24 | 0.00 | 0.00 | 0.00 |
| Solution 2 (ICG) | 2.5 | 1 | 24 | 100.00 | 100.00 | 100.00 |
| Solution 2 (ICG) | 2.5 | 5 | 24 | 99.42 | 99.09 | 99.68 |
| Solution 2 (ICG) | 2.5 | 10 | 24 | 86.38 | 85.01 | 87.70 |
| Solution 2 (ICG) | 2.5 | 20 | 24 | 84.96 | 83.54 | 86.33 |
| Solution 2 (ICG-free) | 0.0 | 1 | 24 | 100.00 | 100.00 | 100.00 |
| Solution 2 (ICG-free) | 0.0 | 5 | 24 | 99.56 | 99.27 | 99.78 |
| Solution 2 (ICG-free) | 0.0 | 10 | 24 | 85.56 | 84.16 | 86.90 |
| Solution 2 (ICG-free) | 0.0 | 20 | 24 | 84.25 | 82.80 | 85.65 |
| Solution 3 (ICG) | 1.0 | 1 | 24 | 100.00 | 100.00 | 100.00 |
| Solution 3 (ICG) | 1.0 | 5 | 24 | 99.77 | 99.55 | 99.92 |
| Solution 3 (ICG) | 1.0 | 10 | 24 | 93.38 | 92.37 | 94.32 |
| Solution 3 (ICG) | 1.0 | 20 | 24 | 93.07 | 92.04 | 94.03 |
| Solution 3 (ICG-free) | 0.0 | 1 | 24 | 100.00 | 100.00 | 100.00 |
| Solution 3 (ICG-free) | 0.0 | 5 | 24 | 99.66 | 99.39 | 99.85 |
| Solution 3 (ICG-free) | 0.0 | 10 | 24 | 92.38 | 91.31 | 93.38 |
| Solution 3 (ICG-free) | 0.0 | 20 | 24 | 90.38 | 89.20 | 91.50 |

| Solution number (dye) | mg/ml | Incub. & illum. time [h] | Follow-up time [h] | Median cell survival [%] | Lower CL [%] | Upper CL [%] |
|---|---|---|---|---|---|---|
| Solution 1 (ICG) | 5.0 | 1 | 72 | 98.47 | 97.95 | 98.91 |
| Solution 1 (ICG) | 5.0 | 5 | 72 | 93.18 | 92.16 | 94.13 |
| Solution 1 (ICG) | 5.0 | 10 | 72 | 18.49 | 16.99 | 20.03 |
| Solution 1 (ICG) | 5.0 | 20 | 72 | 0.00 | 0.00 | 0.00 |
| Solution 1 (ICG-free) | 0.0 | 1 | 72 | 99.12 | 98.72 | 99.44 |
| Solution 1 (ICG-free) | 0.0 | 5 | 72 | 93.40 | 92.40 | 94.33 |
| Solution 1 (ICG-free) | 0.0 | 10 | 72 | 22.08 | 20.48 | 23.72 |
| Solution 1 (ICG-free) | 0.0 | 20 | 72 | 0.00 | 0.00 | 0.00 |
| Solution 2 (ICG) | 2.5 | 1 | 72 | 99.22 | 98.84 | 99.53 |
| Solution 2 (ICG) | 2.5 | 5 | 72 | 97.75 | 97.13 | 98.29 |
| Solution 2 (ICG) | 2.5 | 10 | 72 | 86.41 | 85.04 | 87.72 |
| Solution 2 (ICG) | 2.5 | 20 | 72 | 83.11 | 81.62 | 84.55 |
| Solution 2 (ICG-free) | 0.0 | 1 | 72 | 99.22 | 98.85 | 99.52 |
| Solution 2 (ICG-free) | 0.0 | 5 | 72 | 97.92 | 97.32 | 98.44 |
| Solution 2 (ICG-free) | 0.0 | 10 | 72 | 85.68 | 84.29 | 87.02 |
| Solution 2 (ICG-free) | 0.0 | 20 | 72 | 82.98 | 81.49 | 84.42 |
| Solution 3 (ICG) | 1.0 | 1 | 72 | 99.57 | 99.27 | 99.79 |
| Solution 3 (ICG) | 1.0 | 5 | 72 | 99.20 | 98.81 | 99.51 |
| Solution 3 (ICG) | 1.0 | 10 | 72 | 91.81 | 90.70 | 92.85 |
| Solution 3 (ICG) | 1.0 | 20 | 72 | 91.61 | 90.50 | 92.66 |
| Solution 3 (ICG-free) | 0.0 | 1 | 72 | 99.61 | 99.33 | 99.81 |
| Solution 3 (ICG-free) | 0.0 | 5 | 72 | 99.33 | 98.98 | 99.61 |
| Solution 3 (ICG-free) | 0.0 | 10 | 72 | 92.00 | 90.91 | 93.03 |
| Solution 3 (ICG-free) | 0.0 | 20 | 72 | 90.57 | 89.39 | 91.68 |

## 6.5.4.1 Question four part 1–cell survival

***Table 14:*** Summary of fit for the fourth question part 1–cell survival of the set-up without illumination

| | |
|---|---|
| $R^2$ | 99.81% |
| $R^2$ Adj | 99.78% |
| *s* | 0.0244 |
| Mean of Response | 1.1475 |
| *n* | 288 |

***Table 15:*** Effect tests for the fourth question part 1–cell survival of the set-up without illumination

| Factors and Interactions | Nparm | DF | Sum of Squares | F Ratio | *P-value* |
|---|---|---|---|---|---|
| Solution | 3 | 3 | 17.8240 | 9992.283 | <.0001 |
| Incubation time [min] | 3 | 3 | 40.6134 | 22768.19 | <.0001 |
| Solution * Incubation time [min] | 9 | 9 | 18.1061 | 3383.484 | <.0001 |
| Follow-up time [h] | 2 | 2 | 0.2058 | 173.0898 | <.0001 |
| Solution * Follow-up time [h] | 6 | 6 | 0.0178 | 4.9837 | <.0001 |
| Incubation time [min] * Follow-up time [h] | 6 | 6 | 0.0431 | 12.0873 | <.0001 |
| Solution * Incubation time [min] * Follow-up time [h] | 18 | 18 | 0.0350 | 3.2686 | <.0001 |

***Table 16:*** Median cell survival, lower and upper CL for the interaction between the factors solution, incubation time and follow-up time

| Solution | mg/ml | Incubation time [min] | Follow-up time [h[ | Median cell survival [%] | Lower CL [%] | Upper CL [%] |
|---|---|---|---|---|---|---|
| Solution 1 (ICG) | 5.0 | 1 | 6 | 100.00 | 100.00 | 100.00 |
| Solution 1 (ICG) | 5.0 | 5 | 6 | 94.92 | 94.03 | 95.74 |

| Solution | mg/ml | Incubation time [min] | Follow-up time [h[ | Median cell survival [%] | Lower CL [%] | Upper CL [%] |
|---|---|---|---|---|---|---|
| Solution 1 (ICG) | 5.0 | 10 | 6 | 24.02 | 22.37 | 25.71 |
| Solution 1 (ICG) | 5.0 | 20 | 6 | 0.00 | 0.00 | 0.00 |
| Solution 2 (ICG) | 2.5 | 1 | 6 | 100.00 | 100.00 | 100.00 |
| Solution 2 (ICG) | 2.5 | 5 | 6 | 100.00 | 100.00 | 100.00 |
| Solution 2 (ICG) | 2.5 | 10 | 6 | 90.37 | 89.18 | 91.49 |
| Solution 2 (ICG) | 2.5 | 20 | 6 | 88.79 | 87.52 | 89.99 |
| Solution 3 (ICG) | 1.0 | 1 | 6 | 100.00 | 100.00 | 100.00 |
| Solution 3 (ICG) | 1.0 | 5 | 6 | 100.00 | 100.00 | 100.00 |
| Solution 3 (ICG) | 1.0 | 10 | 6 | 94.58 | 93.66 | 95.43 |
| Solution 3 (ICG) | 1.0 | 20 | 6 | 92.91 | 91.88 | 93.88 |
| Trypan blue | 1.5 | 1 | 6 | 100.00 | 100.00 | 100.00 |
| Trypan blue | 1.5 | 5 | 6 | 100.00 | 100.00 | 100.00 |
| Trypan blue | 1.5 | 10 | 6 | 61.65 | 60.06 | 63.22 |
| Trypan blue | 1.5 | 20 | 6 | 0.00 | 0.00 | 0.00 |
| Solution 1 (ICG) | 5.0 | 1 | 24 | 99.54 | 99.23 | 99.77 |
| Solution 1 (ICG) | 5.0 | 5 | 24 | 93.35 | 92.34 | 94.29 |
| Solution 1 (ICG) | 5.0 | 10 | 24 | 21.16 | 19.58 | 22.78 |
| Solution 1 (ICG) | 5.0 | 20 | 24 | 0.00 | 0.00 | 0.00 |
| Solution 2 (ICG) | 2.5 | 1 | 24 | 100.00 | 100.00 | 100.00 |
| Solution 2 (ICG) | 2.5 | 5 | 24 | 99.42 | 99.09 | 99.68 |
| Solution 2 (ICG) | 2.5 | 10 | 24 | 86.38 | 85.01 | 87.70 |
| Solution 2 (ICG) | 2.5 | 20 | 24 | 84.96 | 83.54 | 86.33 |
| Solution 3 (ICG) | 1.0 | 1 | 24 | 100.00 | 100.00 | 100.00 |
| Solution 3 (ICG) | 1.0 | 5 | 24 | 99.77 | 99.55 | 99.92 |
| Solution 3 (ICG) | 1.0 | 10 | 24 | 93.38 | 92.37 | 94.32 |
| Solution 3 (ICG) | 1.0 | 20 | 24 | 93.07 | 92.04 | 94.03 |
| Trypan blue | 1.5 | 1 | 24 | 100.00 | 100.00 | 100.00 |
| Trypan blue | 1.5 | 5 | 24 | 99.64 | 99.42 | 99.81 |
| Trypan blue | 1.5 | 10 | 24 | 57.75 | 56.14 | 59.35 |
| Trypan blue | 1.5 | 20 | 24 | 0.00 | 0.00 | 0.00 |
| Solution 1 (ICG) | 5.0 | 1 | 72 | 98.47 | 97.95 | 98.91 |
| Solution 1 (ICG) | 5.0 | 5 | 72 | 93.18 | 92.16 | 94.13 |
| Solution 1 (ICG) | 5.0 | 10 | 72 | 18.49 | 16.99 | 20.03 |
| Solution 1 (ICG) | 5.0 | 20 | 72 | 0.00 | 0.00 | 0.00 |
| Solution 2 (ICG) | 2.5 | 1 | 72 | 99.22 | 98.84 | 99.53 |
| Solution 2 (ICG) | 2.5 | 5 | 72 | 97.75 | 97.13 | 98.29 |
| Solution 2 (ICG) | 2.5 | 10 | 72 | 86.41 | 85.04 | 87.72 |
| Solution 2 (ICG) | 2.5 | 20 | 72 | 83.11 | 81.62 | 84.55 |
| Solution 3 (ICG) | 1.0 | 1 | 72 | 99.57 | 99.27 | 99.79 |
| Solution 3 (ICG) | 1.0 | 5 | 72 | 99.20 | 98.81 | 99.51 |
| Solution 3 (ICG) | 1.0 | 10 | 72 | 91.81 | 90.70 | 92.85 |
| Solution 3 (ICG) | 1.0 | 20 | 72 | 91.61 | 90.50 | 92.66 |
| Trypan blue | 1.5 | 1 | 72 | 2.83 | 2.20 | 3.54 |
| Trypan blue | 1.5 | 5 | 72 | 3.51 | 2.81 | 4.28 |
| Trypan blue | 1.5 | 10 | 72 | 60.61 | 58.64 | 62.57 |
| Trypan blue | 1.5 | 20 | 72 | 100.00 | 100.00 | 100.00 |

## 6.5.4.2 Question four part 2–cell survival

***Table 17:*** Summary of fit for the fourth question part 2–cell survival of the set-up without illumination

| | |
|---|---|
| $R^2$ | 82.93% |
| $R^2$ Adj | 79.65% |
| *s* | 0.0200 |
| **Mean of Response** | 1.5447 |
| *n* | 144 |

***Table 18:*** Effect tests for the fourth question part 2–cell survival of the set-up without illumination

| Factors and Interactions | Nparm | DF | Sum of Squares | F Ratio | *P-value* |
|---|---|---|---|---|---|
| Solution | 1 | 1 | 0.0002 | 0.4719 | 0.4934 |
| Incubation time [min] | 3 | 3 | 0.1342 | 112.1122 | <.0001 |
| Solution * Incubation time [min] | 3 | 3 | 0.0021 | 1.7570 | 0.1591 |
| Follow-up time [h] | 2 | 2 | 0.0410 | 51.3404 | <.0001 |
| Solution * Follow-up time [h] | 2 | 2 | 0.0029 | 3.6350 | 0.0293 |
| Incubation time [min] * Follow-up time [h] | 6 | 6 | 0.0469 | 19.5826 | <.0001 |
| Solution * Incubation time [min] * Follow-up time [h] | 6 | 6 | 0.0053 | 2.2158 | 0.0461 |

***Table 19:*** Median cell survival, lower and upper CL for the interaction between the factors solution, incubation time and follow-up time

| Solution | mg/ml | Incubation time [min] | Follow-up time [h[ | Median cell survival [%] | Lower CL [%] | Upper CL [%] |
|---|---|---|---|---|---|---|
| Solution 4 (ICG) | 0.125 | 1 | 6 | 100.00 | 100.00 | 100.00 |
| Solution 4 (ICG) | 0.125 | 5 | 6 | 100.00 | 100.00 | 100.00 |
| Solution 4 (ICG) | 0.125 | 10 | 6 | 100.00 | 100.00 | 100.00 |
| Solution 4 (ICG) | 0.125 | 20 | 6 | 99.79 | 99.58 | 99.93 |
| diluted TB | 0.0375 | 1 | 6 | 100.00 | 100.00 | 100.00 |
| diluted TB | 0.0375 | 5 | 6 | 100.00 | 100.00 | 100.00 |
| diluted TB | 0.0375 | 10 | 6 | 100.00 | 100.00 | 100.00 |
| diluted TB | 0.0375 | 20 | 6 | 99.69 | 99.49 | 99.85 |
| Solution 4 (ICG) | 0.125 | 1 | 24 | 100.00 | 100.00 | 100.00 |
| Solution 4 (ICG) | 0.125 | 5 | 24 | 100.00 | 100.00 | 100.00 |
| Solution 4 (ICG) | 0.125 | 10 | 24 | 100.00 | 100.00 | 100.00 |
| Solution 4 (ICG) | 0.125 | 20 | 24 | 99.86 | 99.68 | 99.97 |
| diluted TB | 0.0375 | 1 | 24 | 100.00 | 100.00 | 100.00 |
| diluted TB | 0.0375 | 5 | 24 | 100.00 | 100.00 | 100.00 |
| diluted TB | 0.0375 | 10 | 24 | 100.00 | 100.00 | 100.00 |
| diluted TB | 0.0375 | 20 | 24 | 99.20 | 98.88 | 99.46 |
| Solution 4 (ICG) | 0.125 | 1 | 72 | 100.00 | 100.00 | 100.00 |
| Solution 4 (ICG) | 0.125 | 5 | 72 | 100.00 | 100.00 | 100.00 |
| Solution 4 (ICG) | 0.125 | 10 | 72 | 99.04 | 98.62 | 99.38 |
| Solution 4 (ICG) | 0.125 | 20 | 72 | 98.59 | 98.10 | 99.02 |
| diluted TB | 0.0375 | 1 | 72 | 100.00 | 100.00 | 100.00 |
| diluted TB | 0.0375 | 5 | 72 | 100.00 | 100.00 | 100.00 |
| diluted TB | 0.0375 | 10 | 72 | 99.34 | 99.05 | 99.58 |
| diluted TB | 0.0375 | 20 | 72 | 98.98 | 98.63 | 99.29 |

## 6.5.5 Question one – morphologic change

***Table 20:*** Summary of fit for the first question–morphologic change of the set-up without illumination

| | |
|---|---|
| $R^2$ | 99.81% |
| $R^2$ Adj | 99.72% |
| *s* | 0.0220 |
| Mean of Response | 0.3386 |
| *n* | 144 |

***Table 21:*** Effect tests for the first question–morphologic change of the set-up without illumination

| Factors and Interactions | Nparm | DF | Sum of Squares | F Ratio | *P-value* |
|---|---|---|---|---|---|
| Solution | 3 | 3 | 10.4328 | 7160.523 | <.0001 |

| Factors and Interactions | Nparm | DF | Sum of Squares | F Ratio | *P-value* |
|---|---|---|---|---|---|
| Incubation time [min] | 3 | 3 | 6.2325 | 4277.653 | <.0001 |
| Solution * Incubation time [min] | 9 | 9 | 7.6378 | 1747.402 | <.0001 |
| Follow-up time [h] | 2 | 2 | 0.0755 | 77.7679 | <.0001 |
| Solution * Follow-up time [h] | 6 | 6 | 0.0096 | 3.2824 | 0.0056 |
| Incubation time [min] * Follow-up time [h] | 6 | 6 | 0.0178 | 6.1096 | <.0001 |
| Solution * Incubation time [min] * Follow-up time [h] | 18 | 18 | 0.0435 | 4.9811 | <.0001 |

***Table 22:*** Median morphologic change, lower and upper CL for the interaction between the factors solution, incubation time and follow-up time

| Solution | mg/ml | Incubation time [min] | Follow-up time [h[ | Median morphologic change [%] | Lower CL [%] | Upper CL [%] |
|---|---|---|---|---|---|---|
| Solution 1 (ICG) | 5.0 | 1 | 6 | 2.27 | 1.58 | 3.09 |
| Solution 1 (ICG) | 5.0 | 5 | 6 | 6.61 | 5.41 | 7.92 |
| Solution 1 (ICG) | 5.0 | 10 | 6 | 81.68 | 79.68 | 83.59 |
| Solution 1 (ICG) | 5.0 | 20 | 6 | 100.00 | 100.00 | 100.00 |
| Solution 2 (ICG) | 2.5 | 1 | 6 | 0.67 | 0.32 | 1.15 |
| Solution 2 (ICG) | 2.5 | 5 | 6 | 1.21 | 0.72 | 1.82 |
| Solution 2 (ICG) | 2.5 | 10 | 6 | 9.76 | 8.31 | 11.31 |
| Solution 2 (ICG) | 2.5 | 20 | 6 | 11.83 | 10.25 | 13.51 |
| Solution 3 (ICG) | 1.0 | 1 | 6 | 0.63 | 0.29 | 1.09 |
| Solution 3 (ICG) | 1.0 | 5 | 6 | 1.08 | 0.62 | 1.67 |
| Solution 3 (ICG) | 1.0 | 10 | 6 | 5.66 | 4.55 | 6.89 |
| Solution 3 (ICG) | 1.0 | 20 | 6 | 7.27 | 6.01 | 8.64 |
| Solution 4 (ICG) | 0.125 | 1 | 6 | 0.39 | 0.14 | 0.77 |
| Solution 4 (ICG) | 0.125 | 5 | 6 | 0.43 | 0.17 | 0.83 |
| Solution 4 (ICG) | 0.125 | 10 | 6 | 0.98 | 0.54 | 1.54 |
| Solution 4 (ICG) | 0.125 | 20 | 6 | 1.50 | 0.94 | 2.17 |
| Solution 1 (ICG) | 5.0 | 1 | 24 | 2.00 | 1.35 | 2.76 |
| Solution 1 (ICG) | 5.0 | 5 | 24 | 7.33 | 6.07 | 8.70 |
| Solution 1 (ICG) | 5.0 | 10 | 24 | 81.18 | 79.17 | 83.12 |
| Solution 1 (ICG) | 5.0 | 20 | 24 | 100.00 | 100.00 | 100.00 |
| Solution 2 (ICG) | 2.5 | 1 | 24 | 1.22 | 0.73 | 1.84 |
| Solution 2 (ICG) | 2.5 | 5 | 24 | 2.13 | 1.47 | 2.93 |
| Solution 2 (ICG) | 2.5 | 10 | 24 | 14.35 | 12.63 | 16.17 |
| Solution 2 (ICG) | 2.5 | 20 | 24 | 15.58 | 13.79 | 17.45 |
| Solution 3 (ICG) | 1.0 | 1 | 24 | 0.73 | 0.36 | 1.22 |
| Solution 3 (ICG) | 1.0 | 5 | 24 | 1.70 | 1.11 | 2.41 |
| Solution 3 (ICG) | 1.0 | 10 | 24 | 6.76 | 5.55 | 8.08 |
| Solution 3 (ICG) | 1.0 | 20 | 24 | 7.25 | 5.99 | 8.61 |
| Solution 4 (ICG) | 0.125 | 1 | 24 | 0.53 | 0.23 | 0.96 |
| Solution 4 (ICG) | 0.125 | 5 | 24 | 0.87 | 0.46 | 1.40 |
| Solution 4 (ICG) | 0.125 | 10 | 24 | 1.43 | 0.89 | 2.09 |
| Solution 4 (ICG) | 0.125 | 20 | 24 | 1.79 | 1.18 | 2.52 |
| Solution 1 (ICG) | 5.0 | 1 | 72 | 2.75 | 1.98 | 3.64 |
| Solution 1 (ICG) | 5.0 | 5 | 72 | 7.17 | 5.93 | 8.53 |
| Solution 1 (ICG) | 5.0 | 10 | 72 | 94.04 | 92.79 | 95.18 |
| Solution 1 (ICG) | 5.0 | 20 | 72 | 100.00 | 100.00 | 100.00 |
| Solution 2 (ICG) | 2.5 | 1 | 72 | 2.48 | 1.76 | 3.33 |
| Solution 2 (ICG) | 2.5 | 5 | 72 | 3.31 | 2.46 | 4.27 |
| Solution 2 (ICG) | 2.5 | 10 | 72 | 14.65 | 12.91 | 16.48 |
| Solution 2 (ICG) | 2.5 | 20 | 72 | 17.27 | 15.40 | 19.22 |
| Solution 3 (ICG) | 1.0 | 1 | 72 | 0.98 | 0.55 | 1.54 |
| Solution 3 (ICG) | 1.0 | 5 | 72 | 2.92 | 2.13 | 3.83 |
| Solution 3 (ICG) | 1.0 | 10 | 72 | 8.43 | 7.08 | 9.88 |
| Solution 3 (ICG) | 1.0 | 20 | 72 | 8.86 | 7.48 | 10.35 |

| Solution | mg/ml | Incubation time [min] | Follow-up time [h[ | Median morphologic change [%] | Lower CL [%] | Upper CL [%] |
|---|---|---|---|---|---|---|
| Solution 4 (ICG) | 0.125 | 1 | 72 | 1.19 | 0.70 | 1.79 |
| Solution 4 (ICG) | 0.125 | 5 | 72 | 1.17 | 0.69 | 1.78 |
| Solution 4 (ICG) | 0.125 | 10 | 72 | 2.72 | 1.96 | 3.60 |
| Solution 4 (ICG) | 0.125 | 20 | 72 | 2.46 | 1.74 | 3.31 |

## 6.5.6 Question two – morphologic change

***Table 23:*** Summary of fit for the second question–morphologic change of the set-up without illumination

| | |
|---|---|
| $R^2$ | 99.92% |
| $R^2$ Adj | 99.89% |
| *s* | 0.0173 |
| Mean of Response | 0.3950 |
| *n* | 72 |

***Table 24:*** Effect tests for the second question–morphologic change of the set-up without illumination

| Factors and Interactions | Nparm | DF | Sum of Squares | F Ratio | *P-value* |
|---|---|---|---|---|---|
| Solution | 1 | 1 | 6.2888 | 20969.55 | <.0001 |
| Incubation time [min] | 3 | 3 | 6.8681 | 7633.737 | <.0001 |
| Solution * Incubation time [min] | 3 | 3 | 5.9406 | 6602.814 | <.0001 |
| Follow-up time [h] | 2 | 2 | 0.0110 | 18.3335 | <.0001 |
| Solution * Follow-up time [h] | 2 | 2 | 0.0003 | 0.4658 | 0.6304 |
| Incubation time [min] * Follow-up time [h] | 6 | 6 | 0.0054 | 3.0258 | 0.0137 |
| Solution * Incubation time [min] * Follow-up time [h] | 6 | 6 | 0.0069 | 3.8072 | 0.0035 |

***Table 25:*** Median morphologic change, lower and upper CL for the interaction between the factors solution, incubation time and follow-up time

| Solution | mg/ml | Incubation time [min] | Follow-up time [h[ | Median morphologic change [%] | Lower CL [%] | Upper CL [%] |
|---|---|---|---|---|---|---|
| Trypan blue | 1.5 | 1 | 6 | 0.90 | 0.56 | 1.32 |
| Trypan blue | 1.5 | 5 | 6 | 1.49 | 1.05 | 2.02 |
| Trypan blue | 1.5 | 10 | 6 | 62.64 | 60.69 | 64.58 |
| Trypan blue | 1.5 | 20 | 6 | 100.00 | 100.00 | 100.00 |
| diluted TB | 0.0375 | 1 | 6 | 0.39 | 0.18 | 0.68 |
| diluted TB | 0.0375 | 5 | 6 | 0.42 | 0.20 | 0.72 |
| diluted TB | 0.0375 | 10 | 6 | 0.80 | 0.48 | 1.20 |
| diluted TB | 0.0375 | 20 | 6 | 1.68 | 1.20 | 2.23 |
| Trypan blue | 1.5 | 1 | 24 | 1.69 | 1.21 | 2.25 |
| Trypan blue | 1.5 | 5 | 24 | 2.28 | 1.72 | 2.92 |
| Trypan blue | 1.5 | 10 | 24 | 62.51 | 60.55 | 64.44 |
| Trypan blue | 1.5 | 20 | 24 | 100.00 | 100.00 | 100.00 |
| diluted TB | 0.0375 | 1 | 24 | 0.72 | 0.42 | 1.10 |
| diluted TB | 0.0375 | 5 | 24 | 0.71 | 0.41 | 1.08 |
| diluted TB | 0.0375 | 10 | 24 | 0.90 | 0.56 | 1.31 |
| diluted TB | 0.0375 | 20 | 24 | 1.31 | 0.89 | 1.80 |
| Trypan blue | 1.5 | 1 | 72 | 2.83 | 2.20 | 3.54 |
| Trypan blue | 1.5 | 5 | 72 | 3.51 | 2.81 | 4.28 |
| Trypan blue | 1.5 | 10 | 72 | 60.61 | 58.64 | 62.57 |
| Trypan blue | 1.5 | 20 | 72 | 100.00 | 100.00 | 100.00 |
| diluted TB | 0.0375 | 1 | 72 | 0.71 | 0.41 | 1.08 |
| diluted TB | 0.0375 | 5 | 72 | 1.05 | 0.68 | 1.49 |

| Solution | mg/ml | Incubation time [min] | Follow-up time [h[ | Median morphologic change [%] | Lower CL [%] | Upper CL [%] |
|---|---|---|---|---|---|---|
| diluted TB | 0.0375 | 10 | 72 | 1.58 | 1.12 | 2.12 |
| diluted TB | 0.0375 | 20 | 72 | 2.41 | 1.83 | 3.06 |

## 6.5.7 Question three – morphologic change

***Table 26:*** Summary of fit for the third question–morphologic change of the set-up without illumination

| | |
|---|---|
| $R^2$ | 99.78% |
| $R^2$ Adj | 99.67% |
| *s* | 0.0254 |
| Mean of Response | 0.4095 |
| *n* | 216 |

***Table 27:*** Effect tests for the third question–morphologic change of the set-up without illumination

| Factors and Interactions | Nparm | DF | Sum of Squares | F Ratio | *P-value* |
|---|---|---|---|---|---|
| Solution number | 2 | 2 | 14.7325 | 11377.85 | <.0001 |
| Dye | 1 | 1 | 0.0069 | 10.6228 | 0.0014 |
| Incubation time [min] | 3 | 3 | 15.4866 | 7973.511 | <.0001 |
| Follow-up time [h] | 2 | 2 | 0.1568 | 121.1035 | <.0001 |
| Solution number * Dye | 2 | 2 | 0.0198 | 15.2806 | <.0001 |
| Solution number * Incubation time [min] | 6 | 6 | 11.5644 | 2977.053 | <.0001 |
| Solution number * Follow-up time [h] | 4 | 4 | 0.0171 | 6.6015 | <.0001 |
| Dye * Incubation time [min] | 3 | 3 | 0.0021 | 1.1015 | 0.3507 |
| Dye * Follow-up time [h] | 2 | 2 | 0.0064 | 4.9316 | 0.0085 |
| Incubation time [min] * Follow-up time [h] | 6 | 6 | 0.0248 | 6.3779 | <.0001 |
| Solution number * Dye * Incubation time [min] | 6 | 6 | 0.0080 | 2.0495 | 0.0628 |
| Solution number * Dye * Follow-up time [h] | 4 | 4 | 0.0017 | 0.6648 | 0.6174 |
| Solution number * Incubation time [min] * Follow-up time [h] | 12 | 12 | 0.0526 | 6.7726 | <.0001 |
| Dye * Incubation time [min] * Follow-up time [h] | 6 | 6 | 0.0031 | 0.8086 | 0.5648 |
| Solution number * Dye * Incubation time [min] * Follow-up time [h] | 12 | 12 | 0.0066 | 0.8541 | 0.5950 |

***Table 28:*** Median morphologic change, lower and upper CL for the interaction between the factors solution number, dye, incubation & illumination time and follow-up time

| Solution | mg/ml | Incubation time [h] | Follow-up time [h] | Median morph. change [%] | Lower CL [%] | Upper CL [%] |
|---|---|---|---|---|---|---|
| Solution 1 (ICG) | 5.0 | 1 | 6 | 2.27 | 1.58 | 3.09 |
| Solution 1 (ICG) | 5.0 | 5 | 6 | 6.61 | 5.41 | 7.92 |
| Solution 1 (ICG) | 5.0 | 10 | 6 | 81.68 | 79.68 | 83.59 |
| Solution 1 (ICG) | 5.0 | 20 | 6 | 100.00 | 100.00 | 100.00 |
| Solution 1 (ICG-free) | 0.0 | 1 | 6 | 0.95 | 0.43 | 1.68 |

| Solution | mg/ml | Incubation time [h] | Follow-up time [h] | Median morph. change [%] | Lower CL [%] | Upper CL [%] |
|---|---|---|---|---|---|---|
| Solution 1 (ICG-free) | 0.0 | 5 | 6 | 5.91 | 4.50 | 7.51 |
| Solution 1 (ICG-free) | 0.0 | 10 | 6 | 75.51 | 72.71 | 78.21 |
| Solution 1 (ICG-free) | 0.0 | 20 | 6 | 99.31 | 98.68 | 99.74 |
| Solution 2 (ICG) | 2.5 | 1 | 6 | 0.67 | 0.32 | 1.15 |
| Solution 2 (ICG) | 2.5 | 5 | 6 | 1.21 | 0.72 | 1.82 |
| Solution 2 (ICG) | 2.5 | 10 | 6 | 9.76 | 8.31 | 11.31 |
| Solution 2 (ICG) | 2.5 | 20 | 6 | 11.83 | 10.25 | 13.51 |
| Solution 2 (ICG-free) | 0.0 | 1 | 6 | 0.68 | 0.26 | 1.31 |
| Solution 2 (ICG-free) | 0.0 | 5 | 6 | 0.87 | 0.38 | 1.57 |
| Solution 2 (ICG-free) | 0.0 | 10 | 6 | 9.89 | 8.07 | 11.88 |
| Solution 2 (ICG-free) | 0.0 | 20 | 6 | 12.23 | 10.21 | 14.40 |
| Solution 3 (ICG) | 1.0 | 1 | 6 | 0.63 | 0.29 | 1.09 |
| Solution 3 (ICG) | 1.0 | 5 | 6 | 1.08 | 0.62 | 1.67 |
| Solution 3 (ICG) | 1.0 | 10 | 6 | 5.66 | 4.55 | 6.89 |
| Solution 3 (ICG) | 1.0 | 20 | 6 | 7.27 | 6.01 | 8.64 |
| Solution 3 (ICG-free) | 0.0 | 1 | 6 | 0.43 | 0.11 | 0.95 |
| Solution 3 (ICG-free) | 0.0 | 5 | 6 | 0.98 | 0.45 | 1.71 |
| Solution 3 (ICG-free) | 0.0 | 10 | 6 | 4.67 | 3.41 | 6.10 |
| Solution 3 (ICG-free) | 0.0 | 20 | 6 | 5.74 | 4.35 | 7.32 |
| Solution 1 (ICG) | 5.0 | 1 | 24 | 2.00 | 1.27 | 2.89 |
| Solution 1 (ICG) | 5.0 | 5 | 24 | 7.33 | 5.89 | 8.91 |
| Solution 1 (ICG) | 5.0 | 10 | 24 | 81.18 | 78.86 | 83.40 |
| Solution 1 (ICG) | 5.0 | 20 | 24 | 100.00 | 100.00 | 100.00 |
| Solution 1 (ICG-free) | 0.0 | 1 | 24 | 1.32 | 0.74 | 2.06 |
| Solution 1 (ICG-free) | 0.0 | 5 | 24 | 6.98 | 5.58 | 8.54 |
| Solution 1 (ICG-free) | 0.0 | 10 | 24 | 78.35 | 75.91 | 80.69 |
| Solution 1 (ICG-free) | 0.0 | 20 | 24 | 99.74 | 99.36 | 99.95 |
| Solution 2 (ICG) | 2.5 | 1 | 24 | 1.22 | 0.67 | 1.94 |
| Solution 2 (ICG) | 2.5 | 5 | 24 | 2.13 | 1.38 | 3.05 |
| Solution 2 (ICG) | 2.5 | 10 | 24 | 14.35 | 12.38 | 16.45 |
| Solution 2 (ICG) | 2.5 | 20 | 24 | 15.58 | 13.53 | 17.74 |
| Solution 2 (ICG-free) | 0.0 | 1 | 24 | 1.63 | 0.98 | 2.44 |
| Solution 2 (ICG-free) | 0.0 | 5 | 24 | 2.38 | 1.58 | 3.35 |
| Solution 2 (ICG-free) | 0.0 | 10 | 24 | 14.94 | 12.93 | 17.07 |
| Solution 2 (ICG-free) | 0.0 | 20 | 24 | 16.74 | 14.63 | 18.96 |
| Solution 3 (ICG) | 1.0 | 1 | 24 | 0.73 | 0.32 | 1.31 |
| Solution 3 (ICG) | 1.0 | 5 | 24 | 1.70 | 1.03 | 2.53 |
| Solution 3 (ICG) | 1.0 | 10 | 24 | 6.76 | 5.38 | 8.29 |
| Solution 3 (ICG) | 1.0 | 20 | 24 | 7.25 | 5.82 | 8.83 |
| Solution 3 (ICG-free) | 0.0 | 1 | 24 | 0.68 | 0.29 | 1.24 |
| Solution 3 (ICG-free) | 0.0 | 5 | 24 | 2.04 | 1.30 | 2.94 |
| Solution 3 (ICG-free) | 0.0 | 10 | 24 | 8.16 | 6.64 | 9.82 |
| Solution 3 (ICG-free) | 0.0 | 20 | 24 | 9.73 | 8.08 | 11.52 |
| Solution 1 (ICG) | 5.0 | 1 | 72 | 2.75 | 1.88 | 3.78 |
| Solution 1 (ICG) | 5.0 | 5 | 72 | 7.17 | 5.75 | 8.74 |
| Solution 1 (ICG) | 5.0 | 10 | 72 | 94.04 | 92.60 | 95.34 |
| Solution 1 (ICG) | 5.0 | 20 | 72 | 100.00 | 100.00 | 100.00 |
| Solution 1 (ICG-free) | 0.0 | 1 | 72 | 2.54 | 1.71 | 3.53 |
| Solution 1 (ICG-free) | 0.0 | 5 | 72 | 6.90 | 5.50 | 8.45 |
| Solution 1 (ICG-free) | 0.0 | 10 | 72 | 88.39 | 86.46 | 90.18 |
| Solution 1 (ICG-free) | 0.0 | 20 | 72 | 100.00 | 100.00 | 100.00 |
| Solution 2 (ICG) | 2.5 | 1 | 72 | 2.48 | 1.66 | 3.47 |
| Solution 2 (ICG) | 2.5 | 5 | 72 | 3.31 | 2.35 | 4.42 |
| Solution 2 (ICG) | 2.5 | 10 | 72 | 14.65 | 12.65 | 16.76 |
| Solution 2 (ICG) | 2.5 | 20 | 72 | 17.27 | 15.13 | 19.52 |
| Solution 2 (ICG-free) | 0.0 | 1 | 72 | 2.51 | 1.69 | 3.50 |
| Solution 2 (ICG-free) | 0.0 | 5 | 72 | 3.24 | 2.29 | 4.35 |
| Solution 2 (ICG-free) | 0.0 | 10 | 72 | 15.03 | 13.01 | 17.16 |
| Solution 2 (ICG-free) | 0.0 | 20 | 72 | 17.46 | 15.32 | 19.72 |

| Solution | mg/ml | Incubation time [h] | Follow-up time [h] | Median morph. change [%] | Lower CL [%] | Upper CL [%] |
|---|---|---|---|---|---|---|
| Solution 3 (ICG) | 1.0 | 1 | 72 | 0.98 | 0.49 | 1.64 |
| Solution 3 (ICG) | 1.0 | 5 | 72 | 2.92 | 2.02 | 3.98 |
| Solution 3 (ICG) | 1.0 | 10 | 72 | 8.43 | 6.89 | 10.11 |
| Solution 3 (ICG) | 1.0 | 20 | 72 | 8.86 | 7.28 | 10.58 |
| Solution 3 (ICG-free) | 0.0 | 1 | 72 | 0.88 | 0.42 | 1.50 |
| Solution 3 (ICG-free) | 0.0 | 5 | 72 | 2.60 | 1.76 | 3.61 |
| Solution 3 (ICG-free) | 0.0 | 10 | 72 | 8.34 | 6.80 | 10.01 |
| Solution 3 (ICG-free) | 0.0 | 20 | 72 | 9.53 | 7.90 | 11.31 |

## 6.5.8.1 Question four part 1–morphologic change

***Table 29:*** Summary of fit for the fourth question part 1–morphologic change of the set-up without illumination

| | |
|---|---|
| $R^2$ | 99.88% |
| $R^2$ Adj | 99.82% |
| *s* | 0.0215 |
| Mean of Response | 0.4840 |
| *n* | 144 |

***Table 30:*** Effect tests for the fourth question part 1–morphologic change of the set-up without illumination

| Factors and Interactions | Nparm | DF | Sum of Squares | F Ratio | *P-value* |
|---|---|---|---|---|---|
| Solution | 3 | 3 | 9.956087 | 7149.494 | <.0001 |
| Incubation time [min] | 3 | 3 | 17.515185 | 12577.7 | <.0001 |
| Solution * Incubation time [min] | 9 | 9 | 9.125542 | 2184.359 | <.0001 |
| Follow-up time [h] | 2 | 2 | 0.063285 | 68.1677 | <.0001 |
| Solution * Follow-up time [h] | 6 | 6 | 0.013173 | 4.7299 | 0.0003 |
| Incubation time [min] * Follow-up time [h] | 6 | 6 | 0.008970 | 3.2206 | 0.0063 |
| Solution * Incubation time [min] * Follow-up time [h] | 18 | 18 | 0.061345 | 7.3420 | <.0001 |

***Table 31:*** Median morphologic change, lower and upper CL for the interaction between the factors solution, incubation time and follow-up time

| Solution | mg/ml | Incubation time [min] | Follow-up time [h[ | Median morph. change [%] | Lower CL [%] | Upper CL [%] |
|---|---|---|---|---|---|---|
| Solution 1 (ICG) | 5.0 | 1 | 6 | 2.27 | 1.58 | 3.09 |
| Solution 1 (ICG) | 5.0 | 5 | 6 | 6.61 | 5.41 | 7.92 |
| Solution 1 (ICG) | 5.0 | 10 | 6 | 81.68 | 79.68 | 83.59 |
| Solution 1 (ICG) | 5.0 | 20 | 6 | 100.00 | 100.00 | 100.00 |
| Solution 2 (ICG) | 2.5 | 1 | 6 | 0.67 | 0.32 | 1.15 |
| Solution 2 (ICG) | 2.5 | 5 | 6 | 1.21 | 0.72 | 1.82 |
| Solution 2 (ICG) | 2.5 | 10 | 6 | 9.76 | 8.31 | 11.31 |
| Solution 2 (ICG) | 2.5 | 20 | 6 | 11.83 | 10.25 | 13.51 |
| Solution 3 (ICG) | 1.0 | 1 | 6 | 0.63 | 0.29 | 1.09 |
| Solution 3 (ICG) | 1.0 | 5 | 6 | 1.08 | 0.62 | 1.67 |
| Solution 3 (ICG) | 1.0 | 10 | 6 | 5.66 | 4.55 | 6.89 |
| Solution 3 (ICG) | 1.0 | 20 | 6 | 7.27 | 6.01 | 8.64 |
| Trypan blue | 1.5 | 1 | 6 | 0.90 | 0.56 | 1.32 |
| Trypan blue | 1.5 | 5 | 6 | 1.49 | 1.05 | 2.02 |
| Trypan blue | 1.5 | 10 | 6 | 62.64 | 60.69 | 64.58 |
| Trypan blue | 1.5 | 20 | 6 | 100.00 | 100.00 | 100.00 |
| Solution 1 (ICG) | 5.0 | 1 | 24 | 2.00 | 1.35 | 2.76 |

| Solution | mg/ml | Incubation time [min] | Follow-up time [h[ | Median morph. change [%] | Lower CL [%] | Upper CL [%] |
|---|---|---|---|---|---|---|
| Solution 1 (ICG) | 5.0 | 5 | 24 | 7.33 | 6.07 | 8.70 |
| Solution 1 (ICG) | 5.0 | 10 | 24 | 81.18 | 79.17 | 83.12 |
| Solution 1 (ICG) | 5.0 | 20 | 24 | 100.00 | 100.00 | 100.00 |
| Solution 2 (ICG) | 2.5 | 1 | 24 | 1.22 | 0.73 | 1.84 |
| Solution 2 (ICG) | 2.5 | 5 | 24 | 2.13 | 1.47 | 2.93 |
| Solution 2 (ICG) | 2.5 | 10 | 24 | 14.35 | 12.63 | 16.17 |
| Solution 2 (ICG) | 2.5 | 20 | 24 | 15.58 | 13.79 | 17.45 |
| Solution 3 (ICG) | 1.0 | 1 | 24 | 0.73 | 0.36 | 1.22 |
| Solution 3 (ICG) | 1.0 | 5 | 24 | 1.70 | 1.11 | 2.41 |
| Solution 3 (ICG) | 1.0 | 10 | 24 | 6.76 | 5.55 | 8.08 |
| Solution 3 (ICG) | 1.0 | 20 | 24 | 7.25 | 5.99 | 8.61 |
| Trypan blue | 1.5 | 1 | 24 | 1.69 | 1.21 | 2.25 |
| Trypan blue | 1.5 | 5 | 24 | 2.28 | 1.72 | 2.92 |
| Trypan blue | 1.5 | 10 | 24 | 62.51 | 60.55 | 64.44 |
| Trypan blue | 1.5 | 20 | 24 | 100.00 | 100.00 | 100.00 |
| Solution 1 (ICG) | 5.0 | 1 | 72 | 2.75 | 1.98 | 3.64 |
| Solution 1 (ICG) | 5.0 | 5 | 72 | 7.17 | 5.93 | 8.53 |
| Solution 1 (ICG) | 5.0 | 10 | 72 | 94.04 | 92.79 | 95.18 |
| Solution 1 (ICG) | 5.0 | 20 | 72 | 100.00 | 100.00 | 100.00 |
| Solution 2 (ICG) | 2.5 | 1 | 72 | 2.48 | 1.76 | 3.33 |
| Solution 2 (ICG) | 2.5 | 5 | 72 | 3.31 | 2.46 | 4.27 |
| Solution 2 (ICG) | 2.5 | 10 | 72 | 14.65 | 12.91 | 16.48 |
| Solution 2 (ICG) | 2.5 | 20 | 72 | 17.27 | 15.40 | 19.22 |
| Solution 3 (ICG) | 1.0 | 1 | 72 | 0.98 | 0.55 | 1.54 |
| Solution 3 (ICG) | 1.0 | 5 | 72 | 2.92 | 2.13 | 3.83 |
| Solution 3 (ICG) | 1.0 | 10 | 72 | 8.43 | 7.08 | 9.88 |
| Solution 3 (ICG) | 1.0 | 20 | 72 | 8.86 | 7.48 | 10.35 |
| Trypan blue | 1.5 | 1 | 72 | 2.83 | 2.20 | 3.54 |
| Trypan blue | 1.5 | 5 | 72 | 3.51 | 2.81 | 4.28 |
| Trypan blue | 1.5 | 10 | 72 | 60.61 | 58.64 | 62.57 |
| Trypan blue | 1.5 | 20 | 72 | 100.00 | 100.00 | 100.00 |

## 6.5.8.2 Question four part 2–morphologic change

***Table 32:*** Summary of fit for the fourth question part 2–morphologic change of the set-up without illumination

| | |
|---|---|
| $R^2$ | 79.37% |
| $R^2$ Adj | 69.49% |
| *s* | 0.0185 |
| **Mean of Response** | 0.1044 |
| *n* | 72 |

***Table 33:*** Effect tests for the fourth question part 2–morphologic change of the set-up without illumination

| Factors and Interactions | Nparm | DF | Sum of Squares | F Ratio | *P-value* |
|---|---|---|---|---|---|
| Solution | 1 | 1 | 0.00171246 | 4.9947 | 0.0301 |
| Incubation time [min] | 3 | 3 | 0.03702881 | 36.0003 | <.0001 |
| Solution * Incubation time [min] | 3 | 3 | 0.00133041 | 1.2935 | 0.2874 |
| Follow-up time [h] | 2 | 2 | 0.01901267 | 27.7268 | <.0001 |
| Solution * Follow-up time [h] | 2 | 2 | 0.00090776 | 1.3238 | 0.2757 |
| Incubation time [min] * Follow-up time [h] | 6 | 6 | 0.00188185 | 0.9148 | 0.4926 |
| Solution * Incubation time [min] * Follow-up time [h] | 6 | 6 | 0.00144672 | 0.7033 | 0.6484 |

***Table 34:*** Median morphologic change, lower and upper CL for the interaction between the factors solution, incubation time and follow-up time

| Solution | mg/ml | Incubation time [min] | Follow-up time [h[ | Median morph. change [%] | Lower CL [%] | Upper CL [%] |
|---|---|---|---|---|---|---|
| Solution 4 (ICG) | 0.125 | 1 | 6 | 0.39 | 0.14 | 0.77 |
| Solution 4 (ICG) | 0.125 | 5 | 6 | 0.43 | 0.17 | 0.83 |
| Solution 4 (ICG) | 0.125 | 10 | 6 | 0.98 | 0.54 | 1.54 |
| Solution 4 (ICG) | 0.125 | 20 | 6 | 1.50 | 0.94 | 2.17 |
| diluted TB | 0.0375 | 1 | 6 | 0.39 | 0.18 | 0.68 |
| diluted TB | 0.0375 | 5 | 6 | 0.42 | 0.20 | 0.72 |
| diluted TB | 0.0375 | 10 | 6 | 0.80 | 0.48 | 1.20 |
| diluted TB | 0.0375 | 20 | 6 | 1.68 | 1.20 | 2.23 |
| Solution 4 (ICG) | 0.125 | 1 | 24 | 0.53 | 0.23 | 0.96 |
| Solution 4 (ICG) | 0.125 | 5 | 24 | 0.87 | 0.46 | 1.40 |
| Solution 4 (ICG) | 0.125 | 10 | 24 | 1.43 | 0.89 | 2.09 |
| Solution 4 (ICG) | 0.125 | 20 | 24 | 1.79 | 1.18 | 2.52 |
| diluted TB | 0.0375 | 1 | 24 | 0.72 | 0.42 | 1.10 |
| diluted TB | 0.0375 | 5 | 24 | 0.71 | 0.41 | 1.08 |
| diluted TB | 0.0375 | 10 | 24 | 0.90 | 0.56 | 1.31 |
| diluted TB | 0.0375 | 20 | 24 | 1.31 | 0.89 | 1.80 |
| Solution 4 (ICG) | 0.125 | 1 | 72 | 1.19 | 0.70 | 1.79 |
| Solution 4 (ICG) | 0.125 | 5 | 72 | 1.17 | 0.69 | 1.78 |
| Solution 4 (ICG) | 0.125 | 10 | 72 | 2.72 | 1.96 | 3.60 |
| Solution 4 (ICG) | 0.125 | 20 | 72 | 2.46 | 1.74 | 3.31 |
| diluted TB | 0.0375 | 1 | 72 | 0.71 | 0.41 | 1.08 |
| diluted TB | 0.0375 | 5 | 72 | 1.05 | 0.68 | 1.49 |
| diluted TB | 0.0375 | 10 | 72 | 1.58 | 1.12 | 2.12 |
| diluted TB | 0.0375 | 20 | 72 | 2.41 | 1.83 | 3.06 |

# 6.6 Tables of the set-up with illumination

## 6.6.1 Question one – cell survival

***Table 35:*** Summary of fit for the first question–cell survival of the set-up with illumination

| | |
|---|---|
| $R^2$ | 97.54% |
| $R^2$ Adj | 97.07% |
| *s* | 0.0302 |
| Mean of Response | 1.3488 |
| *n* | 144 |

***Table 36:*** Effect tests for the first question–cell survival of the set-up with illumination

| Factors and Interactions | Nparm | DF | Sum of Squares | F Ratio | *P-value* |
|---|---|---|---|---|---|
| Solution | 3 | 3 | 3.5789 | 1307.028 | <.0001 |
| Incubation & Illumination time [min] | 1 | 1 | 0.3332 | 365.1303 | <.0001 |
| Solution * Incubation & Illumination time [min] | 3 | 3 | 0.0675 | 24.6442 | <.0001 |
| Follow-up time [h] | 2 | 2 | 0.1806 | 98.9446 | <.0001 |
| Solution * Follow-up time [h] | 6 | 6 | 0.0832 | 15.1964 | <.0001 |
| Incubation & Illumination time [min] * Follow-up time [h] | 2 | 2 | 0.0140 | 7.6455 | 0.0007 |
| Solution * Incubation & Illumination time [min] * Follow-up time [h] | 6 | 6 | 0.0807 | 14.7393 | <.0001 |

***Table 37:*** Median cell survival, lower and upper CL for the interaction between the factors solution, incubation & illumination time and follow-up time

| Solution | mg/ml | Incub. & illum. time [h] | Follow-up time [h] | Median cell survival [%] | Lower CL [%] | Upper CL [%] |
|---|---|---|---|---|---|---|
| Solution 1 (ICG) | 5.0 | 1 | 6 | 89.20 | 87.63 | 90.66 |
| Solution 1 (ICG) | 5.0 | 5 | 6 | 85.99 | 84.25 | 87.64 |
| Solution 1 (ICG) | 5.0 | 1 | 24 | 86.50 | 84.79 | 88.12 |
| Solution 1 (ICG) | 5.0 | 5 | 24 | 71.66 | 69.44 | 73.84 |
| Solution 1 (ICG) | 5.0 | 1 | 72 | 84.83 | 83.04 | 86.54 |
| Solution 1 (ICG) | 5.0 | 5 | 72 | 66.15 | 63.82 | 68.44 |
| Solution 2 (ICG) | 2.5 | 1 | 6 | 96.28 | 95.30 | 97.15 |
| Solution 2 (ICG) | 2.5 | 5 | 6 | 92.33 | 90.98 | 93.58 |
| Solution 2 (ICG) | 2.5 | 1 | 24 | 92.99 | 91.70 | 94.19 |
| Solution 2 (ICG) | 2.5 | 5 | 24 | 91.32 | 89.89 | 92.64 |
| Solution 2 (ICG) | 2.5 | 1 | 72 | 88.80 | 87.22 | 90.29 |
| Solution 2 (ICG) | 2.5 | 5 | 72 | 89.03 | 87.46 | 90.51 |
| Solution 3 (ICG) | 1.0 | 1 | 6 | 99.92 | 99.72 | 100.00 |
| Solution 3 (ICG) | 1.0 | 5 | 6 | 97.13 | 96.26 | 97.89 |
| Solution 3 (ICG) | 1.0 | 1 | 24 | 100.00 | 100.00 | 100.00 |
| Solution 3 (ICG) | 1.0 | 5 | 24 | 97.09 | 96.21 | 97.86 |
| Solution 3 (ICG) | 1.0 | 1 | 72 | 97.95 | 97.20 | 98.59 |
| Solution 3 (ICG) | 1.0 | 5 | 72 | 96.34 | 95.38 | 97.21 |
| Solution 4 (ICG) | 0.125 | 1 | 6 | 100.00 | 100.00 | 100.00 |
| Solution 4 (ICG) | 0.125 | 5 | 6 | 99.89 | 99.66 | 99.99 |
| Solution 4 (ICG) | 0.125 | 1 | 24 | 100.00 | 100.00 | 100.00 |
| Solution 4 (ICG) | 0.125 | 5 | 24 | 98.88 | 98.31 | 99.33 |
| Solution 4 (ICG) | 0.125 | 1 | 72 | 100.00 | 100.00 | 100.00 |
| Solution 4 (ICG) | 0.125 | 5 | 72 | 99.29 | 98.82 | 99.64 |

## 6.6.2 Question two – cell survival

***Table 38:*** Summary of fit for the second question–cell survival of the set-up with illumination

| | |
|---|---|
| $R^2$ | 26.25% |
| $R^2$ Adj | 12.73% |
| *s* | 0.0658 |
| **Mean of Response** | 1.5457 |
| *n* | 72 |

***Table 39:*** Effect tests for the second question–cell survival of the set-up with illumination

| Factors and Interactions | Nparm | DF | Sum of Squares | F Ratio | *P-value* |
|---|---|---|---|---|---|
| Solution | 1 | 1 | 0.0091 | 2.0944 | 0.1530 |
| Incubation & Illumination time [min] | 1 | 1 | 0.0105 | 2.4209 | 0.1250 |
| Solution * Incubation & Illumination time [min] | 1 | 1 | 0.0002 | 0.0555 | 0.8145 |
| Follow-up time [h] | 2 | 2 | 0.0455 | 5.2570 | 0.0079 |
| Solution * Follow-up time [h] | 2 | 2 | 0.0046 | 0.5287 | 0.5921 |
| Incubation & Illumination time [min] * Follow-up time [h] | 2 | 2 | 0.0222 | 2.5702 | 0.0849 |
| Solution * Incubation & Illumination time [min] * Follow-up time [h] | 2 | 2 | 0.0003 | 0.0363 | 0.9644 |

***Table 40:*** Median cell survival, lower and upper CL for the interaction between the factors solution, incubation & illumination time and follow-up time

| Solution | mg/ml | Incub. & illum. time [h] | Follow-up time [h] | Median cell survival [%] | Lower CL [%] | Upper CL [%] |
|---|---|---|---|---|---|---|
| Trypan blue | 1.5 | 1 | 6 | 100.00 | 100.00 | 100.00 |

| Solution | mg/ml | Incub. & illum. time [h] | Follow-up time [h] | Median cell survival [%] | Lower CL [%] | Upper CL [%] |
|---|---|---|---|---|---|---|
| Trypan blue | 1.5 | 5 | 6 | 100.00 | 100.00 | 100.00 |
| Trypan blue | 1.5 | 1 | 24 | 99.89 | 99.25 | 99.96 |
| Trypan blue | 1.5 | 5 | 24 | 99.91 | 99.30 | 99.95 |
| Trypan blue | 1.5 | 1 | 72 | 99.80 | 99.03 | 99.99 |
| Trypan blue | 1.5 | 5 | 72 | 98.81 | 97.36 | 99.69 |
| diluted Trypan blue | 0.0375 | 1 | 6 | 100.00 | 100.00 | 100.00 |
| diluted Trypan blue | 0.0375 | 5 | 6 | 100.00 | 100.00 | 100.00 |
| diluted Trypan blue | 0.0375 | 1 | 24 | 100.00 | 100.00 | 100.00 |
| diluted Trypan blue | 0.0375 | 5 | 24 | 100.00 | 100.00 | 100.00 |
| diluted Trypan blue | 0.0375 | 1 | 72 | 100.00 | 100.00 | 100.00 |
| diluted Trypan blue | 0.0375 | 5 | 72 | 99.31 | 98.13 | 99.91 |

### 6.6.3 Question three – cell survival

***Table 41:*** Summary of fit for the third question–cell survival of the set-up with illumination

| | |
|---|---|
| $R^2$ | 97.58% |
| $R^2$ Adj | 97.11% |
| *s* | 0.0281 |
| **Mean of Response** | 1.3999 |
| *n* | 216 |

***Table 42:*** Effect tests for the third question–cell survival of the set-up with illumination

| Factors and Interactions | Nparm | DF | Sum of Squares | F Ratio | *P-value* |
|---|---|---|---|---|---|
| Solution number | 2 | 2 | 1.4552 | 920.2833 | <.0001 |
| Dye | 1 | 1 | 2.7434 | 3469.858 | <.0001 |
| Incubation & Illumination time [min] | 1 | 1 | 0.4237 | 535.9263 | <.0001 |
| Follow-up time [h] | 2 | 2 | 0.2015 | 127.4039 | <.0001 |
| Solution number * Dye | 2 | 2 | 0.6102 | 385.9042 | <.0001 |
| Solution number * Incubation & Illumination time [min] | 2 | 2 | 0.0276 | 17.4690 | <.0001 |
| Solution number * Follow-up time [h] | 4 | 4 | 0.0256 | 8.0898 | <.0001 |
| Dye * Incubation & Illumination time [min] | 1 | 1 | 0.0118 | 14.9028 | 0.0002 |
| Dye * Follow-up time [h] | 2 | 2 | 0.0410 | 25.9220 | <.0001 |
| Incubation & Illumination time [min] * Follow-up time [h] | 2 | 2 | 0.0013 | 0.8415 | 0.4328 |
| Solution number * Dye * Incubation & Illumination time [min] | 2 | 2 | 0.0740 | 46.8235 | <.0001 |
| Solution number * Dye * Follow-up time [h] | 4 | 4 | 0.0234 | 7.3982 | <.0001 |
| Solution number * Incubation & Illumination time [min] * Follow-up time [h] | 4 | 4 | 0.0184 | 5.8156 | 0.0002 |
| Dye * Incubation & Illumination time [min] * Follow-up time [h] | 2 | 2 | 0.0109 | 6.8670 | 0.0013 |
| Solution number * Dye * Incubation & Illumination time [min] * Follow-up time [h] | 4 | 4 | 0.0737653 | 23.3249 | <.0001 |

***Table 43:*** Median cell survival, lower and upper CL for the interaction between the factors solution number, dye, incubation & illumination time and follow-up time

| Solution number (dye) | mg/ml | Incub. & illum. time [h] | Follow-up time [h] | Median cell survival [%] | Lower CL [%] | Upper CL [%] |
|---|---|---|---|---|---|---|
| Solution 1 (ICG) | 5.0 | 1 | 6 | 89.20 | 87.75 | 90.56 |
| Solution 1 (ICG) | 5.0 | 5 | 6 | 85.99 | 84.38 | 87.52 |
| Solution 1 (ICG-free) | 0.0 | 1 | 6 | 100.00 | 100.00 | 100.00 |
| Solution 1 (ICG-free) | 0.0 | 5 | 6 | 98.22 | 97.58 | 98.77 |
| Solution 2 (ICG) | 2.5 | 1 | 6 | 96.28 | 95.38 | 97.09 |
| Solution 2 (ICG) | 2.5 | 5 | 6 | 92.33 | 91.08 | 93.49 |
| Solution 2 (ICG-free) | 0.0 | 1 | 6 | 100.00 | 100.00 | 100.00 |
| Solution 2 (ICG-free) | 0.0 | 5 | 6 | 98.49 | 97.89 | 98.99 |
| Solution 3 (ICG) | 1.0 | 1 | 6 | 99.92 | 99.73 | 100.00 |
| Solution 3 (ICG) | 1.0 | 5 | 6 | 97.13 | 96.33 | 97.84 |
| Solution 3 (ICG-free) | 0.0 | 1 | 6 | 100.00 | 100.00 | 100.00 |
| Solution 3 (ICG-free) | 0.0 | 5 | 6 | 100.00 | 100.00 | 100.00 |
| Solution 1 (ICG) | 5.0 | 1 | 24 | 86.50 | 84.91 | 88.01 |
| Solution 1 (ICG) | 5.0 | 5 | 24 | 71.66 | 69.60 | 73.68 |
| Solution 1 (ICG-free) | 0.0 | 1 | 24 | 99.91 | 99.72 | 99.99 |
| Solution 1 (ICG-free) | 0.0 | 5 | 24 | 98.88 | 98.35 | 99.31 |
| Solution 2 (ICG) | 2.5 | 1 | 24 | 92.99 | 91.79 | 94.11 |
| Solution 2 (ICG) | 2.5 | 5 | 24 | 91.32 | 90.00 | 92.55 |
| Solution 2 (ICG-free) | 0.0 | 1 | 24 | 99.94 | 99.77 | 100.00 |
| Solution 2 (ICG-free) | 0.0 | 5 | 24 | 98.35 | 97.72 | 98.88 |
| Solution 3 (ICG) | 1.0 | 1 | 24 | 100.00 | 100.00 | 100.00 |
| Solution 3 (ICG) | 1.0 | 5 | 24 | 97.09 | 96.28 | 97.80 |
| Solution 3 (ICG-free) | 0.0 | 1 | 24 | 100.00 | 100.00 | 100.00 |
| Solution 3 (ICG-free) | 0.0 | 5 | 24 | 100.00 | 100.00 | 100.00 |
| Solution 1 (ICG) | 5.0 | 1 | 72 | 84.83 | 83.17 | 86.42 |
| Solution 1 (ICG) | 5.0 | 5 | 72 | 66.15 | 63.99 | 68.27 |
| Solution 1 (ICG-free) | 0.0 | 1 | 72 | 99.37 | 98.96 | 99.68 |
| Solution 1 (ICG-free) | 0.0 | 5 | 72 | 98.10 | 97.44 | 98.67 |
| Solution 2 (ICG) | 2.5 | 1 | 72 | 88.80 | 87.33 | 90.19 |
| Solution 2 (ICG) | 2.5 | 5 | 72 | 89.03 | 87.58 | 90.41 |
| Solution 2 (ICG-free) | 0.0 | 1 | 72 | 99.68 | 99.38 | 99.89 |
| Solution 2 (ICG-free) | 0.0 | 5 | 72 | 97.38 | 96.61 | 98.06 |
| Solution 3 (ICG) | 1.0 | 1 | 72 | 97.95 | 97.26 | 98.54 |
| Solution 3 (ICG) | 1.0 | 5 | 72 | 96.35 | 95.45 | 97.15 |
| Solution 3 (ICG-free) | 0.0 | 1 | 72 | 100.00 | 100.00 | 100.00 |
| Solution 3 (ICG-free) | 0.0 | 5 | 72 | 99.60 | 99.27 | 99.84 |

## 6.6.4.1 Question four part 1–cell survival

***Table 44:*** Summary of fit for the fourth question part 1–cell survival of the set-up with illumination

| | |
|---|---|
| $R^2$ | 97.33% |
| $R^2$ Adj | 96.82% |
| *s* | 0.0315 |
| Mean of Response | 1.3490 |
| *n* | 144 |

***Table 45:*** Effect tests for the fourth question part 1–cell survival of the set-up with illumination

| Factors and Interactions | Nparm | DF | Sum of Squares | F Ratio | *P-value* |
|---|---|---|---|---|---|
| Solution | 3 | 3 | 3.5932 | 1210.32 | <.0001 |
| Incubation & Illumination time [min] | 1 | 1 | 0.2458 | 248.3896 | <.0001 |
| Solution * Incubation & Illumination time [min] | 3 | 3 | 0.1083 | 36.4958 | <.0001 |
| Follow-up time [h] | 2 | 2 | 0.2401 | 121.2945 | <.0001 |
| Solution * | 6 | 6 | 0.0517 | 8.7089 | <.0001 |

| Factors and Interactions | Nparm | DF | Sum of Squares | F Ratio | *P-value* |
|---|---|---|---|---|---|
| Follow-up time [h] | | | | | |
| Incubation & Illumination time [min] * Follow-up time [h] | 2 | 2 | 0.0044 | 2.2226 | 0.1128 |
| Solution * Incubation & Illumination time [min] * Follow-up time [h] | 6 | 6 | 0.0907 | 15.2761 | <.0001 |

***Table 46:*** Median cell survival, lower and upper CL for the interaction between the factors solution, incubation & illumination time and follow-up time

| Solution | mg/ml | Incub. & illum. time [h] | Follow-up time [h] | Median cell survival [%] | Lower CL [%] | Upper CL [%] |
|---|---|---|---|---|---|---|
| Trypan blue | 1.5 | 1 | 6 | 100.00 | 100.00 | 100.00 |
| Trypan blue | 1.5 | 5 | 6 | 100.00 | 100.00 | 100.00 |
| Trypan blue | 1.5 | 1 | 24 | 99.89 | 99.66 | 99.99 |
| Trypan blue | 1.5 | 5 | 24 | 99.91 | 99.69 | 100.00 |
| Trypan blue | 1.5 | 1 | 72 | 99.80 | 99.50 | 99.96 |
| Trypan blue | 1.5 | 5 | 72 | 98.81 | 98.19 | 99.30 |
| Solution 1 (ICG) | 5.0 | 1 | 6 | 89.20 | 87.63 | 90.66 |
| Solution 1 (ICG) | 5.0 | 5 | 6 | 85.99 | 84.25 | 87.64 |
| Solution 1 (ICG) | 5.0 | 1 | 24 | 86.50 | 84.79 | 88.12 |
| Solution 1 (ICG) | 5.0 | 5 | 24 | 71.66 | 69.44 | 73.84 |
| Solution 1 (ICG) | 5.0 | 1 | 72 | 84.83 | 83.04 | 86.54 |
| Solution 1 (ICG) | 5.0 | 5 | 72 | 66.15 | 63.82 | 68.44 |
| Solution 2 (ICG) | 2.5 | 1 | 6 | 96.28 | 95.30 | 97.15 |
| Solution 2 (ICG) | 2.5 | 5 | 6 | 92.33 | 90.98 | 93.58 |
| Solution 2 (ICG) | 2.5 | 1 | 24 | 92.99 | 91.70 | 94.19 |
| Solution 2 (ICG) | 2.5 | 5 | 24 | 91.32 | 89.89 | 92.64 |
| Solution 2 (ICG) | 2.5 | 1 | 72 | 88.80 | 87.22 | 90.29 |
| Solution 2 (ICG) | 2.5 | 5 | 72 | 89.03 | 87.46 | 90.51 |
| Solution 3 (ICG) | 1.0 | 1 | 6 | 99.92 | 99.72 | 100.00 |
| Solution 3 (ICG) | 1.0 | 5 | 6 | 97.13 | 96.26 | 97.89 |
| Solution 3 (ICG) | 1.0 | 1 | 24 | 100.00 | 100.00 | 100.00 |
| Solution 3 (ICG) | 1.0 | 5 | 24 | 97.09 | 96.21 | 97.86 |
| Solution 3 (ICG) | 1.0 | 1 | 72 | 97.95 | 97.20 | 98.59 |
| Solution 3 (ICG) | 1.0 | 5 | 72 | 96.34 | 95.38 | 97.21 |

### 6.6.4.2 Question four part 2–cell survival

***Table 47:*** Summary of fit for the fourth question part 2–cell survival of the set-up with illumination

| | |
|---|---|
| $R^2$ | 30.81% |
| $R^2$ Adj | 18.13% |
| *s* | 0.0646 |
| Mean of Response | 1.5452 |
| *n* | 72 |

***Table 48:*** Effect tests for the fourth question part 2–cell survival of the set-up with illumination

| Factors and Interactions | Nparm | DF | Sum of Squares | F Ratio | *P-value* |
|---|---|---|---|---|---|
| Solution | 1 | 1 | 0.0100 | 2.3849 | 0.1278 |
| Incubation & Illumination time [min] | 1 | 1 | 0.0474 | 11.3476 | 0.0013 |
| Solution * Incubation & Illumination time [min] | 1 | 1 | 0.0100 | 2.3849 | 0.1278 |
| Follow-up time [h] | 2 | 2 | 0.0135 | 1.6140 | 0.2076 |
| Solution * Follow-up time [h] | 2 | 2 | 0.0087 | 1.0373 | 0.3607 |
| Incubation & Illumination time [min] * Follow-up time [h] | 2 | 2 | 0.0135 | 1.6140 | 0.2076 |
| Solution * | 2 | 2 | 0.0087 | 1.0373 | 0.3607 |

| Factors and Interactions | Nparm | DF | Sum of Squares | F Ratio | *P-value* |
|---|---|---|---|---|---|
| Incubation & Illumination time [min] * Follow-up time [h] | | | | | |

***Table 49:*** Median cell survival, lower and upper CL for the interaction between the factors solution, incubation & illumination time and follow-up time

| Solution | mg/ml | Incub. & illum. time [h] | Follow-up time [h] | Median cell survival [%] | Lower CL [%] | Upper CL [%] |
|---|---|---|---|---|---|---|
| diluted Trypan blue | 1.5 | 1 | 6 | 100.00 | 100.00 | 100.00 |
| diluted Trypan blue | 1.5 | 5 | 6 | 100.00 | 100.00 | 100.00 |
| diluted Trypan blue | 1.5 | 1 | 24 | 100.00 | 100.00 | 100.00 |
| diluted Trypan blue | 1.5 | 5 | 24 | 100.00 | 100.00 | 100.00 |
| diluted Trypan blue | 1.5 | 1 | 72 | 100.00 | 100.00 | 100.00 |
| diluted Trypan blue | 1.5 | 5 | 72 | 99.31 | 98.13 | 99.91 |
| Solution 4 (ICG) | 0.125 | 1 | 6 | 100.00 | 100.00 | 100.00 |
| Solution 4 (ICG) | 0.125 | 5 | 6 | 99.89 | 99.66 | 99.99 |
| Solution 4 (ICG) | 0.125 | 1 | 24 | 100.00 | 100.00 | 100.00 |
| Solution 4 (ICG) | 0.125 | 5 | 24 | 98.88 | 98.31 | 99.33 |
| Solution 4 (ICG) | 0.125 | 1 | 72 | 100.00 | 100.00 | 100.00 |
| Solution 4 (ICG) | 0.125 | 5 | 72 | 99.29 | 98.82 | 99.64 |

## 6.6.5 Question five – cell survival

***Table 50:*** Summary of fit for the fifth question–cell survival of the set-up with illumination

| | |
|---|---|
| $R^2$ | 74.82% |
| $R^2$ Adj | 70.20% |
| *s* | 0.0274 |
| Mean of Response | 1.5371 |
| *n* | 72 |

***Table 51:*** Effect tests for the fifth question–cell survival of the set-up with illumination

| Factors and Interactions | Nparm | DF | Sum of Squares | F Ratio | *P-value* |
|---|---|---|---|---|---|
| Solution | 1 | 1 | 0.0010 | 1.2832 | 0.2618 |
| Incubation & Illumination time [min] | 1 | 1 | 0.0820 | 109.1218 | <.0001 |
| Solution * Incubation & Illumination time [min] | 1 | 1 | 0.0010 | 1.2832 | 0.2618 |
| Follow-up time [h] | 2 | 2 | 0.0230 | 15.3093 | <.0001 |
| Solution * Follow-up time [h] | 2 | 2 | 0.0020 | 1.3415 | 0.2692 |
| Incubation & Illumination time [min] * Follow-up time [h] | 2 | 2 | 0.0230 | 15.3093 | <.0001 |
| Solution * Incubation & Illumination time [min] * Follow-up time [h] | 2 | 2 | 0.0020 | 1.3415 | 0.2692 |

***Table 52:*** Median cell survival, lower and upper CL for the interaction between the factors solution, incubation & illumination time and follow-up time

| Solution | mg/ml | Incub. & illum. time [h] | Follow-up time [h] | Median cell survival [%] | Lower CL [%] | Upper CL [%] |
|---|---|---|---|---|---|---|
| BSS | 0.0 | 1 | 6 | 100.00 | 100.00 | 100.00 |
| BSS | 0.0 | 5 | 6 | 100.00 | 100.00 | 100.00 |
| BSS | 0.0 | 1 | 24 | 100.00 | 100.00 | 100.00 |
| BSS | 0.0 | 5 | 24 | 99.34 | 98.93 | 99.65 |
| BSS | 0.0 | 1 | 72 | 100.00 | 100.00 | 100.00 |
| BSS | 0.0 | 5 | 72 | 99.02 | 98.53 | 99.41 |
| Solution 4 (ICG) | 0.125 | 1 | 6 | 100.00 | 100.00 | 100.00 |
| Solution 4 (ICG) | 0.125 | 5 | 6 | 99.89 | 99.66 | 99.99 |

| Solution | mg/ml | Incub. & illum. time [h] | Follow-up time [h] | Median cell survival [%] | Lower CL [%] | Upper CL [%] |
|---|---|---|---|---|---|---|
| Solution 4 (ICG) | 0.125 | 1 | 24 | 100.00 | 100.00 | 100.00 |
| Solution 4 (ICG) | 0.125 | 5 | 24 | 98.88 | 98.31 | 99.33 |
| Solution 4 (ICG) | 0.125 | 1 | 72 | 100.00 | 100.00 | 100.00 |
| Solution 4 (ICG) | 0.125 | 5 | 72 | 99.29 | 98.82 | 99.64 |

## 6.6.6 Question one – morphologic change

***Table 53:*** Summary of fit for the first question–morphologic change of the set-up with illumination

| | |
|---|---|
| $R^2$ | 98.59% |
| $R^2$ Adj | 97.91% |
| *s* | 0.0230 |
| Mean of Response | 0.2549 |
| *n* | 72 |

***Table 54:*** Effect tests for the first question–morphologic change of the set-up with illumination

| Factors and Interactions | Nparm | DF | Sum of Squares | F Ratio | *P-value* |
|---|---|---|---|---|---|
| Solution | 3 | 3 | 1.4168 | 890.9029 | <.0001 |
| Incubation & Illumination time [min] | 1 | 1 | 0.1021 | 192.6667 | <.0001 |
| Solution * Incubation & Illumination time [min] | 3 | 3 | 0.0702 | 44.1514 | <.0001 |
| Follow-up time [h] | 2 | 2 | 0.0887 | 83.6963 | <.0001 |
| Solution * Follow-up time [h] | 6 | 6 | 0.0461 | 14.4790 | <.0001 |
| Incubation & Illumination time [min] * Follow-up time [h] | 2 | 2 | 0.0039 | 3.6664 | 0.0330 |
| Solution * Incubation & Illumination time [min] * Follow-up time [h] | 6 | 6 | 0.0467 | 14.7273 | <.0001 |

***Table 55:*** Median morphologic change, lower and upper CL for the interaction between the factors solution, incubation & illumination time and follow-up time

| Solution | mg/ml | Incub. & illum. time [h] | Follow-up time [h] | Median morphologic change [%] | Lower CL [%] | Upper CL [%] |
|---|---|---|---|---|---|---|
| Solution 1 (ICG) | 5.0 | 1 | 6 | 11.52 | 9.86 | 13.29 |
| Solution 1 (ICG) | 5.0 | 5 | 6 | 15.33 | 13.44 | 17.31 |
| Solution 1 (ICG) | 5.0 | 1 | 24 | 14.04 | 12.23 | 15.96 |
| Solution 1 (ICG) | 5.0 | 5 | 24 | 29.48 | 27.06 | 31.96 |
| Solution 1 (ICG) | 5.0 | 1 | 72 | 15.34 | 13.45 | 17.32 |
| Solution 1 (ICG) | 5.0 | 5 | 72 | 41.09 | 38.46 | 43.75 |
| Solution 2 (ICG) | 2.5 | 1 | 6 | 3.99 | 3.00 | 5.10 |
| Solution 2 (ICG) | 2.5 | 5 | 6 | 7.93 | 6.54 | 9.44 |
| Solution 2 (ICG) | 2.5 | 1 | 24 | 7.29 | 5.95 | 8.75 |
| Solution 2 (ICG) | 2.5 | 5 | 24 | 8.91 | 7.44 | 10.51 |
| Solution 2 (ICG) | 2.5 | 1 | 72 | 11.58 | 9.92 | 13.36 |
| Solution 2 (ICG) | 2.5 | 5 | 72 | 11.50 | 9.84 | 13.27 |
| Solution 3 (ICG) | 1.0 | 1 | 6 | 0.96 | 0.50 | 1.55 |
| Solution 3 (ICG) | 1.0 | 5 | 6 | 2.97 | 2.12 | 3.95 |
| Solution 3 (ICG) | 1.0 | 1 | 24 | 0.85 | 0.43 | 1.42 |
| Solution 3 (ICG) | 1.0 | 5 | 24 | 3.09 | 2.23 | 4.09 |
| Solution 3 (ICG) | 1.0 | 1 | 72 | 2.52 | 1.75 | 3.43 |
| Solution 3 (ICG) | 1.0 | 5 | 72 | 3.75 | 2.79 | 4.83 |
| Solution 4 (ICG) | 0.125 | 1 | 6 | 0.83 | 0.52 | 1.21 |
| Solution 4 (ICG) | 0.125 | 5 | 6 | 0.92 | 0.59 | 0.13 |
| Solution 4 (ICG) | 0.125 | 1 | 24 | 1.19 | 0.81 | 1.63 |
| Solution 4 (ICG) | 0.125 | 5 | 24 | 1.69 | 1.23 | 2.21 |
| Solution 4 (ICG) | 0.125 | 1 | 72 | 1.04 | 0.69 | 1.46 |

| Solution | mg/ml | Incub. & illum. time [h] | Follow-up time [h] | Median morphologic change [%] | Lower CL [%] | Upper CL [%] |
|---|---|---|---|---|---|---|
| Solution 4 (ICG) | 0.125 | 5 | 72 | 1.76 | 1.30 | 2.30 |

## 6.6.7 Question two – morphologic change

***Table 56:*** Summary of fit for the second question–morphologic change of the set-up with illumination

| | |
|---|---|
| $R^2$ | 59.12% |
| $R^2$ Adj | 40.38% |
| *s* | 0.0163 |
| Mean of Response | 0.0915 |
| *n* | 36 |

***Table 57:*** Effect tests for the second question–morphologic change of the set-up with illumination

| Factors and Interactions | Nparm | DF | Sum of Squares | F Ratio | *P-value* |
|---|---|---|---|---|---|
| Solution | 1 | 1 | 0.0020 | 7.4845 | 0.0115 |
| Incubation & Illumination time [min] | 1 | 1 | 0.0001 | 0.4982 | 0.4871 |
| Solution * Incubation & Illumination time [min] | 1 | 1 | 0.0004 | 1.6485 | 0.2114 |
| Follow-up time [h] | 2 | 2 | 0.0030 | 5.6776 | 0.0096 |
| Solution * Follow-up time [h] | 2 | 2 | 0.0010 | 1.8380 | 0.1808 |
| Incubation & Illumination time [min] * Follow-up time [h] | 2 | 2 | 0.0003 | 0.4991 | 0.6132 |
| Solution * Incubation & Illumination time [min] * Follow-up time [h] | 2 | 2 | 0.0024 | 4.5210 | 0.0216 |

***Table 58:*** Median morphologic change, lower and upper CL for the interaction between the factors solution, incubation & illumination time and follow-up time

| Solution | mg/ml | Incub. & illum. time [h] | Follow-up time [h] | Median morphologic change [%] | Lower CL [%] | Upper CL [%] |
|---|---|---|---|---|---|---|
| Trypan blue | 1.5 | 1 | 6 | 0.79 | 0.38 | 1.33 |
| Trypan blue | 1.5 | 5 | 6 | 0.50 | 0.19 | 0.95 |
| Trypan blue | 1.5 | 1 | 24 | 0.94 | 0.50 | 1.54 |
| Trypan blue | 1.5 | 5 | 24 | 1.19 | 0.68 | 1.84 |
| Trypan blue | 1.5 | 1 | 72 | 1.12 | 0.62 | 1.75 |
| Trypan blue | 1.5 | 5 | 72 | 1.47 | 0.89 | 2.19 |
| diluted Trypan blue | 1.5 | 1 | 6 | 0.56 | 0.31 | 0.88 |
| diluted Trypan blue | 1.5 | 5 | 6 | 0.71 | 0.43 | 1.06 |
| diluted Trypan blue | 1.5 | 1 | 24 | 0.73 | 0.44 | 1.09 |
| diluted Trypan blue | 1.5 | 5 | 24 | 0.63 | 0.37 | 0.97 |
| diluted Trypan blue | 1.5 | 1 | 72 | 1.17 | 0.79 | 1.61 |
| diluted Trypan blue | 1.5 | 5 | 72 | 0.71 | 0.28 | 1.31 |

## 6.6.8 Question three – morphologic change

***Table 59:*** Summary of fit for the third question–morphologic change of the set-up with illumination

| | |
|---|---|
| $R^2$ | 98.54% |
| $R^2$ Adj | 97.83% |
| *s* | 0.0216 |
| Mean of Response | 0.2082 |
| *n* | 108 |

***Table 60:*** Effect tests for the third question–morphologic change of the set-up with illumination

| Factors and Interactions | Nparm | DF | Sum of Squares | F Ratio | *P-value* |
|---|---|---|---|---|---|
| Solution number | 2 | 2 | 0.5461 | 583.0151 | <.0001 |
| Dye | 1 | 1 | 0.9717 | 2074.49 | <.0001 |
| Incubation & Illumination time [min] | 1 | 1 | 0.1028 | 219.3817 | <.0001 |
| Follow-up time [h] | 2 | 2 | 0.0775 | 82.7691 | <.0001 |
| Solution number * Dye | 2 | 2 | 0.3790 | 404.6200 | <.0001 |
| Solution number * Incubation & Illumination time [min] | 2 | 2 | 0.0229 | 24.4002 | <.0001 |
| Solution number * Follow-up time [h] | 4 | 4 | 0.0148 | 7.8964 | <.0001 |
| Dye * Incubation & Illumination time [min] | 1 | 1 | 0.0284 | 60.5381 | <.0001 |
| Dye * Follow-up time [h] | 2 | 2 | 0.0308 | 32.9058 | <.0001 |
| Incubation & Illumination time [min] * Follow-up time [h] | 2 | 2 | 0.0006 | 0.6353 | 0.5327 |
| Solution number * Dye * Incubation & Illumination time [min] | 2 | 2 | 0.0322 | 34.3618 | <.0001 |
| Solution number * Dye * Follow-up time [h] | 4 | 4 | 0.0171 | 9.1481 | <.0001 |
| Solution number * Incubation & Illumination time [min] * Follow-up time [h] | 4 | 4 | 0.0238 | 12.7182 | <.0001 |
| Dye * Incubation & Illumination time [min] * Follow-up time [h] | 2 | 2 | 0.0035 | 3.7410 | 0.0285 |
| Solution number * Dye * Incubation & Illumination time [min] * Follow-up time [h] | 4 | 4 | 0.0242 | 12.8941 | <.0001 |

***Table 61:*** Median morphologic change, lower and upper CL for the interaction between the factors solution number, dye, incubation & illumination time and follow-up time

| Solution number (dye) | mg/ml | Incub. & illum. time [h] | Follow-up time [h] | Median morph. change [%] | Lower CL [%] | Upper CL [%] |
|---|---|---|---|---|---|---|
| Solution 1 (ICG) | 5.0 | 1 | 6 | 11.52 | 9.98 | 13.16 |
| Solution 1 (ICG) | 5.0 | 5 | 6 | 15.33 | 13.58 | 17.16 |
| Solution 1 (ICG-free) | 0.0 | 1 | 6 | 1.01 | 0.57 | 1.57 |
| Solution 1 (ICG-free) | 0.0 | 5 | 6 | 2.04 | 1.40 | 2.81 |
| Solution 2 (ICG) | 2.5 | 1 | 6 | 3.99 | 3.07 | 5.02 |
| Solution 2 (ICG) | 2.5 | 5 | 6 | 7.93 | 6.64 | 9.33 |
| Solution 2 (ICG-free) | 0.0 | 1 | 6 | 0.46 | 0.19 | 0.86 |
| Solution 2 (ICG-free) | 0.0 | 5 | 6 | 1.73 | 1.14 | 2.43 |
| Solution 3 (ICG) | 1.0 | 1 | 6 | 0.96 | 0.53 | 1.50 |
| Solution 3 (ICG) | 1.0 | 5 | 6 | 2.97 | 2.18 | 3.87 |
| Solution 3 (ICG-free) | 0.0 | 1 | 6 | 0.72 | 0.36 | 1.20 |
| Solution 3 (ICG-free) | 0.0 | 5 | 6 | 0.90 | 0.49 | 1.43 |
| Solution 1 (ICG) | 5.0 | 1 | 24 | 14.04 | 12.36 | 15.82 |
| Solution 1 (ICG) | 5.0 | 5 | 24 | 29.48 | 27.24 | 0.31.78 |
| Solution 1 (ICG-free) | 0.0 | 1 | 24 | 1.25 | 0.76 | 1.87 |
| Solution 1 (ICG-free) | 0.0 | 5 | 24 | 1.47 | 0.93 | 2.13 |
| Solution 2 (ICG) | 2.5 | 1 | 24 | 7.29 | 6.05 | 8.64 |
| Solution 2 (ICG) | 2.5 | 5 | 24 | 8.91 | 7.55 | 10.38 |
| Solution 2 (ICG-free) | 0.0 | 1 | 24 | 1.00 | 0.57 | 1.56 |
| Solution 2 (ICG-free) | 0.0 | 5 | 24 | 1.96 | 1.33 | 2.71 |
| Solution 3 (ICG) | 1.0 | 1 | 24 | 0.85 | 0.46 | 1.37 |

| Solution number (dye) | mg/ml | Incub. & illum. time [h] | Follow-up time [h] | Median morph. change [%] | Lower CL [%] | Upper CL [%] |
|---|---|---|---|---|---|---|
| Solution 3 (ICG) | 1.0 | 5 | 24 | 3.09 | 2.29 | 4.01 |
| Solution 3 (ICG-free) | 0.0 | 1 | 24 | 0.56 | 0.25 | 1.00 |
| Solution 3 (ICG-free) | 0.0 | 5 | 24 | 1.01 | 0.57 | 1.57 |
| Solution 1 (ICG) | 5.0 | 1 | 72 | 15.34 | 13.59 | 17.17 |
| Solution 1 (ICG) | 5.0 | 5 | 72 | 41.09 | 38.66 | 43.56 |
| Solution 1 (ICG-free) | 0.0 | 1 | 72 | 1.48 | 0.94 | 2.14 |
| Solution 1 (ICG-free) | 0.0 | 5 | 72 | 2.11 | 1.46 | 2.89 |
| Solution 2 (ICG) | 2.5 | 1 | 72 | 11.58 | 10.04 | 13.22 |
| Solution 2 (ICG) | 2.5 | 5 | 72 | 11.50 | 9.96 | 13.14 |
| Solution 2 (ICG-free) | 0.0 | 1 | 72 | 1.42 | 0.89 | 2.07 |
| Solution 2 (ICG-free) | 0.0 | 5 | 72 | 2.87 | 2.09 | 3.75 |
| Solution 3 (ICG) | 1.0 | 1 | 72 | 2.52 | 1.80 | 3.36 |
| Solution 3 (ICG) | 1.0 | 5 | 72 | 3.75 | 2.86 | 4.75 |
| Solution 3 (ICG-free) | 0.0 | 1 | 72 | 1.14 | 0.67 | 1.72 |
| Solution 3 (ICG-free) | 0.0 | 5 | 72 | 1.07 | 0.62 | 1.64 |

## 6.6.9.1 Question four part 1–morphologic change

***Table 62:*** Summary of fit for the fourth question part 1–morphologic change of the set-up with illumination

| | |
|---|---|
| $R^2$ | 98.62% |
| $R^2$ Adj | 97.96% |
| *s* | 0.2316 |
| Mean of Response | 0.2521 |
| *n* | 72 |

***Table 63:*** Effect tests for the fourth question part 1–morphologic change of the set-up with illumination

| Factors and Interactions | Nparm | DF | Sum of Squares | F Ratio | *P-value* |
|---|---|---|---|---|---|
| Solution | 3 | 3 | 1.4778 | 918.7985 | <.0001 |
| Incubation & Illumination time [min] | 1 | 1 | 0.0917 | 170.9451 | <.0001 |
| Solution * Incubation & Illumination time [min] | 3 | 3 | 0.0791 | 49.1875 | <.0001 |
| Follow-up time [h] | 2 | 2 | 0.0939 | 87.5917 | <.0001 |
| Solution * Follow-up time [h] | 6 | 6 | 0.0420 | 13.0468 | <.0001 |
| Incubation & Illumination time [min] * Follow-up time [h] | 2 | 2 | 0.0045 | 4.1990 | 0.0209 |
| Solution * Incubation & Illumination time [min] * Follow-up time [h] | 6 | 6 | 0.0467 | 14.5293 | <.0001 |

***Table 64:*** Median morphologic change, lower and upper CL for the interaction between the factors solution, incubation & illumination time and follow-up time

| Solution | mg/ml | Incub. & illum. time [h] | Follow-up time [h] | Median morph. change [%] | Lower CL [%] | Upper CL [%] |
|---|---|---|---|---|---|---|
| Trypan blue | 1.5 | 1 | 6 | 0.79 | 0.38 | 1.33 |
| Trypan blue | 1.5 | 5 | 6 | 0.50 | 0.19 | 0.95 |
| Trypan blue | 1.5 | 1 | 24 | 0.94 | 0.50 | 1.54 |
| Trypan blue | 1.5 | 5 | 24 | 1.19 | 0.68 | 1.84 |
| Trypan blue | 1.5 | 1 | 72 | 1.12 | 0.62 | 1.75 |
| Trypan blue | 1.5 | 5 | 72 | 1.47 | 0.89 | 2.19 |
| Solution 1 (ICG) | 5.0 | 1 | 6 | 11.52 | 9.86 | 13.29 |
| Solution 1 (ICG) | 5.0 | 5 | 6 | 15.33 | 13.44 | 17.31 |
| Solution 1 (ICG) | 5.0 | 1 | 24 | 14.04 | 12.23 | 15.96 |
| Solution 1 (ICG) | 5.0 | 5 | 24 | 29.48 | 27.06 | 31.96 |
| Solution 1 (ICG) | 5.0 | 1 | 72 | 15.34 | 13.45 | 17.32 |
| Solution 1 (ICG) | 5.0 | 5 | 72 | 41.09 | 38.46 | 43.75 |

| Solution | mg/ml | Incub. & illum. time [h] | Follow-up time [h] | Median morph. change [%] | Lower CL [%] | Upper CL [%] |
|---|---|---|---|---|---|---|
| Solution 2 (ICG) | 2.5 | 1 | 6 | 3.99 | 3.00 | 5.10 |
| Solution 2 (ICG) | 2.5 | 5 | 6 | 7.93 | 6.54 | 9.44 |
| Solution 2 (ICG) | 2.5 | 1 | 24 | 7.29 | 5.95 | 8.75 |
| Solution 2 (ICG) | 2.5 | 5 | 24 | 8.91 | 7.44 | 10.51 |
| Solution 2 (ICG) | 2.5 | 1 | 72 | 11.58 | 9.92 | 13.36 |
| Solution 2 (ICG) | 2.5 | 5 | 72 | 11.50 | 9.84 | 13.27 |
| Solution 3 (ICG) | 1.0 | 1 | 6 | 0.96 | 0.50 | 1.55 |
| Solution 3 (ICG) | 1.0 | 5 | 6 | 2.97 | 2.12 | 3.95 |
| Solution 3 (ICG) | 1.0 | 1 | 24 | 0.85 | 0.43 | 1.42 |
| Solution 3 (ICG) | 1.0 | 5 | 24 | 3.09 | 2.23 | 4.09 |
| Solution 3 (ICG) | 1.0 | 1 | 72 | 2.52 | 1.75 | 3.43 |
| Solution 3 (ICG) | 1.0 | 5 | 72 | 3.75 | 2.79 | 4.83 |

## 6.6.9.2 Question four part 2–morphologic change

***Table 65:*** Summary of fit for the fourth question part 2–morphologic change of the set-up with illumination

| | |
|---|---|
| $R^2$ | 68.78% |
| $R^2$ Adj | 54.48% |
| *s* | 0.0159 |
| **Mean of Response** | 0.0972 |
| *n* | 36 |

***Table 66:*** Effect tests for the fourth question part 2–morphologic change of the set-up with illumination

| Factors and Interactions | Nparm | DF | Sum of Squares | F Ratio | *P-value* |
|---|---|---|---|---|---|
| Solution | 1 | 1 | 0.0062 | 24.4942 | <.0001 |
| Incubation & Illumination time [min] | 1 | 1 | 0.0002 | 0.6025 | 0.4452 |
| Solution * Incubation & Illumination time [min] | 1 | 1 | 0.0020 | 7.9127 | 0.0096 |
| Follow-up time [h] | 2 | 2 | 0.0021 | 4.1074 | 0.0292 |
| Solution * Follow-up time [h] | 2 | 2 | 0.0008 | 1.5813 | 0.2264 |
| Incubation & Illumination time [min] * Follow-up time [h] | 2 | 2 | 0.0002 | 0.3943 | 0.6785 |
| Solution * Incubation & Illumination time [min] * Follow-up time [h] | 2 | 2 | 0.0019 | 3.8531 | 0.0354 |

***Table 67:*** Median morphologic change, lower and upper CL for the interaction between the factors solution, incubation & illumination time and follow-up time

| Solution | mg/ml | Incub. & illum. time [h] | Follow-up time [h] | Median morph. change [%] | Lower CL [%] | Upper CL [%] |
|---|---|---|---|---|---|---|
| diluted Trypan blue | 1.5 | 1 | 6 | 0.56 | 0.31 | 0.88 |
| diluted Trypan blue | 1.5 | 5 | 6 | 0.71 | 0.43 | 1.06 |
| diluted Trypan blue | 1.5 | 1 | 24 | 0.73 | 0.44 | 1.09 |
| diluted Trypan blue | 1.5 | 5 | 24 | 0.63 | 0.37 | 0.97 |
| diluted Trypan blue | 1.5 | 1 | 72 | 0.52 | 0.28 | 0.83 |
| diluted Trypan blue | 1.5 | 5 | 72 | 1.17 | 0.79 | 1.61 |
| Solution 4 (ICG) | 0.125 | 1 | 6 | 0.83 | 0.52 | 1.21 |
| Solution 4 (ICG) | 0.125 | 5 | 6 | 0.92 | 0.59 | 1.31 |
| Solution 4 (ICG) | 0.125 | 1 | 24 | 1.19 | 0.81 | 1.63 |
| Solution 4 (ICG) | 0.125 | 5 | 24 | 1.69 | 1.23 | 2.21 |
| Solution 4 (ICG) | 0.125 | 1 | 72 | 1.04 | 0.69 | 1.46 |
| Solution 4 (ICG) | 0.125 | 5 | 72 | 1.76 | 1.30 | 2.30 |

### 6.6.10 Question five – morphologic change

***Table 68:*** Summary of fit for the fifth question–morphologic change of the set-up with illumination

| | |
|---|---|
| $R^2$ | 64.87% |
| $R^2$ Adj | 48.77% |
| *s* | 0.0149 |
| **Mean of Response** | 0.1016 |
| *n* | 36 |

***Table 69:*** Effect tests for the fifth question–morphologic change of the set-up with illumination

| Factors and Interactions | Nparm | DF | Sum of Squares | F Ratio | *P-value* |
|---|---|---|---|---|---|
| Solution | 1 | 1 | 0.0027 | 12.3026 | 0.0018 |
| Incubation & Illumination time [min] | 1 | 1 | 0.0012 | 5.2927 | 0.0304 |
| Solution * Incubation & Illumination time [min] | 1 | 1 | 0.0005 | 2.3307 | 0.1399 |
| Follow-up time [h] | 2 | 2 | 0.0046 | 10.4109 | 0.0006 |
| Solution * Follow-up time [h] | 2 | 2 | 0.0001 | 0.1247 | 0.8833 |
| Incubation & Illumination time [min] * Follow-up time [h] | 2 | 2 | 0.0002 | 0.4205 | 0.6615 |
| Solution * Incubation & Illumination time [min] * Follow-up time [h] | 2 | 2 | 0.0001 | 1.2424 | 0.3066 |

***Table 70:*** Median morphologic change, lower and upper CL for the interaction between the factors solution, incubation & illumination time and follow-up time

| Solution | mg/ml | Incub. & illum. time [h] | Follow-up time [h] | Median morph. change [%] | Lower CL [%] | Upper CL [%] |
|---|---|---|---|---|---|---|
| BSS | 0.0 | 1 | 6 | 0.51 | 0.29 | 0.80 |
| BSS | 0.0 | 5 | 6 | 0.70 | 0.43 | 1.02 |
| BSS | 0.0 | 1 | 24 | 1.02 | 0.70 | 1.41 |
| BSS | 0.0 | 5 | 24 | 0.92 | 0.61 | 1.29 |
| BSS | 0.0 | 1 | 72 | 1.00 | 0.68 | 1.38 |
| BSS | 0.0 | 5 | 72 | 1.09 | 0.75 | 1.49 |
| Solution 4 (ICG) | 0.125 | 1 | 6 | 0.83 | 0.52 | 1.21 |
| Solution 4 (ICG) | 0.125 | 5 | 6 | 0.92 | 0.59 | 0.13 |
| Solution 4 (ICG) | 0.125 | 1 | 24 | 1.19 | 0.81 | 1.63 |
| Solution 4 (ICG) | 0.125 | 5 | 24 | 1.69 | 1.23 | 2.21 |
| Solution 4 (ICG) | 0.125 | 1 | 72 | 1.04 | 0.69 | 1.46 |
| Solution 4 (ICG) | 0.125 | 5 | 72 | 1.76 | 1.30 | 2.30 |

## 6.7 Tables of the clinical set-up

### 6.7.1 Question one – cell survival

***Table 71:*** Summary of fit for the first question–cell survival of the clinical set-up

| | |
|---|---|
| $R^2$ | 95.79% |
| $R^2$ Adj | 95.01% |
| *s* | 0.0437 |
| **Mean of Response** | 1.3804 |
| *n* | 72 |

***Table 72:*** Effect tests for the first question–cell survival of the clinical set-up

| Factors | Nparm | DF | Sum of Squares | F Ratio | *P-value* |
|---|---|---|---|---|---|
| Solution | 3 | 3 | 2.3375874 | 408.7285 | <.0001 |

| Factors | Nparm | DF | Sum of Squares | F Ratio | *P-value* |
|---|---|---|---|---|---|
| Follow-up time (h) | 2 | 2 | 0.1671781 | 43.8468 | <.0001 |
| Solution * Follow-up time (h) | 6 | 6 | 0.0955111 | 8.3501 | <.0001 |

***Table 73:*** Median cell survival, lower and upper CL for the interaction between the factors solution and follow-up time

| Solution | mg/ml | Follow-up time [h] | Median cell survival [%] | Lower CL [%] | Upper CL [%] |
|---|---|---|---|---|---|
| Solution 1 (ICG) | 5.0 | 6 | 89.87 | 87.62 | 91.92 |
| Solution 1 (ICG) | 5.0 | 24 | 78.34 | 75.33 | 81.20 |
| Solution 1 (ICG) | 5.0 | 72 | 70.22 | 66.91 | 73.43 |
| Solution 2 (ICG) | 2.5 | 6 | 95.92 | 94.40 | 97.22 |
| Solution 2 (ICG) | 2.5 | 24 | 94.41 | 92.67 | 95.94 |
| Solution 2 (ICG) | 2.5 | 72 | 91.63 | 89.55 | 93.50 |
| Solution 3 (ICG) | 1.0 | 6 | 100.00 | 100.00 | 100.00 |
| Solution 3 (ICG) | 1.0 | 24 | 100.00 | 100.00 | 100.00 |
| Solution 3 (ICG) | 1.0 | 72 | 99.10 | 98.30 | 99.65 |
| Solution 4 (ICG) | 0.125 | 6 | 100.00 | 100.00 | 100.00 |
| Solution 4 (ICG) | 0.125 | 24 | 99.88 | 99.50 | 100.00 |
| Solution 4 (ICG) | 0.125 | 72 | 99.89 | 99.52 | 100.00 |

## 6.7.2 Question two – cell survival

***Table 74:*** Summary of fit for the second question–cell survival of the clinical set-up

| | |
|---|---|
| $R^2$ | 55.76% |
| $R^2$ Adj | 48.39% |
| *S* | 0.0267 |
| Mean of Response | 1.5277 |
| *N* | 36 |

***Table 75:*** Effect tests for the second question–cell survival of the clinical set-up

| Factors | Nparm | DF | Sum of Squares | F Ratio | *P-value* |
|---|---|---|---|---|---|
| Solution | 1 | 1 | 0.0235 | 33.0558 | <.0001 |
| Follow-up time (h) | 2 | 2 | 0.0020 | 1.3934 | 0.2638 |
| Solution*Follow-up time (h) | 2 | 2 | 0.0014 | 0.9843 | 0.3854 |

***Table 76:*** Median cell survival, lower and upper CL for the interaction between the factors solution and follow-up time

| Solution | mg/ml | Follow-up time [h] | Median cell survival [%] | Lower CL [%] | Upper CL [%] |
|---|---|---|---|---|---|
| Trypan blue | 1.5 | 6 | 99.53 | 99.18 | 99.79 |
| Trypan blue | 1.5 | 24 | 99.55 | 99.20 | 99.80 |
| Trypan blue | 1.5 | 72 | 99.51 | 99.15 | 99.77 |
| diluted Trypan blue | 0.0375 | 6 | 99.96 | 99.83 | 100.00 |
| diluted Trypan blue | 0.0375 | 24 | 100.00 | 100.00 | 100.00 |
| diluted Trypan blue | 0.0375 | 72 | 99.89 | 99.69 | 99.99 |

## 6.7.3.1 Question three part 1– cell survival

***Table 77:*** Summary of fit for the third question part 1–cell survival of the clinical set-up

| | |
|---|---|
| $R^2$ | 96.29% |
| $R^2$ Adj | 95.61% |
| *s* | 0.0388 |
| Mean of Response | 1.3690 |

| | |
|---|---|
| ***n*** | 72 |

***Table 78:*** Effect tests for the third question part 1–cell survival of the clinical set-up

| Factors | Nparm | DF | Sum of Squares | F Ratio | *P-value* |
|---|---|---|---|---|---|
| Solution | 3 | 3 | 2.0902 | 462.1546 | <.0001 |
| Follow-up time (h) | 2 | 2 | 0.1461 | 48.4628 | <.0001 |
| Solution*Follow-up time (h) | 6 | 6 | 0.1118 | 12.3642 | <.0001 |

***Table 79:*** Median cell survival, lower and upper CL for the interaction between the factors solution and follow-up time

| Solution | mg/ml | Follow-up time [h] | Median cell survival [%] | Lower CL [%] | Upper CL [%] |
|---|---|---|---|---|---|
| Trypan blue | 1.5 | 6 | 99.53 | 99.00 | 99.87 |
| Trypan blue | 1.5 | 24 | 99.55 | 99.02 | 99.87 |
| Trypan blue | 1.5 | 72 | 99.51 | 98.96 | 99.85 |
| Solution 1 (ICG) | 5.0 | 6 | 89.87 | 87.88 | 91.70 |
| Solution 1 (ICG) | 5.0 | 24 | 78.34 | 75.67 | 80.89 |
| Solution 1 (ICG) | 5.0 | 72 | 70.22 | 67.28 | 73.08 |
| Solution 2 (ICG) | 2.5 | 6 | 95.92 | 94.58 | 97.08 |
| Solution 2 (ICG) | 2.5 | 24 | 94.41 | 92.87 | 95.78 |
| Solution 2 (ICG) | 2.5 | 72 | 91.63 | 89.79 | 93.30 |
| Solution 3 (ICG) | 1.0 | 6 | 100.00 | 100.00 | 100.00 |
| Solution 3 (ICG) | 1.0 | 24 | 100.00 | 100.00 | 100.00 |
| Solution 3 (ICG) | 1.0 | 72 | 99.10 | 98.40 | 99.60 |

## 6.7.3.2 Question three part 2– cell survival

***Table 80:*** Summary of fit for the third question part 2–cell survival of the clinical set-up

| | |
|---|---|
| $R^2$ | 15.60% |
| $R^2$ Adj | 1.53% |
| ***s*** | 0.0388 |
| **Mean of Response** | 1.5506 |
| ***n*** | 36 |

***Table 81:*** Effect tests for the third question part 2–cell survival of the clinical set-up

| Factors | Nparm | DF | Sum of Squares | F Ratio | *P-value* |
|---|---|---|---|---|---|
| Solution | 1 | 1 | 0.0003 | 0.1744 | 0.6792 |
| Follow-up time (h) | 2 | 2 | 0.0035 | 1.1638 | 0.3260 |
| Solution*Follow-up time (h) | 2 | 2 | 0.0046 | 1.5213 | 0.2348 |

***Table 82:*** Median cell survival, lower and upper CL for the interaction between the factors solution and follow-up time

| Solution | mg/ml | Follow-up time [h] | Median cell survival [%] | Lower CL [%] | Upper CL [%] |
|---|---|---|---|---|---|
| diluted Trypan blue | 0.0375 | 6 | 99.96 | 99.73 | 99.98 |
| diluted Trypan blue | 0.0375 | 24 | 100.00 | 100.00 | 100.00 |
| diluted Trypan blue | 0.0375 | 72 | 99.89 | 99.57 | 100.00 |
| Solution 4 (ICG) | 0.125 | 6 | 100.00 | 100.00 | 100.00 |
| Solution 4 (ICG) | 0.125 | 24 | 99.88 | 99.54 | 100.00 |
| Solution 4 (ICG) | 0.125 | 72 | 99.89 | 99.57 | 100.00 |

## 6.7.4 Question one – morphologic change

***Table 83:*** Summary of fit for the first question–morphologic change of the clinical set-up

| | |
|---|---|
| $R^2$ | 98.48% |
| $R^2$ Adj | 97.78% |
| *s* | 0.0270 |
| **Mean of Response** | 0.2452 |
| *n* | 36 |

***Table 84:*** Effect tests for the first question–morphologic change of the clinical set-up

| Factors | Nparm | DF | Sum of Squares | F Ratio | *P-value* |
|---|---|---|---|---|---|
| Solution | 3 | 3 | 1.0178648 | 464.3776 | <.0001 |
| Follow-up time (h) | 2 | 2 | 0.0656415 | 44.9211 | <.0001 |
| Solution*Follow-up time (h) | 6 | 6 | 0.0493014 | 11.2463 | <.0001 |

***Table 85:*** Median morphologic change, lower and upper CL for the interaction between the factors solution and follow-up time

| Solution | mg/ml | Follow-up time [h] | Median morphologic change [%] | Lower CL [%] | Upper CL [%] |
|---|---|---|---|---|---|
| Solution 1 (ICG) | 5.0 | 6 | 12.27 | 10.23 | 14.46 |
| Solution 1 (ICG) | 5.0 | 24 | 31.21 | 28.26 | 34.23 |
| Solution 1 (ICG) | 5.0 | 72 | 28.18 | 25.33 | 31.12 |
| Solution 2 (ICG) | 2.5 | 6 | 5.32 | 3.97 | 6.86 |
| Solution 2 (ICG) | 2.5 | 24 | 9.36 | 7.57 | 11.32 |
| Solution 2 (ICG) | 2.5 | 72 | 9.57 | 7.76 | 11.55 |
| Solution 3 (ICG) | 1.0 | 6 | 0.49 | 0.15 | 1.05 |
| Solution 3 (ICG) | 1.0 | 24 | 1.08 | 0.51 | 1.84 |
| Solution 3 (ICG) | 1.0 | 72 | 1.15 | 0.57 | 1.94 |
| Solution 4 (ICG) | 0.125 | 6 | 0.61 | 0.21 | 1.21 |
| Solution 4 (ICG) | 0.125 | 24 | 0.88 | 0.38 | 1.58 |
| Solution 4 (ICG) | 0.125 | 72 | 1.42 | 0.76 | 2.28 |

## 6.7.5 Question two – morphologic change

***Table 86:*** Summary of fit for the second question–morphologic change of the clinical set-up

| | |
|---|---|
| $R^2$ | 47.80% |
| $R^2$ Adj | 26.05% |
| *s* | 0.0139 |
| **Mean of Response** | 0.0840 |
| *n* | 18 |

***Table 87:*** Effect tests for the second question–morphologic change of the clinical set-up

| Factors | Nparm | DF | Sum of Squares | F Ratio | *P-value* |
|---|---|---|---|---|---|
| Solution | 1 | 1 | 0.0002 | 1.1378 | 0.3071 |
| Follow-up time (h) | 2 | 2 | 0.0018 | 4.7771 | 0.0298 |
| Solution*Follow-up time (h) | 2 | 2 | 0.0001 | 0.1486 | 0.8635 |

***Table 88:*** Median morphologic change, lower and upper CL for the interaction between the factors solution and follow-up time

| Solution | mg/ml | Follow-up time [h] | Median morphologic change [%] | Lower CL [%] | Upper CL [%] |
|---|---|---|---|---|---|
| Trypan blue | 1.5 | 6 | 0.57 | 0.34 | 0.87 |
| Trypan blue | 1.5 | 24 | 0.72 | 0.45 | 1.04 |
| Trypan blue | 1.5 | 72 | 1.04 | 0.71 | 1.42 |
| diluted Trypan blue | 0.0375 | 6 | 0.46 | 0.25 | 0.73 |

| Solution | mg/ml | Follow-up time [h] | Median morphologic change [%] | Lower CL [%] | Upper CL [%] |
|---|---|---|---|---|---|
| diluted Trypan blue | 0.0375 | 24 | 0.68 | 0.42 | 1.00 |
| diluted Trypan blue | 0.0375 | 72 | 0.83 | 0.54 | 1.17 |

## 6.7.6.1 Question three part 1– morphologic change

***Table 89:*** Summary of fit for the third question part 1–morphologic change of the clinical set-up

| | |
|---|---|
| $R^2$ | 98.73% |
| $R^2$ Adj | 98.15% |
| *s* | 0.0249 |
| Mean of Response | 0.2427 |
| *n* | 36 |

***Table 90:*** Effect tests for the third question part 1–morphologic change of the clinical set-up

| Factors | Nparm | DF | Sum of Squares | F Ratio | *P-value* |
|---|---|---|---|---|---|
| Solution | 3 | 3 | 1.0442 | 560.8345 | <.0001 |
| Follow-up time (h) | 2 | 2 | 0.0618 | 49.7843 | <.0001 |
| Solution*Follow-up time (h) | 6 | 6 | 0.0516 | 13.8589 | <.0001 |

***Table 91:*** Median morphologic change, lower and upper CL for the interaction between the factors solution and follow-up time

| Solution | mg/ml | Follow-up time [h] | Median morphologic change [%] | Lower CL [%] | Upper CL [%] |
|---|---|---|---|---|---|
| Trypan blue | 1.5 | 6 | 0.57 | 0.21 | 1.11 |
| Trypan blue | 1.5 | 24 | 0.72 | 0.30 | 1.31 |
| Trypan blue | 1.5 | 72 | 1.04 | 0.52 | 1.72 |
| Solution 1 (ICG) | 5.0 | 6 | 12.27 | 10.39 | 14.28 |
| Solution 1 (ICG) | 5.0 | 24 | 31.21 | 28.49 | 33.99 |
| Solution 1 (ICG) | 5.0 | 72 | 28.18 | 25.55 | 30.89 |
| Solution 2 (ICG) | 2.5 | 6 | 5.32 | 4.07 | 6.73 |
| Solution 2 (ICG) | 2.5 | 24 | 9.36 | 7.71 | 11.16 |
| Solution 2 (ICG) | 2.5 | 72 | 9.57 | 7.90 | 11.39 |
| Solution 3 (ICG) | 1.0 | 6 | 0.49 | 0.16 | 1.00 |
| Solution 3 (ICG) | 1.0 | 24 | 1.08 | 0.55 | 1.78 |
| Solution 3 (ICG) | 1.0 | 72 | 1.15 | 0.61 | 1.87 |

## 6.7.6.2 Question three part 2– morphologic change

***Table 92:*** Summary of fit for the third question part 2–morphologic change of the clinical set-up

| | |
|---|---|
| $R^2$ | 48.59% |
| $R^2$ Adj | 27.17% |
| *s* | 0.0203 |
| Mean of Response | 0.0889 |
| *n* | 18 |

***Table 93:*** Effect tests for the third question part 2–morphologic change of the clinical set-up

| Factors | Nparm | DF | Sum of Squares | F Ratio | *P-value* |
|---|---|---|---|---|---|
| Solution | 1 | 1 | 0.0012 | 3.0256 | 0.1075 |
| Follow-up time (h) | 2 | 2 | 0.0031 | 3.7853 | 0.0531 |
| Solution*Follow-up time (h) | 2 | 2 | 0.0031 | 0.3724 | 0.6968 |

***Table 94:*** Median morphologic change, lower and upper CL for the interaction between the factors solution and follow-up time

| Solution | mg/ml | Follow-up time [h] | Median morphologic change [%] | Lower CL [%] | Upper CL [%] |
|---|---|---|---|---|---|
| diluted Trypan blue | 0.0375 | 6 | 0.61 | 0.28 | 1.07 |
| diluted Trypan blue | 0.0375 | 24 | 0.88 | 0.47 | 1.42 |
| diluted Trypan blue | 0.0375 | 72 | 1.42 | 0.88 | 2.09 |
| Solution 4 (ICG) | 0.125 | 6 | 0.46 | 0.18 | 0.87 |
| Solution 4 (ICG) | 0.125 | 24 | 0.68 | 0.33 | 1.16 |
| Solution 4 (ICG) | 0.125 | 72 | 0.83 | 0.43 | 1.35 |

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| **April 2000** | Beginn des Studiums der Humanmedizin an der Johannes-Gutenberg-Universität zu Mainz |
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