Non invasive backscattering light to detection of endothelial cells activity for graft sorting: Proof of consent

Gaëlle Georges, Marion Gil, Laure Siozade Lamoine, Emmanuel Bergeret, Laetitia Abel Tiberini, Carole Deumié

Results in Optics 4 (2021) 100082

Contents lists available at ScienceDirect

Results in Optics

journal homepage: www.elsevier.com/locate/rio

Non invasive backscattering light to detection of endothelial cells activity for graft sorting: Proof of consent

Gaëlle Georges [a], Marion Gil [a], Laure Siozade Lamoine [a,*], Emmanuel Bergeret [b], Laetitia Abel Tiberini [a], Carole Deumié [a]

[a] Aix Marseille Univ, CNRS, Centrale Marseille, Institut Fresnel, F-13013 Marseille, France
[b] Université Clermont Auvergne, CNRS, Laboratoire de Physique de Clermont, F-63000 Clermont-Ferrand, France

ARTICLE INFO

Keywords:
Scattering light
Cornea
Graft sorting
Endothelial cells
Diagnostic

ABSTRACT

In tissue bank, one of the key steps in the corneal grafts sorting is the analysis of the deepest cell layer of the cornea, the endothelium. This analysis consists of counting the density of living cells using trypan blue staining. However, this dye is toxic and the measurement is localized. Then no information is given on the cell's viability of the entire cell layer. This preliminary study proposes to track the kinetics of variation of scattered light during the deturgescence process. The backscattered light of two different sets of cornea, with and without endothelial cells, are measured and the decrease of this parameter as a function of the deturgescence time are different. This noninvasive technique could be used as a criterion for attesting the presence or absence of endothelial cells. These specificities make it possible to preserve the endothelial layer compared to the techniques. The ultimate objective would be to use this measurement as a sorting criterion characterizing endothelial pump activity.

1. Introduction

Outer layer of the eye, cornea closes the eyeball and separates it from the external environment. One of the main characteristics of the cornea is its transparency. This property, which is remarkable and indispensable for good vision, is the consequence of a very particular organized structure that allows low absorption and low scattering of the light that is transmitted through it. The cornea consists of five main layers (Fig. 1). From the anterior face to the posterior face there are: the epithelium (50 μm thick), the Bowman's membrane (12 μm thick), the stroma (500 μm thick), the Descemet's membrane (3 to 10 μm thick) and the endothelium (8 μm thick).

In case of pathologies, the cornea can lose its transparency irreversibly. In these extreme cases, a transplant must be performed. The surgery consists of replacing the damaged tissue or part of it with a healthy graft (Fig. 1(b)).

The grafts are stored by authorized establishments, the corneal banks, where they are screened to sort the tissues. Hypothermia (2–8 °C) is the most commonly applied method of storage. But organ culture is used widely in European eye banks and is the storage method used for the corneal graft studied in this paper (Armitage, 2011). This type of conservation implies that the grafts are kept at a temperature between 31 and 37 °C, in a liquid derived from traditional cell culture media. This type of medium preserves the metabolic activities of the different types of corneal cells for several weeks. This method of preservation induces swelling of the graft due to the natural penetration of fluids into it. To compensate for this swelling, a deturgescence phase is carried out 48 h before the graft in order to evacuate them (Schnitzler et al., 2016).

The quality of the grafts is attested by different criteria (Gareiss-Lok et al., 2020):

- microbiological, virological and epidemiological control: tests are carried out during storage in order to eliminate contaminated or potentially contaminating grafts (Armitage and Easty, 1997);
- tissue transparency control: even though different techniques have been studied in research papers (Ventura et al., 2000, 2005; Weale and Davies/089200 A1, 2004), in most tissue banks, the assessment of transparency is only attested qualitatively by the technician responsible for the control of tissues. The graft is carefully observed for possible opacifications;

* Corresponding author.
E-mail address: laure.siozade@fresnel.fr (L. Siozade Lamoine).

https://doi.org/10.1016/j.rio.2021.100082
Received 20 November 2020; Revised 22 February 2021; Accepted 21 March 2021

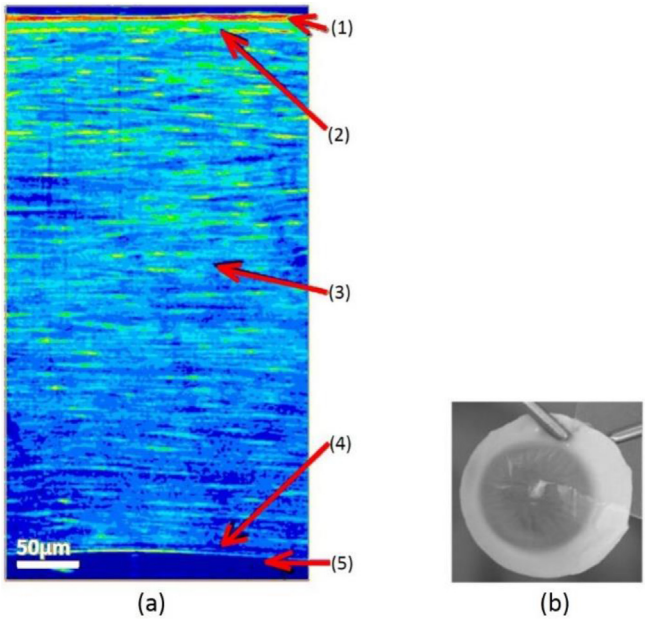


Fig 1. (a) Cross-section of a human corneal graft measured by Full-Field Optical Coherence Tomography (1: epithelium, 2: Bowman's membrane 3: stroma, 4: Descemet membrane, 5: endothelium). (b) Photography of a human corneal graft.

- evaluation of the endothelial layer quality: the evaluation of the endothelium layer in the tissue bank consists of a visual assessment of this layer under a microscope and a count of the number of endothelial cells (which must be greater than 2000/mm^2 in France (Laverne-Acquart, 2013).

This last point is crucial in assessing the quality of the grafts. Indeed, endothelial cells have an essential role in the corneal tissue functioning (evacuation of waste, passage of fluids containing nutrients and oxygen, etc.) which is ensured by a pump and barrier activity that also helps to maintain the hydration rate of the cornea. (Dikstein and Maurice, 1972). An example of optical microscope observation of the endothelial layer is shown in Fig. 2 (Maas-Reijs et al., 1997; Pels and Schuchard, 1983). Since endothelial cells have low contrast in optical observation, they can be immersed in 0.9% NaCl solution (for one to four minutes) to dilate the intercellular spaces and thus increase the contrast around the cells. This osmotic preparation is reversible (Sperling, 1986).

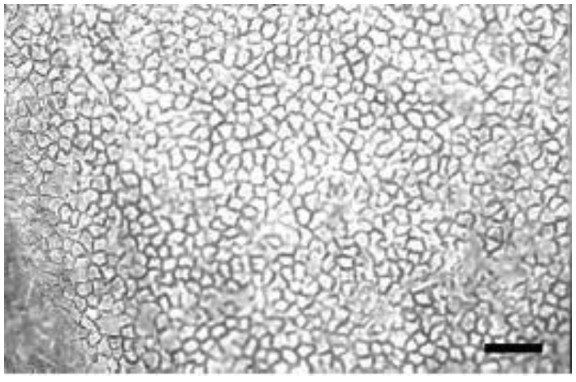


Fig 2. Image (0,1 × 0,1 mm^2) of the endothelium of a cornea preserved in organoculture and observed under optical microscope after dilatation of the intercellular spaces by 0.9% NaCl (scale bar: 100 µm) (Laverne-Acquart, 2013).

This control step is very frequently combined with a trypan blue stain to detect dead or dying cells. Indeed, trypan blue enters passively into all cells but is actively excluded by living cells (Singh et al., 1985). However, the reproducibility of the technique is frequently questioned as it only represents an instantaneous and very localized measurement without prejudging the dynamics of endothelial death during storage over the entire surface of the endothelium. The mortality rate measured with this method at the end of storage does not correlate with cell loss during organoculture (Sperling, 1986) and some non-viable cells with an intact membrane remain trypan blue negative. In addition, studies suggested endothelial toxicity of trypan blue concentrated at 0.1% after five minutes of incubation (Van Dooren et al., 2004). However, there are currently no alternative dyes or other methods to qualify the condition of the endothelium (Van Dooren et al., 2004; Gain et al., 2002; Wilhelm et al., 1995).

An important issue is to propose non-invasive and non-contact sorting method to qualify the endothelium. In this preliminary study, the potential of a technique to dissociate grafts with and without endothelial cells is demonstrated. This method is based on the sensitivity of the light backscattered to very fine changes in corneal graft bulk (Gil et al., 2019). The protocol consists of monitoring the evolution of the intensity scattered by two groups of grafts during deswelling process. One group is made up of samples with endothelial cells, the other is devoid of endothelial cells. The comparison of the behaviors demonstrates the effectiveness of this technique in the qualification of post-mortem corneas.

2. Protocol

Measurements are performed using a scatterometer for angularly resolved measurement of backscattered light by the corneal graft (Gil et al., 2019; Casadessus et al., 2012). The principle of the setup is presented on Fig. 3.

The samples are illuminated by a laser diode with a wavelength of 680 nm at normal incidence in the center of the cornea. The diameter of the beam is about 3 mm. Photodiodes measure the scattered intensity as a function of the scattering angles. From these angular measurements, an integrated parameter, TIS: "Total Integrated Scattering", can be deduce. It is defined as:

$$\mathrm{TIS} = \iint_{\theta}^{\varphi} \mathrm{BRDF}.\cos(\theta,\varphi).\sin(\theta)\mathrm{d}\theta\mathrm{d}\varphi$$

where (θ,φ) are spherical coordinates of the considered point of measurement and BRDF is Bidirectional Reflectance Distribution Function. As the scattered intensity by the cornea is isotropic, the measurements are made only in the incidence plan (φ = 0) (Mar et al., 2009). In order to limit surface effects in the scattering measurement and due to our

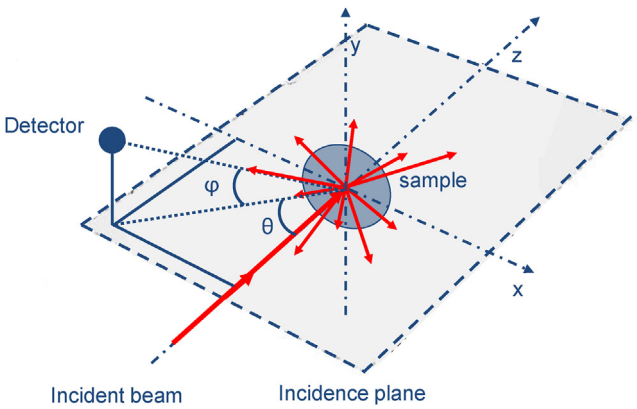


Fig 3. Definition of the measurement configuration.

experimental configuration, the TIS will be calculated over an angular range between 35° and 70° (Gil et al., 2019). The experimental errors on the TIS measurements of a same corneal graft is less than 1%. To limit this errors, the measuring time don't exceed 2 min.

Previous studies have shown the link between corneal edemas and backscattered light (Gil et al., 2019; Casadessus et al., 2012). A same corneal graft is stored in BSS (Balanced Salt Solution) and in Voluven solution (deturgescence media) to control its edema state. The backscattered intensity is measured as a function of the scattered angle and the TIS is extracted. The results are presented in Fig. 4. This figure illustrates the link and the correlation between the edema, the thickness of the corneal graft and the backscattered light.

In this paper, this technique is used in order to test the endothelial cells activity in the deturgescence process.

This study was performed on ten human corneal grafts rejected from the transplant circuit due to insufficient endothelial density, their endothelial cell density (ECD) is less than 2000 cells/mm^2.

The grafts were placed in deturgescence for seven hours after having spent a variable time in the first organoculture medium (nine to twenty days). The presence of graft cells is checked every hour. Endothelial cells are revealed by complete soaking of the graft in saline solution for about three minutes. Trypan blue is not used here. The area analyzed is 100 × 100 μm^2.

These ten human corneal grafts are classified in two categories: presence or absence of endothelial cells (respectively noted ECD > 0 or ECD = 0). Each category includes five grafts. Those qualified as having endothelial cells, correspond to those having a density of endothelial cells not zero throughout the experiment. The value of the endothelial density of the group with ECD > 0 are given in Table 1.

Scattering measurements are performed every hour during the deturgescence process and the first measurement, considered as initial data, is obtained just before being placed in Corneajet. The time of exposure to air to obtain these data does not exceed one minute.

3. Results and discussions

Fig. 3 presents the experimental results obtained of the ten corneal grafts. Graph 5(a) shows the evolution of the TIS (calculated for scattering angles between 35° and 70°) as a function of the time spent by the grafts in deturgencence medium. The red gradient points correspond to the group rated ECD = 0 and the blue gradient points correspond to the other graft that have endothelial cells (ECD > 0.

The first notable observation is that with or without cells, the evolution of light scattered by grafts during deturgescence process goes through two phases. A first phase on which the scattered light progressively decreases until reaching a minimum. And a second phase on which the TIS is almost constant.

Table 1
Endothelial density of the corneal grafts in the group ECD > 0.

Corneal grafts with endothelial cells (ECD > 0)	Endothelial density (cells/mm^2)
1	1300
2	1060
3	1140
4	1120
5	1000

The major difference between the two groups is the time taken to reach this stabilization phase. In the presence of endothelial cells, the decrease of the TIS is more rapid. Less than two hours are enough to reach a minimum and stable level of scattering, against 4 h when the graft has so few cells that they are not visible on the microscope probed area.

The minimum level of scattering reached for the grafts of the group ECD = 0 is higher than in the presence of endothelial cells (group ECD > 0), the difference is about 30%.

The number of endothelial cells is an important parameter in the qualification of grafts. It is therefore essential that characterization techniques affect this density as little as possible.

In order to quantify this kinetics of decrease in scattered intensity during the deturgescence phase, we modeled the data by the following exponential law:

$$\mathrm{TIS} = a e^{-bt} + c$$

It allows to consider the decrease phase in scattering through the term *b*, as well as the final asymptote through the term *c*.

The average trends of the measured behaviors as a function of deturgescence time for the two groups are shown in Fig. 5(b) as a red solid line for the group without cells and a blue solid line for the group with cells. The colored zones represent the 95% confidence interval.

Table 2 shows the mean values (as well as the limits of the 95% confidence interval) of these different parameters for the two groups, EDC = 0 and EDC > 0.

As noted in previous comments, the interest parameter is b which characterize the deturgescence kinetics. A sufficient number of cells (>1000 cells / mm^2) accelerates the decrease in the scattered intensity by the graft by a factor equal to 2 between the two groups.

Measure the decay kinetics of the scattered intensity of the graft during the deturgescence phase could therefore be a non-invasive characterisation method to test the presence or absence of enough endothelial cells. Such a conclusion requires making sure that other parameters cannot influence this parameter b. Those whose outcome we know are:

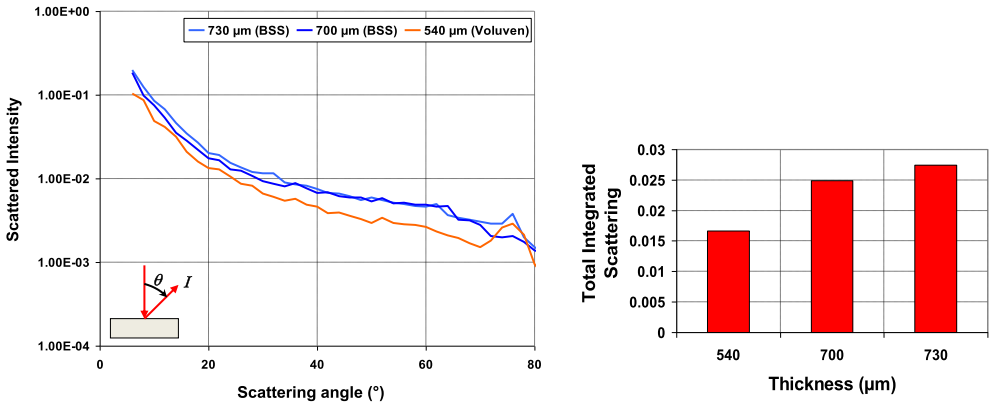
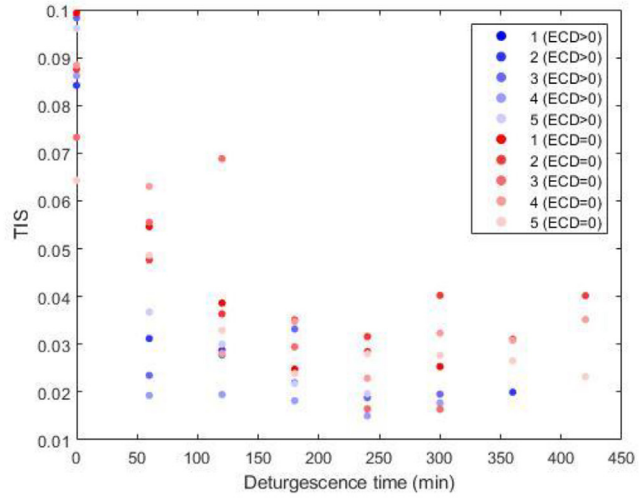
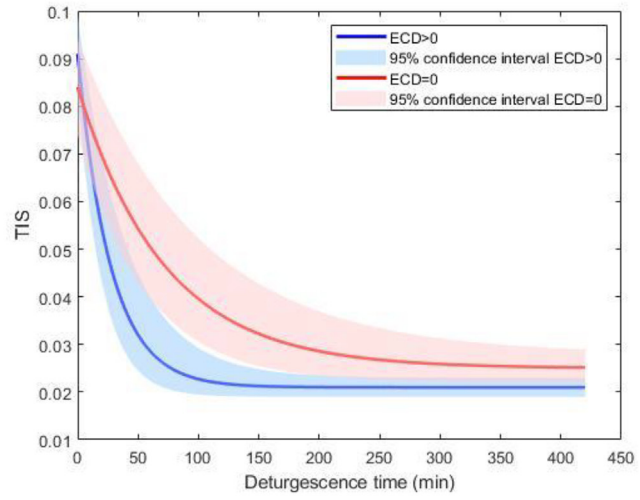


Fig 4. Scattered intensity (left) and TIS (right) of a corneal graft as a function of the scattering angle for different edema states.



(a)



(b)

Fig 5. Evolution of TIS as a function of the deturgescence time for the 10 grafts studied (a) and trend curves of the evolution of TIS of the group of grafts without endothelial cells (ECD = 0) and of the group of grafts with cells (ECD > 0) (b).

Table 2
Mean coefficients and associated 95% confidence interval used for the adjustment of the TIS curves as a function of deturgescence time of the different grafts groups.

Parameters	ECD > 0	ECD = 0
a (without unit)	$(7.0 \pm 0.6)\cdot10^{-2}$	$(5.9 \pm 0.7)\cdot10^{-2}$
b (min^{-1})	$(3.7 \pm 1.2)\cdot10^{-2}$	$(1.4 \pm 0.4)\cdot10^{-2}$
c (without unit)	$(2.1 \pm 0.2)\cdot10^{-2}$	$(2.5 \pm 0.3)\cdot10^{-2}$

- the time spent in the first preservative liquid before being placed in the detergescence medium that is between 9 and 20 days in this study;
- the initial scattering level ($TIS_{initial}$) that varies here between 0.04 à 0.10;
- the maximum relative variation of scattering: this is the relative variation of scattering between the initial TIS and the scattering measured during the stable phase. This parameter vary here between 56% à 81%.

Table 3
Pearson's coefficient (%) of correlation tests between b coefficients and time spent in Corneamax® before deturgescence process, initial scattering measurement, maximum relative scattering variation and donor age.

Parameters	Time spent in the Corneamax®	Initial scattering measurement $TIS_{initial}$	Maximum relative variation in scattering
b coefficients	−0.06	0.35	0.39

Four Pearson correlation tests were performed to identify the linear correlation degree between the b deturgescence kinetics within each group and each of these parameters. The different Pearson coefficients obtained are shown in Table 3.

The results obtained on this small number of samples show no significant correlation between deturgescence kinetics and the different parameters specific to each graft. Considering these results, the parameter presence or absence of endothelial cells seems to be relevant to explain this difference in kinetics between the two groups. Thus, the monitoring of the scattered intensity during this first phase of deturgescence allows to differentiate the group with and without endothelial cells.

As the medium used in the deturgescence process alter the quality of the graft, it is important to reduce this soaking time as much as possible. The scattered intensity is directly related to the quality of transparency of the graft. Monitoring its decrease during the phase could also make it possible to optimize the soaking time and thus reduce the negative impact of the deturgescence medium (CorneaJet® in this study) on the quality of the graft.

4. Conclusion

First results, obtained on ten human corneal grafts rejected from the transplant circuit due to insufficient endothelial cell density (ECD less than 2000 cells/mm^2), show that monitoring the intensity backscattered by the grafts during deturgescence process makes it possible to highlight the presence or absence of endothelial cells via an analysis of deturgescence kinetics. This is an encouraging first step that validates the backscattering as a qualification tool of the quality of the endothelium. This measurement technique has the advantage of being non-invasive and can be performed without contact and without sample invasive preparation. These specificities make it possible to preserve the endothelial layer compared to the techniques used today (particularly the trypan blue staining).

The next step will be to continue this study with one group of grafts suitable and one group rejected for graft in order to verify that the analysis of the scattered intensity allows the two groups to be separated.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The project leading to this publication received funding from Excellence Initiative of Aix-Marseille University - A*MIDEX, a French "Investissements d'Avenir" program.

The authors thank Dr. Picard and Dr. Chateau and the tissue bank service of the Etablissement Français du Sang de Marseille for providing the grafts necessary for the study.

The authors thank the platform CERIMED, "Centre Europeen de Recherche en Imagerie MEDicale" for their collaboration.

References

Armitage, W John, 2011. Preservation of human cornea. Transf. Med. Hemotherapy 38 (2), 143–147. https://doi.org/10.1159/000326632.

Schnitzler, A.C., Salla, S., Hamsley, N., et al, 2016. Role of the endothelial layer in the deswelling process of organ-cultured human corneas before transplantation. Cornea 35 (9), 1216–1221. https://doi.org/10.1097/ICO.0000000000000937.

Gareiss-Lok, A., Dahlström, L., Vermeulen, W., Salla, S., Van Der Wees, J., 2020. European Eye Bank Association Technical Guidelines for Ocular Tissue. www.europeaneyebanks.org.

Armitage, W.J., Easty, D.L., 1997. Factors influencing the suitability of organ-cultured corneas for transplantation. Invest Ophthalmol Vis Sci. 38 (1), 16–24.

Ventura, L., Sousa, S.J., Messias, A.M., Bispo, J.M., 2000. System for measuring the transmission spectrum of 'in vitro' corneas. Physiol Meas. 21 (1), 197–207. https://doi.org/10.1088/0967-3334/21/1/323.

Weale, R.A., Davies, N.P., 2004. Arrangement for measuring the optical quality of the anterior segment of the eye and grating element therefore. Patent WO 2004/089200 A1.

Ventura, L., de Jesus, G.T., de Oliveira, G.C.D., Sousa, S.JF., 2005. Portable light transmission measuring system for preserved corneas. BioMed Eng OnLine 4 (1). https://doi.org/10.1186/1475-925X-4-70.

Laverne-Acquart, S., 2013. Banque de Cornée: 10 ans d'innovations en contrôle qualité du greffon cornéen et projets d'avenir PhD Thesis. University Jean Monnet.

Dikstein, S., Maurice, D.M., 1972. The metabolic basis to the fluid pump in the cornea. J. Physiol. 221 (1), 29–41. https://doi.org/10.1113/jphysiol.1972.sp009736.

Maas-Reijs, J., Pels, E., Tullo, A.B., 1997. Eye banking in Europe 1991–1995. Acta Ophthalmol. Scand. 75 (5), 541–543. https://doi.org/10.1111/j.1600-0420.1997.tb00146.x.

Pels, E., Schuchard, Y., 1983. Organ-culture preservation of human corneas. Doc. Ophthalmol. 56 (1–2), 147–153. https://doi.org/10.1007/BF00154722.

Sperling, S., 1986. Evaluation of the endothelium of human donor corneas by induced dilation of intercellular spaces and trypan blue. Graefes Arch Clin. Exp. Ophthalmol. 224 (5), 428–434. https://doi.org/10.1007/BF02173358.

Singh, G., Böhnke, M., von-Domarus, D., Draeger, J., Lindstrom, R.L., Doughman, D.J., 1985. Vital staining of corneal endothelium. Cornea 4 (2), 80–91. https://doi.org/10.1097/00003226-198502000-00003.

Van Dooren, B.T., Beekhuis, W.H., Pels, E., 2004. Biocompatibility of trypan blue with human corneal cells. Arch Ophthalmol. 122 (5), 736–742. https://doi.org/10.1001/archopht.122.5.736.

Gain, P., Thuret, G., Chiquet, C., et al, 2002. Value of two mortality assessment techniques for organ cultured corneal endothelium: trypan blue versus TUNEL technique. Br. J. Ophthalmol. 86 (3), 306–310. https://doi.org/10.1136/bjo.86.3.306.

Wilhelm, F., Melzig, M., Görscher, T., Franke, G., 1995. Die unterschiedliche Aussagekraft verschiedener Vitalfärbungen des Hornhautendothels [Differential value of various vital stains of corneal endothelium]. Ophthalmologe 92 (4), 496–498.

Gil, M., Soare, S., Georges, G., Lamoine, L.S., Gonzalvez, M., Hoffart, L., Guerin, C., Gard, C., Yin, G.H.W., Deumie, C., 2019. Detection of fine modifications in bulk corneal grafts based on backscattered light quantification. Opt. Commun. 434, 246–252. https://doi.org/10.1016/j.optcom.2018.11.006.

Casadessus, O., Georges, G., Siozade Lamoine, L., Deumié, C., Hoffart, L., 2012. Light scattering from edematous human corneal grafts' microstructure: experimental study and electromagnetic modelization. Biomed. Opt. Express 3, 1793–1810. https://doi.org/10.1364/BOE.3.001793.

Mar, S., Martínez-García, M.C., Blanco-Mezquita, T., Torres, R.M., Merayo-Lloves, J., 2009. Measurement of correlation between transmission and scattering during wound healing in hen corneas. J. Mod. Opt. 56 (8), 1014–1021. https://doi.org/10.1080/09500340902871389.