Journal of Visualized Experiments

Development of a Noninvasive, Laser-Assisted Experimental Model of Corneal Endothelial Cell Loss

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE60542R1
Full Title:	Development of a Noninvasive, Laser-Assisted Experimental Model of Corneal Endothelial Cell Loss
Section/Category:	JoVE Medicine
Keywords:	Nd:YAG Laser; cornea; endothelial cells; Bullous Keratopathy; Cavitation Bubble; disease model
Corresponding Author:	M Dr. Ranjbar Universitat zu Lubeck Sektion Medizin Lübeck, SH GERMANY
Corresponding Author's Institution:	Universitat zu Lubeck Sektion Medizin
Corresponding Author E-Mail:	eye.research101@gmail.com
Order of Authors:	Annekatrin Holzhey
	Svenja Sonntag
	Johannes Rendenbach
	Justus S. Ernesti
	Vinodh Kakkassery
	Salvatore Grisanti
	Fred Reinholz
	Sebastian Freidank
	Alfred Vogel
	M Dr. Ranjbar
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Lübeck, Germany

TITLE:

Development of a Noninvasive, Laser-Assisted Experimental Model of Corneal Endothelial Cell Loss

AUTHORS AND AFFILIATONS:

Annekatrin Holzhey[1,*], Svenja Sonntag[2,*], Johannes Rendenbach[1], Justus S. Ernesti[1], Vinodh Kakkassery[2], Salvatore Grisanti[2], Fred Reinholz[3], Sebastian Freidank[3], Alfred Vogel[3], Mahdy Ranjbar[1,2]

[1]Laboratory for Angiogenesis and Ocular Cell Transplantation, University of Lübeck, Lübeck, Germany
[2]Department of Ophthalmology, University of Lübeck, Lübeck, Germany
[3]Institute of Biomedical Optics, University of Lübeck, Lübeck, Germany

*These authors contributed equally.

Corresponding Author:

Mahdy Ranjbar (eye.research101@gmail.com)

Email Addresses of Co-Authors:

Annekatrin Holzhey (annekatrin.holzhey@uksh.de)
Svenja Sonntag (svenja.sonntag@uksh.de)
Johannes Rendenbach (j.rendenbach@aol.de)
Justus S. Ernesti (justus-satoshi.ernesti@uksh.de)
Vinodh Kakkassery (vinodh.kakkassery@uksh.de)
Salvatore Grisanti (salvatore.grisanti@uksh.de)
Fred Reinholz (reinholz@bmo.uni-luebeck.de)
Sebastian Freidank (freidank@bmo.uni-luebeck.de)
Alfred Vogel (vogel@bmo.uni-luebeck.de)

KEYWORDS:

Nd:YAG laser, cornea, corneal endothelial cells, bullous keratopathy, cavitation bubble, disease model

SUMMARY:

Here, we present a protocol to detach corneal endothelial cells (CEC) from Descemet's membrane (DM) using a neodymium:YAG (Nd:YAG) laser as an ex vivo disease model for bullous keratopathy (BK).

ABSTRACT:

Nd:YAG lasers have been used to perform noninvasive intraocular surgery, such as capsulotomy for several decades now. The incisive effect relies on the optical breakdown at the laser focus. Acoustic shock waves and cavitation bubbles are generated, causing tissue rupture. Bubble sizes and pressure amplitudes vary with pulse energy and position of the focal point. In this study,

enucleated porcine eyes were positioned in front of a commercially available Nd:YAG laser. Variable pulse energies as well as different positions of the focal spots posterior to the cornea were tested. Resulting lesions were evaluated by two-photon microscopy and histology to determine the best parameters for an exclusive detachment of corneal endothelial cells (CEC) with minimum collateral damage. The advantages of this method are the precise ablation of CEC, reduced collateral damage, and above all, the non-contact treatment.

INTRODUCTION:

Transparency of the cornea is essential for the transmission of light to the retina and its photoreceptors[1]. In this regard, a relative state of dehydration is critical to keep the collagen fibers within the corneal stroma correctly aligned. This homeostasis is maintained by corneal endothelial cells (CEC) located on the Descemet's membrane (DM)[2]. The endothelium is the innermost corneal layer. It has an important barrier and pump function, which is crucial for corneal transparency[3]. In contrast to the epithelium, the endothelium is not able to self-renew[4]. Therefore, any cell damage caused by disease or trauma stimulates the remaining endothelial cells to enlarge and migrate, to cover resulting defects and to maintain corneal functionality[5]. However, if the CEC density falls below a critical threshold, decompensation of the endothelium leads to an edema, resulting in blurred vision and discomfort or even severe pain[4]. Despite the availability of drugs to relieve symptoms, currently the only definitive treatment in these cases is corneal transplantation, which can be performed in the form of a full-thickness graft or a lamellar endothelial transplantation. The latter procedure is available as Descemet's membrane endothelial keratoplasty (DMEK) as well as Descemet's stripping automated endothelial keratoplasty (DSAEK)[6]. However, the protection of remaining CEC and enhancing their survival could be an alternative target, which needs an adequate disease model to test potential therapeutic drugs.

Current CEC loss disease models focus on the destruction of the endothelium through the injection of toxic agents (e.g., benzalkonium chloride) into the anterior chamber or by mechanical abrasion of the cells using an invasive descemetorhexis technique[7,8]. While these models are well established, disadvantages such as general inflammatory response and imprecise collateral damage exist. Therefore, these models are more likely to represent final stages of the disease, when the above-mentioned surgical options are inevitable.

With advances in cellular treatment strategies such as stem cells and gene therapy, the application of these cellular therapies could be useful in early stages of CEC loss[9]. Subsequently, we need a model that represents these earlier stages of the disease more adequately. In this regard, cell culture models have improved over the last decade but are still limited in their validity, as cells in vitro cannot come close to replicating the complex interactions that occur between the different cell types within the cornea[10]. Therefore, ex vivo and in vivo disease models are still in high demand and improving the existing ones is of utmost interest.

Noninvasive, intraocular surgery by photodisruption using a neodymium:YAG (Nd:YAG) laser has become a routine procedure for ophthalmologists worldwide since its introduction in the late 1970s[11]. Photodisruption relies on nonlinear light absorption leading to the formation of plasma,

generation of acoustic shock waves, and creation of cavitation bubbles, whenever the application site is located in a liquid environment[12]. In general, these processes contribute to the intended effect of precise tissue cutting. However, they can also be the source of unnecessary collateral damage limiting the local confinement of laser surgery[13].

The prediction of resulting mechanical effects has significantly improved through characterization of the shock wave propagation and cavitation course. It is our goal to target CEC with as little damage to surrounding tissue as possible to provide a noninvasive, laser-assisted experimental disease model for the early stages of CEC loss. For this purpose, it is necessary to determine the optimal pulse energies and positions of the focal spots of the laser.

PROTOCOL:

All procedures involving animal tissue follow the guidelines of the local Animal Care and Ethics Committee.

1. Preparation of organ culture and laser treatment

1.1. Obtain freshly enucleated porcine eyes from the local abattoir. Keep them cool (4 °C) in Dulbecco's modified Eagle medium (DMEM) with high glucose, supplemented with L-glutamine, sodium pyruvate, penicillin/streptomycin (1%), and porcine serum (10%), henceforth referred to in this article as full medium.

1.2. Remove extracellular tissues with scissors and soak the eyes in 5% povidone-iodine ophthalmic solution for 5 min before placing them in sterilized phosphate-buffered saline (PBS) until use.

1.3. Screen eyes for major anterior segment pathologies, such as corneal scarring, edema, and other opacities with a spectral-domain optical coherence tomography device (**Table of Materials**).

1.4. Position the eyes in front of a slit-lamp unit equipped with a Nd:YAG laser (**Table of Materials**), which has a wavelength of 1,064 nm and a focal spot diameter of 10 µm in air.

NOTE: For optimal positioning a 3D-printed holding apparatus is used, which was designed to hold the eye firm, without putting too much pressure on it (**Figure 1**).

1.5. Use a magnification of 12x and deflect the illumination to visualize the individual corneal layers.

1.6. Set the pulse energy (e.g., 1.6 mJ) and focus point (e.g., 0.16 mm) for selective ablation of endothelial cells.

1.7. Place a clear cornea paracentesis close to the limbus and inject viscoelastic (**Table of Materials**) to stabilize the anterior chamber.

1.8. Excise the laser-treated central cornea using an 8 mm trephine.

1.9. Place the excised cornea in a well of a 12-well plate with the endothelial site facing upwards and incubate the specimen in 3 mL of full medium at 37 °C for up to 3 days.

NOTE: Potential cytoprotective agents can be added to the medium during this step.

2. Preparation for histology

2.1. Prepare Sorensen's buffer with a pH of 7.4 containing 19.6 mL of 133 mM KH_2PO_4 and 80.4 mL of 133 mM Na_2HPO_4.

2.2. Remove the medium from the cornea-containing well and fixate the tissue for 20 min at room temperature (RT) using methanol-free paraformaldehyde (4%) in Sorensen's buffer.

2.3. Place tissue in 20% sucrose in PBS until tissue sinks (1 h) and then in 30% sucrose in PBS overnight at RT. Take care to avoid contact with bubbles and the air surface interface. Embed tissue in optimal cutting temperature (OCT) compound and store at -80 °C.

2.4. Cut sections 10 µm thick using a cryostat at -27 °C.

NOTE: A camel hairbrush is useful to help guide the emerging section over the knife blade.

2.5. Transfer the section to a microscope slide by touching the slide to the tissue within 1 min of cutting it to avoid freeze-drying of the tissue. Store the slides at -80 °C.

3. Hematoxylin and eosin (H&E) staining

3.1. Air dry sections for several minutes to remove moisture.

3.2. Stain with filtered 0.1% Mayers hematoxylin for 10 min in a 50 mL tube.

3.3. In a Coplin jar, rinse in cool running ddH_2O for 5 min and dip in 0.5% eosin 10x.

3.4. Dip in ddH_2O until the eosin stops streaking and then dip in 50% (10x) as well as 70% (10x) EtOH.

3.5. Equilibrate in 95% EtOH (30 s) and 100% EtOH (60 s) before dipping in xylene several times.

3.6. Finally mount and coverslip the specimen before taking images using a light microscope.

REPRESENTATIVE RESULTS:

Using the procedure presented here, we treated eyes with a Nd:YAG laser, evaluating different pulse energies (1.0−4.6 mJ) and positions of focal points (distance from the posterior surface of the cornea: 0.0−0.2 mm) to find the optimal parameters. Multiple replicates (n = 3) were evaluated for each constellation of the laser parameters (12 x 21).

In addition to the above-mentioned protocol, specimen was analyzed with a two-photon microscope before fixation and H&E staining. The two-photon microscope used a solid-state, mode-locked 80 MHz Ti:sapphire laser with a tuning range of 690−1b040 nm and a mean laser output of >900 mW at 800 nm as light source. It delivered pulses with a width of approximately 150 fs to the sample. Images were taken with a microscope objective (20x/0.95) at a wavelength of 730 nm and 30 mW of laser power.

Two-photon as well as light microscopy images were independently reviewed by 3 reviewers, who were blinded to experimental settings and had to assign the images to three categories: (1) no damage, (2) too much damage, or (3) right amount of damage (**Figure 2** and **Figure 3**). Based on their evaluation a heatmap was calculated (**Figure 4**). Using this heatmap it is possible to select the right constellation of laser parameters to selectively ablate CEC with minimal damage to surrounding tissue (green). Results show that the focal point of the laser must be at least 0.15 mm behind the corneal endothelium for the lowest pulse energy (1.0 mJ) tested. For pulse energies higher than 2.9 mJ, the longest focal distance tested (0.2 mm) is still too close to the endothelium.

FIGURE LEGENDS:

Figure 1: Experimental setup. (**A**) Eyes were fixed in a partially 3D-printed holding apparatus, which allowed precise alignment with respect to the laser beam. (**B**) Before laser treatment, the tissue was evaluated with an anterior segment optical coherence tomography device to check for major anterior segment pathologies. Positions of focal laser points are indicated with exemplary for 0.0 mm (black asterisk), 0.1 mm (red asterisk), and 0.2 mm (blue asterisk).

Figure 2: Two-photon microscopy. Results ranged from no damage at all (**A**), extensive collateral damage (**B**) to selective ablation of endothelial cells (**C**). The red arrowhead shows a ruptured Descemet's membrane, and green arrowheads indicate selective ablation of CEC-clusters. Scale bar = 100 µm.

Figure 3: Histology. Hematoxylin and eosin staining confirmed the damage range from no damage at all (**A**), extensive collateral damage (**B**) to selective ablation of endothelial cells (**C**). The red arrowhead shows a ruptured Descemet's membrane, and green arrowheads indicate selective ablation of CEC-clusters. Scale bar = 100 µm.

Figure 4: A heatmap showing the probability of selective CEC damage. In this regard, the pulse energy as well as the position of the Nd:YAG laser focal point must be taken into account. Excessive damage is shown in red, and the desired portion of damage is shown in green.

DISCUSSION:

The results of this pilot study indicate that a Nd:YAG laser can be used to selectively ablate corneal endothelial cells when appropriate parameters for energy dose and focus point position are chosen.

As the endothelial function is important for corneal transparency and safeguarding the cornea from stromal edema, models of endothelial dysfunction play an important role in the development of anti-edematous drugs or surgical procedures. There are several established in vitro models for mimicking the in vivo situation[10,14,15], but as generally known, in vitro models cannot completely imitate the influences of enzymes and cytokines or the effect of cell-cell-interaction. In contrast, this newly developed ex vivo model of endothelial cell loss offers the possibility to monitor different states of the disease and corneal decompensation in the natural environment.

As the cornea in our model is excised with a trephine after the laser procedure, the physiology of the cornea changes and similar to the in vitro situation, regional interactions are significantly hampered. However, the local interface remains intact and especially the cellular communication to other cells within the cornea persists. Our study revealed that under these circumstances the cornea maintains its function for three days in culture, which offers sufficient time to observe and evaluate potential therapeutic agents. On the other hand, longtime healing processes cannot be investigated.

In our model, we used porcine eyes as larger quantities of those can be easily obtained. Furthermore, porcine eyes mimic human eyes very well. In contrast to rabbits that are regularly used for experiment, porcine eyes have a Bowman's membrane. Nevertheless, the corneal thickness of the porcine eye differs considerably from the human eye. In particular, the porcine stroma is much thicker and there is no difference between central and peripheral thickness in porcine corneas[16]. It must also be considered that human CEC do not have any healing capability while this regenerative feature is reported in some animals, such as pigs and rabbits[17,18]. Our study did not focus on wound healing processes, but this difference should be kept in mind when translating results. As the wound healing of animal CEC does not exceed four days, it might still be observed in our cultured tissues even if they last only for three days.

Comparing our experimental setup with previous studies, the applied energy levels were similar[19]. Instead of focusing only on the endothelium, different locations of focal points were evaluated. Therefore, the main difference of our setup to earlier studies is its potential to induce specific endothelial damage without damaging either DM or stromal tissue. Accurate monitoring of energy dose and focus position enables selective CEC ablation without causing shock wave damage to other parts of the cornea. Also, we have not noticed stromal or subepithelial edema directly after laser treatment[20].

A previous study showed that temperatures of ~40 °C can lead to CEC damage[21]. We did not measure the induced temperature, but it might be of interest for further studies. Moreover, the effect of different types of laser systems should be evaluated. Earlier studies showed a difference

between laser induced circumscribed CEC damage and other CEC injuries[19,22]. This might also limit the comparability to human tissue and diseases because laser induced damage seems to be followed by different disease development processes. Interestingly, the wound healing after laser application stopped at the burned DM in earlier studies[19]. The source of injury might be less important if the DM remains intact.

Higher energy levels can pose a higher risk of extended injury[22]. Our results show that using higher energy levels (<2.9 mJ) for selective CEC damage requires a tighter focus range. While the damage to CEC was mechanically applied in previous studies[17], laser-induced damage used here has a more precise dosing of the lesion and can be easily used in in vivo studies.

It should be noted that only a small number of eyes have been treated and examined according to this protocol. As mentioned above, there might be interindividual differences in the response to the laser treatment as well as differences between the corneas of different animal species. Since CEC damage can be produced at different focal positions, certain thresholds should not be exceeded to avoid extended damage.

Finally, the lesions generated in this study were placed in the central part. An earlier study of porcine eyes showed accelerated wound healing in the corneal periphery, which might be due to its proximity to limbal stem cells[17] as the corneal thickness in porcine eyes does not differ between regions. It should be evaluated in future studies whether laser parameters should be adjusted when switching from the center of the cornea to the periphery.

In conclusion, our study introduces a noninvasive model for further investigations on CEC dysfunction. Limitations of ex vivo or in vivo studies in terms of usage of animal instead of human tissue remain and must be considered when interpreting the results.

ACKNOWLEDGMENTS:
We thank Christine Örün and Jan A. M. Sochurek for their help with experimental methods.

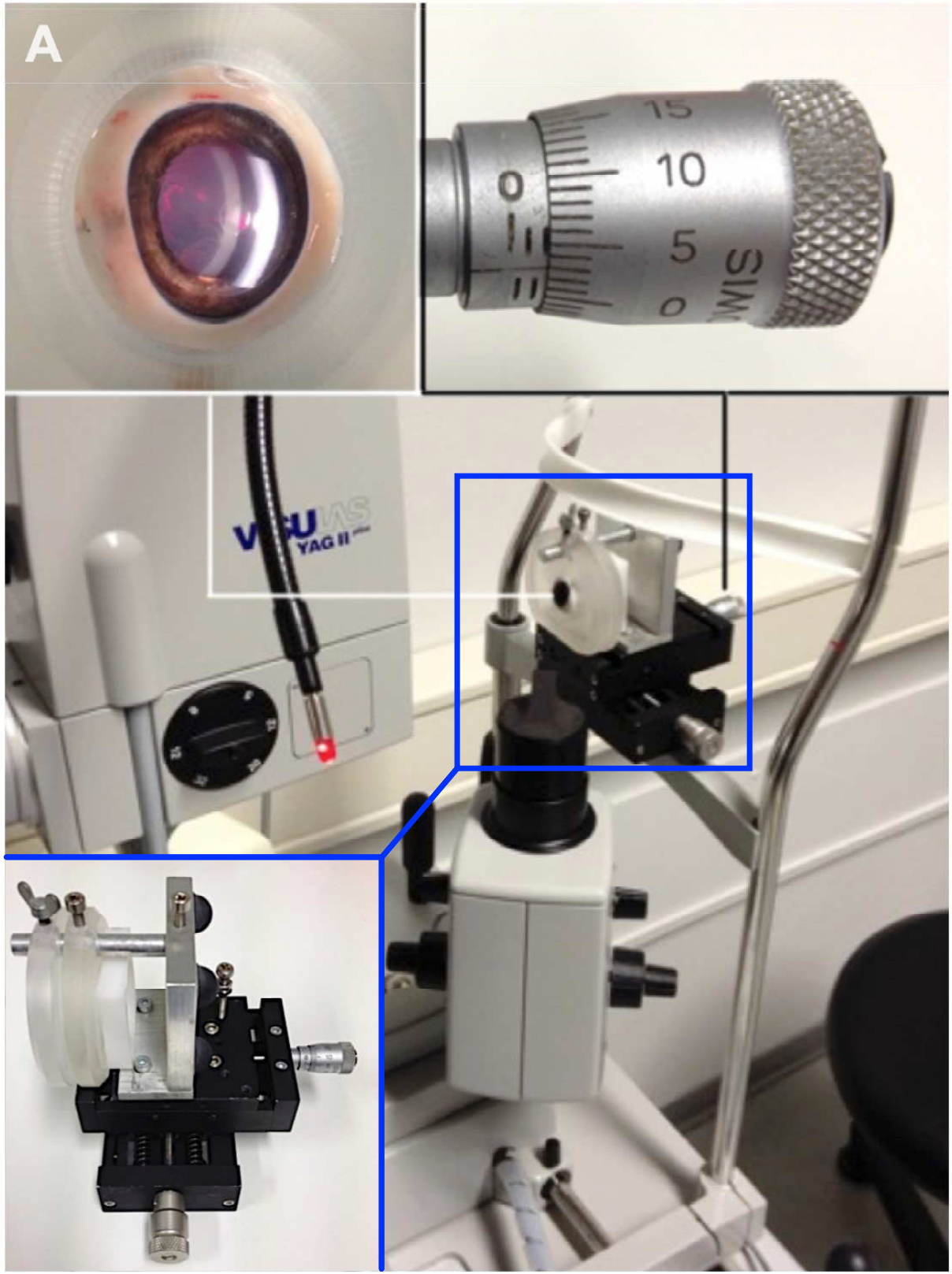
DISCLOSURES:
The authors have nothing to disclose.

REFERENCES:
1. DelMonte, D.W., Kim, T. Anatomy and physiology of the cornea. *Journal of Cataract and Refractive Surgery*. **37** (3), 588–598 (2011).
2. Edelhauser, H.F. The balance between corneal transparency and edema: the Proctor Lecture. *Investigative Ophthalmology & Visual Science*. **47** (5), 1754–1767 (2006).
3. Tuft, S.J., Coster, D.J. The corneal endothelium. *Eye (London, England)*. **4** (Pt 3), 389–424 (1990).
4. Bourne, W.M. Biology of the corneal endothelium in health and disease. *Eye*. **17**, 912–918 (2003).
5. He, Z. et al. 3D map of the human corneal endothelial cell. *Scientific Reports*. **6**, 29047 (2016).

6. Gain, P. et al. Global Survey of Corneal Transplantation and Eye Banking. *JAMA Ophthalmology*. **134** (2), 167–173 (2016).
7. Schwartzkopff, J., Bredow, L., Mahlenbrey, S., Boehringer, D., Reinhard, T. Regeneration of corneal endothelium following complete endothelial cell loss in rat keratoplasty. *Molecular Vision*. **16**, 2368–2375 (2010).
8. Bredow, L., Schwartzkopff, J., Reinhard, T. Regeneration of corneal endothelial cells following keratoplasty in rats with bullous keratopathy. *Molecular Vision*. **20**, 683–690 (2014).
9. Bartakova, A., Kunzevitzky, N.J., Goldberg, J.L. Regenerative Cell Therapy for Corneal Endothelium. *Current Ophthalmology Reports*. **2** (3), 81–90 (2014).
10. Zhao, B. et al. Development of a three-dimensional organ culture model for corneal wound healing and corneal transplantation. *Investigative Ophthalmology & Visual Science*. **47** (7), 2840–2846 (2006).
11. Aron-Rosa, D., Aron, J.J., Griesemann, M., Thyzel, R. Use of the neodymium-YAG laser to open the posterior capsule after lens implant surgery: a preliminary report. *Journal - American Intra-Ocular Implant Society*. **6** (4), 352–354 (1980).
12. Vogel, A., Hentschel, W., Holzfuss, J., Lauterborn, W. Cavitation bubble dynamics and acoustic transient generation in ocular surgery with pulsed neodymium: YAG lasers. *Ophthalmology*. **93** (10), 1259–1269 (1986).
13. Vogel, A., Schweiger, P., Frieser, A., Asiyo, M.N., Birngruber, R. Intraocular Nd:YAG laser surgery: laser-tissue interaction, damage range, and reduction of collateral effects. *IEEE Journal of Quantum Electronics*. **26** (12), 2240–2260 (1990).
14. Zhu, Q., Zhu, Y., Tighe, S., Liu, Y., Hu, M. Engineering of Human Corneal Endothelial Cells In Vitro. *International Journal of Medical Sciences*. **16** (4), 507–512 (2019).
15. Li, Z. et al. Nicotinamide inhibits corneal endothelial mesenchymal transition and accelerates wound healing. *Experimental Eye Research*. **184**, 227–233 (2019).
16. Pescina, S. et al. Development of a convenient ex vivo model for the study of the transcorneal permeation of drugs: histological and permeability evaluation. *Journal of Pharmaceutical Sciences*. **104** (1), 63–71 (2015).
17. Smeringaiova, I. et al. Endothelial Wound Repair of the Organ-Cultured Porcine Corneas. *Current Eye Research*. **43** (7), 856–865 (2018).
18. Yamashita, K. et al. A Rabbit Corneal Endothelial Dysfunction Model Using Endothelial-Mesenchymal Transformed Cells. *Scientific Reports*. **8** (1), 16868 (2018).
19. Schubert, H.D., Trokel, S. Endothelial repair following Nd:YAG laser injury. *Investigative Ophthalmology & Visual Science*. **25** (8), 971–976 (1984).
20. Zhang, W. et al. Rabbit Model of Corneal Endothelial Injury Established Using the Nd: YAG Laser. *Cornea*. **36** (10), 1274–1281 (2017).
21. McCally, R.L., Bonney-Ray, J., de la Cruz, Z., Green, W.R. Corneal endothelial injury thresholds for exposures to 1.54 micro m radiation. *Health Physics*. **92** (3), 205–211 (2007).
22. Nash, J.P., Wickham, M.G., Binder, P.S. Corneal damage following focal laser intervention. *Experimental Eye Research*. **26** (6), 641–650 (1978).

Figure1

Click here to access/download;Figure;Figure_1_Rev.eps



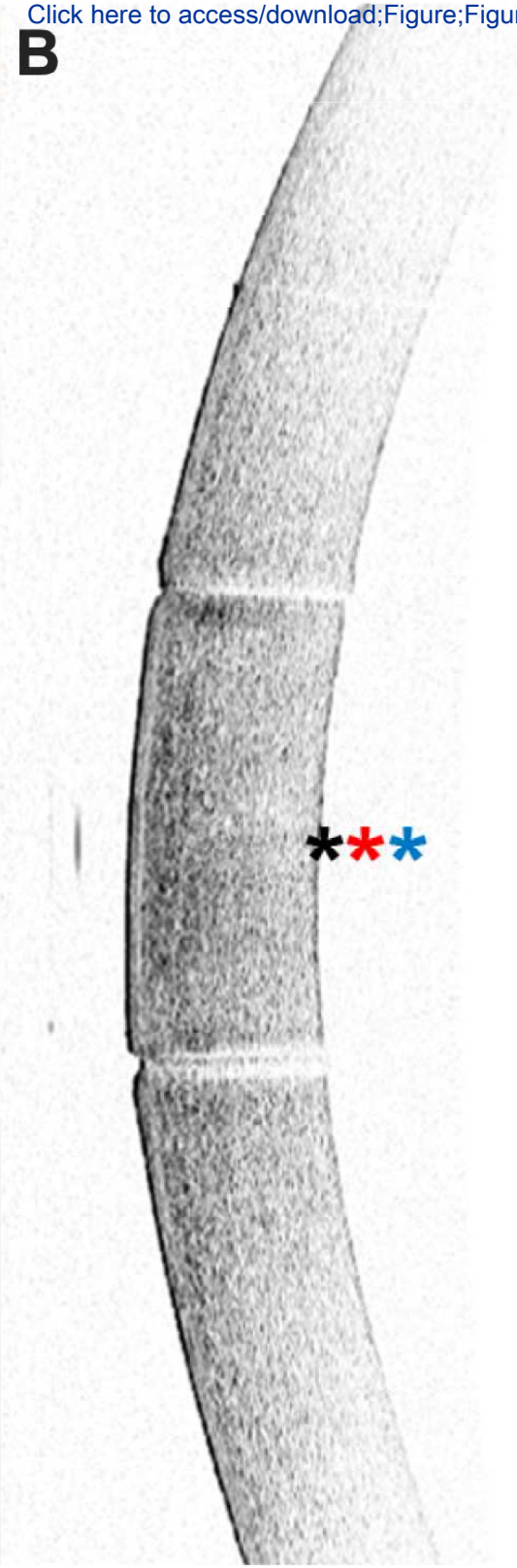


Figure2

Click here to access/download;Figure;Figure_2_Rev.eps

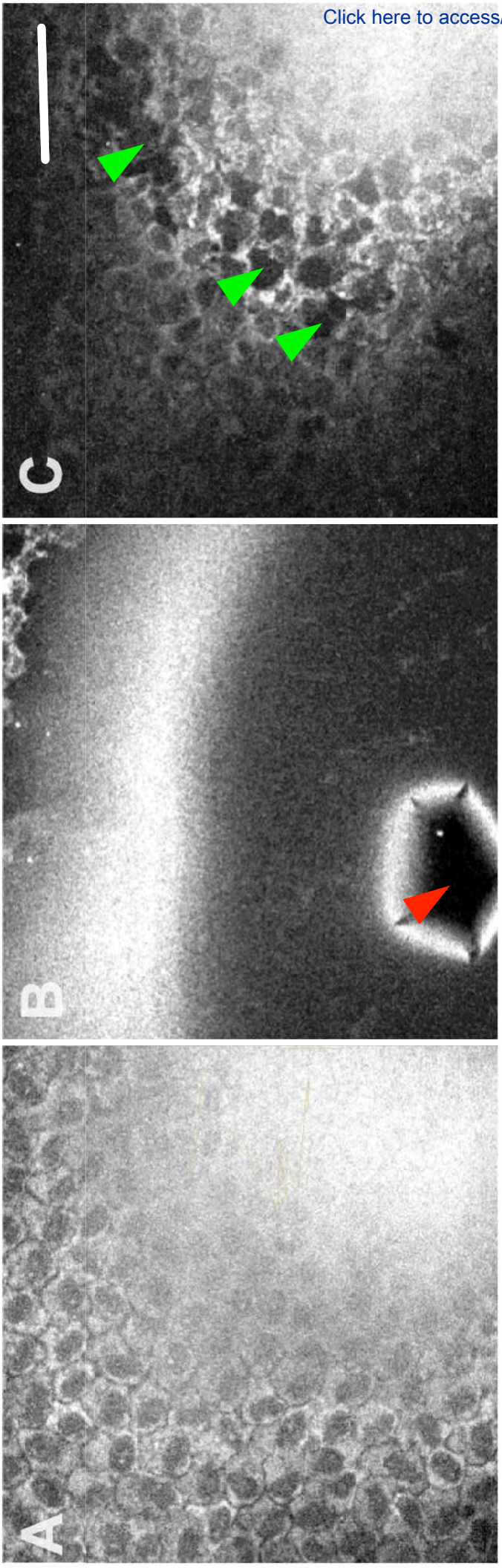


Figure3

Click here to access/download;Figure;Figure_3_Rev.eps

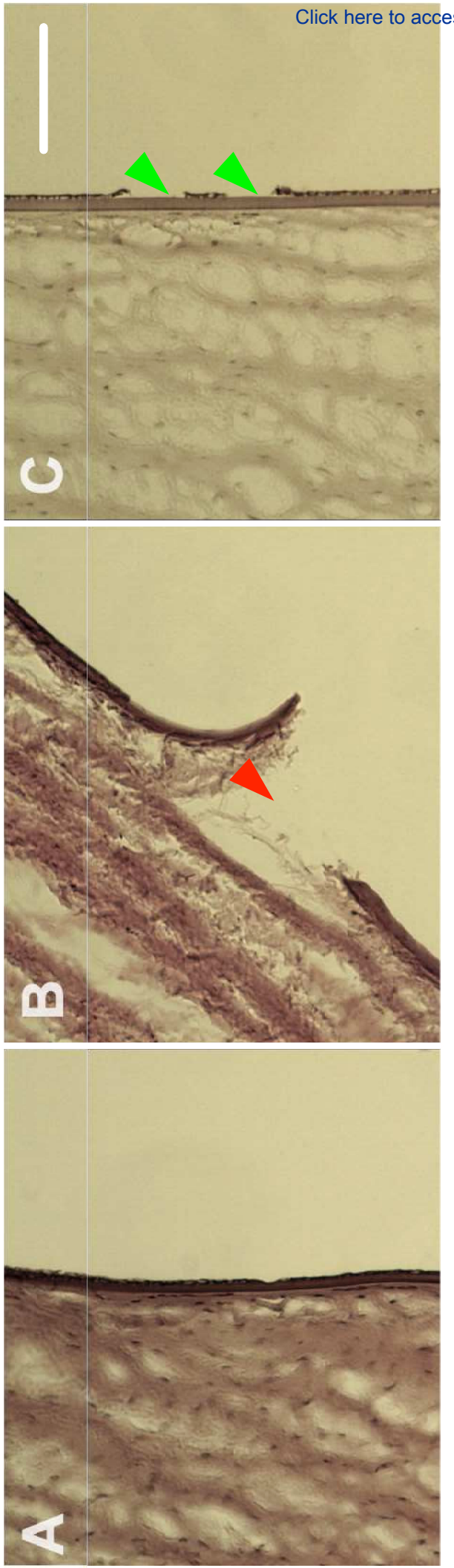
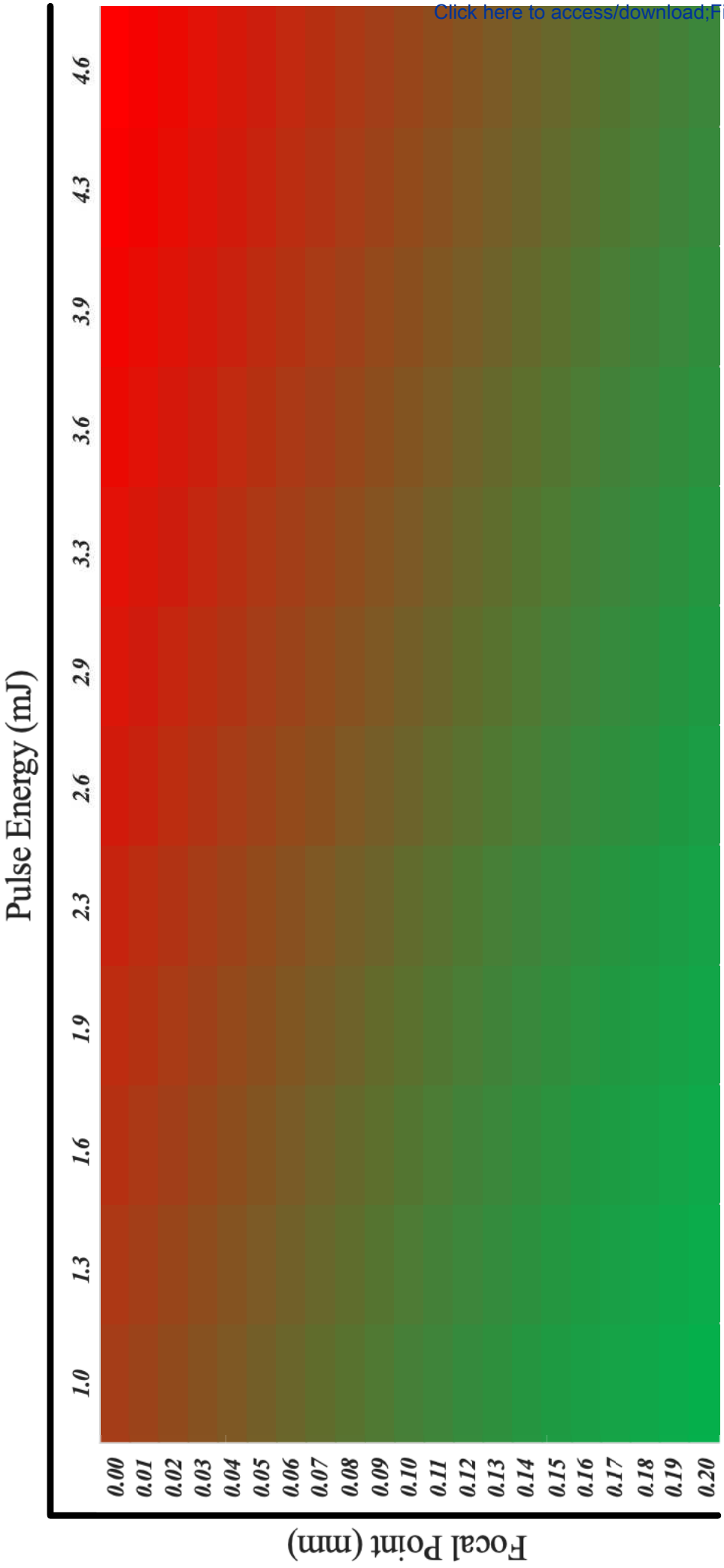


Figure4

Click here to access/download;Figure;Figure_4_Rev.eps



Name of Material/ Equipment	Company
BARRON VACUUM TREPHINE	Katena
Cryostat	Leica
Dulbecco's Modified Eagle's Medium - high glucose	PAA
Eye holder	Self
Inverted Microscope	Leica
KH_2PO_4	Merck
Na_2HPO_4	Merck
Nd:YAG laser	Zeiss Meditec
OCT Tissue Tek	Sakura Finetechnical
Penicillin-Streptomycin	Sigma-Aldrich
Phosphate Buffered Saline (PBS)	Gibco
Porcine serum	Sigma-Aldrich
Spectral-domain optical coherence tomograph	Heidelberg Engineering
Tissue culture plate 12-well	Sarstedt
Two-Photon Microscope	JenLab
Viscoelastic	OmniVision

Catalog Number

K20-2058

CM 3050S

E-15009

N/A

DMI 6000 B

529568

1065860500

visuLAS YAG II plus
4583

P4333

10010056

12736C

Spectralis

833921

DermaInspect

Methocel

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article: Development of a noninvasive, laser-assisted experimental model of corneal endothelial cell loss

Author(s): Hatthey, Sonntag, Rendenbach, Ernesti, Hallhausen, Gwarth, Reinhold, Freidank, Vogel, Ranjbar

Item 1: The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via:

☒ Standard Access ☐ Open Access

Item 2: Please select one of the following items:

☒ The Author is **NOT** a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of The Journal of Visualized Experiments; "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and(c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

4. **Retention of Rights in Article.** Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. **Grant of Rights in Video – Standard Access.** This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. **Grant of Rights in Video – Open Access.** This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this **Section 6** is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. **Government Employees.** If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. **Protection of the Work.** The Author(s) authorize JoVE to take steps in the Author(s) name and on their behalf if JoVE believes some third party could be infringing or might infringe the copyright of either the Author's Article and/or Video.

9. **Likeness, Privacy, Personality.** The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

10. **Author Warranties.** The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

11. **JoVE Discretion.** If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

12. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

13. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication of the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

14. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement is required per submission.

CORRESPONDING AUTHOR

Name:	MAHDY RANJBAR
Department:	Dpt. of ophthalmology
Institution:	University of Lübeck
Title:	MD
Signature:	
Date:	18th June 2019

Please submit a **signed** and **dated** copy of this license by one of the following three methods:

1. Upload an electronic version on the JoVE submission site
2. Fax the document to +1.866.381.2236
3. Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02140

Dear Professor Cao,
Dear Reviewers,

First of all, I'd like to thank you for your constructive revision, thoughtful comments, reasonable suggestions, and appreciation of our manuscript. We tried to answer all questions to the best of our knowledge and edited the manuscript.

Thank you for still considering our now revised manuscript for JoVE.

With best wishes and appreciation for your time,

Mahdy Ranjbar

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

R1: The manuscript has been proofread. Spelling as well as grammar issues were modified to the best of our knowledge.

2. Please revise lines 41-44 and 127-129 to avoid textual overlap with previously published work.

R2: Lines 41-44 have been revised. However, the technical description in lines 127-129 is difficult to rephrase as it simply states the characteristics of the device.

3. Authors and affiliations: Please provide an email address for each author in the manuscript.

R3: Email addresses have been added.

4. Please add a Summary section before the Abstract section to clearly describe the protocol and its applications in complete sentences between 10−50 words: "Here, we present a protocol to …"

R4: The first sentence of our abstract, which summarizes our manuscript very well, has been moved to a separate summary section before the abstract section.

5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials. You may use the generic term followed by "(Table of Materials)" to draw the readers' attention to specific commercial names. Examples of commercial sounding language in your manuscript are: Spectralis®, Heidelberg Engineering GmbH, Carl Zeiss Meditec AG, Methocel, Tissue Tek, JenLab GmbH, etc.

R5: The manuscript has been modified accordingly.

6. All methods that involve the use of human or vertebrate subjects and/or tissue sampling must include an ethics statement. Please provide an ethics statement at the beginning of the protocol section indicating that the protocol follows the guidelines of your institution.

R6: The required statement has been added.

7. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. Step 1 followed by 1.1, followed by 1.1.1, etc. Each step should include 1−2 actions and contain 2−3 sentences. Use subheadings and substeps for clarity if there are discrete stages in the protocol. Please refrain from using bullets, dashes, or indentations.

R7: The protocol has been reformatted.

8. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Alternatively, add references to published material specifying how to perform the protocol action. See examples below.

R8: The protocol has been modified.

9. Line 86: Please provide the composition of antiseptic solution. If it is purchased, please list it in the Table of Materials.
R9: The information has been added.

10. Line 96: Please describe how to adjust the pulse energy and focus point. What is the desired effect here?
R10: The sentence has been rephrased.

11. Line 100: Once cornea per well? Please also specify the incubation temperature and regenerative additives used in this step.
R11: The sentence has been rephrased.

12. Line 106: What container is used for fixation?
R12: The sentence has been rephrased.

13. Line 108: At what temperature is this done?
R13: The information has been added.

14. Line 117: Please describe how histological staining is done.
R14: The information has been added.

15. Please reference Figure 1 in the protocol.
R15: The reference has been added.

16. Please describe how to obtain data presented in Figure 4 in the protocol.
R16: The information has been added in the representative results section.

17. Figure 1: Please indicate what each color asterisk represents? Which focal length?
R17: The description has been added.

18. Figure 2 and Figure 3: Please describe what the arrowheads point to in the figure legend.
R18: The description has been added.

19. Please submit each figure as a vector image file to ensure high resolution throughout production: (.psd, ai, .eps., .svg).
R19: Images have been reformatted to .eps

20. Table of Materials: Please ensure that it has information on all relevant supplies, reagents, equipment and software used, especially those mentioned in the Protocol. Please sort the materials alphabetically by material name.
R20: The materials have been reordered.

Reviewer 1:

It would be useful if authors can provide a mechanical drawing of the porcine eye holder. The schematic could be part of Figure 1.

R: The porcine eye holder consists of different parts, which were partially 3D-printed and specifically arranged to hold the eye firm, without putting too much pressure on it. Instead of the suggested mechanical drawing we added a bigger image of the device to illustrate the setup.

Microscopy in Fig. 2 cannot be duplicated by many investigators since 2-photon microscopes are not commonly available in most research institutions. Perhaps authors could discuss alternative approaches to assess/tune the laser to ablate endothelial cells alone without damage to the adjacent cells and tissue.

R: We are aware that 2PM is not available everywhere. Therefore, we demonstrate in Figure 3 that the ablation of endothelial cells is also visible using common light microscopy after H&E staining.

Line 137 - How was the heat map calculated? Instead of graphical representation, is it more useful to provide the actual values of probability?

R: We used a single-blind review system, in which 3 reviewers who were blinded to the treatment parameters had to assign two-photon as well as histology images to three categories (no damage, too much damage, selective damage). Based on their evaluation the heatmap was calculated.

If possible, reproduce the histology micrographs at higher resolution in Fig. 3.

R: The resolution has been upscaled.

Reviewer 2:

1. In the abstract, the following phrase: "Precise application of lesions" is not self-explanatory. Please use a better phrase to relay your message.
R1: The sentence has been rephrased.

2. Line 48, it is better to state full corneal transplant, endothelial transplantation in the forms of DMEK and DSAEK rather stating lamellar graft.
R2: The sentence has been rephrased according to your suggestion.

3. Line 83, need to specify what DMEM abbreviation stands for.
R3: It has been written out in full.

4. Line 1010, what do you mean by regenerative additives of interest for up to 3 days? What do you mean? Did you already tell us what the regenerative additives of interests are?
R4: The sentence has been rephrased. We just wanted to state, that during these 3 days potential therapeutic agents can be added to the medium.

5. It is difficult to know how many eyes in total were used in this study based on the energy level and distance form the endothelium.
R5: In total we used 3 eyes for every constellation (12x21). 756 eyes in total were evaluated. This information has been added to the representative results section.

6. Was there any collateral damage to descemets membrane? Was the damage to descemet membrane unacceptable.? Only CEC without basement membrane insult was the target of your study?
R6: Yes, damage to Descemet's membrane (DM) ranged from small bumps to total rupture. During the review of the images minor damage to DM was acceptable, as long as there was no partial or complete rupture. Actually, minor damage to DM would be ideal for a disease model as this is common early stages of these diseases.

7. Author need to provide some range of energy and corresponding posterior distance from the endothelium that caused no damage except the desired specific insult to the CEC and nothing else. Please provide these in the result section instead of simply referring the reader to the figures.
R7: The range is illustrated in the heatmap, however we now added the required information also in the results section.

8. Was desired damage defined in terms of single cell damage or clusters of few cells without collateral damage?
R8: Clusters. We rephrased some passages for clarity.

9. Can author share some of the safe YAG laser setting in the discussion for other investigators who are interested in this field.
R9: Referring to R7.

10. Congratulate the authors on the great work. As mentioned in vivo animal study needs to verify the validity of their laser setting.
R10: Thanks for your appreciation of our efforts.

11. I think authors need to share their energy threshold and distance threshold that caused only CEC damage and then emphasize that the safety and validity of their work in the actual in vivo system needs to be cautiously performed.
R11: Referring to R7+R9.

12. I am curious to know about your custom designed holding apparatus. Such detail in the method section needs published references or more detailed information in the method section about the apparatus.
R12: The porcine eye holder consists of different parts, which were partially 3D-printed and specifically arranged to hold the eye firm, without putting too much pressure on it. We added a bigger image of the device to illustrate the setup.

13. For examples in the table of material, the holding apparatus company or manufacturing should be reflected.
R13: Referring to R12.