

In vivo keratotomy by mid-infrared femtosecond laser resonant with amide vibrational mode

Corresponding Author: Professor Houkun Liang

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The manuscript presents an innovative in vivo MIR keratotomy technique utilizing a 6.1 μm femtosecond laser, which corresponds to the amide-I resonant mode. The authors successfully demonstrate the experimental feasibility of deep keratotomy using the proposed MIR technique, exhibiting reduced collateral damage compared to conventional NIR femtosecond lasers under comparable ablation conditions. The study also provides a comprehensive evaluation of the efficacy, healing process, biological safety, and postoperative complications associated with this approach, suggesting that MIR femtosecond laser technology could represent a novel pathway for ophthalmic surgery and broaden the scope of femtosecond laser-assisted keratotomy. The results are compelling and possess substantial scientific significance and potential for clinical application.

However, there are still several points that require further clarification before the manuscript can be considered for acceptance by Communications Medicine:

1. **Insufficient Analysis of Limitations:** While the manuscript emphasizes the advantages of the proposed technique, the discussion on its limitations and challenges is insufficient. Could the authors elaborate on any technical difficulties or potential barriers encountered during the experimental implementation? Are there any specific drawbacks or risks that might hinder its clinical translation? A deeper analysis of these aspects would offer a more balanced evaluation and provide readers with a clearer understanding of the feasibility and potential limitations of adopting the MIR keratotomy approach in clinical settings.

2. **Mechanistic Elaboration on Amide-I Resonance:** The manuscript briefly references the amide-I resonant mode as a key component of the proposed technique. To enhance the clarity and scientific rigor of the manuscript, could the authors provide a more detailed explanation of the specific mechanisms by which the amide-I resonance influences the keratotomy process? Additionally, including more literature and references on the use of amide-I or similar resonant modes in laser-based medical applications would help contextualize the significance of this mode in the broader field of laser surgery, further highlighting the novelty and contribution of this work.

Reviewer #2

(Remarks to the Author)

The manuscript titled "In vivo keratotomy by mid-infrared femtosecond laser resonant with amide vibrational mode" investigated MIR keratotomy by employing 6.1 μm femtosecond laser coinciding to the amide-I resonant mode. While it was an interesting topic, several issues need attention to improve the manuscript.

1. Through the systematical investigation of the healing process by histological and fluorescein staining imaging, it is revealed that deep corneal incisions ablated by MIR fs laser can heal in 3 days with structurally intact epithelium, stromal and endothelial layers by identifying the optimal ablation parameters. The corneal endothelium is composed of a single layer of cells. The morphology of endothelial cells is closely related to their function, and such changes cannot be assessed merely through sectioning.

2. Additionally, the authors have observed the histological images of eyeball tissues from different groups at 3 days, 7 days, and 1 month after MIR fs laser ablation. It would be beneficial to include corresponding functional indicators to provide a comprehensive evaluation of corneal recovery.

3. In the Methods section, there is a lack of information regarding the number of samples for each experimental group and the statistical methods used for data analysis. It is crucial to include these details to allow for a thorough assessment of the

results' reliability and validity.

4. NIR femtosecond lasers encounter challenges such as prolonged vision healing time and postoperative astigmatism due to scattering by edematous tissues, and endothelial cells distortion by mechanical impact. More importantly, MIR lasers with long wavelengths significantly mitigate optical scattering, making them well-suited for deep or penetrating keratotomy in edematous corneas. However, the authors conducted their research only in normal mice, whereas the clinical application of this technology is likely to be mostly in edematous corneas. Comparing the application effects of NIR and MIR fs lasers in edematous corneas is of great significance for MIR fs lasers to become a new path for addressing deep or penetrating keratotomy in the future.

Version 1:

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(Remarks to the Author)

After reviewing the revised manuscript and the authors' responses, the authors have adequately addressed the concerns raised in the initial review.

Regarding technical limitations, the authors have expanded on the challenges of MIR transmission, particularly optical fiber constraints, and proposed potential solutions such as anti-resonant hollow-core fiber (AR-HCF). This provides a more balanced assessment of the technique's feasibility for clinical applications.

For amide-I resonance, the authors have clarified its role in keratotomy, distinguishing their mechanism from conventional thermal confinement and detailing the dual energy absorption process involving protein denaturation and water vaporization. The inclusion of additional references further strengthens the scientific context.

These revisions incorporate feedback based on both my comments and those of the fellow reviewer, enhancing the manuscript's clarity and depth, making it suitable for acceptance in its current form.

Reviewer #2

(Remarks to the Author)

The authors have done an excellent job addressing many of the issues brought up by the reviewers. There are two additional comments :

1. The authors should explicitly distinguish between keratectomy (tissue removal) and keratotomy (incision creation) in their terminology. Currently, the excimer laser serves as the primary tool for keratectomy (ablative procedures), while the NIR FS laser functions as the key instrument for keratotomy (precise stromal cutting). This terminological distinction is essential for accurately characterizing the mechanistic differences between these complementary laser technologies in corneal surgery. (lines 66-68)

2. The MIR femtosecond laser offers significant advantages in corneal transplantation and refractive surgery, particularly for edematous or cloudy corneas. We are eager to know whether the laser can achieve precise intrastromal keratotomy rather than a full-thickness incision, which is crucial for lamellar keratoplasty. (lines 562-565)

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20 February, 2025

Dear Editor,

Thank you for your help in handling our Manuscript, entitled “In vivo keratotomy by mid-infrared femtosecond laser resonant with amide vibrational mode”. We have carefully studied through all the comments, provided operational details, experimental mechanisms, analysis, and new experimental data. Below we provide a point-by-point response to the reviewers’ comments and the corresponding list of revisions we have made. We believe that with these changes our manuscript has now been improved and we hope it can now meet the standards and criterion for publication in Communications Medicine.

Yours sincerely,

Houkun Liang

Senior member of Optica (formally OSA)

Chair professor of School of Electronics and Information Engineering,
Sichuan University,

A handwritten signature in black ink, consisting of a series of fluid, connected strokes that form a stylized representation of the name 'Houkun Liang'.

We thank the reviewers for the constructive comments of our work and useful suggestions for improving the manuscript. Please find below the response to all the comments.

Reviewer report 1

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Response: We thank the reviewer for the recognition of the manuscript and the constructive suggestions. For the purpose of applying MIR-fs lasers to clinical surgical settings, we consider that the development of portable hand tools is a critical challenge.

Thus, it entails the design of transmission system for the femtosecond laser with special wavelength. It is necessary to select the optical fiber with low transmission loss to MIR wavelength. Currently, there is a limited variety of commercial fibers capable of transmitting MIR laser with good efficiency, mainly chalcogenide glass fiber, fluoride glass fiber, and hollow waveguides. Despite of the low transmission loss, chalcogenide and fluoride glasses could not transmit femtosecond pulses due to large material dispersion. Hollow waveguides with special metal coating emerge as a more favourable choice. At least two hollow waveguide products (from Laser Components and Guiding Photonics) have already been employed in medical spectroscopic applications. However, hollow waveguides with metal coating have relatively low damage threshold, and could not handle pulses with hundreds mW average power and tens MW peak power. These present challenges in both research and implementation of future clinical applications. As a potential solution, we are committed to waveguide research of anti-resonant hollow-core fiber (AR-HCF), specifically designed for the delivery of MIR fs pulses, as shown in Fig. R1. As shown by a scanning electron microscope cross-sectional image in Fig. R1(a), the fiber consists of 7 cladding elements and light is guided in the hollow core air, which has a diameter of 113 μm . This not only eliminates the extra dispersion imposed to the femtosecond pulse, but also assures a superior damage threshold. Fig. R1(b) reveals the transmission loss between 5 to 6.5 μm , which marked by red stars. The measured fiber losses at wavelength of 6.1 μm is 7.6 dB/m, which ensures sufficient output energy for tissue ablation. Clear sign of ablation is observed in the *in vitro* image of eye ball, as shown in set of Fig. R1(b). We believe that

the application of the AR-HCF further underscores the potential of MIR-fs laser for keratotomy. We have added the challenge and prospect regarding applying the system to clinical setting in the revised manuscript.

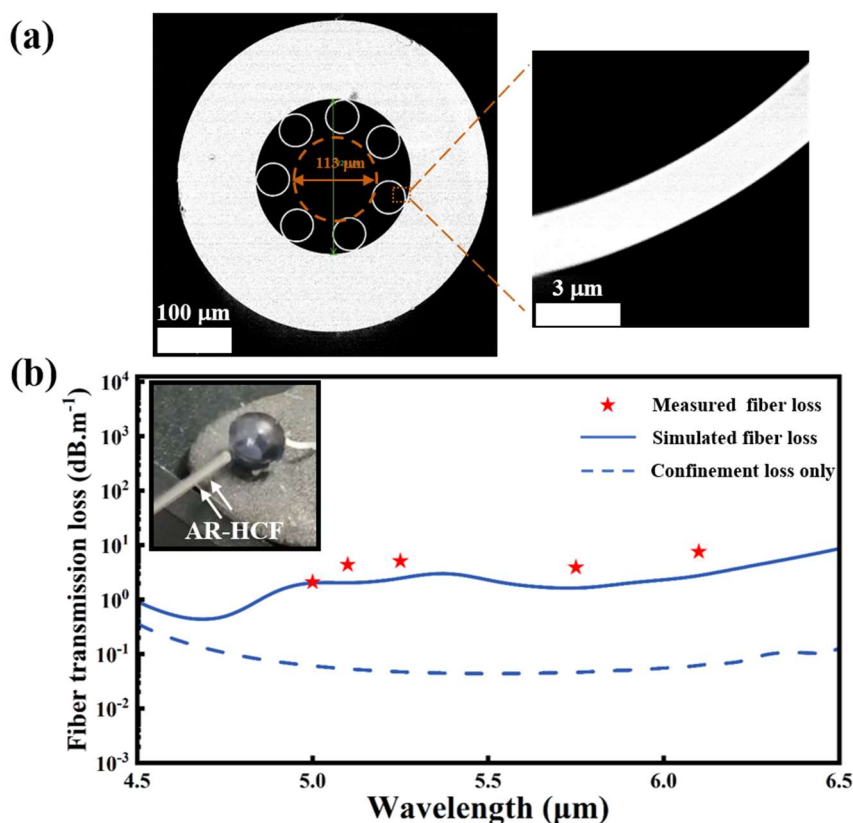


Fig. R1 (a) A cross-sectional image of the AR-HCF captured using a scanning electron microscope, with a magnified image of cladding on the right. (b) Normalized transmission spectrum of the AR-HCF. The measured loss with centres at 5, 5.1, 5.25, 5.75 and 6.1 μm (red stars). Inset shows the *in vitro* corneal ablation based on AR-HCF, as a conceptual demonstration.

Please see changes in Line 573-579 and 698-699 in the revised manuscript.

2. Mechanistic Elaboration on Amide-I Resonance: The manuscript briefly references the amide-I resonant mode as a key component of the proposed technique. To enhance the clarity and scientific rigor of the manuscript, could the authors provide a more detailed explanation of the specific mechanisms by which the amide-I resonance

influences the keratotomy process? Additionally, including more literature and references on the use of amide-I or similar resonant modes in laser-based medical applications would help contextualize the significance of this mode in the broader field of laser surgery, further highlighting the novelty and contribution of this work.

Response: We thank for the meaningful suggestion from the reviewer. Generally, the mechanism of laser ablation is described by the thermal confinement model: water molecules in tissues absorb photon energy, then vaporize and explode, causing decomposition of biomolecules, finally leading to tissue removal. On the other hand, keratotomy based on the amide resonance wavelength differs from the single thermal confinement model, and it has a composite energy distribution mechanism. As characterized by the FTIR analysis of corneal tissue presented in Fig 2 in our manuscript, the main components of the tissue, namely water and protein, exhibit strong absorption of 6.1 μm laser. Laser with this wavelength can simultaneously excite the resonance of water and protein, which involves two main processes. The first process entails the protein absorbing photon energy, resulting in an increase of its temperature to about 60 °C. This causes the structural transitions from highly ordered arrays of crosslinked triple helices with high tensile strength to amorphous gelatin, a far less resilient material. The second process occurs when water molecules absorb photo energy, causing a further increase of temperature to vaporization and explosion. Notably, the denaturation of protein or collagen (melting of collagen) occurs more rapidly than that of water because of the lower temperature threshold. Consequently, when the collagen is melted and transformed into a less resilient structure, it is easier to be decomposed with the explosive vaporization of water, which leads to an efficient ablation with minimal collateral damage. We have presented a more comprehensive description of ablation mechanism and added the following references related to resonant modes laser-based medical applications in revised manuscript.

[1] G. S. Edwards, "Mechanisms for soft-tissue ablation and the development of alternative medical lasers based on investigations with mid-infrared free-electron lasers," *Laser Photonics Rev.* **3**, 545-555 (2009).

[2] K. M. Joos, J. H. Shen, D. J. Shetlar, and V. A. Casagrande, "Optic nerve sheath

fenestration with a novel wavelength produced by the free electron laser (FEL)," *Lasers Surg. Med.* **27**, 191-205 (2000).

[3] J. H. Shen, J. A. Harrington, G. S. Edwards, and K. M. Joos, "Hollow-glass waveguide delivery of an infrared free-electron laser for microsurgical applications," *Applied Optics* **40**, 583-587 (2001).

[4] W. S. Sun, J. H. Shen, D. J. Shetlar, and K. M. Joos, "Endoscopic goniotomy with the free electron laser in congenital glaucoma rabbits," *Journal of Glaucoma* **9**, 325-333 (2000).

[5] R. J. Shah, J. H. Shen, and K. M. Joos, "Endoscopic free electron laser technique development for minimally invasive optic nerve sheath fenestration," *Lasers Surg. Med.* **39**, 589-596 (2007).

Please see changes in Line 97-101, 658-659 and 666-668 in the revised manuscript.

We thank the reviewers for the constructive comments of our work and useful suggestions for improving the manuscript. Please find below the response to all the comments.

Reviewer report 2

The manuscript titled "In vivo keratotomy by mid-infrared femtosecond laser resonant with amide vibrational mode" investigated MIR keratotomy by employing 6.1 μm femtosecond laser coinciding to the amide-I resonant mode. While it was an interesting topic, several issues need attention to improve the manuscript.

1. Through the systematical investigation of the healing process by histological and fluorescein staining imaging, it is revealed that deep corneal incisions ablated by MIR fs laser can heal in 3 days with structurally intact epithelium, stromal and endothelial layers by identifying the optimal ablation parameters. The corneal endothelium is composed of a single layer of cells. The morphology of endothelial cells is closely related to their function, and such changes cannot be assessed merely through sectioning.

Response: We thank the meaningful suggestion from the reviewer. In order to evaluate the morphology and function of corneal endothelial cells following MIR-fs laser ablation, ZO-1 and Na/K-ATPase are used to conduct immunofluorescence staining on the cornea, which characterized the structural integrity and regulation ability of osmotic pressure, respectively. After MIR-fs keratotomy on left eyes of mice, corneas are harvested from different groups on 1, 3, and 7 days post-operation. Corneas are subsequently fixed in 4% paraformaldehyde for 30 min, thoroughly washed with phosphate buffered saline (PBS), blocked with the mixed solution of 5% normal goat serum (NGS), 0.5% Triton X-100 and PBS for 1 h at room temperature. Corneas are then incubated with primary antibodies Anti ZO-1 rabbit (Proteintech, China) and Anti Na/K-ATPase mouse (Sigma-Aldrich Chemicals, USA) at 4 °C overnight, and corneas are incubated with secondary antibodies Goat-rabbit 488 and Goat-mouse 555 (Jackson ImmunoResearch, USA) respectively at 4 °C overnight. Corneal tissues are mounted endothelial side up on a slide and are imaged using fluorescent microscope. ImageJ

software (National Institutes of Health) is used to measure the endothelial cells density and fluorescence intensity, and the ratio between left and right eyes are subsequently calculated. As shown in Fig. R1, compared to the control group, the endothelial cells in study groups on 1 day post-operation exhibit irregular shapes instead of typical hexagons, and there are blurred cell boundaries in some areas. The density of endothelial cells decreased, with the ratios of three study groups to the control groups being 0.79 ± 0.09 , 0.78 ± 0.03 and 0.86 ± 0.14 , respectively. This may be attributed to endothelial cell barrier dysfunction. In addition, the expression levels of Na/K-ATPase in Group1 and Group2 are lower than those in the control groups, with the fluorescence intensity ratios as 0.80 ± 0.07 and 0.70 ± 0.06 , respectively. As the corneal incision heals, the function of endothelial cells gradually recuperates. 7 days after operation, the corneal endothelium exhibits a continuous and flat layered structure. The ratios of endothelial cells density in each study groups to that in control group are 0.96 ± 0.03 , 0.99 ± 0.03 and 0.98 ± 0.03 , respectively. Notably, the density in Group 2 increased significantly ($P<0.05$). The fluorescence intensity ratios are 1.15 ± 0.05 , 1.11 ± 0.09 and 1.08 ± 0.09 , respectively, with Group 2 showing a significant increase ($P<0.01$). These results indicate that under the ablative parameters established above, the morphology and function of endothelial cells can be restored as the cornea heals. We have added the evaluation study of endothelial cells in revised manuscript.

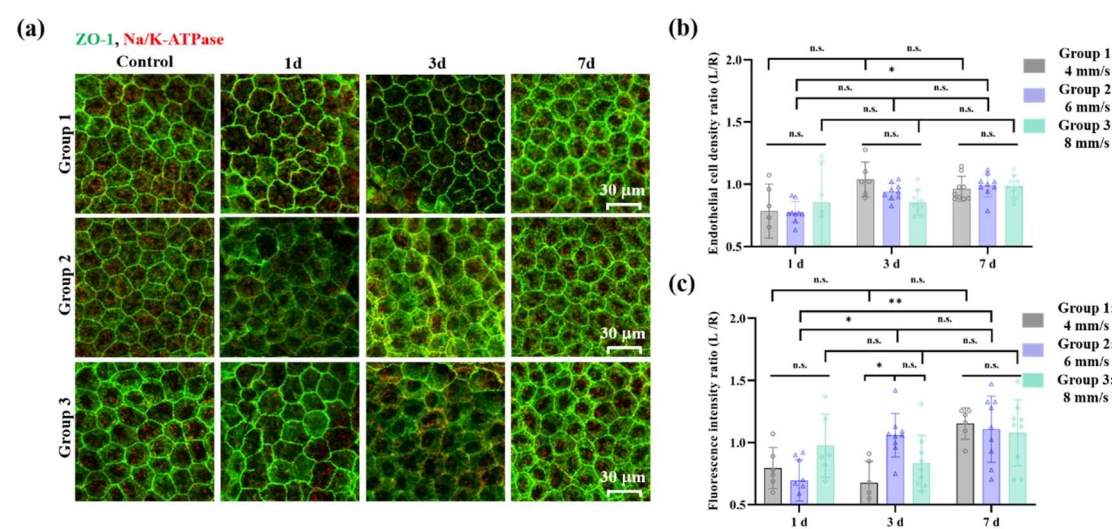


Fig. R1 Immunofluorescence images of endothelial cells healing after keratotomy using MIR-fs laser at different time points. (a) The expression of ZO-1 (green) and Na/K-ATPase (red) in the mouse

corneal endothelium. Analysis of corneal endothelial cells density based on ZO-1 staining (b) and quantization of Na/K-ATPase-related fluorescence intensity (c), * $P < 0.05$, ** $P < 0.01$, n.s., not significant.

Please see changes in Line 31-36, 197-208, 443-458, 460-464 and Figs. 5(c-e) in the revised manuscript.

2. Additionally, the authors have observed the histological images of eyeball tissues from different groups at 3 days, 7 days, and 1 month after MIR fs laser ablation. It would be beneficial to include corresponding functional indicators to provide a comprehensive evaluation of corneal recovery.

Response: Thanks for the suggestion for reviewer. As an *in vivo* supplement for histological images of eyeball tissues at different time points after MIR-fs laser ablation, optical coherence tomography (OCT) is carried out on the cornea of mice at 1, 3 and 7 days post-surgery. After anaesthesia, an anterior segment OCT system (Spectralis OCT, Heidelberg) equipped with 25D lens is utilized. Subsequently, the depth of corneal incisions in each group is measured using ImageJ, serving as an indicator for evaluating corneal recovery. As shown in Fig. R2, on the first day after surgery, the corneal defects in each group are distinctly visible (dark areas in the cornea). Moreover, there are significant differences in the depth of incisions producing by different ablative parameters. Lower laser scanning speed is found to achieve deeper ablation, which is consistent with previously described results. 3 days after surgery, as the corneal healing, the depth of dark areas presented in OCT images of each group decreased significantly ($P < 0.0001$). 7 days later, corneal defect has further diminished, and there is no significant difference in depth among the groups. Additionally, no corneal epithelial defects are detected in any study groups. We have added the characterization of OCT in the revised manuscript.

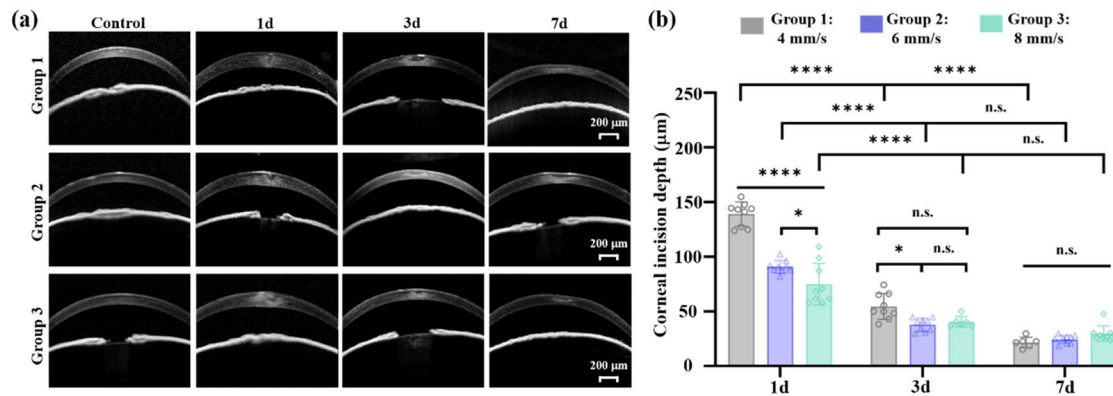


Fig. R2 OCT characterization of corneal healing following MIR-fs laser keratotomy. (a) OCT images of cornea at different time points. The dark area in the central cornea represents the incision generated by MIR-fs laser. (b) Corneal incision depth in mice. * $P<0.05$, **** $P<0.0001$, n.s., not significant.

Please see changes in Line 31-36, 197-200, 433-442 and Figs. 5 (a, b) in the revised manuscript.

3. In the Methods section, there is a lack of information regarding the number of samples for each experimental group and the statistical methods used for data analysis. It is crucial to include these details to allow for a thorough assessment of the results' reliability and validity.

Response: We apologize for the insufficient description of experimental methods and data. In the methods section of revised manuscript, we have provided detailed information regarding the number of samples and statistical analysis.

Please see changes in Line 188, 196, 199-200, 211, 228 and 234-237 in the revised manuscript.

4. NIR femtosecond lasers encounter challenges such as prolonged vision healing time and postoperative astigmatism due to scattering by edematous tissues, and endothelial cells distortion by mechanical impact. More importantly, MIR lasers with long wavelengths significantly mitigate optical scattering, making them well-suited for deep or penetrating keratotomy in edematous corneas. However, the authors conducted their research only in normal mice, whereas the clinical application of this technology is

likely to be mostly in edematous corneas. Comparing the application effects of NIR and MIR fs lasers in edematous corneas is of great significance for MIR fs lasers to become a new path for addressing deep or penetrating keratotomy in the future.

Response: We thank for the meaningful suggestion from the reviewer. In order to compare the application effects of NIR and MIR fs lasers in edematous corneas, keratotomy using these two lasers is conducted for *in vitro* edematous corneas under the same energy parameters as mentioned in the manuscript. Eyeballs are harvested and immersed immediately in hypotonic solution for 20 h, and edematous corneas are ablated as soon as possible. Figs. R3 (a-d) present the photos of edematous corneal incisions at different wavelengths, and Figs. R3(e-h) are corresponding histological images. In edematous cornea, the accumulation of water enlarges the separation between collagen bundles in the matrix, leading to a disordered structure, a closer distance between the lens and cornea, and decrease in corneal transparency, as shown in Figs. R3(a, e). Notably, to study the effect of deep keratotomy, the scanning speed is reduced to 2 mm/s to extend the action time on deep tissue. When corneal ablation is performed at wavelength of 6.1 μm with a pulse energy of 2 μJ , a deep incision can still be formed and almost penetrates the corneal layer due to the extended action time, as shown in Figs. R3 (b, f). When the wavelength is switched to 1.03 μm , with a pulse energy of 3 μJ , only shallow scratch is observed. The extension of action time fails to increase the incision depth, but only causes collateral thermal damage around the incision, as shown in Figs. R3 (c, g). Further increasing the pulse energy of the 1.03 μm laser to 16 μJ deepens the incision. However, this also results in a much larger area of thermal damage compare to that in normal corneal keratotomy, which may be due to the scattering of light in watery cornea. In addition, severe deformation of lens is observed in Figs. R3 (d, h), which may be attributed to the extrusion between cornea and lens in turgid eyeball, as well as the mechanical shock induced by high intensity NIR laser. These results indicate that NIR laser has limitations in deep keratotomy for edematous cornea. In contrast, MIR laser with low scattering in tissue offers more advantages, which ensure smaller collateral damage while achieving deep incision. We have added the ablative results of edematous cornea in the revised manuscript.

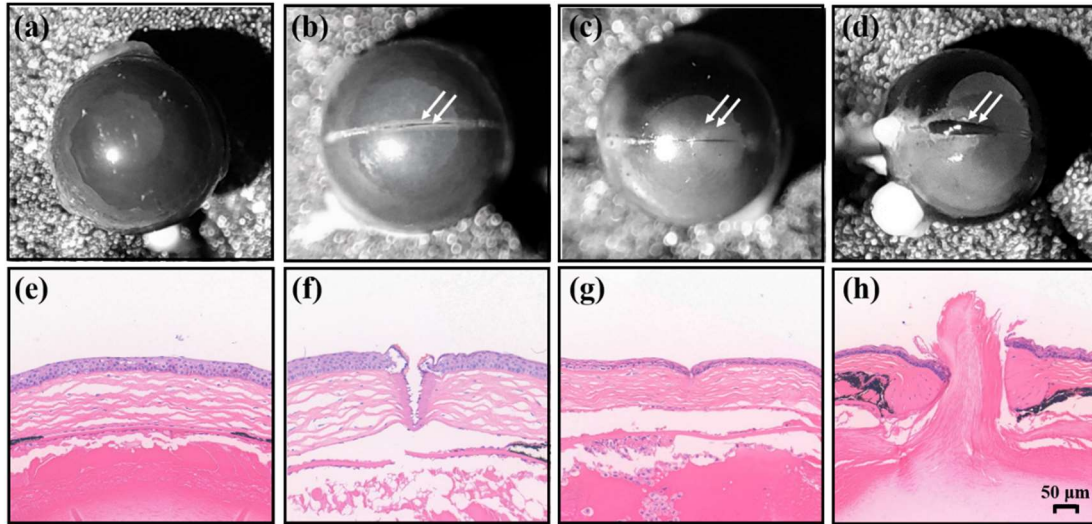


Fig. R3 Comparison of edematous corneal ablation by employing MIR fs laser and the conventional NIR fs surgical laser. Photos (a-d) and corresponding histological images (e-h) of corneal incisions under different laser wavelength and pulse energy. (a, e) Edematous cornea. (b, f) When corneal ablation is performed at a wavelength of 6.1 μm with a pulse energy of 2 μJ , a sharp penetrating incision can still be formed. (c, g) As the laser wavelength is switched to 1.03 μm , with 3 μJ pulse energy, only a shallow scratch with epithelial ablation is observed. (d, h) Further increasing the pulse energy of the 1.03 μm femtosecond laser to 16 μJ deepens the incision; however, severe corneal collateral damage and deformation of lens are caused. The incisions are marked by white arrows.

Please see changes in Line 37-38, 166-170, 287, 311-323, 325-328, 334-338, 562-563 and Figs.2 (j-q) in the revised manuscript.

2 April, 2025

Dear Editor,

Thank you for your help in handling our Manuscript, entitled “In vivo keratotomy by mid-infrared femtosecond laser resonant with amide vibrational mode”. We have carefully studied through all the comments and revised the manuscript. Below we provide a point-by-point response to the reviewers’ comments and the corresponding list of revisions we have made. We believe that with these changes our manuscript has now been improved, and we hope it can now meet the standards and criterion for publication in Communications Medicine.

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Response: We are sorry for the unclear description regarding corneal surgery. We have carefully reviewed the statement and made an explicit distinction between keratectomy and keratotomy in the manuscript.

Please see changes in Line 60-62 and 64-68 in the revised manuscript.

2. The MIR femtosecond laser offers significant advantages in corneal transplantation and refractive surgery, particularly for edematous or cloudy corneas. We are eager to know whether the laser can achieve precise intrastromal keratotomy rather than a full-thickness incision, which is crucial for lamellar keratoplasty. (Lines 562-565)

Response: We thank for the question from the reviewer. The MIR femtosecond laser at wavelength of 6.1 μm resonates with amide and hydroxyl, causing strongly absorption by the corneal tissue while reducing optical transmission and scattering. Therefore, the MIR laser keratotomy is a process of linear energy transfer starting from surface towards interior of the cornea, as only an extremely small amount of energy can be directly transmitted to the deeper layers of the tissue. This mechanism offers the advantage of MIR laser in performing deep keratotomy for highly scattering tissue such

as edematous corneas. Regarding the precise control of incision depth, our simulation and experiment have demonstrated that MIR femtosecond laser can achieve micrometer-level precision incision with minimal collateral damage by adjusting the energy and exposure time.