Comparative Study of Wound Healing in Rat Skin Following Incision With a Novel Picosecond Infrared Laser (PIRL) and Different Surgical Modalities

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Background and Objective: As a result of wound healing the original tissue is replaced by dysfunctional scar tissue. Reduced tissue damage during surgical procedures beneficially affects the size of the resulting scar and overall healing time. Thus the choice of a particular surgical instrument can have a significant influence on the postoperative wound healing. To overcome these problems of wound healing we applied a novel picosecond infrared laser (PIRL) system to surgical incisions. Previous studies indicated that negligible thermal, acoustic, or ionization stress effects to the surrounding tissue results in a superior wound healing.

Study Design/Materials and Methods: Using the PIRL system as a surgical scalpel, we performed a prospective wound healing study on rat skin and assessed its final impact on scar formation compared to the electrosurgical device and cold steel. As for the incisions, 6 full-thickness, 1-cm long-linear skin wounds were created on the dorsum of four rats using the PIRL, an electrosurgical device, and a conventional surgical scalpel, respectively. Rats were euthanized after 21 days of wound healing. The thickness of the subepithelial fibrosis, the depth and the transverse section of the total scar area of each wound were analyzed histologically.

Results: After 21 days of wound healing the incisions made by PIRL showed minor scar tissue formation as compared to the electrosurgical device and the scalpel. Highly significant differences ($P < 0.001$) were noted by comparing the electrosurgical device with PIRL and scalpel. The transverse section of the scar area also showed significant differences ($P = 0.043$) when comparing PIRL (mean: 141.46 mm^2; 95%CI: 105.8–189.0 mm^2) with scalpel incisions (mean: 206.82 mm^2; 95%CI: 154.8–276.32 mm^2). The subepithelial width of the scars that resulted from using the scalpel were 1.3 times larger than those obtained by using the PIRL (95%CI: 1.0–1.6) though the difference was not significant ($P < 0.083$).

Conclusions: The hypothesis that PIRL results in minimal scar formation with improved cosmetic outcomes was positively verified. In particular the resection of skin tumors or pathological scars, such as hypertrophic scars or keloids, are promising future fields of PIRL application. Lasers Surg. Med. 48:385–391, 2016.

Conflict of Interest Disclosures: All authors have completed and submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest and have disclosed the following: R.J. Dwayne Miller is cofounder of AttoDyne Inc (manufacturer of the PIRL system) and is the author of a patent related to the mechanism of PIRL laser ablation. All other authors declare no conflicts of interest. There has been no financial relationship with any sponsoring organization.

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INTRODUCTION

The end result of the wound healing process in adults is the replacement of the original tissue by dysfunctional scar tissue which consists mostly of collagen. Wound healing can be differentiated into three distinct stages: inflammation, proliferation, and remodeling [1].

In contrast to adult wound healing, tissue damage occurring during the maturation process of an embryo results without any remaining scars [2,3]. This difference in wound healing has been attributed to the degree of inflammation which takes place during the first stage of wound healing. There is an incomplete or even missing immune response to tissue damage in embryonic wound healing, which some studies have associated with faster and scarless wound healing [4–7]. These results have led to the hypothesis that reduced tissue damage, which results in decreased inflammatory reactions, has a beneficial effect on the size of the resulting scars and on the time of healing. The choice of the surgical instrument and the degree of tissue damage it inflicts can therefore have a significant influence on postoperative wound healing.

Today, the scalpel is classified as the gold standard for performing skin incisions or excisions in surgery. The use of the electrosurgical device as an alternative to the scalpel is also wide-spread but has the disadvantage of significantly damaging the surrounding tissue [8–10].

Recently, the research group of R.J. Dwayne Miller demonstrated a method to convert matter of a liquid or solid aggregate phase directly into a gaseous aggregate phase by using an innovative Mid-IR picosecond laser [11]. The complete confinement of the deposited energy inside the ablation volume is ensured by a physical process denoted as desorption by impulsive vibrational excitation (DIVE). No significant amount of energy is transferred to the surrounding tissue, neither in thermal form nor by acoustic shockwaves, or by ionizing radiation. During DIVE ablation, an ultrafast laser pulse is used to selectively excite the strongly absorbing vibrational modes of water molecules located in the tissue on a time scale faster than their thermalization time. Using the PIRL-laser system that emits 400 ps pulses at 1 kHz repetition rate and 3 μm wavelength, the water molecules inside the irradiated tissue are converted into the gas phase on a picosecond time scale. The entire ablation process takes place before nucleation and cavitation effects within the ablation zone can occur. In addition, the effective frequency of any thermally excited acoustic modes is in the GHz range, which are attenuated within micron propagation distances such that all the laser energy is confined to the region of interest with the minimum energy required to drive the ensuing ablation and material removal [12–14].

Initial applications of the novel PIRL-scalpel have shown a reduction of collateral damage zones to a minimum in both soft and densely calcified tissue.

In this study, following the cutting of tissue with the PIRL-scalpel, common scalpel, and electrosurgical device, the healing process is compared on a macroscopic and histologic scale. We investigate beyond the proliferation phase and report the wound healing process in the remodeling phase in a rat model for each incision method.

MATERIALS AND METHODS

Animals

The experiments were supervised by the institutional animal welfare officer, and approved by the local licensing authority (Amt für Gesundheit und Verbraucherschutz; Hamburg, Germany) under the project No. 16/12.

Four female Wister rats weighing between 400 and 600 g were used. The animals were anesthetized prior to surgical procedures with ketamin (80–100 mg/kg KG) and xylazine (5 mg/kg KG) for general anesthesia. Buprenorphine per os (0.03 mg/ kg KG) were given 1 hour prior to the surgery and from then every 8–12 hours for analgesia for 72 hours postoperatively. In addition to the Buprenorphine, Metamizol (100 mg/kg KG every 6 hours) was given via the drinking water for 1 week. In order to prevent postoperative wound infections, Enrofloxacin 10% (100 mg/l drinking water) was administered via the drinking water for 1 week.

After surgical recovery the animals were held separately to prevent manipulation of the wounds by each other for 10 days. In order to monitor pain and wound infection, the animals were inspected every 12 hours for the first 72 hours following incision creation and every other day thereafter. All animals remained in good health over the entire time of the experiment and no excessive inflammation or severe infections were noted at the site of incisions or elsewhere.

Surgical Procedure

Following appropriate anesthesia, the dorsum (operative field) and the belly (placement of the neutral electrode of the electrosurgical device) of each rat were shaved with an electric shaver. After marking the cutting line on every rat, two 1 cm long-incisions were made for each cutting method (6×1 cm paravertebral incisions, three incisions per side).

The incisions were made paravertebral along the longitudinal axis and perpendicular to the skin cleavages lines of rats [15]. In addition, the incisions were cut at least 1 cm apart. For each incision, full thickness dorsum skin cuts were made in sterile conditions with the PIRL (Attodyne Lasers Inc., Toronto, Canada), a conventional electrosurgical device (KLSmartin ME 411, KLS Martin Group, Tuttlingen, Germany) and a 15 scalpel (B. Braun Aesculap AG, Tuttlingen, Germany). The incisions were made through the cutis and subcutis. Thereby, slight tension was applied to the skin, which allowed for depth control under direct vision. In all incisions the back muscle was defined as the landmark to stop the incision. The order of the surgical instrument was not rotated between the animals.

The PIRL was operated in a 10-mm linear scan mode with a scan speed set to 200 mm/second and an average laser pulse energy of 420 μJ at 1 kHz repetition rate. The laser beam was kept in focus on the tissue by a fast-response autofocusing optic, which ensured a constant beam waist of 190 μm on the tissue during all cuts.

The resulting incision wounds were all closed immediately and in the same manner with two stitches of non-absorbable 5.0 Ethilon sutures (Johnson & Johnson Medical GmbH, Ethicon Germany, Norderstedt). All Animals were euthanized at day 21 after wounding. Immediately following euthanasia, the dorsum was shaved again and all wounds were photo-documented and harvested. The harvested wounds were clamped on cork and fixed in phosphate buffered containing 3.5% formaldehyde.

Histology

Each formalin fixed scar was divided in two halves by cutting it perpendicular to the incision line in the middle of the scar.

To minimize the influence of the sutures to the wound healing process, the staining was performed on 4 μm formalin fixed paraffin embedded sections, which were extracted from the middle of the wound. The tissue was stained with hematoxylin and eosin (H.E.) as well as with Masson-Goldner trichrome (Merck, Darmstadt, Germany) [16]. The stained samples were then scanned using the MIRAX SCAN (Carl Zeiss Microimaging GmbH, Jena, Germany). Microscopic measurement and histological examination were carried out using Pannoramic Viewer software (3DHISTECH Kft., Budapest, Hungary). In all Masson-Goldner trichrome stained sections, the width of the subepithelial fibrosis and the depths of the wound were measured. In addition, a line was charted all around the fibrosis-zone and the area was calculated.

The measurements were performed by three different examiners, who were experienced in histological evaluations. The observers were blinded, whereas all of them were involved in the study and could have possibly recognized some stained samples.

Statistical Analysis

All outcome measures (area/width/depth) were analyzed separately, while the modeling was analogous. A linear mixed model was used with respect to the cluster structure resulting from the setting of the study. To fulfill the assumptions of the model, log-transformed data of all outcome variables were analyzed.

The three devices were modeled as fixed effects in order to make a comparison, while the observer, the rats and the single cuts were included as random effects to control their potential variability. The following structure resulted from the experimental setting: all observers evaluated all cuts within each rat, therefore the cuts were modeled nested in the rats and the rats are crossed through the observers. Using the Likelihood-ratio test, the significance of the fixed effect was tested and individual contrasts for pairwise comparisons were performed, the resulting effects with 95% confidence intervals and *P*-values were reported.

In order to visualize the variability between the observers, a model with a fixed effect for the observer (hence, no more as random effect) as an interactor for the devices was performed. The model based marginal means with the corresponding 95% confidence intervals for the fixed effects were represented.

These analyses were conducted with StataCorp. 2015. Stata Statistical Software: Release 14. College Station, TX: StataCorp LP.

RESULTS

In total, 24 incisions using three different cutting instruments were performed on four female Wister rats. The macroscopic evaluation of the PIRL cutting edges following wounding showed a very precise shape, with no signs of carbonization (Fig. 1). The wound margins of the scalpel incision also appeared clean. Carbonization, a sign of heat generation, was observed at the cutting surface of the electrosurgery wound.

After 3 weeks of wound healing all wounds appeared non-irritated/-inflammed and completely epithelial attached without any signs of infection. The scars caused by using the electrosurgical device appeared wider, more reddened, and clearly inducted under the skin level compared to the scars caused by the scalpel and the PIRL. The scars caused by using the scalpel or the PIRL were obviously narrower and only slightly under the skin level. Some of the scars induced by the PIRL were difficult to identify and define macroscopically (Fig. 2).

By using hematoxylin and eosin staining, the histological examination of the scars showed a fibrosis zone in all 18 slices (Fig. 3). All instruments made a complete cut through the skin. There was no significant difference in the depth of the scars (comparison of devices: 0.348) between the instruments; the estimated depth for the electrosurgical device was 1.29 μm (95%CI: 1.14 μm; 1.45 μm); for PIRL 1.44 μm (95%CI: 1.28 μm; 1.63 μm) and for the scalpel 1.34 μm (95%CI: 1.19 μm; 1.51).

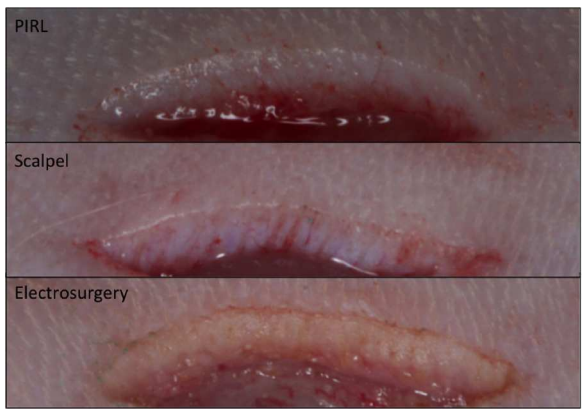

Fig. 1. Macroscopic view of three 1 cm full thickness skin incisions after the use of the PIRL, scalpel, and electrosurgical device.

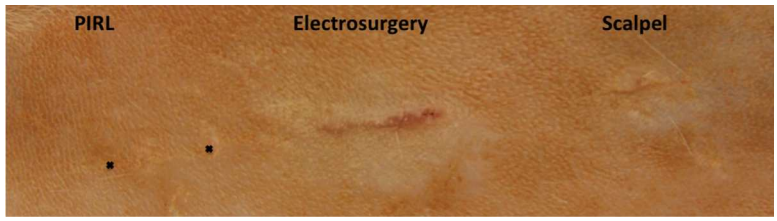

Fig. 2. Macroscopic view of the dorsum of a rat with three differentially induced scars. Incisions of 1 cm were made 3 weeks before by the scalpel, the electrosurgical device and the PIRL (between the crosses). Less visible scarring at the PIRL-incision in comparison to the scalpel and the electrosurgical device.

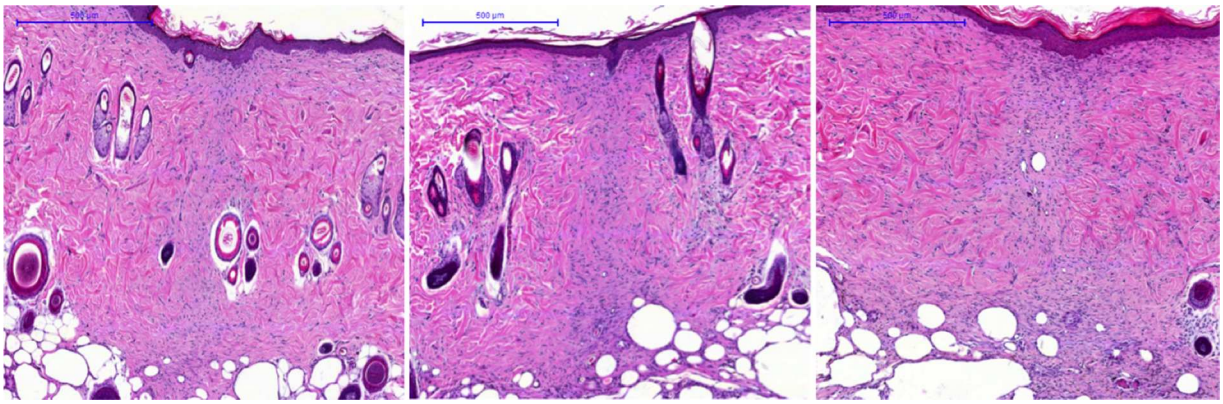

Fig. 3. Hematoxylin and Eosin staining after full thickness skin incisions performed by PIRL (**a**), scalpel (**b**), and electrosurgical device (**c**) after 3 weeks wound healing (pictured scale: 500 μm; magnification: 5×).

In all cuts the fibrosis zone can be described as being rich in cells with some isolated vessels and funnel shaped with a maximum width in the sub-epithelial tissue. The connective tissue fibers were densely packed. The fibers were not scattered and undulated in structure but formed parallel bundles. By using Masson-Goldner staining the typical unorganized tissue structure of the scars was easily observed (Fig. 4). All wounds healed both macroscopically and microscopically and re-epithelialized completely.

The estimated mean from all investigators of the sub-epithelial scar width for the PIRL was 0.32 mm (95%CI: 0.25; 0.42 mm), for the scalpel 0.41 mm (95%CI: 0.31; 0.52 mm) and for the electrosurgical device 0.8 mm (95%CI: 0.62; 0.1 mm) (Fig. 5). Compared to the PIRL and the scalpel, the electrosurgical device showed a significantly larger scar width ($P < 0.001$ for both). The scar width was 2.5 times larger as compared to the PIRL (95%CI: 1.9; 3.2). The scars caused by using the scalpel were 1.3 times larger compared to the PIRL

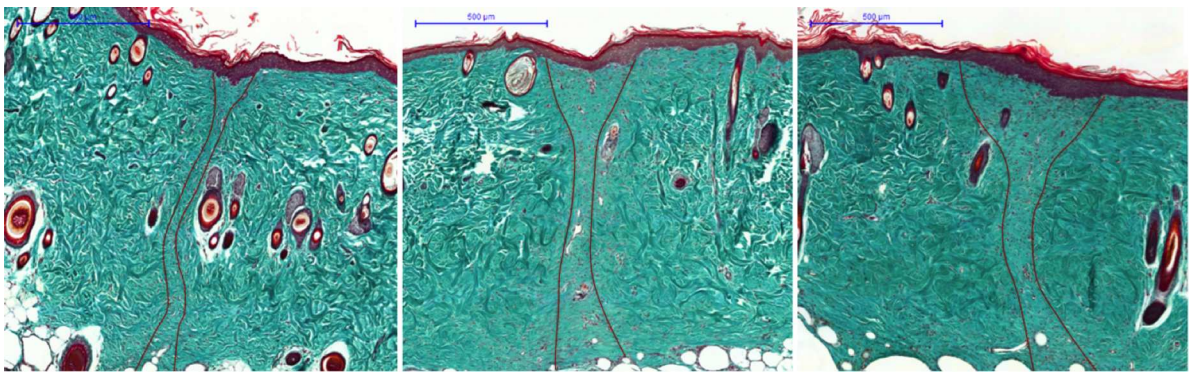

Fig. 4. Masson-Goldner staining after full thickness skin incisions performed by PIRL (**a**), scalpel (**b**), and electrosurgical device (**c**) after 3 weeks wound healing (marking: scar area; pictured scale: 500 μm; magnification: 5×).

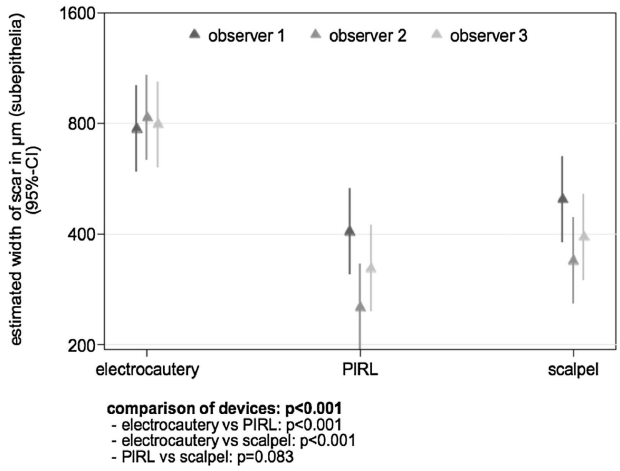

Fig. 5. Mean and 95% confidence interval of the subepithelial width of scarred tissue caused by full thickness skin incisions (three different methods) and measured on Masson Goldner staining after 3 weeks of wound healing (three different observers).

(95%CI: 1.0; 1.6) though the difference was not significant ($P < 0.083$). Taking account of all investigators, the estimated mean of the transverse section of the total scar area for the PIRL was 141.4 mm^2 (95%CI: 105.8; 189.0 mm^2), for the scalpel 206.82 mm^2 (95%CI: 154.8; 276.32 mm^2), and for the electrosurgical device 516.35 mm^2 (95%CI: 386.47; 690.37 mm^2) (Fig. 6). Comparing both the electrosurgical device as well as to the scalpel to the PIRL showed high significant differences ($P < 0.001$). The scar area of the electrosurgical device compared to the PIRL was 3.7 times larger (95%CI: 2.5; 5.3). There was also a significant difference between the PIRL and the scalpel ($P = 0.043$). Here the scar area caused by the scalpel was 1.5 times larger than the area of the PIRL (95%CI: 1.0; 2.1).

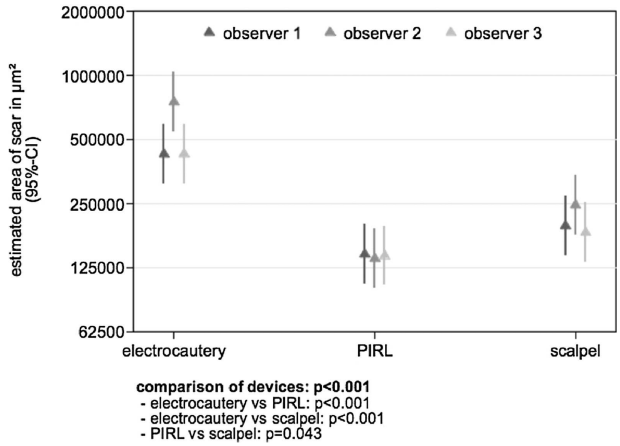

Fig. 6. Mean and 95% confidence interval of the area of scarred tissue caused by full thickness skin incisions (three different methods) and measured on Masson Goldner staining after 3 weeks of wound healing (three different observers).

DISCUSSION

In this study, wound healing of the skin was quantified for comparison after cutting the tissue with PIRL, scalpel, and electrosurgical device by using a rat model.

Ex vivo soft tissue incisions made with PIRL have demonstrated nearly complete absence of thermal injury and much narrower cutting gaps when compared to CO_2 laser and scalpel [17,18]. Infrared thermography has shown minimal *ex vivo* skin, mucosa, bone, and cartilage temperature rise during ablation using PIRL in contrast to Er:YAG-Laser [19–21]. Further, the detection of protein activity and intact higher order protein structure in the ablation-aerosol emphasizes the soft character of the DIVE-ablation process [22,23].

A preliminary wound healing study showed that after 2 weeks the wounds created by the PIRL showed a significantly narrower scar width in the skin as compared to those created using the Er.YAG-Laser or the scalpel [24]. This work made a quantitative comparison of the expression of signaling factors involved in healing and showed that PIRL reduced the expression level of these factors. However, the study was limited due to the lack of a histopathological characterization of the wound healing beyond the proliferation phase.

In this study, the macroscopic and histological process is analyzed within the remodeling phase and comparisons are made to using the scalpel and for the first time the commonly used electrosurgical device. By comparing all three surgical instruments after 21 days of wound healing, the incisions made by the PIRL showed minor scar formation compared to the electrosurgical device and indicates an advantage of the PIRL compared to the scalpel. Highly significant differences were found by comparing the electrosurgical device with the PIRL and the scalpel.

In contrast to the transverse section of the scar area, which showed significant differences comparing the PIRL with the scalpel, no significant differences were found by comparing the width of the scars. Although the PIRL-caused scar width close to the epithelial layer was 30% less.

The Masson Goldner stain specifically labels collagen fibers and thus simplifies the measurement of the fibrosis zone. Located between the close-packed and scarred tissue and the undulated scattered tissue there is a transition zone, which can be interpreted differently by different observers and could in principle limit the assessment of the scar area. However, all investigators of the study had congruent outcomes in measuring the median of the scar extent. The smallest values were denoted from the PIRL and the largest by the electrosurgical device incised samples.

While PIRL has the ability to make precise, non-traumatic cuts, several studies have observed mechanical stress imparted to tissue following scalpel incisions. Consecutively the wound margins and the collagen fibers appeared fringed with a lateral damage zone of

100–400 μm. However, for the PIRL a lateral damage zone of only 9–29 μm is described [18,24]. After making incisions in pig skin by using an electrosurgical device, a much larger zone of thermal coagulation necrosis (450–650 μm) was detected and described. Furthermore, electrosurgical incisions revealed a decreasing wound strength, increasing inflammation, and pronounced scar formation [8,10].

The hypothesis that minor tissue damage has a beneficial effect on wound healing is fully consistent with the present results. However, the direct damage of the surrounding tissue and the inflammatory phase as well as the long-term development were not investigated in this study. The remodeling of the scar will last for several months and only long-term examinations can make reliable assertions about the aesthetic value [25]. Therefore, an estimation of the scar only 21 days after skin damage is limited in its significance. However, it has to be considered that the wound healing process in animals such as rats is accelerated and consequently days ahead of human wounds at the same point of time [26].

Rats have been widely used as a research model for skin wound healing and a broad knowledge base on rat wound healing exists [27]. Although rat skin wound healing does not entirely imitate human skin wound healing, because of the different skin morphologies. Hence, the transferability is limited.

The electrosurgical device performs hemostasis by coagulation. Without a thermal effect to the surrounding tissue, the PIRL-scalpel does not perform hemostasis.

However, in this study, we did not observe any severe bleedings while cutting the skin with the PIRL. Smaller bleedings did not influence the cutting process significantly.

To minimize tissue bleedings, vasoconstricting substances can be injected prior to the incision as it is frequently utilized in surgical procedures.

Although the DIVE cutting process enables tissue dissection with absolutely no collateral damage, this high standard has not yet been completely achieved. The deviance can be explained by an imperfect match of beam parameters and the beam quality of the laser system to the DIVE parameters. For optimal DIVE ablation, the beam has to have the precise wavelength centered at the water absorption peak, the pulse duration has to be on the time scale of the thermalization time of the vibrational modes of water, and the fluence of the laser beam has to be above ablation threshold over the entire beam area [14]. These criteria are not perfectly met by the current laser configuration. The measured wavelength is slightly off the absorption peak. Due to the limited beam quality of the laser system ($M^2 \sim 10$) the beam could only be focused on a 190 μm spot size with a significant part of the spot being below tissue ablation threshold within the side wings of the beam. Optimizing the laser parameters to even better meet the DIVE condition will certainly decrease the collateral damage to the tissue and promises an even better wound healing.

The PIRL is an instrument that can be controlled with very high precision. Contrary to other existing systems, investigations on the wound healing provide results that indicate an advantage over cold instruments such as a scalpel.This confirms the hypothesis that PIRL is an innovative surgical tool that allows a precise and tissue-converting surgery with minimal scar formation and distinctive cosmetic outcome. In particular the resection of skin tumors or pathological scars like hypertrophic scars or keloids are promising fields of application.

In order to clinically implement PIRL as a surgical tool, further wound healing studies are necessary. It is essential to evaluate the optimal laser parameters which are associated with minimum scarring.

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