Supplementary Materials: Controlling laser irradiation with tissue temperature feedback enhances photothermal applications: *ex-vivo* evaluation on bovine liver

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Analysis of the Temperature-Time Responses

Generally, the temperature-time response of our samples showed a logarithmic characteristic during the irradiation phase. We observed regions with a more abrupt change of curvature in some of the low power groups (especially 3.4 W & 65 °C and 3.4 W & 75 °C groups, corresponding to longer exposure times) leading to slight deviations from the logarithmic model. We were not able to generalize the characteristics of these changes. The change of temperature-time response curves may be due to the modifications of optical and thermal characteristics the tissues undergo at these temperature values, which are also varying among samples.

In contrast, the optical characteristics of the tissue are not relevant in the temperature decrease phase. Consequently, this phase displays an exponential decay. We expect that the temperature decrease curves mainly depend on the total heat deposited to the tissue, the thermal conductivity and dissipation characteristics of the tissue, and the temperature gradient between the measurement spot, the surrounding tissue, and the ambient environment.

In the clinical setting, we expect that the perfusion of the tissue will have a cooling effect increasing the time constant of the heating phase and decreasing the time constant of the cooling phase. Hence, we expect that the heating phase photothermal tissue characteristic differences would be exacerbated and the cooling phase thermal tissue characteristic differences would be subdued further emphasising the requirement of a tissue temperature controlled irradiation system.

Further Discussion on Fixed-Dose Results

The analysis of the achieved tissue surface temperature values at the end of the laser exposure (T_{final}) is provided in Table 3. The average final temperature standard deviation and the average final temperature range ($T_{final\,(max)} - T_{final\,(min)}$) are 5.6 °C and 19.8 °C, respectively. These values are larger than the desired target temperature windows for all photothermal laser application modalities. Hence, these results suggest that dosimetry based clinical photothermal laser applications are not viable for ensuring patient safety and treatment success.

Thermal Denaturation Experiment

Evaluation of Thermal Denaturation Area & Statistical Analysis

The use of bovine liver tissue enabled us to gauge the thermal effect of laser exposure by evaluating the extent of thermal denaturation diameter and depth. We evaluated the thermal denaturation in our samples by macroscopical inspection of color change and opacification (whitening). We photographed thermal denaturation diameter and depth of the irradiated samples using cross polarization imaging technique to eliminate reflections that can complicate the evaluation process [33]. A camera fitted with a prime macro lens and a circular polarizer (linear polarizer and quarter-wave plate combination) was placed perpendicularly at a fixed distance above the sample. The light source (flash) combined with a linear polarizer was placed at an oblique angle (45°) to further avoid surface reflections. The thermal denaturation depth images were taken after dissecting the sample along the center of the thermal denaturation zone. We used fixed magnification, shutter speed, f-number, ISO, and flash intensity for all photographs; and aligned a scale to the sample surface as a reference. A MATLAB code was composed to facilitate the blinded acquisition of manual measurements by 3 evaluators by marking the perceived thermal denaturation boundary on displayed images on a computer. We averaged the measurements of all 3 evaluators for each sample.

We performed one-way analysis of variance (ANOVA) of thermal denaturation zone measurements for each set of 3 groups with a common parameter (3 laser powers + 3 target temperatures). Scheffe's procedure was used to determine statistical differences between groups in the *post hoc*

multiple comparison test after the one-way ANOVA conducted in MATLAB. Two-way ANOVA was performed to evaluate the interaction between laser power and target temperature on thermal denaturation zone. Significance level was set at $p < .05$ for all statistical tests.

Results of Thermal Denaturation Experiment

The thermal denaturation diameter and depth values of temperature controlled laser irradiation are provided as a box and whiskers plot in Fig. S1. Additionally, Fig. S2 depicts corresponding representative thermal denaturation area photographs.

One-way ANOVA

One-way ANOVA results indicate that during surface temperature controlled laser irradiation target temperature has a statistically significant effect on denaturation diameter ($p < .001$) and depth ($p < .005$) in all three power groups. On the other hand, laser power has a statistically significant effect on denaturation diameter only in 55 °C ($p = .015$) and 65 °C ($p = .007$) target temperature groups. Additionally, laser power has no statistically significant effect on denaturation depth in any target temperature group.

Post Hoc Test Using Scheffe's Procedure

Figure S1 contains the *post hoc* tests conducted using Scheffe's procedure for denaturation diameter ($n = 16$) and depth ($n = 13$). The mean and standard deviation values for thermal denaturation diameter and depth are also incorporated in Table S1.

Target temperature creates statistically significant differences in denaturation diameters between all groups for all laser powers. Yet, we only observe significant differences in the denaturation diameters between lowest and highest laser power groups (3.4 W and 10.2 W) for target temperatures of 55 °C and 65 °C. There is no statistically significant difference between laser power groups for 75 °C target temperature in the surface temperature controlled application.

There are statistically significant differences in denaturation depths between 55 °C and 75 °C target temperature groups for all laser powers. Additionally, there is a statistically significant difference between 65 °C and 75 °C target temperature groups for 6.8 W. There is no statistically significant difference in denaturation depths between any laser power groups for any target temperature group.

Two-way ANOVA

Two-way ANOVA results reveal that there is no interaction between target temperature and laser power using our surface temperature controlled laser irradiation system with respect to thermal denaturation diameter ($p = .996$) and depth ($p = .897$).

Discussion of Thermal Denaturation Zone

As it can be deduced from Figs. S1 and S2, and Table S1, the thermal denaturation size clearly increases with rising target temperature values for all power groups. This is expected for two reasons. First, higher target temperatures require longer exposure durations allowing more thermal energy dissipation to surrounding tissues. Second, a high temperature at the measurement spot also corresponds to a high spatio-thermal gradient resulting in faster thermal energy dissipation and expansion of the thermal damage. There is also a trend for an increase in thermal denaturation size with decreasing laser power for all target temperature groups. A low laser power results in an extended exposure duration to reach target temperature. Thus, it provides more time for heat dissipation to surrounding tissues, increasing the thermal denaturation size.

One-way ANOVA results indicate that target (surface) temperature has a statistically significant effect on denaturation diameter and depth. But, there is no statistically significant effect of laser power on denaturation diameter in 75 °C target temperature groups and on denaturation depth in any of the target temperature groups. Evaluation of the *post hoc* (multiple comparison) tests using Scheffe's procedure reveals further insight into the capabilities of our system. The most crucial interpretation is that our surface temperature controlled medical laser system can successfully regulate thermal denaturation diameter (by creating significant differences between groups) using

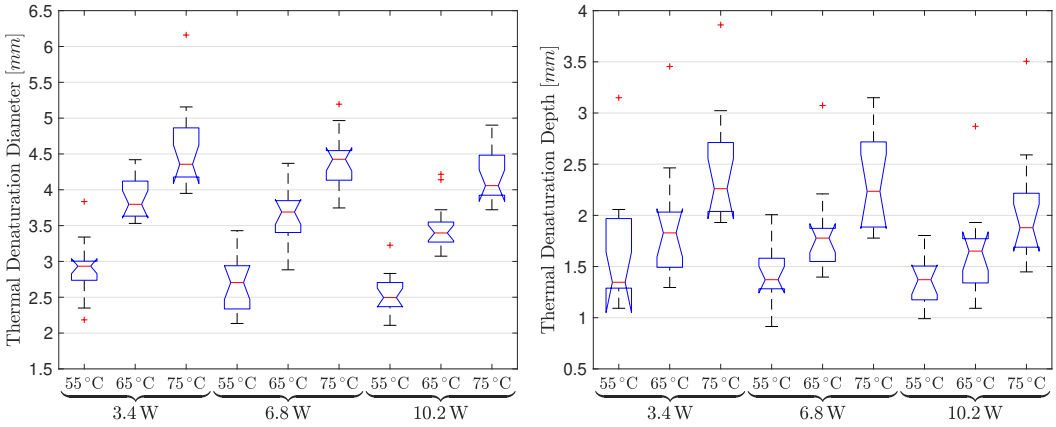

Figure S1. Box and whiskers plot of thermal denaturation diameters ($n = 16$) and depths ($n = 13$). Red horizontal lines are group medians. Blue boxes mark interquartile ranges. Red '+' signs represent outliers. Whiskers indicate most extreme data that is not an outlier.

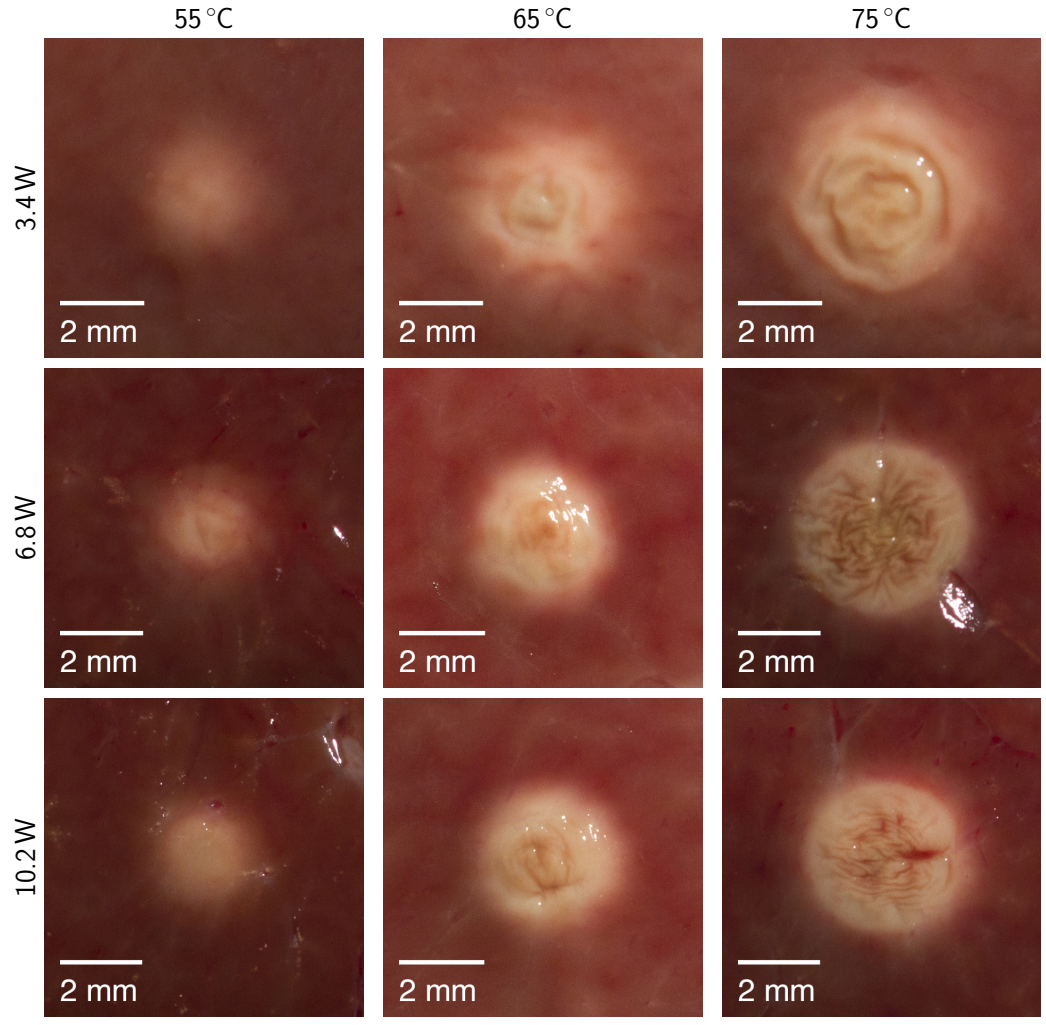

Figure S2. Exemplary bovine liver photothermal denaturation photographs.

Table S1. Results of post hoc tests using Scheffe's procedure after one-way ANOVA of thermal denaturation diameter ($n = 16$) and depth ($n = 13$) values. Each row represents multiple comparisons conducted for the common parameter provided in the first column. Solid lines connecting two groups represent a statistically significant difference ($p < .05$).

	Denaturation Diameter ($\mu \pm \sigma$) [mm]			Denaturation Depth ($\mu \pm \sigma$) [mm]		
	55 °C	65 °C	75 °C	55 °C	65 °C	75 °C
3.4 W	2.90 ± 0.38	3.85 ± 0.28	4.52 ± 0.56	1.65 ± 0.56	1.93 ± 0.59	2.47 ± 0.55
	$p < .001$	$p < .001$	$p < .001$	$p = .474$	$p = .063$	$p = .003$
6.8 W	2.68 ± 0.39	3.64 ± 0.38	4.37 ± 0.38	1.44 ± 0.28	1.85 ± 0.44	2.35 ± 0.48
	$p < .001$	$p < .001$	$p < .001$	$p = .052$	$p = .013$	$p < .001$
10.2 W	2.53 ± 0.27	3.47 ± 0.32	4.18 ± 0.38	1.37 ± 0.23	1.68 ± 0.44	2.04 ± 0.55
	$p < .001$	$p < .001$	$p < .001$	$p = .198$	$p = .107$	$p = .001$

	3.4 W	6.8 W	10.2 W	3.4 W	6.8 W	10.2 W
55 °C	2.90 ± 0.38	2.68 ± 0.39	2.53 ± 0.27	1.65 ± 0.56	1.44 ± 0.28	1.37 ± 0.23
	$p = .228$	$p = .454$	$p = .016$	$p = .383$	$p = .897$	$p = .188$
65 °C	3.85 ± 0.28	3.64 ± 0.38	3.47 ± 0.32	1.93 ± 0.59	1.85 ± 0.44	1.68 ± 0.44
	$p = .216$	$p = .317$	$p = .007$	$p = .918$	$p = .688$	$p = .446$
75 °C	4.52 ± 0.56	4.37 ± 0.38	4.18 ± 0.38	2.47 ± 0.55	2.35 ± 0.48	2.04 ± 0.55
	$p = .667$	$p = .473$	$p = .113$	$p = .846$	$p = .341$	$p = .132$

target temperature as a parameter. Additionally, it is capable of decreasing the effects of laser power on denaturation diameter and depth. Target temperature was less effective in regulating the denaturation depth. The reason for this phenomenon may be the fact that our system measures and controls tissue surface temperature.

Considering the two-way ANOVA results, we can irrefutably state that our temperature controlled laser irradiation system successfully eliminates the interaction between laser power and target temperature values (controlled variables) with respect to thermal denaturation diameter and depth. Conventional (fixed-dose) laser applications exhibit a strong interaction between these controlled variables.

The rate of temperature increase has a major role in controlling the resulting thermal effect successfully. Higher values of $^{dT}/_{dt}$ are associated with narrower gradients of thermal damage and provide more control over the denaturation area when using temperature feedback control [2,24]. The representative thermal denaturation area photographs depicted in Fig. S2 illustrate this phenomenon clearly. Higher laser power groups (corresponding to high $^{dT}/_{dt}$) exhibit a more defined thermal denaturation border than the lower laser power groups. A high $^{dT}/_{dt}$ also means reaching the target temperature faster, which enables us to minimize the exponential phase of the thermal damage progression. Conversely, a low $^{dT}/_{dt}$ allows the heat to diffuse into surrounding tissues for a longer duration (exponential phase), resulting in a less controlled thermal buildup due to the preponderate changes in optical and thermal characteristics. However, an extremely high $^{dT}/_{dt}$ value may prove hard to regulate thermally using a control system with limited response time. A momentary overshoot of tissue temperature may cause excessive thermal damage, especially when the target temperature value is high.

During surface temperature controlled application, tissues with different temperature increase curves receive different amounts of energy until the target temperature is reached. A difference in the delivered dose can lead to changes in the extent and size of thermal denaturation. The modulation of laser power to regulate the temperature-time response of tissue may be beneficial in controlling the thermal damage and the resulting denaturation zone. Dosimetry studies may reinforce efficiency and effectiveness of temperature- and temperature-change-rate-controlled medical laser systems, by providing feasible $^{dT}/_{dt}$ ranges and ideal initial laser power values specific to different tissues.

Study Limitations of Thermal Denaturation Experiment

Ideally, thermal damage is evaluated histologically with the help of staining techniques. Hematoxylin and eosin (H&E) or trichrome stains are commonly used to visualize thermally denatured collagen in tissues, which is a surrogate marker for thermal damage [34]. Collagen can also be utilized in polarization microscopy by observing loss of its birefringence due to thermal denaturation [35,36]. A recent trend for the evaluation of thermally treated tissues is viability staining methods. Tetrazolium based stains [*e.g.*, triphenyltetrazolium chloride (TTC) and nitroblue tetrazolium chloride (NBT)] interact with active cellular enzymes and mitochondrial cofactors to demarcate viable tissues [34,36]. Denaturation of these enzymes and disruption of mitochondrial processes due to thermal damage (non-viable tissues) will result in unstained (native colored) tissues.

Unfortunately, none of the above mentioned techniques were suitable for the evaluation of our samples. Liver tissues contain very little amounts of collagen ruling out H&E and trichrome staining as well as polarization microscopy techniques. Abattoir regulations in our country prevent acquisition of tissues at early stages of the process and render the tissues incompatible with viability staining methods (due to immediate cooling and prolonged acquisition time) as per the procedure described by Beatty *et al.* (2015). Therefore, we evaluated the thermal denaturation in our samples by macroscopical inspection of color change and opacification (whitening).