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FULL ARTICLE

Qualitative tissue differentiation by analysing the intensity ratios of atomic emission lines using laser induced breakdown spectroscopy (LIBS): prospects for a feedback mechanism for surgical laser systems

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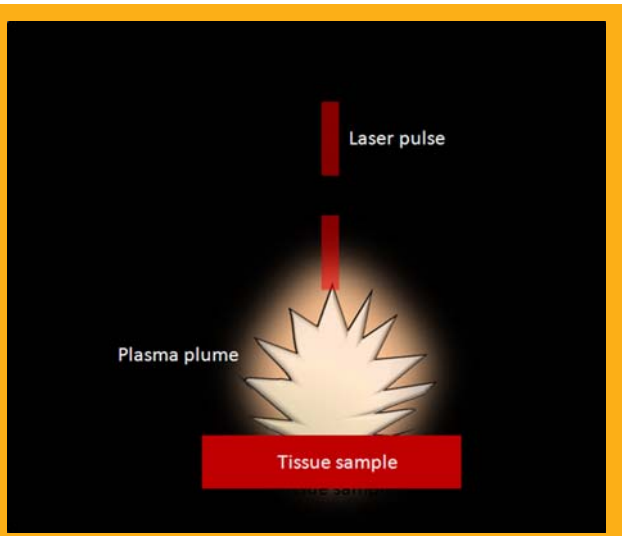
The research work presented in this paper focuses on qualitative tissue differentiation by monitoring the intensity ratios of atomic emissions using 'Laser Induced Breakdown Spectroscopy' (LIBS) on the plasma plume created during laser tissue ablation. The background of this study is to establish a real time feedback control mechanism for clinical laser surgery systems during the laser ablation process. *Ex-vivo* domestic pig tissue samples (muscle, fat, nerve and skin) were used in this experiment. Atomic emission intensity ratios were analyzed to find a characteristic spectral line for each tissue. The results showed characteristic elemental emission intensity ratios for the respective tissues. The spectral lines and intensity ratios of these specific elements varied among the different tissue types. The main goal of this study is to qualitatively and precisely identify different tissue types for tissue specific laser surgery.



Plasma plume formation during laser tissue interaction.

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1. Introduction

Although laser surgery is a well-established procedure with various advantages, the technique provides no information about the type of tissue being ablated at the bottom of the cut. As a result, critical structures such as blood vessels, connective tissues and nerve tissues are prone to iatrogenic damage due to their unintentional exposure to high power laser light [1–3]. The lack of information concerning the kind of tissue which is ablated by the surgical laser system limits its application as a general surgical tool. Therefore, to expand the range of possible applications for laser surgery, there is a need for a feedback control system that can remotely provide accurate real-time information on the currently processed tissue. Especially in the head and neck area, the importance of preserving crucial anatomical structures becomes apparent due to their proximity. When establishing an optical feedback mechanism for laser surgery, the differentiation between nerve and fat is of high interest. The peripheral nerves are surrounded by a myelin sheath which inherits high amounts of fat tissue. In addition, most nerves are surrounded by loose connective tissue which again comes with high amounts of fat tissue. Therefore, to preserve nerve tissue during laser surgery, it is important to differentiate between fat and nerve tissue. Damage to nerve tissue in the head and neck region may yield major functional and aesthetic impact on the patient [4, 5]. A real time identification of the currently ablated tissue would allow to establish a feedback mechanism for a tissue-specific laser ablation process [6].

Different approaches on tissue differentiation have been described in literature [1–3, 6–12]. However, all of these techniques share some limitations concerning real-time accurate, specific and remote tissue differentiation during laser ablation that limit their use for a laser surgery feedback control system [6]. Therefore, the aim of this study was to present a first step towards a real time laser surgery feedback system by differentiating and identifying fat, muscle, nerve and skin tissues using Laser Induced Breakdown Spectroscopy.

In recent years, 'Laser Induced Breakdown Spectroscopy' (LIBS) as an analytical technique has drawn some attention for various elemental analysis applications [13]. The technique can be used to identify elements and determine their concentrations in solids, liquids and gases [14]. High spatial resolution and absolute quantification, down to concentration levels of a few parts-per-million, can be achieved with this technique [15]. Furthermore, LIBS can be used in the real time analysis of multiple elements under open air conditions while requiring very small samples [16]. Due to its advantages, such as real-time applicability, minimal-invasiveness and high chemical specificity [17, 18], it provides potential regarding biomedical applications including clinical tissue diagnostics and detection of pathogenic microorganisms. It can be used for the analysis of different tissue compositions including dental hard-tissue, blood and other body fluids [19, 20]. The technique can also be used in the detection of microorganisms [21] such as bacteria, mould and yeast [19].

The LIBS technique uses high power laser pulses that are delivered to the surface of the target samples. The high irradiance of the laser light generates plasma from the surface of the samples. Different radiative processes are observed during the life-time of the plasma, one of which is atomic emission, resulting from the relaxation of excited electrons in the atoms presented in the plasma [22–24]. Each atomic element has characteristic emission lines corresponding to the energy difference of its transitions. Therefore, emissions of different wavelengths from different elements in the plasma are observed in the process. Since the atoms found in the plasma are mostly the atoms from the processed samples, monitoring the emissions from these atoms is used to qualitatively and quantitatively identify the different target samples [25]. The emissions at different wavelengths provide qualitative information whereas the intensity level of the emissions provides quantitative information [22, 26].

The chemical composition in terms of elemental concentration of the target tissues is specific for each type of tissue [27]. This composition is stable in each particular tissue type. The main composing elements of fat, muscle, nerve and skin are Carbon (C), Hydrogen (H), Oxygen (O), Nitrogen (N), Sodium (Na), Chlorine (Cl) and Sulphur (S) [27]. Potassium (K) is also present in muscle, nerve and skin tissues [27]. Here, only few elements account for the majority of the mass of biological tissues. O, C, H and N are the major constituent elements [27]. Mainly the concentrations of the different elements vary from tissue to tissue. Therefore, intensity levels of LIBS spectra have been used to provide information about the concentration of the elements present in the biological sample [15]. These differences in concentration can be used to differentiate between tissue samples of similar elemental composition that do not have unique identifying elements. Based on this information, it can be predicted that the LIBS signals from all the tissues investigated in this study will have common emission lines that may differ in intensity levels due to the difference in their quantitative elemental composition. In a previous study on tissue differentiation using LIBS, the authors have demonstrated a successful differentiation of the target tissues with high sensitivity and specificity using a statistical approach [6]. The spectra recorded during the experiments showed that the intensity levels of the atomic emissions differ according to the tissue

type, even though the emission lines were similar for all the tissues.

In this study, the result of the intensity ratio analysis among the atomic emissions is used to achieve a differentiation of the tissues. Unique intensity ratio values among the emission lines are evaluated to characterize and identify each tissue type. It is the goal of this study to use the intensity ratio analysis of the atomic emission lines obtained during the LIBS experiments to differentiate between the *ex-vivo* tissue samples of domestic pigs.

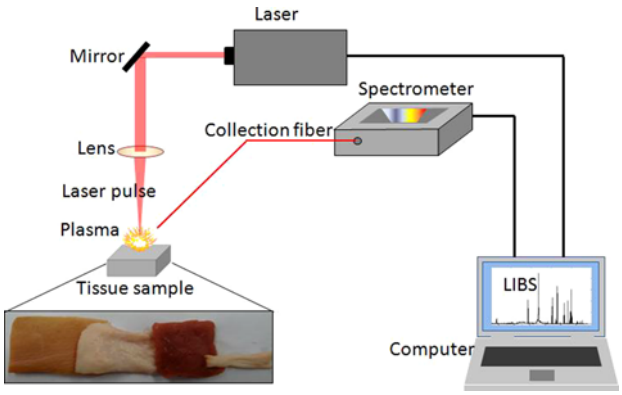

Figure 1 Schematics of the LIBS experimental setup [6].

2. Methodology

Four different types of tissue (fat, muscle, nerve and skin) were taken from six bisected heads of *ex-vivo* domestic pigs, 6 months of age on average (24 tissue samples in total). The bisected pig heads were obtained from the local slaughterhouse. Therefore, approval of ethics committee was not necessary. Tissue samples were prepared manually using a scalpel. The average dimension of the tissue samples was 3×3 cm. The thickness ranged from 0.7–1.1 cm. The nerve tissue (N. infraorbitalis) was extracted from the perineural sheath to allow for a measurement of pure nerve tissue fibres. Due to its anatomical features, it had an average length of 5 cm and a diameter of 0.5 cm. After preparation, the tissue samples were carefully washed with sterile saline solution to remove all superficial contaminations including clotted blood particles without mechanical irritation of the biological samples. The LIBS spectra of the tissue samples were collected on the day of slaughter with a maximum ex-vivo time of 5 hours. In order to mimic the real conditions of an operation room, all the measurements were performed in an open environment under normal stray light conditions and without a chamber. Chambers are usually used in LIBS to perform the experiments in different gas environments (inert gas, vacuum and air). In general, it is reported that the effect of the ambient gas is on the expansion of the plume [28].

2.1 Laser induced optical breakdown spectroscopy for tissue differentiation

The experimental setup consisted of an Excimer laser (Ar-F, ExistarTM M100, Tui Laser AG, Germany, $\lambda = 193$ nm, pulse duration 28 nm, spot size $= 0.6 \times 0.4$ mm and energy per pulse 38 mJ) focused by a convex lens (focal length = 100 mm) for plasma formation. A software controlled motorized stage capable of moving in three dimensions was used to move the samples to the focal point of the focusing lens. A single optical fiber (NA = 0.22, 1000 µm core, multimode, Thorlabs) was used as a detector fiber to collect the light of the generated plasma from the tissue samples and deliver it to a spectrometer (QE65000®, spectral range: 350–1100 nm, spectral resolution: 6 nm, integration time: 8 ms, Ocean Optics, USA). The spectrometer was operated in free running mode without delay at 8 ms integration time.

In order to remove any contaminants and the saline solution on the surface of the tissues, five laser pulses were sent to each tissue spot in all the experiments. After the 5th laser shot, LIBS spectra from each tissue type were collected and saved. During all measurements, samples were irradiated by focusing the laser pulses on the surface of the sample at a repetition rate of 10 Hz. To get sufficient data for the analysis, 100 LIBS spectra were collected from different spots of each sample (fat, muscle, nerve and skin). Therefore, 400 LIBS spectra were collected from each pig. A total of six pigs were investigated in the experiments providing 2,400 LIBS spectra. The Spectra Suite software (Ocean Optics, Florida, USA) was used to run the spectrometer for the LIBS spectra collection. To visualize the data from the experiments, Origin software (8.1 G) was used.

2.2 Data analysis

To determine the elemental composition of the samples, the atomic emissions in the LIBS spectra of the tissue samples were first mapped with the NIST atomic emission database [29]. In all measurements of the tissues, 13 peaks (atomic emissions) were found to be reproducible both within the spectra of each tissue and in all the different tissue types (Figure 2). To obtain ratio features from the spectra of the target tissues, ratio analysis was performed on

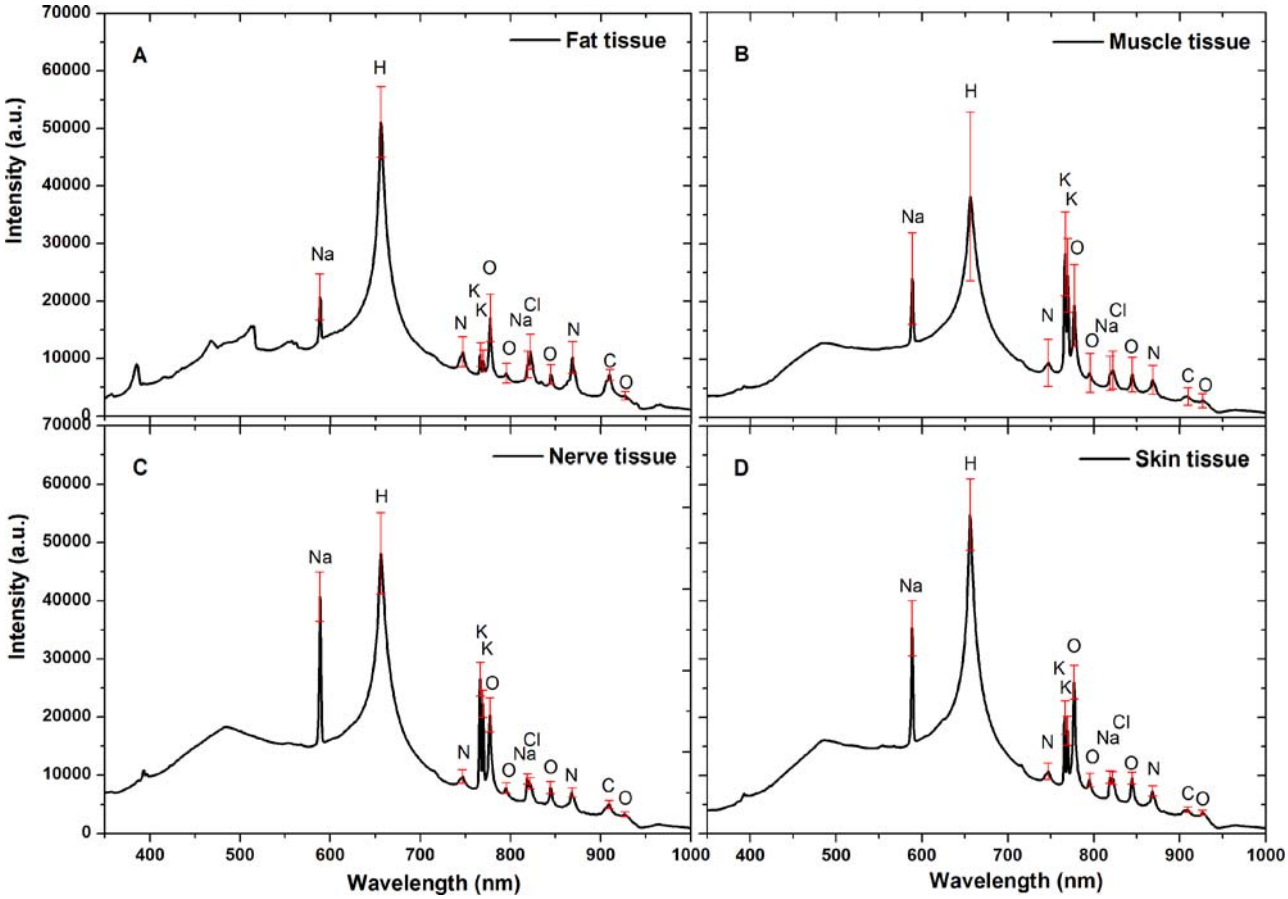

Figure 2 Mean LIBS spectrum of 100 spectra of (**A**) fat; (**B**) muscle; (**C**) nerve and (**D**) skin tissues of pig 6 with elements and intensity deviation of their emissions [6].

the intensity values of the atomic emissions. Therefore, different combinations of ratios among these intensity values were evaluated in order to find those which were reproducible and at the same time gave a comparable value for a given tissue. Using the 13 peaks, 69 different ratio combinations among the monitored elements were investigated (Table 2). Before the ratios were evaluated, the spectra from all the tissues of all the investigated animals were first normalized. Intensity ratios between the selected emissions were then calculated for the spectra of all the tissue types of all the animals (100 spectra of each tissue type and four tissue types of six pigs). The mean value and the standard deviation of each ratio were calculated over the 100 signals of each tissue type from all the pigs. The intensity ratio values of the selected emissions for each tissue were then visualized by plotting the mean ratio versus the index number (Table 2) given to each ratio combination. The standard deviations of the ratio values are presented as error bars in the graphs (Figure 3).

3. Results and discussion

The obtained LIBS spectra of skin, muscle, fat and nerve showed specific atomic emissions resulting from the elements composing these samples. The peaks corresponding to the atomic emission lines of the elements in the tissue samples are shown in Figure 2. The peaks in the spectra were observed to have different intensity levels. The reason for this is assumed to be due to the different concentration levels of the respective elements in the different tissues. This concludes that even though the same elements are present in skin, muscle, fat and nerve tissues, the tissues may be differentiated according to the concentration levels of these elements.

The monitored elements in the LIBS spectra of fat, muscle, nerve and skin tissue were C, H, O, Cl, Na, K and N. Table 1 compares the elements monitored in the experiments to the elemental composition of the samples found in the literature [27]. Since, the experiments were performed in open air conditions, signals from H, O and N may have been

Table 1 Comparison of the elements expected and those monitored using LIBS experiments of fat, muscle, nerve and skin tissues.

Sr. No	Environment	Fat		Muscle		Nerve		Skin	
		Literature	LIBS	Literature	LIBS	Literature	LIBS	Literature	LIBS
1	H	H	H	H	H	H	H	H	H
2	C	C	C	C	C	C	C	C	C
3	N	N	N	N	N	N	N	N	N
4	O	O	O	O	O	O	O	O	O
5	–	Na	Na	Na	Na	Na	Na	Na	Na
6	–	S	–	S	–	S	–	S	–
7	–	Cl	Cl	Cl	Cl	Cl	Cl	Cl	Cl
8	–	–	K	K	K	K	K	K	K
9	–	–	–	P	–	P	–	P	–

enhanced by the presence of these elements in the air. However, the effect of the air is equal for all tissue samples and was neglected. Moreover, the elements monitored in the investigated tissues were found in all the samples of the six pigs.

Figure 2 shows the majority of elements found in the tissue samples that could be visualized in the LIBS spectra. The presence of Potassium (K) in fat tissue is in disagreement with the chemical composition of fat tissue described in literature. This may result from the blood perfusion of fat tissue, leading to accumulation of K. In this study, Sulphur (S) and Phosphorus (P) which are meant to exist as trace elements in the tissues, could not be identified [27].

However, all elements that could be monitored are in agreement with those described in literature. In addition, the intensity values of the atomic emissions in the LIBS spectra of the tissues showed varying values, using the experimental parameters described, indicating a difference in the concentration of the constituent elements. Therefore, the intensity variation of the peaks was used to perform an intensity ratio analysis among the emission peaks to differentiate between the tissue classes.

In the ratio analysis, 69 different ratios were calculated between the intensity values of the 13 atomic emission lines of each tissue type's LIBS signal for all of the 6 pigs investigated (Table 2). When more than one pair of the same elements is shown in the table, this indicates ratio values of emissions of these elements at different wavelengths. Figure 3 shows an example of the mean values of the 69 ratios including their standard deviation, calculated from the atomic emission intensities obtained from the soft tissue's LIBS spectra of pig 6. The horizontal *x*-axis in the graphs represents the given index number of each ratio obtained from two emission lines. The assigned index numbers of all the ratio combinations showing which element ratio pair belongs to which index is given in Table 2.

The vertical *y*-axis shows the average value of each calculated ratio from 100 LIBS signals. Several ratio combinations are presented with different values in each of the tissues for the given experimental parameters. Based on these differences in ratio values, differentiation among the tissue samples could be performed. The ratios of Na to C (index No. 10), K to Na (index No. 34) and O to C (index No. 51), present in each tissue, can be taken as a promising reference point to differentiate between the skin, muscle, fat and nerve tissues investigated in this study. The level of the intensity ratios of Na to C, K to Na and O to C are marked with rectangles on the ratio plot of the four tissues of pig 6 in Figure 3. In the obtained results, muscle was seen to produce more unstable peaks and hence relatively unstable ratio values in comparison to the other tissue samples which can be seen from the error bars in its peaks. This could be due to a more inhomogeneous structure of the muscle tissue in relation to the other tissue samples. Muscle tissue inherits a certain percentage of intramuscular conjunctive tissue e.g. collagen [30]. The distribution and amount of the connective tissue in the samples could thus account for the higher variance in the ratio values.

Figure 4(A) shows the values of the ratios of the Na : C pair in all of the 6 pigs investigated. The results of the analysis indicate that the ratio values of this pair lie within a certain range when looking at fat and nerve tissue. The average Na to C intensity ratio value of fat tissue varies between 2.08 to 4.57 for all of the 6 pigs. In this ratio range, only fat tissue is present. Similarly, the average intensity ratio of Na to C of nerve tissue varies between 5.49 to 8.19 in all of the pigs. However, in pig 1 muscle tissue showed a relatively lower value, overlapping the range of nerve tissue. In general, muscle tissue showed a relatively high variation of Na : C ratio in all the samples. This may be due to the before mentioned inhomogeneous composition. The mean value of the Na to C ratio of skin tissue varies between

Table 2 The given index numbers of 69 pairs of ratio combinations between the elements monitored during LIBS experiments of fat, muscle, nerve and skin.

Index No.	Ratio Pair	Index No.	Ratio Pair	Index No.	Ratio Pair	Index No.	Ratio Pair	Index No.	Ratio Pair	Index No.	Ratio Pair	Index No.	Ratio Pair
1	Na : H	11	Na : O	21	H : C	31	Na : O	41	K : O	51	O : C	61	Cl : O
2	Na : N	12	H : N	22	H : O	32	K : O	42	K : Na	52	O : Na	62	Cl : Na
3	Na : K	13	H : K	23	N : K	33	K : O	43	K : Cl	53	O : Cl	63	Cl : C
4	Na : K	14	H : K	24	N : K	34	K : Na	44	K : O	54	O : Na	64	Cl : O
5	Na : O	15	H : O	25	N : O	35	K : Cl	45	K : Na	55	O : C	65	O : N
6	Na : O	16	H : O	26	N : O	36	K : O	46	K : C	56	Na : Cl	66	O : C
7	Na : Cl	17	H : Na	27	N : Na	37	K : Na	47	K : O	57	Na : O	67	N : C
8	Na : O	18	H : Cl	28	N : Cl	38	K : C	48	O : Na	58	Na : N	68	N : O
9	Na : N	19	H : O	29	N : O	39	K : O	49	O : Cl	59	Na : C	69	C : O
10	Na : C	20	H : Na	30	N : C	40	K : O	50	O : Na	60	Na : O		

8.60 and 14.02. The mean ratio value of this pair showed to also be in the range of muscle tissue in four of the investigated animals (pig 2, pig 4, pig 5, pig 6). Therefore, it is difficult to differentiate between skin and muscle tissue in four of the animals (pig 2, pig 4, pig 5, pig 6). This is due to the fact, that the intensity ratio for the Na : C pair is within the same range. However, skin has different ration feature from nerve and fat tissue. Concluding from that, ratio values of emission intensities of Na to C can be used to differentiate between fat, skin and nerve tissue, as well as between fat, nerve and muscle tissue.

The analysis of the ratio of the intensity values of K to Na emissions is shown in Figure 4(B). The results show that the mean value of the intensity ratio of K to Na emissions from skin tissues of all six pigs varies between 1.96 and 3.08. Similarly, the mean value of the intensity ratio from the emissions of the same pair of elements obtained from muscle tissues of all six pigs varies between 2.91 and 7.51. There-

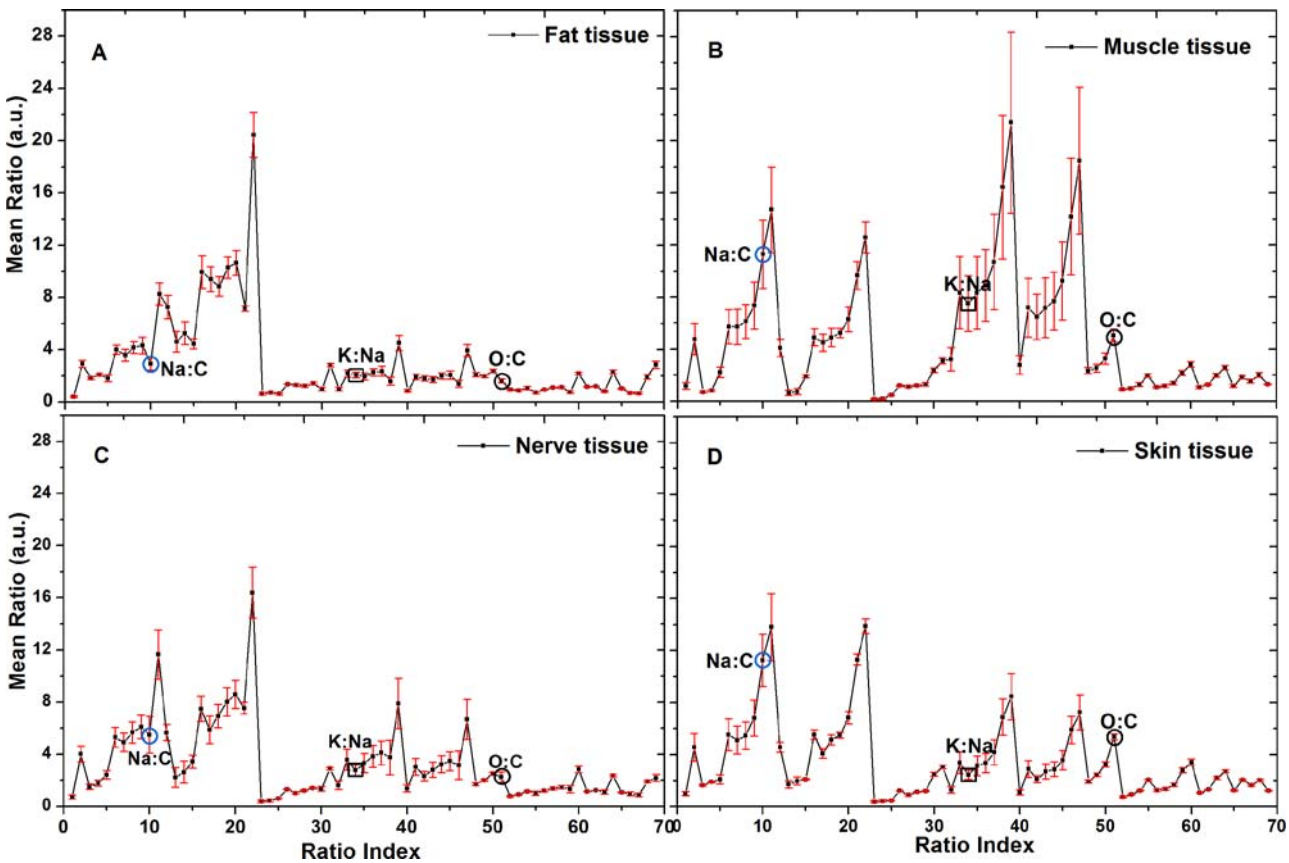

Figure 3 Intensity ratio plots of (**A**) fat; (**B**) muscle; (**C**) nerve; and (**D**) skin of pig 6.

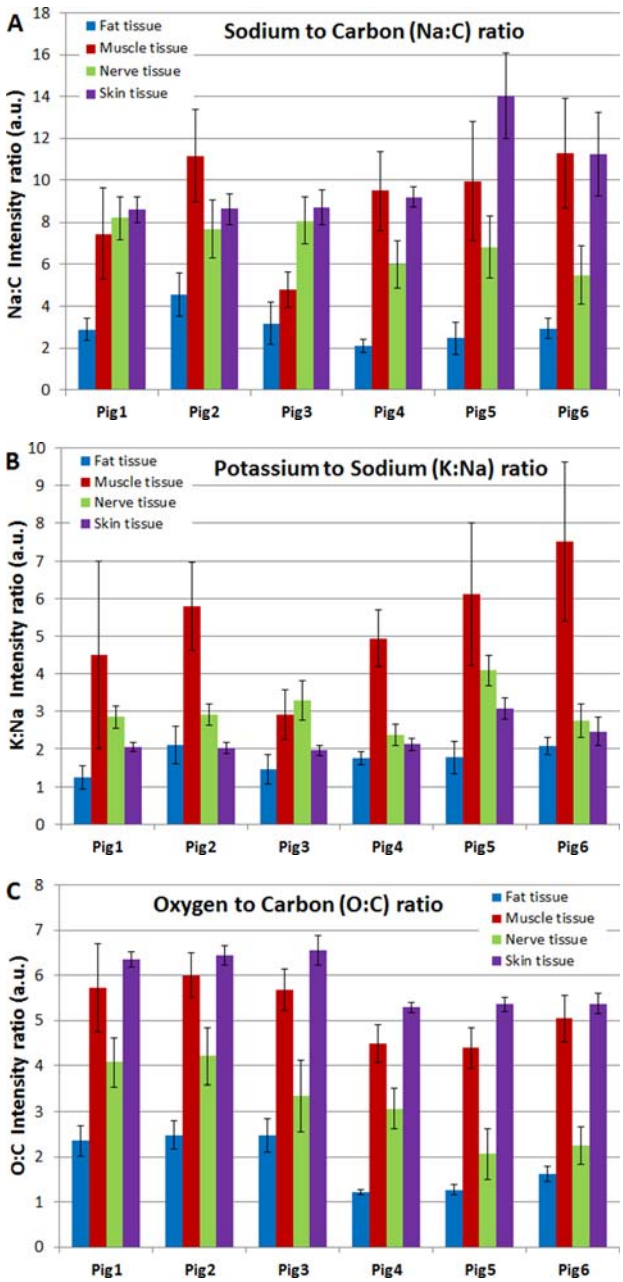

Figure 4 Mean ratio values of emission intensities of elements in fat, muscle, nerve and skin tissues of six pigs, (**A**) Emission intensity ratio of Na to C, (**B**) Emission intensity ratio of K to Na, (**C**) Emission intensity ratio of O to C.

fore, the results of Na to C and K to Na ratios can be used to differentiate between the investigated tissues, given the same experimental parameters are used and the reproducibility of the spectra from muscle tissue is improved. Figure 4(B) shows a similar trend in the mean values of the intensity ratios of K to Na emissions within the tissues of five pigs. The mean values are highest for muscle tissue with the exception of pig 3, in which the muscle signals were shown to have the poorest reproducibility. Nerve tissue values scored second highest, followed by skin tissue and fat tissue. Similarly, the investigation of O to C average ratio values (Figure 4(C)) shows a stable trend within each pig. In general, fat tissue scored the lowest in the three ratio combinations of most of the pigs considered in this study. The trend of ratio values of K to Na showed skin tissue scoring the second lowest in five of the pigs. Muscle tissue, followed by nerve tissue, scored the highest in the K to Na ratio values. The trend in the ratio values of O to C emissions showed the highest values in skin samples, followed by muscle, nerve and fat tissues.

As elaborated on above, a focus in this study was laid on the crucial tissue pair nerve/fat as this is one of the key elements for a successful implementation of the feedback system in a laser surgical environment. It becomes apparent that the differentiation of the tissue pair nerve/fat is difficult when regarding the microscopical structure of these tissues: In our study, the infraorbital nerve of a domestic pig was used. As in most of the nerve branches, a myelin sheath encircles the nerval structure, containing up to 75% lipids which also is the major cell population in fatty tissue [31]. This implies that the differentiations of these structures are hampered by their superficial similarity. Nevertheless, the approach used in this study could overcome this problem. As shown in the results, the ratio value of Na to C of nerve and fat tissues range from 5.49 to 8.19 and 2.08 to 4.57 for all of the 6 pigs, respectively. Therefore, these two ratio combinations can be used to differentiate between nerve and fat.

The elements monitored in the LIBS spectra obtained from fat, muscle, nerve and skin tissues, using the parameters specified, were C, H, O, Cl, Na, K and N. All these elements were monitored in all the tissue samples from all the investigated animals and are in agreement with elements reported to be found in these tissues in literature. The analysis on the average ratio values among the intensity emissions of the elements monitored in the LIBS experiments of the soft tissues show promising ability to differentiate among the samples. For such an analysis, emission intensity ratios of Na to C, K to Na, and O to C suggest the best differentiation performance. However, further attempts to improve reproducibility of the spectra collected from muscle tissue are essential. Overall, there is a high level of variance in the LIBS spectra of the majority of the tissues. Hence, an attempt to reduce this error may provide better and more robust ratio analysis results. Several factors could be responsible for the poor reproducibility of the LIBS signals observed in the investigated tissues. These factors may include laser parameters and target sample properties [32]. Temporal and spatial energy fluctuations of the pulses may affect the process. Additionally, sample surface topol-

ogy and density can affect plasma formation, which in turn affects the LIBS signals [32]. Therefore, thorough investigation of these factors is important.

In this paper, the potential of LIBS as a robust, fast and minimally-invasive alternative method for qualitative tissue differentiation with a promising performance was demonstrated. By monitoring the ratios of the intensity of emission lines in the plasma plume created from each laser pulse, a simple and straightforward approach for a real-time feedback mechanism for laser surgery has been described in the current study. Thus, the experimental setup described in this study may help broaden the field of application and improve the security of clinical laser surgery systems by preventing iatrogenic damage of blood vessels, connective tissue and nerve tissue during complex laser surgery procedures. Differentiation among the four tissue types, at this stage, can also be used for validation in subsequent experiments on samples where all the tissue types are together in their original intact state. This study was also able to show the differentiation between nerve and fat tissue, critical for effective laser surgery application.

4. Conclusions

This preliminary study demonstrates a successful application of a simple LIBS setup for tissue differentiation under *ex-vivo* conditions by monitoring the atomic intensity lines acquired from the samples. The elements monitored in the LIBS spectra of fat, muscle, nerve and skin tissue were C, H, O, Cl, Na, K and N. Moreover, all the elements monitored in the investigated tissues were the same and were equally found in all of the six animal's samples. The elemental composition of the tissues determined using LIBS were again found to be in agreement with those described in literature. The results of ratio analysis of the intensity of atomic emissions show that they provide a wide range of promising ratio values for successful tissue differentiation. Therefore, we conclude that the proposed method can provide qualitative information at atomic level during transition from one tissue layer to another during laser cutting in order to avoid damage of critical tissues. However, further studies need to be performed to improve the reproducibility of the signals and study its applicability on larger numbers of animals and different animal models.

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Author biographies Please see Supporting Information online.

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