Weak biophoton emission after laser surgery application in soft tissues: Analysis of the optical features

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cover letter here. Please note, if you are submitting a revision of your manuscript, there is an opportunity for you to provide your responses to the reviewers later; please do not add them to the cover letter.	please find enclosed the manuscript entitled “Weak biophoton emission after laser irradiation in soft tissues: analysis of the optical features” by Federico Boschi and co-workers for review and possible publication in the Journal of Biophotonics. In the manuscript we report, for the first time, the detection and characterization of the light emitted by soft tissues after (up to 1 hour!) laser irradiation. The content of the manuscript is original and it has not been published or accepted for publication, either in whole or in part, in any form. No part of the manuscript is currently under consideration for publication elsewhere. The authors report that Gabriel Segalla is employed at the OROTIG S.r.l.company which lent the laser equipment for the experiments, as declared in the manuscript. We hope this manuscript could fit the aims of the Journal of Biophotonics. On behalf the authors, sincerely yours, Federico Boschi

Weak biophoton emission after laser irradiation in soft tissues: analysis of the optical features

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Keywords: thermoluminescence, optical imaging, diode laser, laser surgery, Cerenkov luminescence.

Abstract

Laser scalpels are nowadays becoming largely diffuse for surgery, cutting or ablating living biological tissue, replacing for some applications the traditional surgical scalpel. Laser scalpels are generally used to concentrate in a very small sized area the light energy which is converted in heat by tissues. Depending on the temperature reached in the area different effects are visible in the tissues. The irradiated surface is also source of light emission and this process is employed in laser induced fluorescence imaging and spectroscopy. In these cases the light emission is collected during the irradiation process. Here we report the discovery and characterization of the light emitted by soft mammalian biological tissues from seconds to hours after laser irradiation. A laser diode commercially available for medical and dentistry applications working at 808 nm was used. The

irradiated tissues (red meat, chicken breast and fat) were found sources of light emission in the visible range, well detectable with a commercial Charge Coupled Device camera. The time decay of the light emission, the laser power effects and the spectral features in the range 500-840 nm in the different tissues are here reported.

1. Introduction

Laser scalpels are worldwide used for many biomedical applications including dermatology ophthalmology, otorhinolaryngology, lithotripsy, oncology and neurosurgery [1, 2].

The interaction between laser beam and biological tissues depends on the specific laser parameters and the tissue characteristics [3, 4], but in laser medicine the photon energy conversion is frequently based on heating. The subsequent thermal relaxation depends on the thermal properties of the irradiated tissues and on the temperature gradient between irradiated and non-irradiated regions [5]. Indeed, laser emitted photons travelling in biological tissues are absorbed by the tissues components (chromophores, proteins, enzyme and water) and the energy is converted as heat [6].

The effects of the thermal reaction induced by laser irradiation are reported in literature [6, 7]. Up to 42 °C no measurable effects are observable; from 42 to 50 °C hyperthermia occurs (with tissue necrosis if hyperthermia lasts for several minutes); at 60 °C starts a visible paling of tissue due to the denaturation of proteins and collagen which is responsible of the coagulation of tissue and necrosis of the cells. At temperature higher than 80 °C cell membrane permeability is drastically increased, altering the chemical concentration equilibrium; at 100 °C water molecules in the tissues start to vaporize causing the formation of gas bubbles and thermal decomposition of tissue fragments. The inter- and intra- cellular water content is vaporized with the consequent production of photo-ablative effects and dissociation of large tissue elements (wounds) [7, 8]. Reaching 150 °C blackening reveals the carbonization of the tissues, accompanied by the production of smoke; at higher temperature, around few hundred of degrees Celsius, melting may occur.

The cooling, or thermal diffusion, is responsible for heat flow in the tissues and is correlated with the extent of tissue damage [6]. In particular, for diode laser emitting in the range 800-1100 nm, the relative low light absorption of hemoglobin, water and melanin in this wavelength range results in a widely spread thermal damage zone of several millimeters [9, 10] and the penetration depth in soft tissues is almost three order of magnitude greater than for the CO_2 laser wavelength (approximately 0.015 mm [11]) which is the gold standard for soft tissue surgery [12, 13].

Laser irradiation is applied to study the chemical composition of samples via the so called laser induced fluorescence (or laser induced spectroscopy) [14]. In biomedicine it is applied to discriminate normal from cancerous tissue [15] This technique is based on the detection of light simultaneously with the irradiation.

In our knowledge, the study of the seconds-to-hours retarded laser-induced luminescence in biological tissues is not reported previously in literature. We have called this phenomenon laser-induced retarded luminescence (LIRL). This study arises from a specific application, more precisely the light emission from the margins of anatomical resections obtained with the electric scalpel was reported and it could overlap the Cerenkov light emission due to the radioactive uptake in the biological resections [16]. Cerenkov luminescence guided surgery was introduced by our group to evaluate the burdens of malignancies in patient injected with radiopharmaceuticals before surgery [17]. Cerenkov luminescence imaging (CLI) is based on the detection of photons in the visible range coming from the interaction between beta particles and tissues, [18]. CLI can be applied in the oncological field where beta emitting radiotracers are widely used to image or treat cancer masses. It is noteworthy that ex vivo surgical specimens can be imaged with a portable Charge Coupled Device (CCD) camera directly in a surgery room in almost real time. Defining tumor burdens during surgery is indeed extremely important for the surgeon allowing to evaluate almost in real time the outcome and to predict quality and duration of the patient's life.

Here we report the experimental data of the interaction between the light beam of a laser scalpel, developed for medical and dentistry surgical applications, and biological tissues as cause of light emission from the tissues. We investigated time decay, line profile and spectral features of the emitting area around the laser irradiated soft tissues. The study of the molecular mechanisms responsible for the light emission are beyond the aims of this paper.

2. Material and Methods

2.1 Laser surgery

The analysed biological tissues were fresh red meat (beef), white meat (chicken breast) and fat (beef). They were irradiated with a laser beam at 808 nm to induce about 1 mm deep wounds. The LD-10U AlGaAs diode LASER EightoEight (Orotig, Italy) was chosen because it has been specifically developed and designed for medical-dentistry applications. The 808 nm wavelength

owns very good hemostatic properties, thanks to the excellent absorption coefficient of both hemoglobin and melanin, with advantages for the treatment of soft tissues of the oral cavity.

The laser emits, through a flexible optical fiber, a pulsed or a continuous laser beam (808 nm ± 10 nm) up to a max power of 10 W (± 20% at the fiber). The optical fiber (200 μm diameter) is composed by a core (silica) and a cladding (hard polymer). The nominal beam divergence (20.5° = 0. 36 rad) allows to treat very thin/small tissue areas. During the experimental sessions, continuous wavelength mode was used with three different power levels (3, 6 and 9 W). The laser was used in contact with the tissue. The tip of the optical fibre was passed on the tissues three-four times for each wound, for a total amount of time estimable in 4-5 seconds.

2.2 Thermal imaging

Thermal images were acquired with FLIR i7 camera (FLIR Systems, Inc. Wilsonville, Oregon, USA), 140x140 pixels, field of view 25° x 25°, accuracy 2%, thermal sensitivity 0.10°C, minimum focus distance = 0.6 m. The temperature measurement mode applied to the images was "spot" (in the centre) with correction for emissivity and reflected temperature. The spot measurement does not represent the temperature where the laser is focused, but corresponds to an average temperature evaluated inside a few millimetres radius circle (greater than point where the laser beam is focused).

2.3 Luminescent imaging

Luminescence acquisitions of the biological tissues started just after the end of laser treatment. Luminescent images were acquired by using the IVIS Spectrum optical imager (Perkin Elmer, Massachusset, USA). The IVIS Spectrum is based on a cooled (-90 ˚C) back-thinned, back-illuminated CCD camera. The CCD has an active array of 1920 x 1920 pixels with a dimension of 13 microns. Images were acquired and analysed with Living Image 4.5 (Perkin Elmer) and were corrected for dark measurements. Image parameters for the time-decay measurement were: exposure time = 60 s, f=1, binning B=8 and Field of View (FoV) = 6.6 cm. A 180 s delay was inserted between the second and the third images and all the subsequent images. Image parameters for the spectral measurement were: exposure time = 300 s, f=1, B=16 and FoV = 6.6 cm. No delay was inserted between the images in this case. The spectral measurements were corrected for the

time decay of the light signal. Region of interests (ROIs) were traced manually on the luminescence images, to obtain the average radiance (p/s/cm2/sr) emitted by the biological surface.

2.3 SEM imaging

Specimens around the wounds were collected with a surgery scalpel and were fixed with Na-cacodylate buffer (pH 7.4), dehydrated in ascending ethanol and hexamethyldisilazane, sputter-coated with pure gold in an Emitech K550 apparatus and mounted on appropriate stubs with carbon-based conductive adhesive. All specimens were observed under an FEI XL-30 FEG scanning electron microscopy (SEM) operated at 7–15 kV. Pictures were directly acquired in digital format as 1424 x 968-pixel grayscale TIFF images.

3. Results

3.1 Luminescence in soft tissues

In the experimental setup described above the laser beam focused on biological tissues induces wounds that behave like bioluminescent sources of light. Luminescence emissions are well detectable with a commercial optical instrument for in vivo small animal imaging. Photographs of the wound and their luminescence images are shown in Fig. 1.

In the same experimental conditions fat shows the highest signal, followed by chicken breast and red meat. In all the three types of specimens the light emission from the surface reaches the value of 10^4 p/s/cm2/sr.

The optical fibre tip flows well on the surface of red meat and chicken breast producing thin wounds (the laser emission is optimized for red meat). Instead, during fat treating, we noticed that the movement of the optical fibre tip was hampered and a thin film of liquid fat formed on the irradiated point. Furthermore, wound profile resembles a crater instead a thin wrinkle as in the other samples.

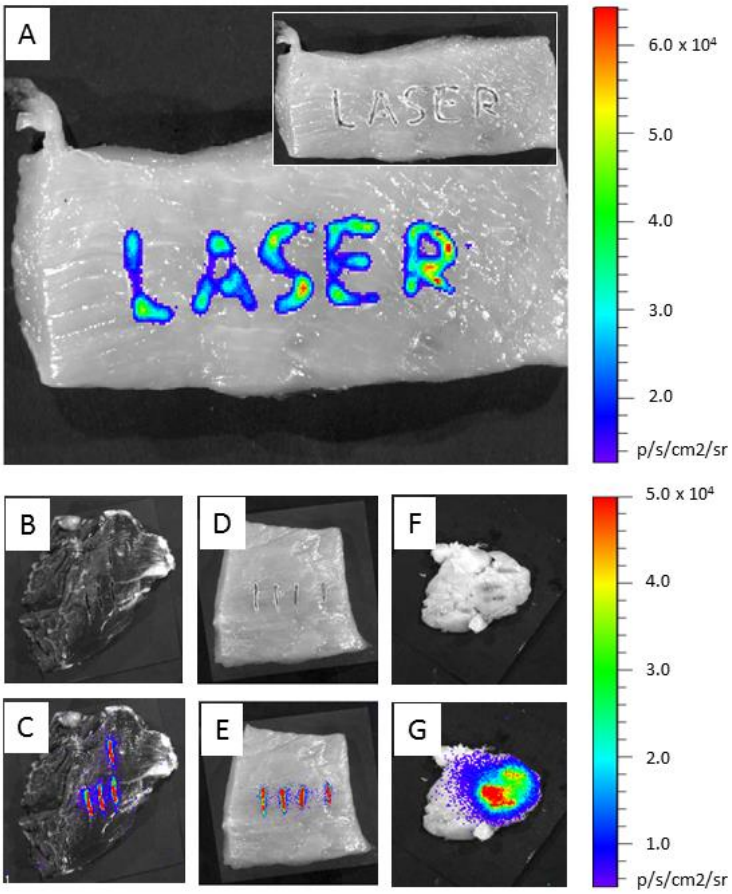

Fig. 1 Light emission from chicken breast irradiated with laser beam (A). The word "LASER" is written on the chicken breast (a photograph of the wound can be seen in the small insert) and the light emitted by the letters is clearly visible. Photographs of laser wounds and the corresponding luminescence emissions in red meat (A, B), chicken breast (D, E) and fat (F, G) obtained with laser power 3 W.

In order to measure the temperature reached in the laser spot on the biological tissues a thermocamera was used. Due to the minimum focus distance (0.6 m) the measurement is thus a mean value in a region much greater than the laser spot. Therefore the measurement given by the thermocamera can be considered as a qualitative information. In any case, a slight dependence of the temperature with the power laser was noticed. In case of 9 W power laser reached a temperature 12-15 °C greater than the ones obtained at 3 W. The thermal images with the temperature values measured in the region around the wounds are shown in Fig. 2.

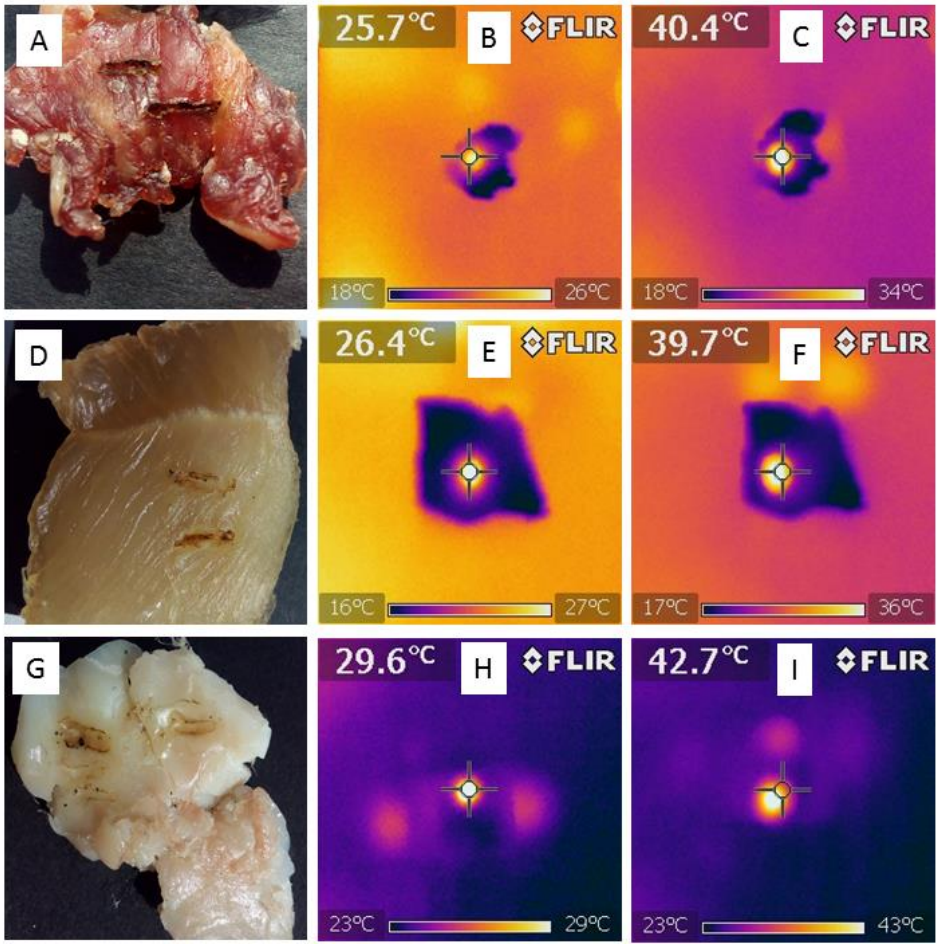

Fig. 2 Photographs of laser wound taken with commercial digital camera (left column) and with thermal camera after laser beam exposure with respectively 3 W (middle column) and 9 W (right column) in red meat (A-C), chicken breast (D-F) and fat (G-I).The temperature values correspond to the average temperature measured in the central spot corresponding to a circle of about 2 cm of radius.

3.2 Luminescence time decay

The luminescence signal is well visible in the luminescent images. The signal decreases along time in all the samples and, but using all the laser powers, one hour after laser treatment the signal is still visible, remaining in the order of 10^3 p/s/cm2/sr. The decrease of the average radiance measured for all the soft tissues and for all the laser power is shown in Fig. 3. The data are normalized to the first measured value. Red meat shows the steeper decrease followed by chicken breast; fat shows a slowest decrease instead. It was not found a clear relationship between the maximum average radiance and power, as we will see in the Discussion section.

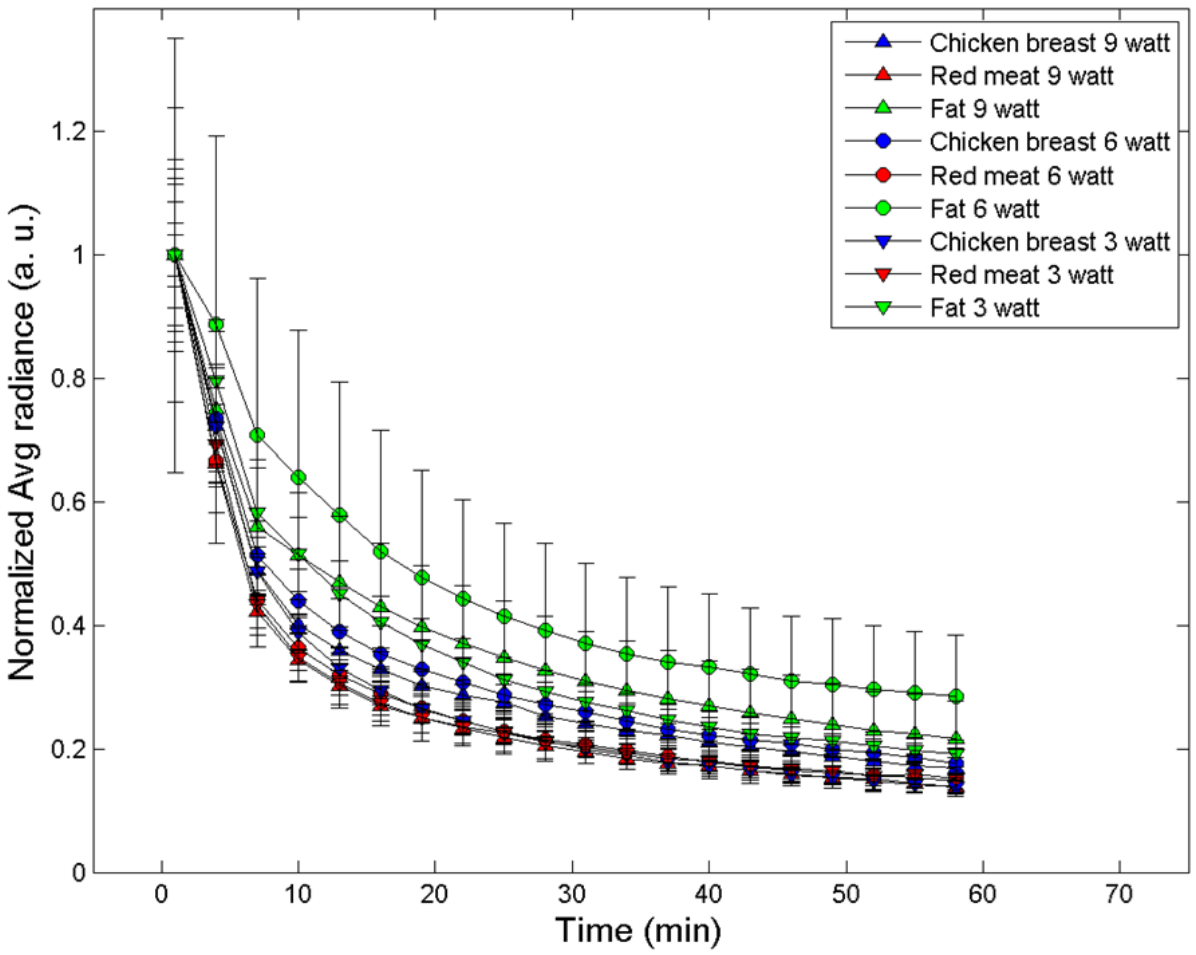

Fig. 3 Average radiance of the light emission vs. time for the three soft tissues and for the three laser powers (3, 6 and 9 W) used. Data are normalized with respect to the first measured value.

In order to describe the time fading of the light signal the data were fitted with one phase decay and two phase decay models with GraphPad Prism software (version 5.0).

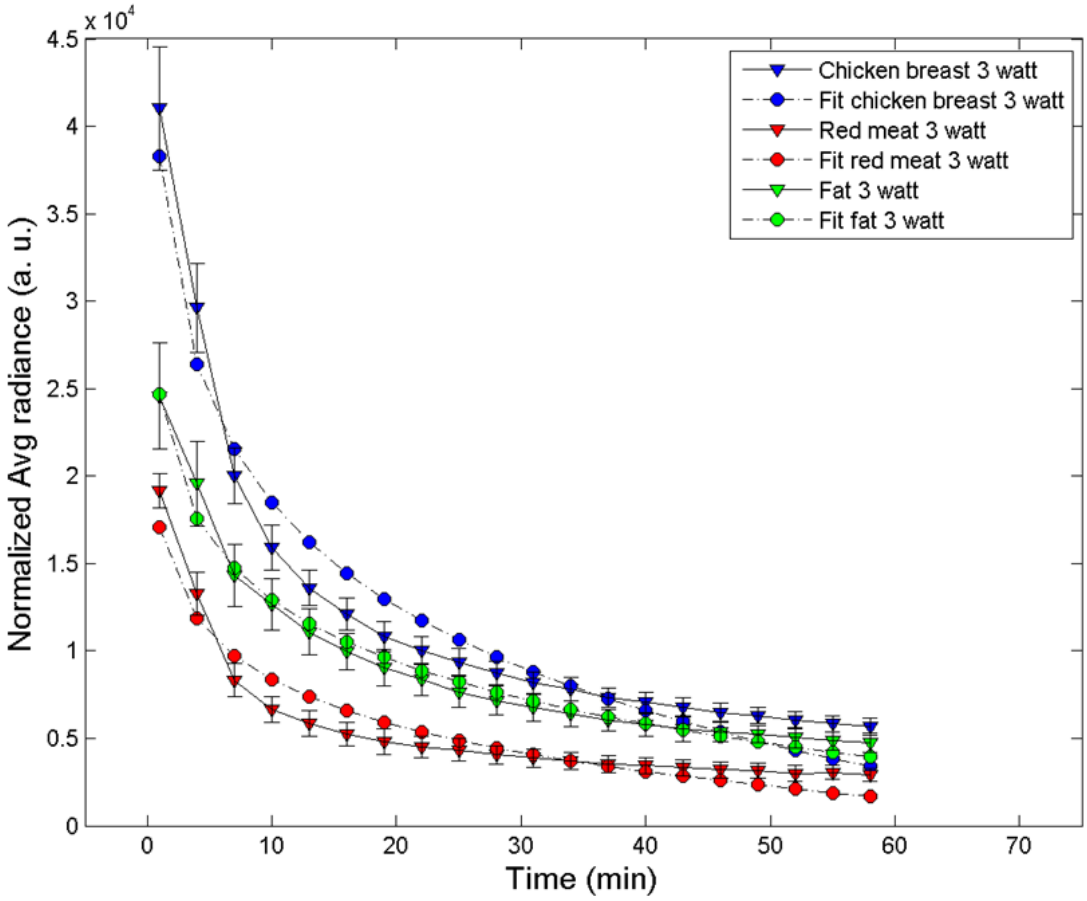

Fig. 4 Light intensity measurements in soft tissues (red meat, chicken breast and fat) after irradiation with 3 W laser power and the corresponding two phase decay curves obtained by data fitting.

The values of half life of the light signal intensity obtained by the data fitting for the different biological samples and laser powers are reported in Table 1. It shows a good agreement between measured and fitting data, especially for the two phase decay model.

Table1 The half life of the light signal in red meat, chicken breast and fat obtained by data fitting with two models (one phase decay and two phase decay).

		ONE PHASE DACAY		TWO PHASE DACAY		
Biological sample	**Laser power (W)**	**Half life (min)**	**R square**	**Slow half life (min)**	**Fast half life (min)**	**R square**
Red meat	**3**	**5.6**	**0.969**	**21.9**	**3.1**	**0.979**

	6	**5.9**	**0.965**	**19.9**	**2.7**	**0.985**
	9	**5.5**	**0.966**	**19.8**	**2.7**	**0.987**
Chicken breast	**3**	**6.8**	**0.970**	**22.5**	**3.8**	**0.983**
	6	**7.4**	**0.982**	**24.2**	**2.9**	**0.979**
	9	**6.6**	**0.962**	**30.8**	**3.3**	**0.979**
Fat	**3**	**10.1**	**0.975**	**18.8**	**3.8**	**0.984**
	6	**12.7**	**0.978**	**21.8**	**7.6**	**0.979**
	9	**13.1**	**0.973**	**23.5**	**2.9**	**0.982**

3.3 Line profiles

The intensity of the light signal was measured on the luminescence images along a line (3 pixels thick) traced perpendicularly to the laser wound. The profiles are reported in Fig. 5 and they refers to the data obtained just after surgery (thick lines) and 30 min late (thin lines).

Red meat and chicken breast showed a very sharp signal (Fig. 4A, B) with a FWHM of 0.16 cm and 0.17 cm respectively. In fat sample a signal with a broad peak was found with a FWHM of 0.39 cm (Fig. 4C). Thirty minutes after surgery no differences in the line profiles are visible in all the samples with respect to the profiles obtained just after surgery.

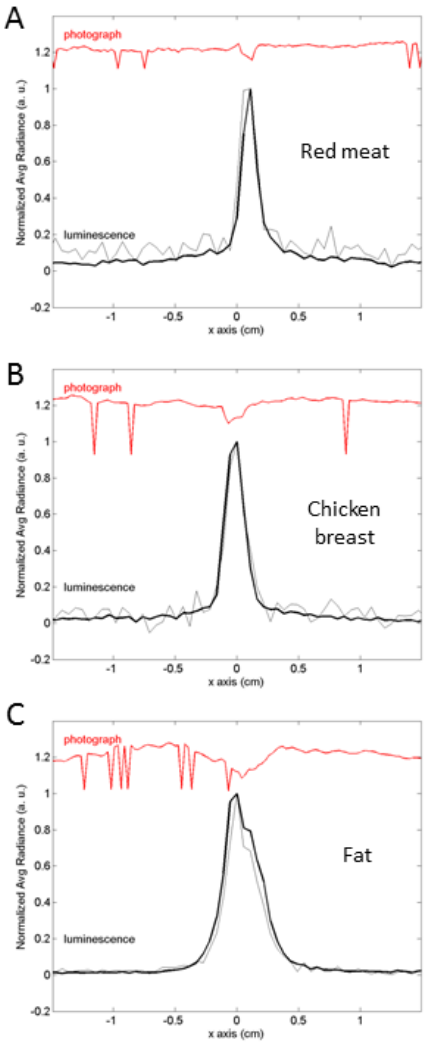

Fig. 5 Normalized line profile of the luminescence signal measured perpendicularly to the wound in red meat (A), chicken breast (B), fat (C) after irradiation with 3 W laser power. The thick line represents the profile measured just after the irradiation, the thin one the profile measured 30 min after irradiation. No significant difference in the light profile are visible in the time course. Sharp profiles are visible in particular in red meat and chicken breast. Red lines on the top of the panels represents (in arbitrary units) the light intensity measured on the photographs in order to localize the luminescent areas with respect the wounds.

3.4 Spectral features

The spectral emissions analyzed in the 500-850 nm region are shown in Fig. 8, the signal decay corrected spectra are normalized at 500 nm. Red meat shows a broad spectral emission peaked

around 700 nm; fat emission is broader than red meat with a maximum in the range 600-700 nm, instead chicken breast presents an almost uniform emission on the entire spectral range.

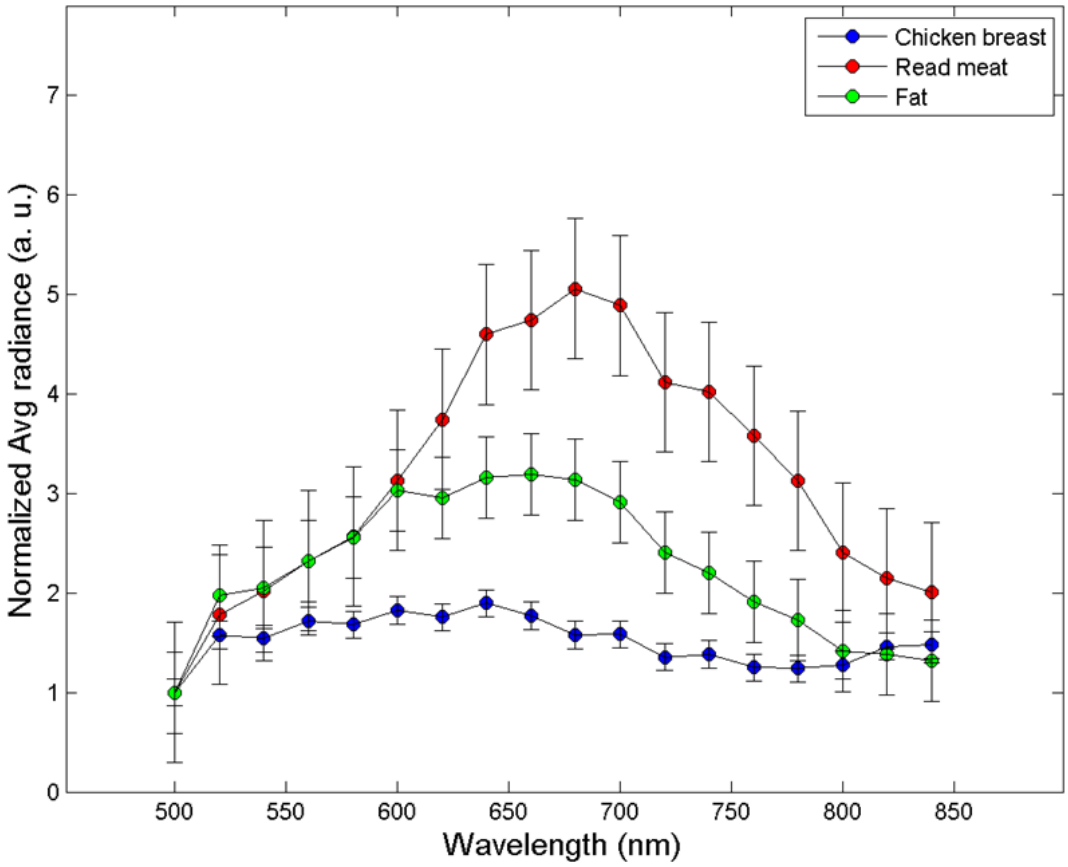

Fig. 6 Spectral emission over the 500 – 840 nm range measured in red meat, chicken breast and fat after irradiation with 3 W laser power. The spectra, corrected for time decay, are normalized at 500 nm.

3.5 SEM images

SEM analysis show that the laser-induced lesions are well visible all kind of tissues. In red meat the lesion has a corrugate appearance and the fibers near the wound are curved (Fig. 7A). The muscular tissue close to the lesion itself appears morphologically well preserved (Fig. 7B), while at the bottom of the lesion many melting points are shown (Fig. 7C). In chicken breast the laser causes a wound clearer than the ones observed in red meat samples (Fig. 7D). Again, as in red meat samples, the wound surrounding tissues are morphologically preserved and no heat-related

alterations are appreciated (Fig. 7E). At the bottom of the lesion widespread signs of melting, similar to foamy areas, are well recognizable (Fig. 7F).

The lesion overview shows a complete diastase in the fatty tissue caused by the laser beam (Fig. 7G). The structure of the adipose tissue is no longer recognizable: the heat-related alteration propagates far from the lesion and areas of fused adipose tissue are observed along the laser passage surface (Fig. 7H). Approximately 2 mm away from laser injury molten tissue is observed (as if it was dropped over the connective component) (Fig. 7I).

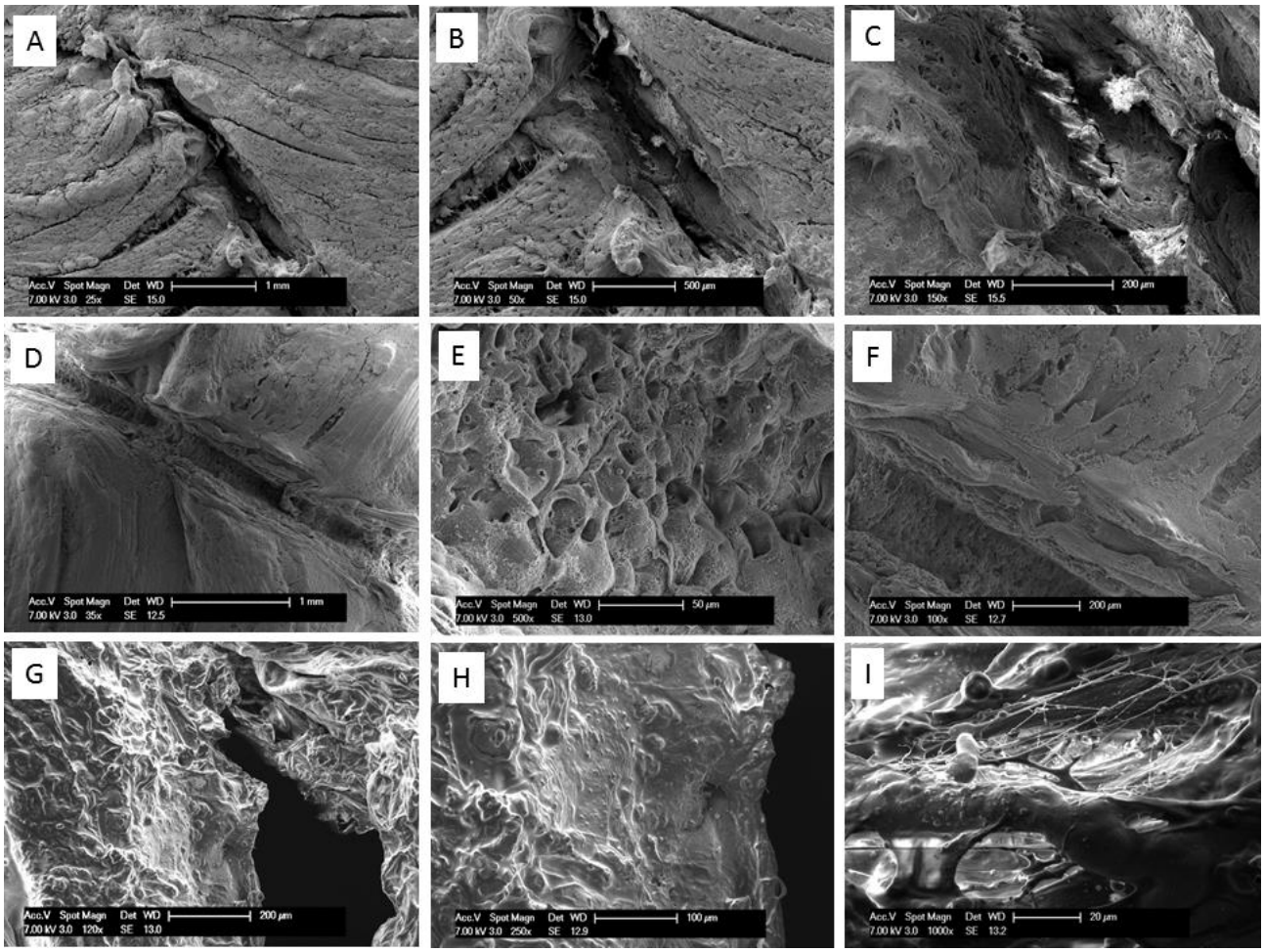

Fig. 7 Scanning electron micrographs of the laser wounds in red meat (A-C), chicken breast (D-F) and fat (G-I) after irradiation with 3 W laser power.

Discussion

Soft tissues irradiated by laser light at 808 nm are sources of light in the visible range lasting seconds-to-hours after irradiation. The emission was observed in the range 500-840 nm and the

spectrum was found peaked for red met and fat with a maximum around 650-700 nm, and almost flat for chicken breast.

Generally, the treatment protocol for surgery applications suggests the use of diode lasers in continuous or pulsed modality through contact or non-contact application of the optical fiber on tissue [19]. We used the laser in continuous wave and in contact modality to release high energy dose to the tissues. It is known that diode lasers emitting in the 810-1100 wavelength region are poorly absorbed by soft tissues [9, 20] and are not ideal for cutting. However, when the tip of the glass fiber is charred, the char is enlighten by the laser beam and the subsequent heat is transferred to the tip. Thus, the soft tissue is cut not by the laser beam but by the hot glass tip [13, 21]. SEM images confirmed the high temperature reached in the core of the wounds. To establish that the cutting effects are due principally to the heat and not to the laser light we inserted a glass microscope slide between the optical fiber tip and the tissue during irradiation and we noticed that no wounds were produced in the tissue and subsequently no LIRL emission was detectable from the irradiated tissues after removing the glass microscope; only a pale spot on the irradiated surface of the tissue was visible. Thus, we verified that in our experimental setup the energy of the laser is principally deposited in the tissues as heat. Many researches focused their attention on the heat diffusion from the cutting edge to the surrounding tissues. Our main result indicates that part of the deposited energy by the laser beam can leave the tissues in the form of visible-NIR light from seconds to hours after irradiation. This process contributes to the thermal cooling of the irradiated area.

The dosimetry for continuous wave applications is characterized by power, irradiation time and spotsize [22]. In our experimental procedure the irradiation time and the time elapsed between surgery and the beginning of the imaging acquisitions were difficult to be controlled due to the number of passages and the velocity of the optical fiber tip necessary to produce a wound. We tried our best to standardize the procedure employing, for example, only one operator.

Our results showed that LIRL is few or not affected by the laser power. This is in agreement with the observations of Welch et al. who found that change in laser power generally does not alter laser-tissue interactions, affecting more the times in which the effects are produced than the reached temperature [22].

The dimensions of the luminescent sources indicated by the FWHM measurements are higher than the wound sizes valuable in photographs and in SEM images. This is due to the photon scattering

which is responsible of the blurring of the luminescence source and it prevents the fine localization of the light sources with the morphological findings.

Recently, weak light emission of chicken breast tissues induced by heating obtained by direct contact between sample and conventional welding device or by high intensity focused ultrasound (HIFU) was reported by our group [23]. The signal remains detectable after 30 minutes from heating and the range of the half life using a one phase decay curve is within 4-8 minutes. The half life of the light signal induced in chicken breast is thus comparable with the values shown in table 1 obtained using a laser. This is a further experimental evidence that the main cause of the weak light production is the heating of the tissue (e.g. not the laser light) and is independent by the heating modality since we obtain similar light signal half life values using three completely different heating methods.

To explain biophoton emission after an heat shock, the generation of reactive oxygen species (ROS) was invoked by Kobayashi and co-workers [24], but further investigations to explain the origin of the light emission are needed. Finally, understanding the processes responsible of the luminescence could add new information regarding the interaction between heat ~~a~~nd tissues and the heat/energy exchange from irradiated and surrounding tissues

Conflict of interest

Gabriel Segalla is employed at the OROTIG S.r.l.company

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