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Detection of epidermoid squamous-cell carcinoma by laser induced autofluorescence – preliminary results

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Abstract: *Epidermoid squamous-cell carcinoma is by far the most common malignant tumor of larynx (95-96%), representing 1.6-2% of cancers in men and 0.2-0.4% of malignancies in females, with a world growing incidence and a slight dominance in urban areas. By exposing cells and tissues to UV light, the excitation of naturally occurring chromophores occurs in part by non-radiative deactivations, in part via fluorescence emission. Using laser induced autofluorescence (LIAF) in natural tissues not impregnated with photosensitizers as a noninvasive autofluorescence technique for both diagnosis and intraoperative assessments of laryngeal cancer we can improve the tumor malign identification in vivo. A total of three laryngeal biopsies (i.e. three pairs of tissues, each pair containing a healthy and a tumor tissue sample extracted from the same patient) were considered in this study. The samples were collected from patients previously diagnosed with stage T3 laryngeal carcinoma. Immediately after the surgery fragments of normal tissue and neoplastic tissue were collected, fragments of which later, after freezing, were sectioned in 25-30 μm thickness slices and stretched to quartz slides. All samples were subjected to controlled laser irradiation using a pulsed diode laser (λ=375nm, pulse width=87ps, frequency 31MHz) and the autofluorescence and its lifetime were collected using two optical fibers (inner diameter 400μm and 1500μm, respectively) positioned in a 45^{o} geometry. The signals were recorded using a spectrograph and a photo-sensor module, the output of which was fed to a digital oscilloscope.*

We assessed the impact of laser induced autofluorescence and autofluorescence lifetime measurements in order to identify the differences between healthy and tumoral laryngeal tissue and outlining them, in terms of differences between the laser autoinduced fluorescence averaged intensity. The results determined the usefulness of laser induced spectroscopy in the diagnosis of laryngeal squamous cell carcinoma, discriminating between the malignant and normal tissue by analyzing the differences in spectral autofluorescence intensity and autofluorescence lifetime.

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Keywords: *Laser induced autofluorescence, autofluorescence lifetime, laryngeal squamos cell carcinoma*

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INTRODUCTION

Cancer is a growing dilemma in the developed countries. Laryngeal carcinoma is the most common cancer of the head and neck. Epidermoid squamous-cell carcinoma is by far the most common malignant tumor of larynx (95-96%), representing 1.6-2% of cancers in men and 0.2-0.4% of malignancies in females, with a world growing incidence and a slight dominance in urban areas [1].The main risk factors are smoking, alcohol consumption, harmful dietary habits, exposure to the irritant chemical substances [2].

Early identification and pre-operative evaluation of laryngeal carcinoma and its precursor lesions are essential for function-preserving surgical treatment. Accurate determination of the site and extent of carcinomas is very important for planning therapy and establishing prognosis [3]. Attempts to optimize diagnostic methods to obtain more sensitive detection and precise description of laryngeal pathology remain a challenge for the otolaryngologist [4].

Microlaryngoscopy with biopsy is the standard diagnostic procedure for the detection of laryngeal carcinoma. Anyway, the microscopic view added to the palpation of the larynx is not always enough to the evaluation and precise description of precursor lesions. Some new techniques, like videostroboscopy, contact endoscopy, confocal endomicroscopy, optical coherence tomography, narrow-band imaging and fluorescence endoscopy have been developed to come up with the detailed examination and the histopatological information about mucosal lesions [5,6].

Fluorescence is a optical phenomenon of the light emission by some molecules after their excitation by the light at a particular wavelength. These fluorescent molecules are called fluorophores. Every fluorophore exhibits its characteristic absorption and emission bands whose shapes and positions of their maxima on the excitation and emission light wavelength axes depend on the physical and chemical properties of its nearest environments. The interest in the use of fluorescence in medical diagnostics has grown during the last decades. Fluorescence spectroscopy provides information on the biochemical and morphological aspects of different tissues [7].

The procedures using the fluorescence spectroscopy for the differential diagnosis of malignant lesions are based upon the differences in the autofluorescence emission due to the pathological changes of the tissue. These changes include the modifications of tissue architecture (tissue layer loss or thickening), light absorption and scattering by neovasculature, and the alterations of metabolic status of the tissue such as changes of the concentration and distribution of fluorophores [8]. As a result, recorded fluorescence emission spectra of cancerous lesions reveal differences in fluorescence intensity and fluorescence band shapes, when compared to healthy tissue.

MATERIALS AND METHODS

The spectroscopic measurements were investigated in three larynxes from laryngectomized cancer patients treated in ENT Clinic, Carol Davila Central University Emergency Military Hospital, Bucharest. Histological analysis of the tumor tissues revealed laryngeal epidermoid squamous-cell carcinoma (SCC) in both cases, stage T3 (Figure 1). Immediately after the surgery (total laryngectomy), the larynx was carefully washed in saline solution to remove any blood contamination.

After that, fragments of normal tissue and neoplastic tissue were collected, fragments of which later, after freezing, were sectioned in 25-30 μm thickness slices and stretched to glass slides (reconfirmed histological) and stored at -80ºC for further examination. All participants provided written consent to participate in the experiment. The study was approved by the local ethics committee.

Experimental setup (Figure 2) was performed using laser beam exposure of the samples a picosecond pulsed laser diode (Alphals, type PicoPower LD-375-50) emitting at 375 nm with 31MHz pulse repetition rate, 87 ps full time width at half-maximum laser pulses, and the average power of about 0.5 mW. A laser beam of 1.6x1.8 mm (elliptical) diameter was

sent to the sample in double-pass vertical irradiation geometry through a 355 nm dichroic mirror and a totally reflecting mirror.

Two types of measurements on tissue samples were performed: laser induced autofluorescence (LIAF) and autofluorescence life-time. The autofluorescence signal was collected using two optical fibers positioned each, at 45^{o} in the same plane with respect to the pumping laser beam.

Figure 1. Larynx after surgical ablation for SCC (T3)

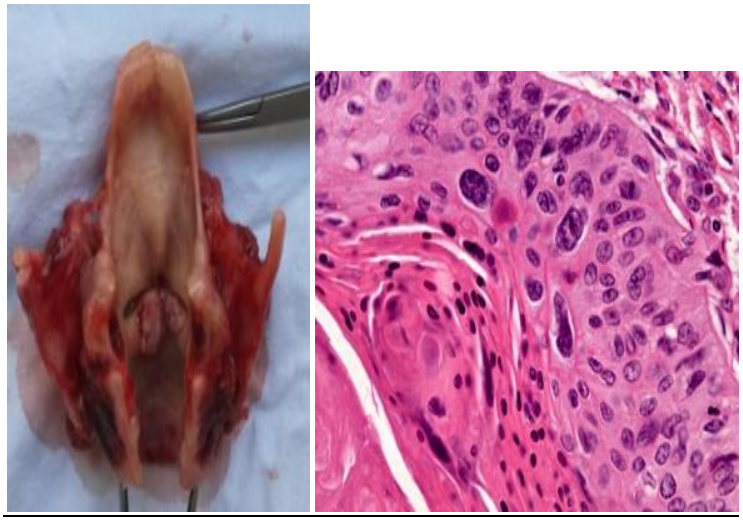
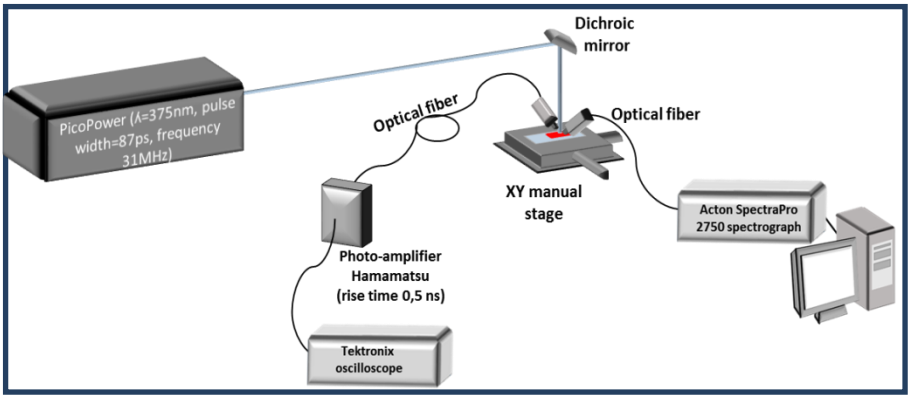


Figure 2: Experimental setup



The optical fiber with the core diameter of 400 µm collects the signal for LIAF spectrum analysis; another fiber of 1,500 µm collects the remitted signal for autofluorescence life-time analyses. The LIAF signals were recorded using a spectrograph (Princeton Instruments, type Acton SpectraPro 2750) with an optical resolution of 0.08 nm and; a photosensor module (Hamamatsu, type H6780-20) having 0.78 ns rise time, was used for LIAF life-time measurements and its output was fed to a digital oscilloscope (Tektronix, type DPO7254). The signal acquisition by spectrograph and photosensor module was synchronized with the laser pulse through a pulse delay generator (SRS, DG 535). During the LIAF measurements, the probe (sample and substrate/slide) was placed on a XY manual stage with a displacement resolution of 5 µm. Each type of measurement was performed collecting the LIAF signal from three different spots for each sample. For each healthy and tumoral tissue two slides with the same type of samples were prepared.

RESULTS

The LIAF spectra of the healthy and tumoral tissues are presented in Figures 3-5 and are compared in terms of peak intensity (*I*), Area (*A*) under fluorescence spectral distribution curve and ratio between the intensity and the corresponding full width at half maximum (*I/FWHM*) signal.

In case I, two healthy tissues of different thicknesses, 20 µm and 30 µm, were compared. Figures 3A and 3B presents the LIAF spectra of the three spots from the duplicate samples and Figure 3C shows the corresponding averaged spectra. The peaks of LIAF spectra are placed between 456 – 462 nm for the 30-µm tissue thickness and between 460-463 nm for the 20-µm tissue thickness. In Figures 3A and 3B are observed variations in peak intensity, area and I/FWHM and approximately the same values for FWHM. These variations are, dominantly and most probably, due to different concentrations of fluorophores in the samples. The comparison between the averaged spectra of 20 µm and 30 µm shows 11.4% autofluorescence intensity, a 9.5 % area and a 14.4% I/FWHM increase in 30 µm with respect to 20 µm case.

Figure 3. Case I. LIAF intensity for healthy tissue for different thickness: A) 20 µm and B) 30 µm; collected in three spots of the duplicate samples and the averaged spectrum; C) LIAF intensity of the averaged spectra from the 20 µm and 30 µm thickness samples.

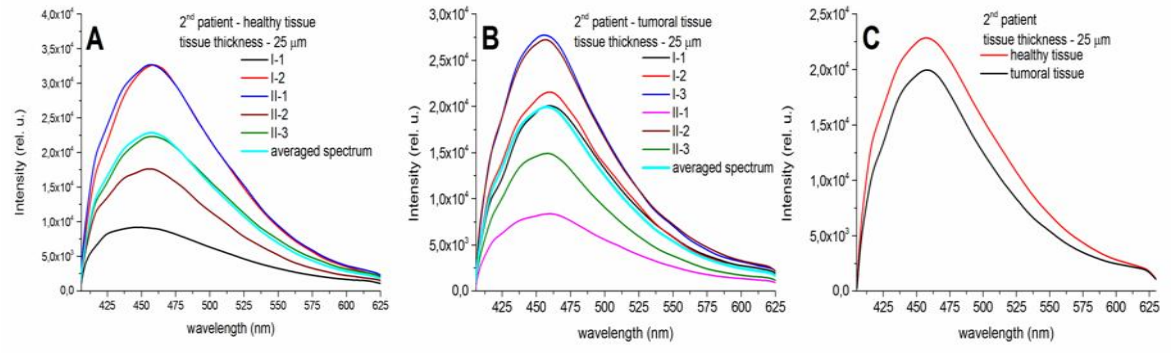
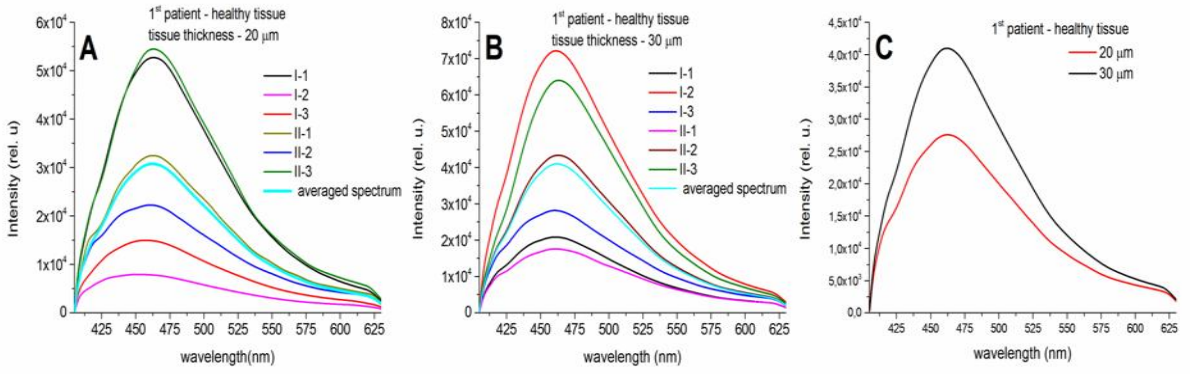


Figure 4. Case II. LIAF intensity for 25 µm tissue thickness: A) healthy and B) tumoral; collected in three spots of the duplicate samples and the averaged spectrum; C) LIAF intensity of the averaged spectra from the healthy and tumoral samples.



In case II, one healthy and one tumoral tissue of 25-µm thickness were analyzed. Figures 4A and 4B present the LIAF spectra of the three spots from the duplicate samples and the corresponding averaged spectrum (Figure 4C). The peaks of LIAF spectra are placed between 445 – 462 nm for healthy tissue and between 456 – 459 nm for tumoral tissue. In Figures 4A and 4B, are observed variations in peak intensity,

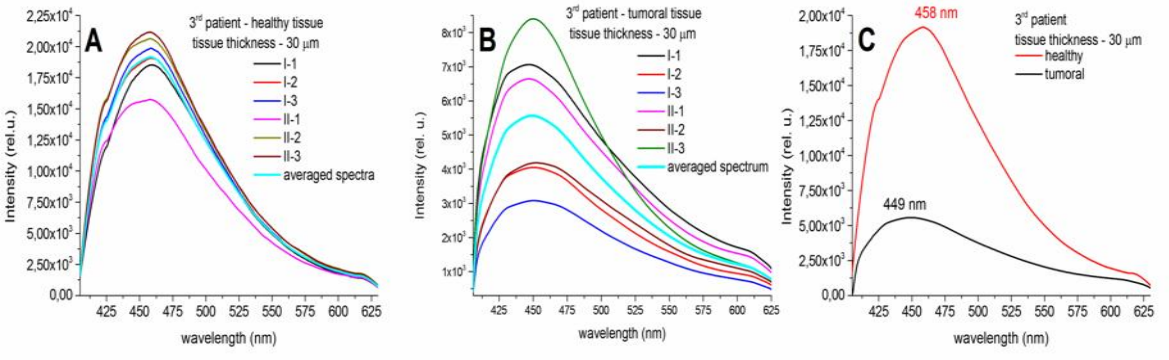
area and I/FWHM and approximately the same values for FWHM.

In general, the maximum value of LIAF's intensity is smaller for the cancerous tissue (with 16.7%) than the healthy one. The area and the I/FWHM ratio of LIAF for cancerous tissue is also smaller than for healthy tissue (with 17.1% and 4.7%).

In case III, one healthy and one tumoral tissue of 30-µm thickness were analyzed. Figures 5A and 5B present the LIAF spectra of the three spots from the duplicate samples and the corresponding averaged spectrum (Figure 5C). The peaks of LIAF spectra are located between 457 – 458 nm for healthy tissue and between 456 – 459 nm for tumoral tissue. In Fig. 5A and 5B, are observed variations in peak intensity, area and I/FWHM, but smaller than those from case II, and approximately the same values for FWHM.

In Figure 5C, the maximum value of LIAF's intensity is smaller for the cancerous tissue (with 71%) than the healthy one. The area of LIAF for cancerous tissue is also smaller than healthy tissue (with 66.7%). At the same time, maximum spectrum for tumor sample is shifted to blue with nine nm (449-458 nm) than healthy tissue and the I/FWHM ratio decreases with 74.2% when comparing the healthy tissue with the tumoral one.

Figure 5. Case III. LIAF intensity for 30 µm tissue thickness: A) healthy and B) tumoral; collected in three spots of the duplicate samples and the averaged spectrum; C) LIAF intensity of the averaged spectra from the healthy and tumoral samples



The computed parameters based on the experimental data extracted from LIAF spectra for the 2nd and 3rd patient healthy and tumoral tissues are shown in Table 1.

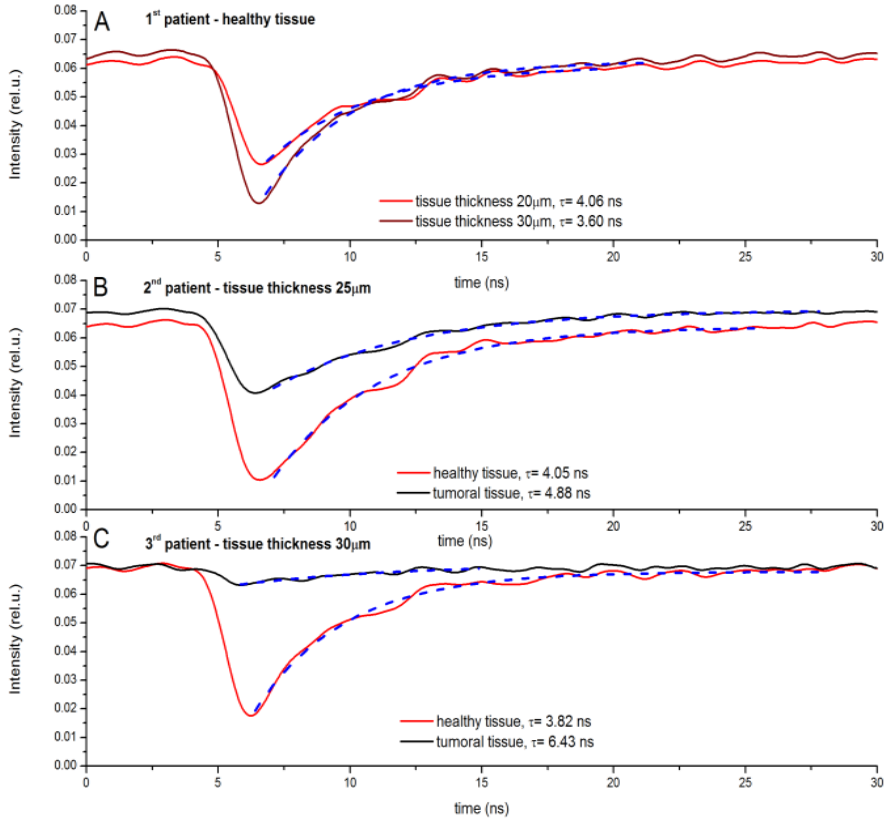
The results indicate that values of LIAF's parameters (wavelength, intensity, area, I/FWHM) are helpful and complementary diagnostic tools in detection of laryngeal carcinoma. These variations are due to the different concentrations and distribution of fluorophores in the samples.

Table 1. Parameters extracted from LIAF spectra for the 2nd and 3rd patient healthy and tumoral tissues (rel. u. are different from one column to the other; the same values of rel.u. are used for different patients)

Patient	Tissue	λ_{peak} (nm)	I_{max} (rel. u.)	FWHM (nm)	Area (nm x rel.u.)	I/ FWHM
2nd	Healthy	457	2.29×10^4	107.48	2.58×10^6	212.6
	Tumoral	457	2×10^4	98.5	2.13×10^6	202.5
3rd	Healthy	458	1.92×10^4	101.69	2.07×10^6	188.5
	Tumoral	449	5.56×10^4	114.6	6.87×10^6	48.56

Regarding LIAF's life-time (Figure 6), performed measurements show that the life-time of the healthy tissue decreases with thickness increasing, both for first patient case (Figure 6A) and for the second and third patients (Figures 6B and C, respectively). In Figure 6A, the life-time value decreases from 4.06 ns for the tissue of 20 µm thickness to 3.6 ns for that of 30 µm thickness, both values being obtained by averaging 4 samplings.

Figure 6. LIAF's life-time. A) 1st patient, healthy tissue at two thickness, 20 and 30 µm; B) 2nd patient, healthy and tumoral tissues at 25 µm tissue thickness; C) 3rd patient, healthy and tumoral tissues at 30 µm tissue thickness; blue dash line indicates the exponential decay fit function whose time constant τ represents life-time of the analyzed process.



It is to mention that the life-time values of healthy tissue for second and third patients are similar with those for the first patient considered for the same tissue thickness. For both patients exhibiting developed malignant tumor, the life-time values were higher for the cancerous tissues than the healthy samples (4.88 vs 4.05 ns for second patient; 6.43 vs 3.82 ns for third patient) as we see in Figures 6B and C.

DISCUSSION

The prognosis for patients with laryngeal cancer is significantly better with early detection of malignant lesions. Therefore, otolaryngologists look for supplementary techniques, as laser-induced autofluorescence, which may improve accuracy of real time cancer detection.

Malzahn et al. [9] have demonstrated high sensitivity of autofluorescence endoscopy in the diagnosis of early laryngeal cancer compared with light endoscopy alone. In the study of Zargi et al. [10] the sensitivity and specificity of described method were 86.9% and 82.8%.

The aim of the presented study was to analyze the applicability of laser-induced autofluorescence measurements to the differentiation of the changes

in the cancerous and healthy tissue of the larynx. For the measurements, it was used a laser diode emitting pulses of picosecond duration and λ = 375 nm. The chosen wavelength was found to excite the endogenous fluorophores: tyrosine (max. emission at 310 nm), tryptophan (max. emission at 340 nm), oxidized form of nicotinamide adenine dinucleotide (NAD+) (max. emission at 460 nm), the collagen cross-links (max. emission at 420–460 nm), the reduced form of nicotinamide adenine dinucleotide (NADH) (max. emission at 460nm), and the oxidized form of flavin adenine dinucleotide (FAD) (max. emission at 520 nm) [11]. The levels of the mentioned fluorophores, especially NADH and FAD, provide a strong emission signal and are significantly altered in cancerous cells, enabling the differentiation of the healthy and neoplastic tissues by autofluorescence measurements [12, 13, 14].

In both cases of the study, the LIAF's intensities of the cancerous tissue samples are lower in comparison with the adjacent healthy tissue samples. These results are in accordance with other studies where the decrease of intensity may be a consequence of reduced concentration of oxidized flavoproteins, the breakdown of collagen cross-links, the thickening of the cancerous tissue or the blood absorption (neovascularisation, inflammation)[15,16]. Neoplastic cells infiltrating submucosa express matrix metalloproteinases activity, which participate in breaking collagen cross-links [17].

We also observed in both cases that the area of LIAF for cancerous tissue is smaller than healthy tissue, the value of the ratio Intensity/ Full width at half maximum (I/FWHM) decreases in cancerous tissue sample and maximum spectrum for tumor sample is shifted towards blue. As for LIAF's life-time, in both cases the values were higher for cancerous tissues than healthy samples. Measurement of these parameters gives to our study originality, as well as the experimental setup.

Our results indicate that LIAF's parameters values (intensity, area, I/FWHM, life-time) are potentially a very helpful, complementary, diagnostic tool in detection of laryngeal carcinoma. The clinical prospective research on a great number of patients should be performed to establish utility of these measurements in everyday practice.

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