Optical Spectroscopic Methods to Discriminate in-Vitro Hodgkin Cancerous and Normal Tissues

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ABSTRACT— Simultaneous laser induced-breakdown spectroscopy (LIBS) and acoustic response techniques as well as Laser induced fluorescence (LIF) are applied to investigate the abnormal lymph tissues due to Hodgkin disease. The spectral shift in the emissive fluorescence of the cancerous tissues has been observed respect to the normal ones. Regarding LIBS, the concentrations of Ca and Na trace elements have been identified to be higher in the cancerous samples. In addition, the acoustic response of cancerous tissues has been elevated against healthy ones. The distinct differences in the spectra are taken into account for early and the rapid identification and diagnosis.

KEYWORDS: LIF, LIBS, Acoustic response, Hodgkin disease, Cancerous tissues, Rhodamine 6G, Spectral shift.

I. INTRODUCTION

Cancer is known as one of the major cause of gradual death. The most important factors in improving the survival rate are as follow: reliable diagnosis for early detection, early treatment, and follow up after treatment. Hence, there is a profound clinical need for diagnostic tools to achieve these goals [1].

Cancerous lymphomas are a relatively common type of malignancy in Middle East. Among all the cancers in Iran, lymphoma finds high abundance such that after esophagus cancer, it demonstrates an increasing prevalence. Because of the advances in the management of such kind of cancer and lack of any defined prophylaxis, the diagnosis at the initial stages of occurrence could be a vital importance.

Lymphoma considered as the most common blood cancer. The two main forms of lymphoma are Hodgkin lymphoma (HL) and non-Hodgkin lymphoma (NHL). Lymphoma occurs when lymphocytes, a type of white blood cell, abnormally grow. The body carries a couple of main types of lymphocytes that can develop into lymphomas: B-lymphocytes (B-cells) and T-lymphocytes (T-cells). At progressive stages, the cancerous lymphocytes migrate to the other organs, including the lymph nodes, spleen, bone marrow and blood. These can accumulate to form tumors. Non-Hodgkin lymphoma is the most common abnormal proliferation of the lymph as a part of the immune system. Hodgkin lymphoma is not as common as NHL while HL type may be successfully cured according to current treatment choices **[2].**

Recently, the application of advanced technologies, such as single cell polymerase chain reaction (PCR) and gene expression array analysis to study the Hodgkin's disease have yielded novel insights into the pathogenesis of the tumor. In addition, the Hodgkin's tumors harbour the Epstein-Barr virus (EBV) whose genome is monoclonal. The EBV virus contributes to the development of Hodgkin's disease in some cases [3].

Pre-neoplastic and malignant diseases are accompanied by local metabolic and architectural changes at cellular and subcellular levels. Subsequently, this could

affect the optical properties such as the scattering, absorption and fluorescence properties of the tissue. Therefore, optical spectroscopy could reveal functional information to identify focal (pre-) cancerous lesions. Furthermore, the optical methods offer several significant advantages over the routine clinical imaging methods. In fact, laser as a non-ionizing coherent radiation, is utilized to discriminate the optical properties of soft tissues [1].

Fluorescence spectroscopy analyzes the fluorescence emissions from the sample, usually by means of ultraviolet light to excite the molecules of certain compounds. The absorbed photons in the molecular transition reemit at longer wavelengths due to Stoke shift. Steady state and time-resolved LIF spectroscopy are taken into account as a renowned characterization method in biological studies [4]-[10].

Laser-induced breakdown spectroscopy (LIBS) relies on the atomic emissions which uses a highly energetic laser shot as a coherent source. The laser is focused on the sample to form plasma after atomizing the material. In principle, LIBS analyzes any matter regardless of its physical state i.e. solid, liquid or gas. This is upgraded as a popular and promising technique for the atomic analysis of trace elements as well as major constituents of biomaterials. It can identify the trace element concentration as low as ppm scale [11].

Furthermore, Photoacoustic signals are employed as another diagnostic tool for the cancer tissues that has been developed accompanying several imaging protocols both in vivo and in vitro during the last decades [12]. Photoacoustic spectroscopy mainly deals with the acoustic response following the laser absorption. Here, the shock waves due to laser induced plasma generation exhibit significant contribution.

In this work, the interactive combination of LIF, LIB and acoustic response are employed to diagnose the lymph malignancy quantitatively, particularly based on the distinct spectral differences. First, the spectral shifts of emissive spectra of cancerous tissues are investigated against normal ones after Rd6G staining. Then, the trace elements of two distinct types of tissues were assessed by means of LIBS. Moreover, the acoustic signals of tissues have been simultaneously recorded during optical data measurement given by plasma on the target surface.

II. METHODS AND RESULTS

A. *Tissue sample preparation*

Healthy and Hodgkin cancerous in vitro samples were investigated accordingly. Tissue samples of different patients were fixed in the formalin after biopsy, and then sliced in small pieces of $0.5\times0.5\times2$ mm^3. Before taking LIF spectra, the samples were immersed in Rd6G dye solutions for 30 minutes, and then were dried and exposed to the laser beam. Afterwards, the subsequent fluorescence data acquisition was carried out.

B. *Rhodamine 6G*

Rhodamine 6G is a highly fluorescent fluorophore from Rhodamine dye family with chemical formula of $C_{27}H_{29}ClN_2O_3$.

Table 1 Some optical properties of Rd6G.

Chemical formula	$C_{27}H_{29}ClN_2O_3$
Appearance	Red (powder)
Mass	479.02 g/mol
Density	1.26 g/cm^3
Absorption peak wavelength (in ethanol)	530 nm
Emission peak wavelength (in ethanol)	556 nm
Quantum efficiency	0.95

C. *Laser- induced fluorescence*

There are several endogenous fluorophores in the tissues that generate autofluorescence, such as keratin, melanin, nicotin amide adenine dinucleotide (NADH), porphyrin, and flavin adenine dinucleotide (FAD). Such macromolecules have been used for tissue visualization and cancer diagnosis. Various stages of tumor growth are correlated with

metabolic activity, concentration and spatial distribution of endogenous fluorophores, which are reflected in autofluorescence spectra. Those are prone for diagnostic purposes [5].

Although many of the studies show promising results for NADH and FAD autofluorescence in tumor differentiation, these fluorophores still burden some inherent limitations. Since the absorption and emission peaks of NADH (340 and 460nm, respectively) and FAD (450 and 520nm, respectively) lie in the visible spectrum, thus experiments may face difficulties in deep- tissue imaging due to short tissue penetration. In addition, these studies are unable to discriminate different types of cancer. Furthermore, NADH and FAD fluorescence signal ought to compete with numerous other fluorophores' absorption and scattering events. The restrictions induce inaccuracy, estimating the individual contributions of each fluorophore in the combined spectral acquisition [5].

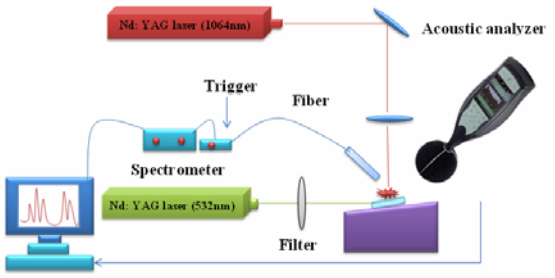

Fig. 1 Experimental setup of LIBS, LIF and acoustic analyzer using a couple of lasers: A CW laser for LIF and Q-switch pulsed laser for LIBS.

In order to overcome the above-mentioned drawbacks leading to enhance the detection specificity, the fluorophores were replaced by exogenous agents. In particular, near-infrared (NIR) organic dyes have shown great potential for fluorescence imaging of cancer tissues, owing to the low autofluorescence undertaking deep light penetration in tissues within the NIR therapeutic window. Currently, the readily available exogenous class of NIR dye compounds such as phthalocyanine, hematoporphyrin and Indocyanine green (ICG) [13] are being used within the window interval 600-1100 nm, for in vitro and in vivo cases.

Here, we have utilized Rd6G to investigate the spectral shift differentiation between normal and cancerous tissues. A CW Nd:YAG laser (100 mW) with SHG at 532 nm, is employed as a coherent optical source in LIF experiments. Fig 1 illustrates the schematic arrangement of the experiment.

The R6G is employed to enhance the fluorescence process leading to a better spectral resolution. Indeed, the Rd6G absorption peak locates at the Nd: YAG laser' second harmonic. The fluorescence emissions were collected by AvaSpec spectrometer (2048 pixel CCD detector array and 0.4 nm spectral resolutions) while the laser amplitude has been suppressed using interference filter. Fig ٢ depicts the averaged fluorescence emission of normal and cancerous tissues.

A significant red shift ranging 8-10 nm took place due to the fluorescence emission of cancerous host respect to that of healthy tissues for a certain dye solution (1mM).

The spectral shift accounts for the strongly reabsorption events in hybrid medium. There are a couple of mechanisms which give rise to high reabsorption. At first, longer random walk of scattered photons due to higher natural abundance of the nanoparticles in medium, secondly, a large Forster constant which both lead to the high reabsorption and eventually the sensible spectral shift.

Furthermore, the LIF spectra illustrate a significant red shift in terms of dye concentrations [14]. The relative red shift of cancerous tumor respect to healthy one enhances with dye concentration.

It is believed that cancerous tumors contain a large number of microcavities that enable them to capture more dye molecules inside. The tendency of the dye molecule accumulation in the cancerous tissues is mainly due to the high permeability of the damaged cells membrane respect to the healthy ones. In fact, the higher

dye concentrations give rise to the larger red spectral shift [14].

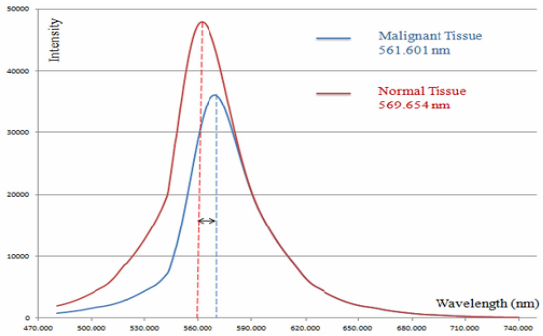

Fig. 2 LIF spectra of Hodgkin cancerous and healthy tissues, indicating a significant red shift (~ 8nm) for cancerous tissues.

D. *Laser induced breakdown spectroscopy*

In the case of LIBS, second harmonic generation of Q-switched Nd: YAG laser (532nm) with 100 mJ/pulse energy was employed as the source of excitation. Fig. 1 depicts the experimental setup.

Regarding LIBS technique, the characteristic emissions of the constituents in the cancerous and normal tissues are statistically distinguished. This arises from relative changes in the concentration of elements, particularly calcium and sodium trace [15]-[18].

Ca I characteristic lines at 422.872 nm, 396.988 nm, 393.405 nm for the healthy and cancerous tissues are illustrated in Fig 3. It indicates that the amplitude of the characteristic emissions attributing to the cancerous tissues is significantly enhanced, mainly due to the elevation in population of Ca content. Similarly, sodium characteristic line at 589.59 nm taken from cancerous samples is notably higher than healthy ones as shown in Fig. 4.

Kumar *et al.* [15] observed the intensity of the Ca, Al lines at 394.4 nm and 396.15, respectively in dog to be stronger in malignant tissue as well. Nasiadek et al. [19] has found a significant increase in Ca^{2+} concentration and also the rise in Mg level in cancerous uterine comparing to non-neoplastic tissues. The same authors reported a significant increase in Mg and Mg/Ca ratio in uterine myoma, too. The rise in the calcium content could be due to the enormous uncontrolled divisions of the cancerous cells.

Ronald *et al.* [20] have analyzed MRI images to investigate the amount of sodium deposited in breast cancer tissues. They have found that the Na abundance in the lesions takes higher values than normal ones. El-Hussein et al. [***16***] have shown the distinct differences of calcium and magnesium spectral lines' intensities in LIBS spectra of non-neoplastic, malignant breast and colorectal tissue samples as well.

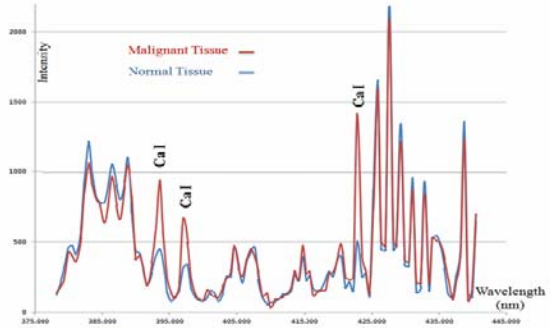

Fig. 3 The LIBS averaged spectra of Hodgkin cancerous and healthy tissues, which exhibits a notable difference in Ca I characteristic emission intensities.

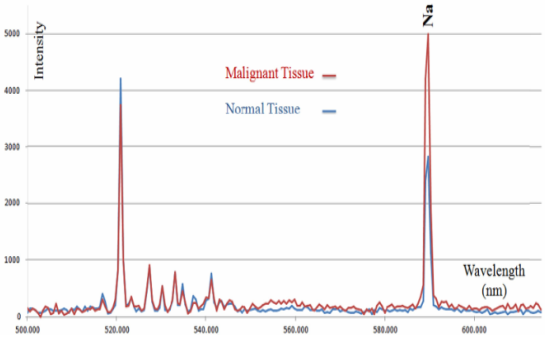

Fig. 4 The LIBS averaged spectra of Hodgkin cancerous and healthy tissues, which exhibit a notable difference in Na I characteristic emission intensities.

Therefore, LIF and LIBS techniques offer valuable data taken from corresponding spectra. These are sufficient optical sets of data to discriminate the tissues by means of dual findings i.e. spectral shift in LIF spectra accompanying significant amplitude alteration

due to Ca I and Na I characteristic lines during LIBS of cancerous tissues.

E. *Acoustic signals*

On the other hand, the acoustic response arises from the laser induced plasma as an evidence to verify the diagnostic data taken from the other techniques. A 2260 Brüel and Kjaer sound level meter and acoustic analyzer, 20Hz–20kHz with 45Hz resolution, was employed to plot the acoustic spectra.

Fig. 5 depicts the cancerous tissue that undergoes a notable rise in the acoustic signal respect to the healthy one. Relative higher Ca population and tissue porosity are evidences that attest the change of acoustic impedance to such an extent that the acoustic signals drastically differ over 20Hz– 20kHz in cancerous tissues. The evidence of numerous microcavities of cancerous samples may significantly magnify this event. The proliferation of cancerous cells respect to the normal ones make the shock waves trap in the mirocavities to give out higher dB level.

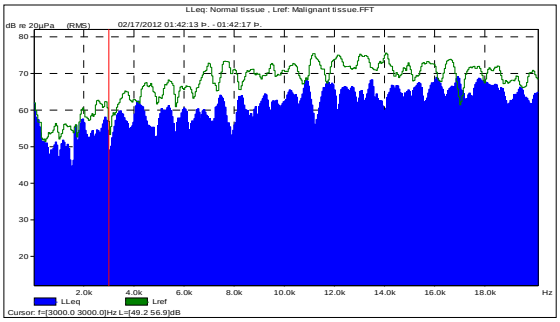

Fig. 5 The averaged acoustic signals of healthy and Hodgkin lymphoma cancerous tissues, which display the higher signals in the cancerous tissues.

Three interactive spectroscopic techniques perform to diagnose the cancerous tissues. They verify the likelihood of occurrence of the cancerous tumors based on the detection of the acoustic signals, determination of the trace elements and the spectral shift measurements in the same time. The data taken from the methods follow an algorithm to enhance the reliability of the optical cancer diagnosis according to a typical flow chart similar to Fig. 6.

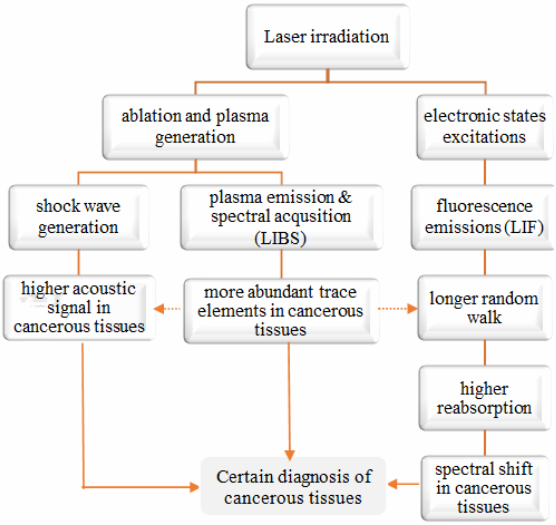

Fig. 6 The flow chart indicates the link of the three techniques leading to a reliable diagnosis of the cancerous tumors.

Fig. 7 summarizes the significant findings and the indications.

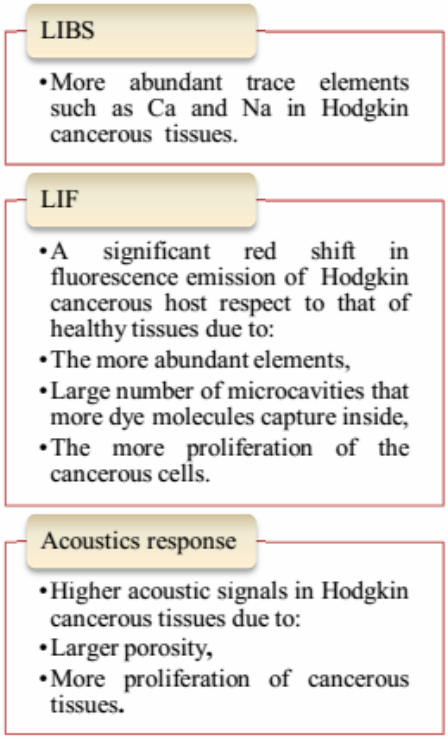

Fig. **7** The flow chart explaining the significant findings and indications of the current spectroscopic methods.

III. CONCLUSION

Optical emission spectra taken from LIBS exhibit that the abundance of trace elements such as Ca I, Na I are significantly higher in cancerous tissues than those appear in the normal tissues. Furthermore, healthy and cancerous of tissues were dyed in R6G solutions, and subsequently the fluorescence spectra were obtained. Spectral shift in LIF spectra due to the cancerous tissues against the normal ones have been lucidly recorded. In addition, the acoustic response spectra of shock waves after the laser- induced plasma formation demonstrate the stronger signals in cancerous tissues than normal ones.

The preliminary results for in- vitro investigation attest that the link of three methods, with bio-compatible fluorophores such as ICG (Indocyanine green) promises a novel in- vivo diagnostic method for early and reliable diagnosis of lymphoma.

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