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Optical Fine Needle Biopsy in Hepatocellular Carcinoma Mouse Model

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ABSTRACT

The paper describes the results of experimental studies using custom developed optical biopsy system for diagnostics *in vivo* during the procedure of fine needle aspiration biopsy. Experimental studies were conducted in laboratory mice with inoculated hepatocellular carcinoma. The measurements were carried out using fluorescence spectroscopy and diffuse reflectance spectroscopy methods to reveal metabolic and morphological changes in tissues. The results show that the developed approach is sensitive for cancer detection. Quantified differences in the maximum of fluorescence spectra and diffuse reflectance spectra between tumor and normal tissues were demonstrated and approved with morphological analysis.

Keywords: optical biopsy, liver cancer, hepatocellular carcinoma, fine needle aspiration biopsy, fluorescence spectroscopy, diffuse reflectance spectroscopy

1. INTRODUCTION

According to the statistic data of World Health Organization, cancer is the second leading cause of death in the world¹. One of the most difficult types of cancer pathology is liver cancer. Primary liver cancer is the sixth most common in the world and the fourth most lethal among other types of malignant neoplasms, while there is a tendency to increase the number of reported cases of this disease². It is also known that the liver is the organ that most often has metastases caused by malignant neoplasms in other organs³. One of the factors that improves the prognosis in patients with liver tumors is the possibility of earlier diagnosis to improve the effectiveness of treatment. Despite the rapid technical and methodological development of medicine, the diagnosis of liver cancer (especially early one) is associated with certain difficulties.

Currently, histological and cytological examinations remains required for liver cancer diagnosis. Tissues samples are usually obtained from several areas of suspicious neoplasm during fine needle aspiration biopsy (FNAB) procedure⁴. FNAB allows for obtaining material using fine needle with a normal or cutting edge without significant disruption of tissues. However, this method requires 1-2 weeks waiting for the results, while the surgeons are interested in getting information about the state of the tissues of the affected organ during this period to plan further treatment. Therefore, the search for new methods to determine tissues state in real-time remains relevant. Another problem is the risk of acquiring non diagnostic biopsy samples. While the examination itself has high sensitivity and specificity, some factors can lead to obtaining uninformative material. To introduce a biopsy needle into the tumor, FNAB is performed under ultrasound, CT and MRI control. However, involuntary movements of a patient may cause a shift of tissues in the area of interest. The size of the affected area can be too small; so ultrasound, CT and MRI can poorly visualize it.

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The error caused by an incorrectly defined area of interest as well as cancer heterogeneity lead to 15-29 % of undiagnosed samples⁵⁻⁷. The uninformative result often requires performing another one procedure, which is an increased risk to patients' health.

One of possible solutions is application of optical biopsy. This is rapidly developing area of biophotonics methods, which does not require to remove a tissue sample from investigated area and to wait several days for results. Application of spectroscopic methods can improve needle targeting and provide real-time information on various parameters of morphology and metabolism of biological tissues *in vivo*. Combined application of these methods seems promising for providing complex information about interconnected parameters of tissues state.

One of the methods widely used to study the metabolic activity of cells in healthy and pathological tissues is fluorescence spectroscopy (FS). This method is based on excitation of fluorescence in biological tissue by monochromatic radiation of the near ultraviolet or visible range and registration of a spectrum obtained for further analysis. Fluorescence spectroscopy (FS) allows detecting metabolic changes by comparing intensities depending on the content of specific fluorophores. Mitochondrial coenzymes nicotinamide adenine dinucleotide (NAD) and flavin adenine dinucleotide (FAD) have autofluorescence spectra, which changes caused by various pathologies can be registered *in vivo*^{8,9}. Both coenzymes are involved in synthesis of adenosine triphosphate that provide energy for other biochemical reactions. These substances undergo oxidation and reduction reactions, and reduced NAD (NADH) and oxidized FAD demonstrate the greatest contribution to tissue fluorescence spectra. Changes in the intensity of NADH and FAD fluorescence are associated with pathological processes, including oncological ones¹⁰.

Another widespread spectroscopic method is diffuse reflectance spectroscopy (DRS). DRS is used to evaluate changes at the cellular and tissue levels by analyzing the absorption of light by different tissue chromophores¹¹. In particular, this method is sensitive to the blood content in tissues and its oxygen saturation (oxygenated, deoxygenated). The data obtained using DRS channel can also be used to compensate the effect of light absorption on fluorescence spectra in case of high blood content¹².

The development of optical biopsy for malignant neoplasms detection requires reliable and reproducible optical measurements of normal and pathological tissues. For further implementation of the technology in clinical practice, it can be tested using test objects, cells cultures and tissue slices or performing measurements in laboratory animals. In this work, liver cancer was modeled in laboratory mice to imitate previously developed methodology of comparison of data obtained from intact liver and neoplasm tissues¹³.

Therefore, the aim of this work was to study the possibilities of liver cancer detection *in vivo* using custom developed optical biopsy system for FNAB.

2. MATERIALS AND METHODS

The measurements were carried out using custom developed optical biopsy setup (Fig. 1) implementing FS and DRS channels^{14,15}. The LED with a wavelength of 365 nm and a laser diode with a wavelength of 450 nm were used to excite NADH and FAD autofluorescence. The output power at the end of the optical probe for the sources used was no more than 1.5 mW and 3.5 mW, respectively¹⁶. CCD spectrometer FLAME-T-VIS-NIR-ES (Ocean Optics, USA) was used to record fluorescence and diffuse reflectance spectra in the range of 350-1000 nm. To attenuate the backscattered radiation from monochromatic sources FGL400 and FGL495 filters (Thorlabs, Inc., USA) with cutoff wavelengths of 400 nm and 495 nm respectively were introduced in receiving channel before the spectrometer. DRS channel included broadband tungsten halogen lamp HL-2000-FHSA (Ocean Optics, USA) with a range of 360-2400 nm.

Delivery of radiation from the sources and collection of secondary optical radiation from biological tissue was carried out using a specially designed fine-needle optical probe. The probe with outer diameter of 1 mm was designed to be compatible with standard 17.5 G fine needle. The probe contains 10 optical fibers. The central fiber (200 μm) is used for collecting radiation and transmitting it to the spectrometer. Another 9 fibers (100 μm) placed around the central ones (3 fibers for each source) are used to create uniform illumination during measurements. The end of the fiber probe has a 20° bevel optimized for the collection of maximum back reflected light. The numerical aperture of the fibers is 0.22.

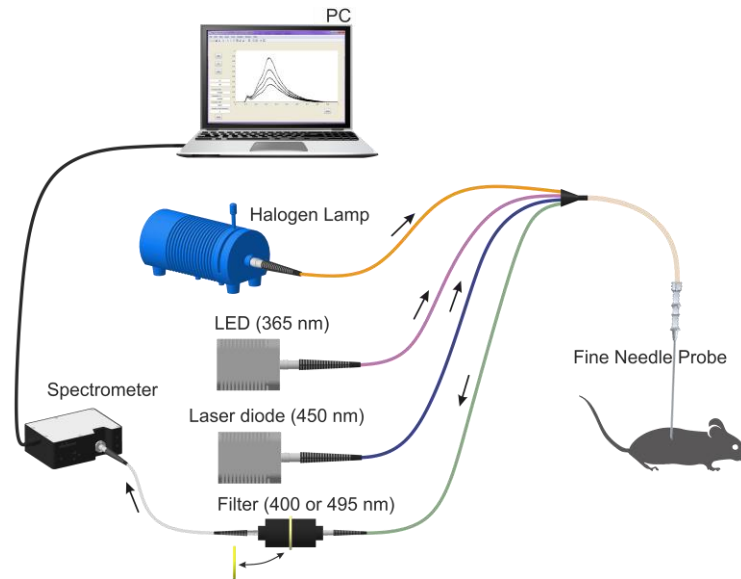


Figure 1. The scheme of experimental optical biopsy system.

Experimental studies were conducted in BDF (C57Bl6xDBA) laboratory mice (male, $n=3$, 20 weeks). Experimental studies were conducted in accordance with the principles of Good Laboratory Practice¹⁷. The studies were approved by the Ethics Committee of Orel State University named after I.S. Turgenev (protocol No. 12 of 06.09.2018). The animals were kept in separate rooms animals under controlled environmental conditions (20-26°C, relative humidity of 30-70%). The animals were fed *ad libitum* with a balanced granulated feed for rodents in accordance with daily physiological needs. Upon arrival at the vivarium, the veterinarian gave an initial assessment of the animals' condition and performed daily inspections during the two-week quarantine.

The H33 mouse hepatocellular carcinoma culture was implanted by injection in a volume of 100 μl (50000 cells/ μl) per mouse. The mice were anesthetized with Zoletil 160-190 μl /mouse. The surgery included fixing the animal in the back position, preparing for the opening of the abdominal cavity, longitudinal pararectal laparotomy and opening the abdominal cavity in the liver area. The cells were implanted by injecting a suspension of cells through a syringe into the medial lobe of the liver. During 2 months after the surgery, an examination of liver area was performed every three days. Changes in the weight and size of each animal were being recorded. When the weight increased by more than 15% of the normal weight, a daily examination and observation of the animal's behavior was performed. In the case of deterioration of the animal condition, the experimental measurements were performed the following day.

Before the measurements, the animal was anesthetized with Zoletil in the standard dosage. The animal was fixed on a special platform in the back position. A laparotomy was performed and the entire abdominal cavity was opened. The general state of the abdominal cavity and the presence of tumor in the liver and other organs were assessed. Series of optical measurements (20 fluorescence spectra for each source, 100 diffuse reflectance spectra) were carried out in several areas of intact liver and tumor.

3. RESULTS AND DISCUSSION

The fluorescence and diffuse reflectance spectra obtained were normalized by the maximum intensity measured and averaged for further analysis. The diffuse reflectance spectra were previously normalized by the spectra of the halogen lamp recorded from spectralon. The analyzed parameters were maximums of fluorescence intensity and normalized diffuse reflectance intensities.

During the carcinogenesis process, two of three mice had multiple tumors developed in liver and peritoneum. Tumor cell inoculation in the third mice had led to the growth of the large solid tumor in liver (16x8 mm). First two mice demonstrated similar differences between liver and tumor spectra. Typical averaged spectra recorded in these cases are shown in Figure 2.

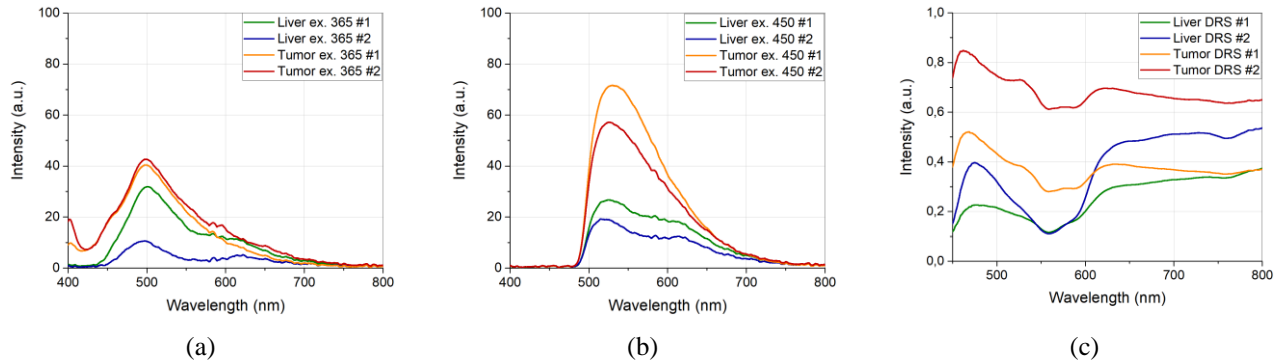


Figure 2. Typical averaged fluorescence (a, b) and diffuse reflectance (c) spectra of intact liver (green line) and tumor (red line) of mice 1 and 2.

It was observed that fluorescence intensity increased in tumor tissues compared with intact parenchyma. However, this result can be caused not only by atypical metabolic activity of malignant cells, but also by the changes in blood content. The fluorescence spectrum is a superposition of the contributions of a number of fluorophores (NADH, FAD, collagen, bilirubin, porphyrin, lipofuscin, etc.), which also depends on the presence of various absorbers, such as hemoglobin and bile. The spectra obtained by DRS channel demonstrated the general decrease of light absorption in tumor, especially in the range of 500-800 nm. The increased amount of blood can affect the values of absolute fluorescence intensities.

Both areas demonstrated the presence of absorption peaks at 560 and 760 nm associated with deoxyhemoglobin. The tumor spectra also have oxyhemoglobin peak of 580 nm, while deoxyhemoglobin peaks in liver has more expressed shape, which can be a sign of decreased oxygen saturation in intact liver parenchyma. It is associated with prevalent amount of venous blood caused by additional amount of venous blood brought by portal vein to the liver from other abdominal organs¹⁸. The presence of even small lesions can change liver blood flow and increases the proportion of arterial blood in their tissues¹⁹.

The typical spectra recorded in the mice with large tumor are demonstrated in Figure 3. It was noticed that diffuse reflectance spectra of this tumor has strongly pronounced peaks of oxyhemoglobin absorption at 540 and 580 nm. Another feature of obtained fluorescence spectra is significant content of porphyrins, in particular protoporphyrin IX, which has its fluorescence peak at 635-640 nm range. The accumulation of porphyrins can serve as another sign of active tumor development²⁰.

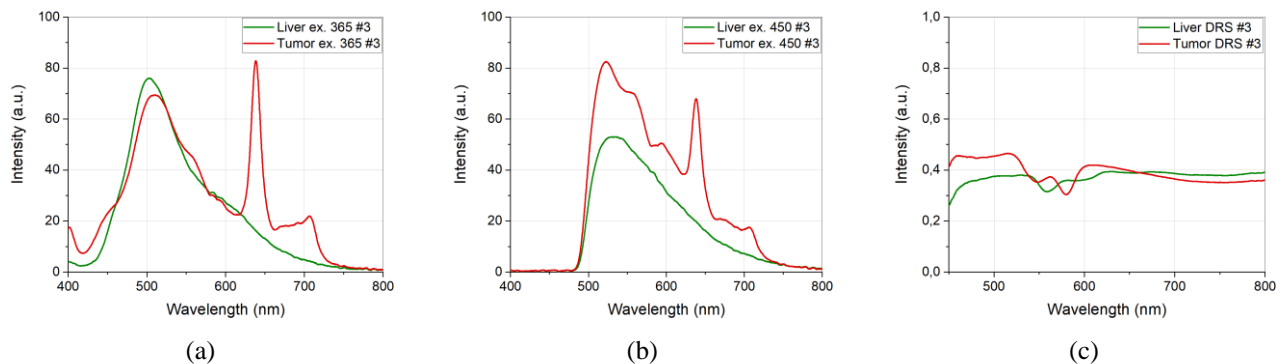


Figure 3. Typical averaged fluorescence (a – ex. 365 nm, b – ex. 450 nm) and diffuse reflectance (c) spectra of mouse 3 with the large tumor.

The studies were followed by the procedure of histological examination. The morphological picture of the studied tumor corresponds to the liver tissue with the presence of complexes of poorly differentiated adenocarcinoma (Fig. 4a). The tumor cells demonstrated high mitotic activity resulting in significant infiltrative growth not only in the liver parenchyma, but also intestinal loops and retroperitoneal space (Fig. 4b). The tumor was made of solid cluster of malignant cells.

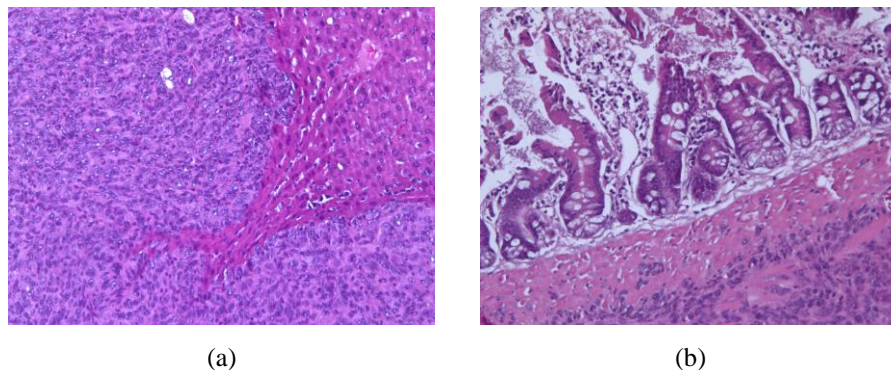


Figure 4. Histological examination of tumor tissues with haematoxylin and eosin staining: tumor node of liver at x10 (a), intestine at x20.

The results showed that FS results could indicate the metabolic changes induced by the growth of tumor. In its turn, DRS method provides the information about tissues blood supply, which is important sign because of blood supply anatomy and physiology in liver. The obtained spectra also indicate that the processes of carcinogenesis occurred individually in each mouse. Namely, we suppose that the tumor described in Figures 3 and 4 was at a later stage of its development. The size and degree of tumor distribution can affect the results as well. Diffuse reflectance spectra of large tumor node showed marks of more significant morphological changes, which required more oxygenated blood from arteries to grow.

4. CONCLUSION

The optical biopsy methods are promising to be applied in the clinical practice as they allow one to obtain valuable diagnostic information about tissue metabolism and morphology in real time, which can be important in cancer treatment. In particular, the results of preliminary studies of multimodal approach using FS and DRS methods during standard biopsy in laboratory animals demonstrate the possibility of differentiating between healthy and malignant tissues.

The results also show the necessity of further research in laboratory animals for more understanding of carcinogenesis phases itself and their influence on obtained data. These measurements together with other methods of testing of developed experimental setup will be taken into account when planning and conducting the stage of clinical studies. Thus, the proposed approach will provide the basis for collecting the database of spectral data and developing the automatic classifier for analysis of different stages and types of tumors for minimally invasive diagnostics procedures.

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