

Use of fluorescence spectroscopy for diagnosis of hypoxia and inflammatory processes in tissue

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In vivo laser-induced fluorescence spectroscopy (LIFS) is primarily used in oncology for the diagnosis of malignant tumors. This paper provides background for and describes experiments modeling nonmalignant local hypoxia and inflammation. LIFS techniques were used to assess the dynamics of induced fluorescence from endogenous porphyrins in the first case and the Photosens exogenous photosensitizer in the second case. In both cases, the fluorescence intensity was observed to be higher in the pathological area than in an intact area. This provides a strong impetus for taking a second look at the use of LIFS in oncology and also provides the foundation for a promising *in vivo* diagnosis method for ischemic hypoxia and inflammatory processes in areas other than oncology. © 2016 Optical Society of America.

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INTRODUCTION

Laser-induced fluorescence spectroscopy (LIFS) [1] has become a promising technique for noninvasive (*in vivo*) diagnosis of soft biological tissue. It has long been known that certain physiological reactions (and disruption of normal metabolism, especially that associated with pathological processes) affect the relative concentrations of various natural (endogenous) fluorophores. In biology and medicine, these fluorophores are studied using LIFS [2]. One important natural fluorophore of this type found in tissue is porphyrin [3]. Approximately 100 years ago, development of malignant neoplasms was found to be associated with the accumulation of porphyrins in tumor tissue [4,5]. In the late 20th century, this led to the development of photodynamic therapy (PDT) and fluorescence diagnosis of tumors, including intra-operational fluorescence-based navigation based on the ability of tumor cells to accumulate elevated concentrations of porphyrin, porphyrin derivatives, and other exogenous (produced elsewhere, e.g., intravenously) photoactive substances—photosensitizers [6,7].

However, the reason for the accumulation of porphyrin and other photosensitizers in tumors is not yet fully understood [3,8,9]. The basic hypothesis is that porphyrin is accumulated from the bloodstream by actively proliferating cancer cells with low pH [3,7,9]. However, there is reason to believe that all cells (not just cancer cells) accumulate porphyrin if they are in a state of chronic hypoxia [9,10]. The decrease in pH is not the only possible explanation for the additional accumulation of porphyrins in cells under hypoxia; another possibility is a direct increase in the porphyrin synthesis substrate succinyl CoA, which is a catabolic product of protein, fat, and carbohydrates [11]. It is formed via oxidative decarboxylation

of α -ketoglutaric acid and is then hydrolyzed, with conjugate transfer of the thioether bond to guanosine diphosphate (GDP), resulting in the formation of guanosine triphosphate (GTP). These are precisely the processes that may become more active and increase generation of succinyl-CoA during hypoxia.

It is well known that in most cases, the cells in malignant neoplasms become hypoxic as tumors grow [9]. This provides a strong impetus for taking another look at the capabilities and effectiveness of PDT and LIFS in oncology. The locations inhabited by the pool of actively dividing cancerous cells may not be the same locations inhabited by the hypoxic tumor cell pool. In this case, LIFS errors and reduced PDT effectiveness are unavoidable. Moreover, tumor growth, especially in Stages III–IV, is also often accompanied by ischemia and inflammation; however, we did not obtain any reliable experimental data on the evolution of fluorescence due to porphyrins or other photosensitizers under the influence of these nonmalignant processes, even though it is theoretically possible for porphyrins or other photosensitizers to accumulate under such conditions. For example, tissue damage typically causes inflammation, which leads to disturbed blood flow, and changes in the composition of blood and connective tissue in the form of alteration, exudation, and proliferation [12]. According to the literature, the processes of respiration and adenosine triphosphoric acid synthesis proceed more slowly in the inflammatory tissues, and the oxidation-reduction status of the cells is affected, along with the concentrations of fluorescent forms of endogenous fluorophores, such as NAD(P)H, flavoproteins, collagens, etc. [3]. It can therefore be assumed that inflammation and ischemia may also lead to increased accumulation of porphyrin and other photosensitizers in tissue.

This study came to be focused on an analysis of *in vivo* induced fluorescence of endogenous and exogenous fluorophores in tissue under ischemic conditions and under inflamed conditions. Unlike [10], which involved chronic hypoxia, this study looked at the accumulation of endogenous porphyrins in acute ischemic hypoxia modeled by mechanical occlusion of vessels. An experimental study of the accumulation of Photosens (an aluminum phthalocyanine-based exogenous photosensitizer) under model inflammatory conditions was also performed.

MATERIALS AND METHODS

In all experiments, the fluorescence was recorded using an LAKK-M laser diagnostic system [13] (Fig. 1). The laser excitation beam is delivered to the tissue under study and the fluorescence radiation is delivered back to the diagnostic system via a fiber-optic cable that is part of the LAKK-M system. The fluorescence is excited at wavelengths of 532 nm (solid-state laser) for experiments involving ischemia and 635 nm (semiconductor laser) for experiments involving inflammation. The porphyrin and aluminum phthalocyanine fluorescence was recorded on the 640–800 nm wavelength range. Typical fluorescence spectra at excitation wavelengths of 532 nm and 635 nm for tissues with excess porphyrin accumulation are

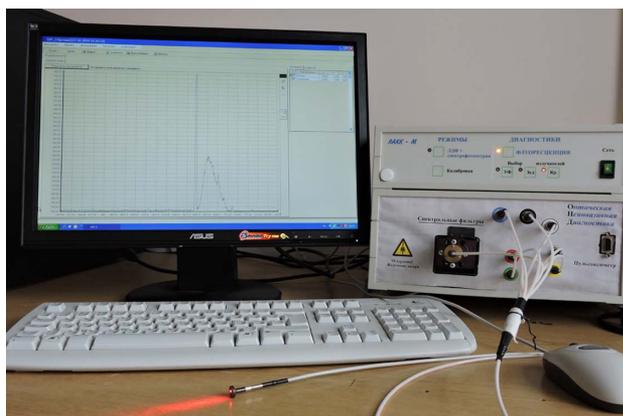
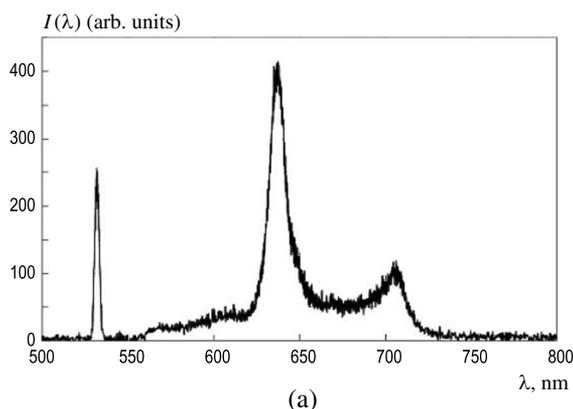


FIG. 1. LAKK-M diagnosis system and measurement procedure.



(a)

shown in Figs. 2(a) and 2(b). The peaks at wavelengths of 640 nm and 710 nm correspond to the fluorescence peaks for porphyrin.

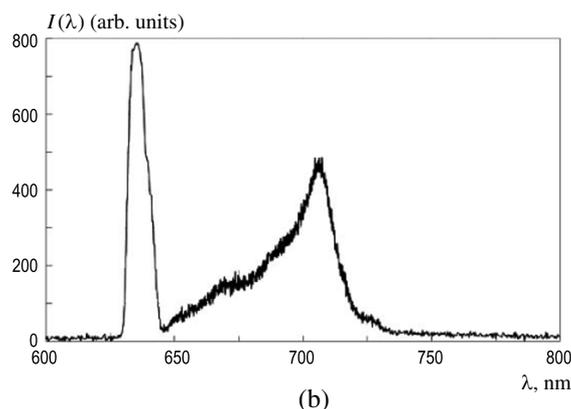
In order to compare the fluorescence spectra obtained under various conditions, the spectra were mathematically converted into tissue fluorophore content indices $\eta(\lambda_f)_{\lambda_e}$ (see [1]), where

$$\begin{aligned} \eta(\lambda_f)_{\lambda_e} &= I_f(\lambda_f) / (I_f(\lambda_f) + I_{\text{laser}}(\lambda_e)) \\ &= 1 / (1 + I_{\text{laser}}(\lambda_e) / I_f(\lambda_f)). \end{aligned} \quad (1)$$

where $I_f(\lambda_f)$ is the fluorescence signal intensity at the fluorescence wavelength λ_f , $I_{\text{laser}}(\lambda_e)$ is the recorded intensity of laser radiation back-scattered by the tissue at the fluorescence excitation wavelength λ_e , reduced by a factor of β ($\beta \approx 10^3$ [13]). This representation of the results enables the spectral data to be transformed to a compact range of values from 0 to 1 and enables for the optical density of the tissues to be taken into account via comparison with the back-scattered source signal [1].

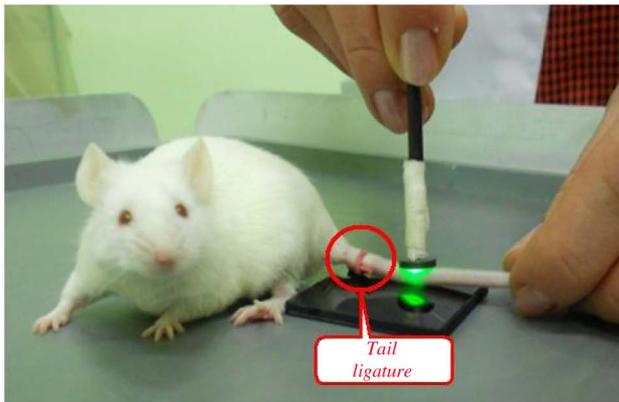
In the first series of experiments, ischemic hypoxia of tissue was modeled by applying a ligature (a cord that impedes blood circulation) to the tails of Wistar experimental mice ($N = 3$) for a period of up to 48 hours. The variation in the endogenous porphyrin fluorescence signal from the surface of the animal's tail skin was measured along the length of the tail [Fig. 3(a)] as ischemia and necrosis developed in the tissues.

In the second series of experiments, inflammation was induced in the haunch muscles of two groups of white non-linear mice ($N = 12$) by intramuscular injection of 0.25 mL of a suspension of microcapsules with a CaCO_3 core in a bioresorbable polyelectrolyte envelope. All mice were given intra-haunch injections of Photosens photosensitizer at a rate of 0.5 mg/kg [Fig. 3(b)]. In the first group of mice, the fluorescence of the Photosens photosensitizer in the injected haunch (relative to the intact, healthy haunch) was studied. In the second group of experimental animals, the extremity under study was amputated for visual inspection of the injection site 1, 3, 11, and 25 days after introduction of the microcapsules. These animals were first euthanized using a lethal dose of the anesthetic Uretan—200 mg/kg of body weight.



(b)

FIG. 2. Typical fluorescence spectra of biological tissue at excitation wavelength (a) 532 nm and (b) 635 nm.



(a)



(b)

FIG. 3. Procedure for (a) introduction of Photosens photosensitizer and (b) collection of fluorescence spectra.

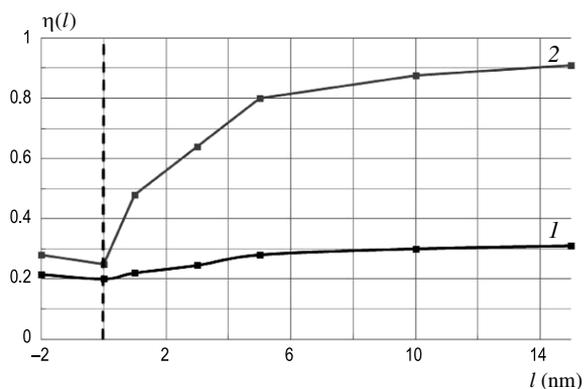


FIG. 4. Fluorescence signal intensity from tail skin prior to occlusion (I) and 24 hours after occlusion (2). $\eta(l)$ is the tissue fluorophore concentration index, l is the distance along the tail from the ligature, and the vertical dashed line indicates the point at which the ligature was applied.

All experimental studies were performed¹ in compliance with the principles of the Declaration of Helsinki on humane treatment of animals, as well as the humane principles set forth in the European Communities Directive (86/609/EC) and USSR Ministry of Health Order No. 755 of 12 August 1977, *On Measures for Further Improvement of Organizational Operations Involving Experimental Animals*.

RESULTS AND DISCUSSION

In the ischemic hypoxia model, fluorescence of endogenous porphyrins from the surface of the animal's tail was observed to gradually increase at wavelengths of 640–710 nm as ischemia developed. The indicator describing the porphyrin content of the mouse tail tissue for $\lambda_f = 640$ nm increased with distance from the occlusion along the tail, correlating with the amount of damage to the tail from ischemia (Fig. 4). In actual fact, theoretically, the concentration at which oxygen

¹The work involving the experimental animals was performed by biophysicist O. D. Smirnova.

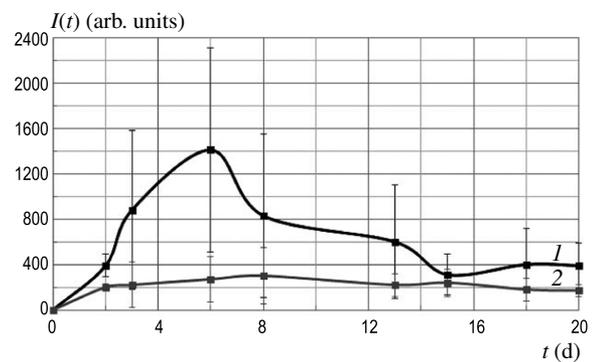


FIG. 5. Intensity of fluorescence signal as a function of time after introduction of nonencapsulated CaCO_3 cores (I —intact haunch, 2 —at injection site). $I(t)$ is the fluorescence intensity, and t is the time after introduction.

and glucose enter the cells should drop with increasing distance from the point of occlusion, and cell metabolites should be excreted at a lower rate. Thus, the level of ischemic hypoxia should increase with distance down the tail. The resulting LIFS data indicate that the level of tissue damage for porphyrins in acute ischemic hypoxia is well modeled via a method using the index $\eta(640)_{532}$. Porphyrins accumulate in the skin in higher concentrations under occlusive ischemia conditions than under normal circulatory conditions.

In a series of experiments designed to study fluorescence of an exogenous photosensitizer (Photosens) under conditions where inflammation was modeled by intramuscular introduction of micro-encapsulated calcium carbonate, the intensity of the fluorescence signal from the surface of the femur immediately above the inflamed spot was much higher than the value of this indicator for an intact femur (Fig. 5). This experiment showed the following characteristic changes in the fluorescence signal: 1 hour after introduction of the photosensitizer, the fluorescence signal increased, and it was observed to die out within 2–3 days, and then increase again (Fig. 6). This behavior presumably corresponds to the typical phases in the development of inflammation [12,14]. For example, the reduced fluorescence signal on days 2 and 3 may correspond

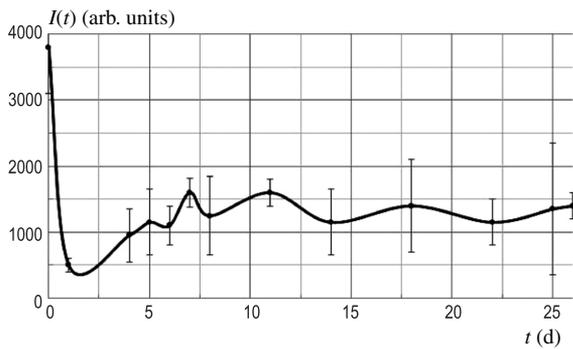


FIG. 6. Fluorescence signal intensity as a function of time for inflammation. $I(t)$ is the fluorescence intensity, and t is the time after introduction.

to the development of edema (exudate) during the first phase of inflammation, while the increase on days 6 and 7 corresponds to the end of the second, proliferative phase. Generally speaking, in all cases, the fluorescence signal intensity at the microcapsule injection site turned out to be higher than in the intact haunch; this was observed for three weeks.

This may have been due to the fact that the tissue was hypoxic and due to an inflammatory reaction to damage resulting from mechanical introduction of the preparation into the tissue, as demonstrated using the second group of experimental mice. Euthanasia of the experimental animals in the second group followed by resection of the area where the preparation was introduced into the muscle indicated the presence of bulk nonresorbed calcinate at the injection site; the calcite had remained there for five or more weeks (Fig. 7). The volume of microcapsules introduced (0.25 mL) was quite large relative to the tissue volume of a typical mouse haunch. These muscle deposits, which primarily consisted of calcium salts, amounted to as much as 25 mg. Deposits in this amount and with this composition will inevitably lead to reactive inflammation. Deposition of calcium salts in such quantities should affect anatomical structure and adversely impact blood and lymph microcirculation in the haunch muscle (as in fact observed



FIG. 7. Calcium and calcium/polymer clumps (nodules) at the injection site.

upon resection), causing tissue hypoxia. Thus, in this case, inflammation was accompanied by hypoxia.

Thus, it has been experimentally shown that tissue damage associated with ischemia and inflammation is typically associated with elevated induced fluorescence levels and additional local accumulation of both endogenous and exogenous fluorophores in the vicinity of the inflammation or ischemia, thereby confirming our hypothesis stated in the introduction to this paper. Moreover, the data obtained indicate that there is a good correlation between the extent of tissue damage associated with ischemia or inflammation and the index for concentration of fluorophore in tissue $\eta(\lambda_f)_{\lambda_e}$, and it is likely to be possible to assess and determine the extent to which these processes have developed in tissue based on the levels of $\eta(\lambda_f)_{\lambda_e}$. On the one hand, this provides a strong basis for taking another look at the use of LIFS results in oncology. Highly oxygenated cancer cells may turn out not to be visible *in vivo* using LIFS or in real time against the background of strongly fluorescing hypoxic and inflamed regions in the tumor. On the other hand, the increased accumulation of fluorophores documented in this paper provides an opportunity to develop techniques for noninvasive rapid diagnosis of local inflammatory processes, ischemia, and hypoxia using LIFS in other areas of medicine.

CONCLUSION

In this paper, we experimentally demonstrated the phenomenon of elevated fluorescence from endogenous and exogenous fluorophores in *in vivo* tissue using an endogenous porphyrin and aluminum phthalocyanine, in the case of occlusive ischemia and model inflammation, respectively. The fluorescence was recorded using a technique, process, and instrumentation frequently used in the performance of oncological procedures (PDT), fluorescence-based intra-operational navigation for surgical removal of brain tumors, etc. However, this study did not include any malignant neoplasms in tissues of experimental animals. All processes studied were nonmalignant. The results obtained indicate that ischemia, hypoxia, and inflammation must be taken into account when interpreting LIFS results in oncology. On the other hand, the fact that we have demonstrated enhanced accumulation of fluorophores during ischemia and inflammation provides an opportunity to develop techniques for noninvasive rapid diagnosis of local inflammatory processes, ischemia, and hypoxia, as well as methods for using LIFS to assess the effectiveness of therapy directed at these states in a wide variety of clinical settings.

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