Fluorescence lifetime of indocyanine green in molecular and nano-forms in the cellular model of a brain tumor in vitro

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Abstract. Any surgical intervention to a central nervous system requires special accuracy and selectivity of the effect on the cancer cells. The application of laser-spectroscopic methods provides a unique opportunity to non-invasively determine the most significant parameters that characterize the tissue states. Moreover, non-invasive state assessment of the brain tumor tissue and surrounding tissues is essential for performing a relapse-free operation. Indocyanine Green (ICG) is a photosensitizer approved for clinical practice and has absorption peak in the near-infrared range corresponding to the spectral transparency window of biological tissue. Also, the aqueous colloidal solution of ICG aggregates was used for spectroscopic properties research in glioma tumor cells. The comparative analysis of ICG in molecular and nanoforms demonstrate the difference between spectral values which allow distinguishing monomers and aggregates in tumor tissue.

1. Introduction
The treatment of malignant glial tumors is a significant problem for neurosurgeons and neuro-oncologists. The primary approach of treating brain gliomas is a surgical intervention, which aims to remove malignant tissues as much as possible within the functional boundaries. Surgical resection of tumor requires compliance with the principle of minimizing surgical trauma, maximizing the preservation of adjacent brain structures, which should provide an improved quality of patient life. Thus, surgical treatment, radiation and chemotherapy are the main treatment tactics in this category of patients [1]. Noticeably, relapses of this tumor type occur in a big part of patients regardless of the initial treatment tactics, for the reason that methods for preventing metastatic brain damage have not been developed yet. Rapid growth of primary and metastatic gliomas causes neurological disorders and results in damage to brain structures which could lead to early disability and high mortality [2]. The indicator of the treatment effectiveness is the level of the quality of the patient’s life due to the lack of alternative
methods that lead to partial or complete control over the pathological processes in the brain cells. Thus, the steady and widespread increase in morbidity rates, early disability, and high mortality of the active and able-bodied population, as well as the unsatisfactory results of standard combined treatment (surgical treatment plus radiation therapy [3, 4]), prompted the search for ways to improve the treatment effectiveness of brain tumors through the development of new combined methods, which led to the relevance of this study.

Laser spectroscopic methods with time resolution provide a unique opportunity to determine the composition of tumor tissue by the change in the photosensitizer (PS) fluorescence lifetime. This difference is due to the different PS accumulation and intracellular processing by cells with different phenotypes [5]. One of the important advantages is the high speed of information processing. The wide range of physiological and morphological parameters available for analysis by optical spectroscopy is equally important. These methods allow identifying an unambiguous correspondence between the recorded signal, such as absorption, fluorescence or various scattering types caused by substances originally inherent in nerve tissues and cells, and contrasting those with externally introduced markers. Additionally, it indicates small and rapid functional changes in the studied tissues and also shows a strong correlation between deep metabolic and structural changes that occur in the development of brain pathologies [6].

Non-invasive state assessment of the brain tumor tissue and surrounding tissues is essential for performing a relapse-free operation. Laser-fluorescent methods and devices with picosecond time resolution appear to be the most promising for in vivo video-fluorescent monitoring of biotissue. Molecules and nanoparticles with fluorescent properties, photo-cytotoxic effect, and selective accumulation by various cell structures and tumor tissues in comparison with the surrounding tissues can significantly increase the diagnostic informativeness of the approach and enhance the therapeutic effect due to the selective cytotoxic effect on cancer cells.

Laser radiation in the near-infrared region penetrates deeper into the biological tissue due to the features of its absorption (minimal absorption and insignificant heating) in this spectral range. Optical imaging in the near-infrared range of the biological tissue transparency window (700-900 nm) has a significant perspective for in vivo imaging due to reduced light scattering by the tissue and increased penetration depth. Near-infrared fluorescent indicators, such as ICG, are promising for fluorescent diagnostics due to its significant penetration depth, which can reach 1-2 cm. ICG is one of the most common dyes approved for clinical use, used in surgery for fluorescent imaging in the infrared range [7, 8]: mapping of sentinel lymph nodes; intraoperative visualization of head and neck lesions [9]. Organic dye based on J-aggregates of ICG opens up truly unique opportunities in the development of new technologies for sensing and imaging based on fluorescence [10].

2. Materials and Methods

The monolayer culture of rat C6 glioma cell line was used as an experimental model. The cell model was cultured in RPMI-1640 medium with the addition of 9% fetal bovine serum. The monolayer was incubated at 37 °C in a 5% CO₂ atmosphere. The viability of the cells used in the experiments was 95% at least. The cells were incubated with ICG in molecular and nanoforms during 24 hours. After incubation, the cells were washed twice from unbound PS via buffer and trypsinized to produce a cell suspension. Then the cells in 1 ml eppendorf were spinned at around 1500 rpm for 5 min. The centrifugation is necessary for fluorescence signal registration from the cell surface.

The fluorescence signal for spectroscopic curves analysis was obtained via spectroscopy system based on diffuse-reflected method. For research of fluorescence kinetics measuring system based on a streak-camera Hamamatsu C10627-13 with a time resolution of 15 ps, laser with 637 nm excitation wavelength and 100 ps pulsewidth, coupled to a fiber-optic spectrometer was developed. The system utilizes time-correlated single photon counting approach. The description of the system is presented in the article [11].

3. Results and Discussion
The accumulation analysis of ICG in cells was assessed according to the fluorescence intensity level in the PS emission band. The fluorescence spectra were obtained from a cell surface with 785 nm excitation wavelength. Different incubation time of tumor cells with ICG molecular form illustrate a practically stable level of fluorescence and the same form of spectral lines (fig.1).

Figure 1. The fluorescence spectra of ICG molecular form from tumor cells.

The dynamics of ICG NP accumulation in tumor cells was obtained for estimating the rate of ICG NP accumulation. Significantly, the various fluorescence extents were observed with ICG nanoforms in dynamics (fig.2). Spectral curve consists of monomer peak with maximum at 848 nm and J-aggregate peak with maximum at 910 nm. The proportion changes of fluorescence intensity which correspond to monomer and J-aggregate peaks were obtained in dynamics with 785 nm excitation wavelength.

Figure 2. Fluorescence spectra of ICG nanoform from tumor cells.

The fluorescence profile was approximated by the pseudo-Voigt function as a linear combination of the Gaussian and Lorentz distributions [12]. Figure 4 illustrates an example approximation of ICG NP fluorescence spectra accumulated in tumor cells.
Figure 3. Pseudo-Voigt approximation example of ICG NP fluorescence curve.

The approximation allows obtaining the data of integral intensity of monomer and J-aggregate peaks (fig.4a). The change of the monomers and J-aggregate intensities with time can be noted.

Figure 4. a) Integral dependence of the spectral curve of ICG nanoform correspond to monomers and J-aggregates, b) ratio of J-aggregate peak to monomer peak.

It is noted that over time, the peak of fluorescence corresponding to the J-aggregates of ICG in cells increases for the first 3 hours, which indicates the different rate of accumulation. For the next 6 hours the ratio remains constant(fig.4b).

The fluorescence spectra of the molecular and the colloidal solution of ICG were obtained with 633 nm excitation wavelength. A distinct fluorescence peak with a maximum at 700 nm is observed, corresponding to the H-aggregates of ICG, during the excitation colloidal solution of ICG NPs. At the same time, when the molecular form of ICG is irradiated, two fluorescence peaks are observed, corresponding to the H-aggregates and ICG monomers at the wavelengths of 700 nm and 845 nm, respectively.

The fluorescence decay kinetics were obtained via the streak camera (fig.6).

The fluorescence lifetime of ICG in different forms was obtained at different optical ranges. For molecular ICG the spectral range was from 790 nm to 860 nm, which corresponds to ICG monomers. For ICG colloidal solution the fluorescence lifetime data were registered from 700 nm to 730 nm which correspond to H-aggregates of ICG.
Figure 5. Fluorescence spectra of ICG in molecular and nanoform at 633 nm excitation.

Figure 6. Fluorescence decay kinetics spectrum obtained from C6 cell surface with a) ICG in molecular form, b) ICG in nanoform.

Figure 7. a) The dependence of fluorescence lifetime value to ICG concentration, b) the dependence of fluorescence lifetime amplitude ratio to ICG concentration.
Mathematical processing of obtained spectrum demonstrate the presence of two fluorescence lifetimes for molecular form of ICG is 0.22±0.04 ns and 0.63±0.01 ns and also two fluorescence lifetimes for nanoform of ICG is 0.28±0.03 ns and 1.21±0.08 ns (fig. 7a). The fluorescence lifetime of ICG in molecular and nanoform does not change by the concentration growth. The results illustrate the difference between fluorescence lifetime values of various ICG forms. The first component of the fluorescence lifetime of ICG molecular form is 0.2 times lower than the first component of ICG NP. However, the second component of the fluorescence lifetime of ICG molecular form is almost 2 times lower than ICG NP. The amplitude ratio of ICG mol fluorescence lifetime decreases with concentration growth, while the values of amplitude ratio of ICG NP fluorescence lifetime maintain the same level.

4. Conclusion
The research demonstrates the contrast of spectral characteristics of ICG in monomers and aggregate type. Additionally, the transformation from monomers to J-aggregates was observed during accumulation in tumor cells. The analysis of fluorescence kinetics shows the difference between fluorescence lifetime which allows separating ICG forms in biological tissue. However, ICG demonstrates the stability of fluorescence lifetime with concentration growth. ICG nanoparticles have huge perspectives for fluorescent diagnostics of brain tumors due to the spectroscopic properties and enhance the conservation of healthy brain tissue.

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