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RESEARCH ARTICLE

# Study of possibility of cell recognition in brain tumors

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**Abstract** The brain has an exceptionally high requirement for energy metabolism, with glucose serving as the exclusive energy source. Cancers, including glioblastoma, have a high glucose uptake and rely on aerobic glycolysis for energy metabolism. The alternation of high-efficiency oxidative phosphorylation to a low-efficiency aerobic glycolysis pathway (Warburg effect) provides macromolecules for biosynthesis and proliferation. Current research indicates that the specific metabolism in the tumor tissue and normal brain tissue in the glioma allows the use of 5-aminolevulinic acid (5 ALA)-induced protoporphyrin IX (PpIX) and methylene blue (MB) to monitor and correct the development of the tumor. The focus is on the detection of the differences between tumor cells and tumor-associated macrophages/microglia using spectroscopic and microscopic methods, based on the fluorescent signals and the difference in the drug accumulation of photosensitizers (PSs). Since 5 ALA has long been used effectively in the clinic for fluorescent surgical navigation, it was employed as an agent to identify the localization of tumor tissue and study its composition, particularly tumor and immune cells (macrophages), which have also been shown to actively accumulate PpIX. However, since PpIX is photodynamically active, it can be considered effective as the main target of tumor tissue for further successful photodynamic therapy. MB was employed to visualize resident microglia, which is important for their activation/deactivation to prevent the reprogramming of the immune cells by the tumor. Thus, using two drugs, it is possible to prevent crosstalk between tumor cells and the immune cells of different geneses.

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## 1 Introduction

Brain tumors are associated with cerebral edema, blood–brain barrier breakdown, and neuroinflammation, which are characterized by an enhanced resistance to cell death. From the discovery of the Krebs cycle to mitochondrial oxidative phosphorylation, medicine has recorded significant progress in cellular metabolism research. Despite the significant advancements in modern medicine, cancer remains the most devastating disease, and effective therapeutic interventions are desperately needed [1,2]. Integrative molecular, cellular, and metabolic approaches might provide a better understanding of neurodegenerative disorders and cancers, thus leading to the development of novel therapeutics for these incurables. Abnormal metabolism is the main characteristic of many disorders. Cancer cells are phenotypically heterogeneous, given their different origins and the inter-tumor instability [3–5]. Nonetheless, cancer cells have long been known to have characteristic alterations in their metabolism. Reprogramming of energy metabolism has recently been proposed as an emerging cancer hallmark. Accordingly, novel therapeutic targets on metabolic pathways have been explored for the treatment of cancer.

According to the latest data, the immune system, i.e., immunocompetent cells: both resident (microglia) and newly arrived, plays the most significant role in the invasion and progression of tumors, because of the disrupted blood–brain barrier (macrophages). Immune system agents, which, according to some sources, account for approximately 60% of the total tumor volume [6,7], are reprogrammed by the tumor, causing them to protect the tumor.

Thus, to increase the effectiveness of the treatment of brain tumors, the acute problem of rapid and objective comprehensive assessment of the tissue state during surgery or laser-induced therapy sessions must be solved. Laser-spectroscopic methods provide a unique opportunity to non-invasively determine the most significant parameters for identifying the tissue state and to control the presence of immunocompetent cells in the tissue, particularly the dynamics of accumulation and elimination of drugs and photosensitizers (PSs), which signal the severity of the disease and the likely direction of its development. Most tumor cells are known to efficiently accumulate PSs; however, it has been reported that immunocompetent cells can accumulate PSs up to 10 times more, particularly tumor-associated agents [7,8]. By choosing drugs and PSs that can accumulate in cells of different geneses, we can divide the cellular composition of tissue using spectral methods. The 5-aminolevulinic acid (5 ALA)-induced protoporphyrin IX (PpIX) agent is widely used in the clinic for surgical navigation of brain tumors; thus, it was used in this work to identify the location of rat brain tumor tissues [7,8]. Methylene blue (MB) has a high affinity toward brain and nerve tissues when administered systemically or supravitally. Consistently, MB has been found to actively bind to mitochondria and enter the mitochondrial matrix in a manner enhanced by the mitochondrial membrane potential. This high affinity of MB toward nerve and cancer tissues leads to its application for staining neuronal structures and cancer tissues in both clinical and histochemistry studies. Thus, MB has been used as a dye for resident microglia [9,10]. Using this approach, researchers attempted to separate the cells of the immune system, namely resident cells and system cells, originating from outside due to the violation of the blood–brain barrier. However, since PpIX accumulates in both tumor cells and the immune cells that make up the tumor tissue, additional functional morphological staining is required to separate these cells [11,12]. At present, the proposed PSs are used for two different purposes: 5 ALA is used for fluorescent navigation in brain tumor removal surgeries, while MB is used to control inflammation in the brain area and to activate and deactivate microglyphs. The novelty of the proposed approach is the combined simultaneous effect of two PSs to influence the different components of tumor tissue. These include cancer cells and immunocompetent cells, which are inflammatory agents. Thus, with the systemic administration of PSs, because of the variety of cellular metabolism and drug accumulation not only by tumor cells but also by immunocompetent cells, it is possible to evaluate the cellular composition of pathological tissue based on the differences in the fluorescence spectra. The control methodology in this study was morphological staining.

The renewed interest in the Warburg effect positions metabolism as an emerging highly interesting target for anti-cancer drug development. There are concerns that cancer and immune cells may exploit the signaling redundancy and crosstalk to tackle the anabolic challenge. Indeed, despite the advancement of our understanding of cancer metabolism, our knowledge of the metabolic signaling regulation is limited; however, it can be assumed that dyes and PSs can be involved in the metabolic reprogramming of cancer and immune cells. Further studies are warranted to determine the high plasticity of the metabolic network in cancer cells.

## 2 Materials and methods

### 2.1 Biological materials

To conduct this study, a series of experiments was performed on adult female Wistar rats. The rats weighed 200–220 g at the beginning of the experiment, with glioblastoma multiforme simulated by the stereotactic implantation of $5 \times 10^5$ C6 glioma cells into the right cerebral hemisphere [13]. Dynamic magnetic resonance imaging of the rat brain to assess the dynamics of the intracranial tumor development was performed on a BioSpec 70/30 tomograph (Bruker, Germany), with a constant magnetic field of 7 T. A PS was injected into the femoral vein of rats under ketamine anesthesia (bodyweight: 100 mg/kg).

To analyze the brain tissue at the microscopic level, cryosections were prepared [14,15].

### 2.2 Perfusion protocol for the rats

- The animal was anesthetized by parenteral injection. The anesthetic agent was approved by a veterinarian for usage in rats.
- The anesthetized rat was placed and immobilized on a working pad.
- The skin and abdominal muscles were incised from the abdominal cavity to the thoracic cavity.
- The left ventricle, apex of heart, and right atrium were identified. The perfusion needle was positioned at the apex and perpendicularly inserted into the left ventricle. The right atrium was clipped.
- Perfusion began at a slow and steady rate. Successful perfusion was noted as the perfusate replaced the blood within the vessels. The perfusate can be saline or formaldehyde solution.

### 2.3 Protocol for rat brain removal

- The perfused rat neck was cut at the level of the first cervical vertebrae to free the head from the rest of the body.
- The skin on the skull was incised on the midline using a pair of scissors and flipped over the eyes.
- The tip of a small pair of scissors was inserted into the foramen magnum, and the skull was cut above the ear; this process was repeated on the other side.
- Both incisions were extended toward the nose and cut across the frontal bones.
- The top of the cranium was lifted and removed using forceps.
- The brain was freed from meninges. The olfactory bulb, optic nerves, and other cranial nerves were cut, and the brain was gently lifted out from the skull.
- The brain was transferred to a Petri dish and immersed in cold saline/phosphate buffered saline (PBS).

### 2.4 Protocol for preparation of frozen sections

- The specimen was immersed in an embedded medium (Colorless Neg-50 Medium) and frozen on the specimen chunk in a fast-freezing device or a cooling chamber of Microm HM 560.
- The specimen chunk was clamped together with the frozen specimen into the specimen holder.
- A chamber and specimen temperature ( −15°C) were selected, corresponding to the consistency of the specimen that should be sectioned.
- The front end of the specimen holder was approached by the knife carrier using a handwheel or an automatic approach system. After the specimen and the knife are adjusted, the specimen is trimmed.
- After trimming, sectioning at the selected thickness (10–20 μm) is initiated.
- The sections from the knife surface to the slides are retrieved using a brush or tweezers.
- Fixation, staining, and coverslipping of section slides.

### 2.5 Giemsa stain protocol for frozen section

- When the frozen tissue section is complete, the tissue on the slide is picked up and air-dried for 5–10 min.
- The slide is immersed in the fixative solution (10% neutral buffered formalin) for 5 min.
- The fixative solution is washed off by PBS for 5 min.
- Incubation with May–Grunwald solution for 5–7 min.
- Washing with PBS.
- Incubation with Giemsa solution for 10–15 min.
- Washing with PBS, and incubation in PBS for 5 min.
- The slide and mount are dried.

### 2.6 Studied photosensitizer

An effective endogenously formed photodynamic agent that has been widely used in clinical practice for the treatment and diagnosis of cancer (particularly gliomas of high malignancy) is 5 ALA-induced PpIX [5,6]. PpIX is an intermediate product of the chain of heme synthesis from 5 ALA. The 5 ALA-induced PpIX agent is characterized by high fluorescence contrast and increased accumulation in some types of rapidly proliferating tissues, characterized by a lack of ferrochelatase. The lack of the ferrochelatase enzyme in tumor cells, compared to normal cells, results in the accumulation of PpIX not only inside the cell but also in the intercellular space. PpIX accumulates and remains in significant quantities in the tumor for several hours, while in normal cells it quickly turns into a photoinactive heme under the action of ferrochelatase.

In this work, preliminary studies were carried out using the Alasens® pharmaceutical powder (NIOPIK, Russia) on a model of experimental animals with induced C6 glioma. A sterile aqueous solution of PS for application (concentration $c$ = 100 mg/kg in an animal, which was injected into the femoral vein of rats under ketamine anesthesia) was prepared 1 h before use by dissolving the required amount of Alasens® powder in a 5% sterile solution of sodium bicarbonate.

MB is a synthetic basic dye, an organic chloride salt having 3,7-bis(dimethylamino)phenothiazin-5-ium as the counterion. It stains negatively charged cell components, such as nucleic acids; when administered in the lymphatic bed of a tumor during oncologic surgery, MB may stain lymph nodes draining from the tumor, thereby aiding in the visual localization of tumor sentinel lymph nodes.

To evaluate the potential effects of MB on brain microglia, an I.V. tail vein injection of low-dose MB (bodyweight: 2 mg/kg) was administered to rats. The MB (1% solution, 10 mg/mL) was diluted 100 times, and 100 μL was injected.

Three groups of animals participated in the survey:

- Experimental animals intravenously injected with 5 ALA-induced PpIX.
- Animals intravenously injected with MB and 5 ALA-induced PpIX.
- Control group of animals without drugs.

There were five animals in each group, based on which all the results presented in this paper were statistically averaged.

### 2.7 Study of fluorescence spectra using a laser scanning microscope

A study of drug accumulation in the cellular structures of the brain was carried out by cryosections using confocal microscopy (LSM-710-NLO Carl Zeiss, Germany).

We used a method for recording PS fluorescence attachment for a confocal laser scanning microscope, which facilitated the estimation of the distribution of PS at different points of the test sample based on cryosections of the brain of the experimental animals obtained directly after *in vivo* spectra measurements. The actual localization (accumulation region) and the fluorescence spectrum of PS or endogenous fluorophores are estimated by analyzing the obtained images using fluorescence microscopy. Studies of the fluorescence kinetic characteristics were carried out using the LSM-710-NLO laser scanning microscope (Carl Zeiss, Germany). The samples were excited using Chameleon Ultra II femtosecond pulsed laser (80 MHz, pulse duration 140 fs, wavelength range 690–1060 nm, Coherent Inc., USA). Different modes of excitation and fluorescence registration were used to separate the two drugs: 5 ALA was excited by 514 nm laser, and the range of registration was 600–665 nm; MB was excited by 488 and 633 nm lasers, and the registration range was 617–735 nm. The cryosections, after morphological Giemsa stain, were examined in passing white light.

Images were obtained in the following scan modes: 20× lens, scan area 400 μm × 400 μm, resolution 1024 pixel × 1024 pixel, and scan speed 1.2–3.2 μs/pixel. The total image acquisition time was 18 s. The average power density measured using a Coherent power meter (USA) at the sample position was 5.4 mW; a scanning spot of 10 μm in diameter had a power density of 7 $kW/cm^2$.

## 3 Results and discussion

The brain tumor tissue is a complex system that may contain cells of various geneses, particularly immune cells, i.e., resident cells (microglia) or incoming cells (macrophages/tumor-associated macrophages). Their detection is important to assess, analyze, and predict the development of the tumor process. In the case of glial tumors, the immune system protects the growth and development of the tumor; this makes the defenders (tumor-associated microglia/macrophages) an important target for destruction during therapy. In this regard, it is important to address the issue of the specific localization of the accumulation of the PS, namely whether tumor or immune cells are more likely to be the center for the accumulation of the PS. To identify the type of cells that are the centers of accumulation, "mirror" slices were identified: one slice was examined by spectral analysis of the fluorescence signal to identify the topography of the distribution of the PS, while the second slice was examined using functional coloration according to Giemsa stain. This facilitates the determination of the genesis of a cell in the light of its color and morphology to distinguish between tumor cells and immunocompetent cells.

A study of a group of experimental animals who were intravenously injected with 5 ALA-induced PpIX showed a contrast in the local accumulation of PpIX in rat brain tumor 2 h after injection. The experiments on the animals were concluded at the time of maximum drug accumulation in the glioma, which was monitored *in vivo* spectrally and locally in the striatum area, through a functional opening in the skull that was induced at the time of the tumor implantation. At maximum accumulation, brain tissue cryosections were obtained, which were examined for PpIX accumulation in individual cellular structures. Figure 1 shows that the PS was distributed evenly in the brain tumor tissues and was not detected in normal tissues of healthy brain regions. The spectral analysis shown in Fig. 2 confirms that the tumor tissue contains PpIX, which is fully spectrally matched by excitation fluorescence with laser radiation at 514 nm, and the fluorescence signal registering in the range of 600–665 nm.

A detailed analysis of the glioma was conducted around the blood vessel, which attracts interest as a port of landing and access for non-resident macrophages to the brain and directly to tumor tissue. Functional spectral analysis has shown that PpIX accumulates as effectively around the blood vessel as it does in the surrounding tumor tissue; however, functional staining has shown that there are cells around the vessel whose morphology is similar to that of immune genesis (Fig. 3). This staining method prevents the

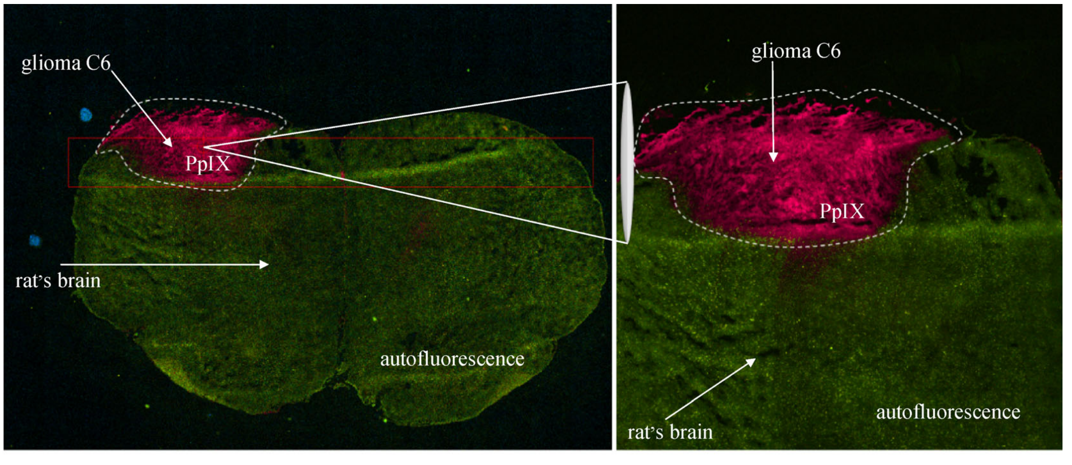


**Fig. 1** Image of a rat brain cryosection obtained using a fluorescence microscope: pink areas indicate fluorescence in the range of 660–665 nm, which corresponds to the accumulation of PpIX; green areas represent tissue autofluorescence

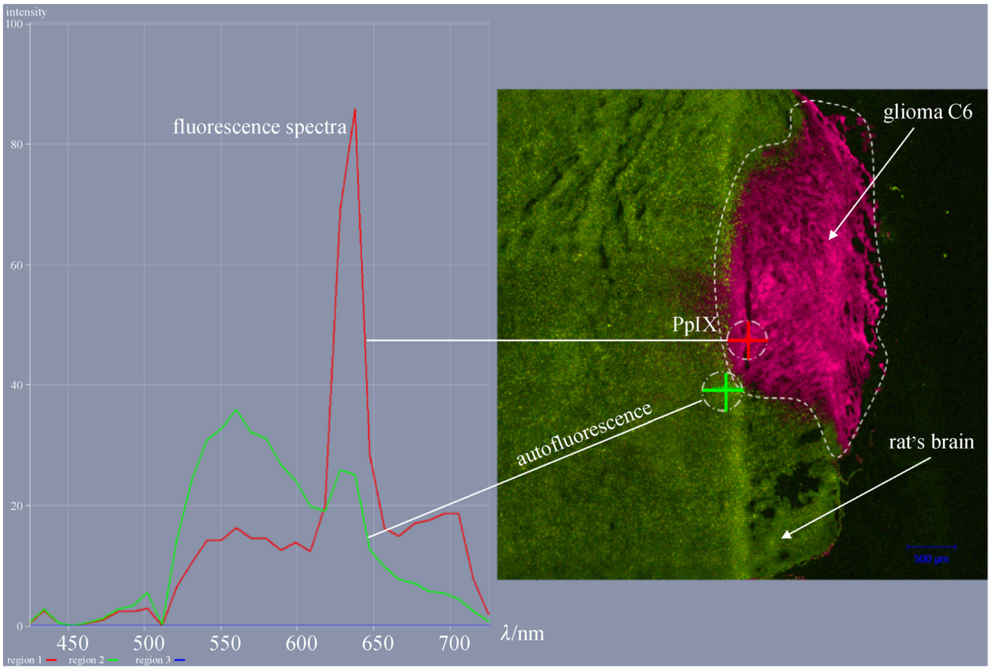


**Fig. 2** Image of a rat brain cryosection obtained with a fluorescence microscope: pink areas indicate fluorescence in the range of 660–665 nm, which corresponds to the accumulation of PpIX; green areas indicate tissue autofluorescence. Spectral characteristics in the marked area are presented according to the area

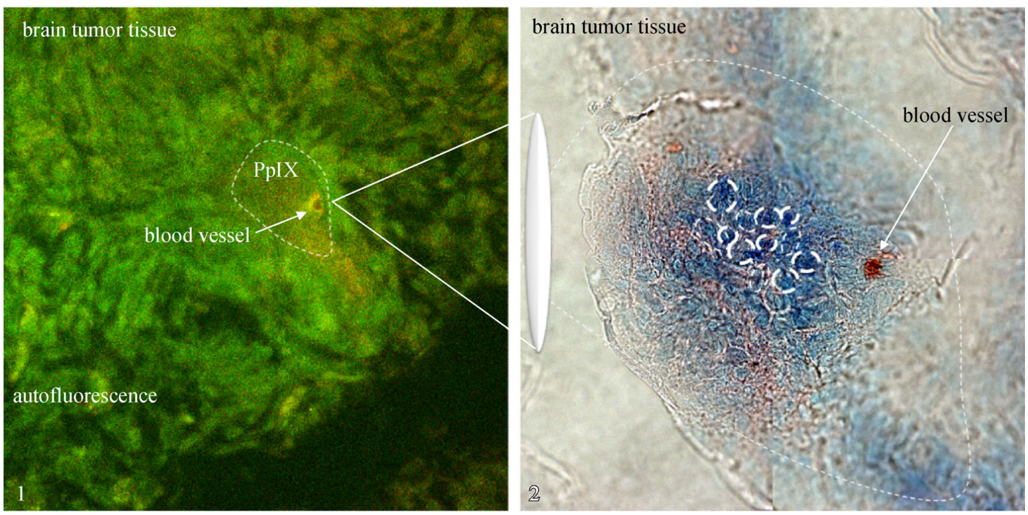


**Fig. 3** Microscopic image of the tumor tissue area around the blood vessel in a fluorescent signal (1) and the light after Giemsa stain (2). A comparison of the pink fluorescent areas of PpIX accumulation and areas corresponding to the blue light areas: tumor cells; dark-blue areas: immune cells

precise determination of whether these cells are resident microglia or newly arrived macrophages; however, this localization was chosen due to the known fact that the tumor macrophages enter the tumor through blood vessels, thereby overcoming the disturbed blood–brain barrier in the tumor area. Thus, knowing the migration paths of macrophages that have been reprogrammed by the tumor, we assume that in Fig. 3, the dark-blue clusters of rounded cells represent the incoming macrophage tumor, while the light blue clusters of elongated cells directly represent the cells of glioma C6. Thus, we can conclude that PpIX, which accumulates in the tumor tissue, is contained in both glioma cells and tumor-associated macrophages, making them an available target for photodynamic therapy in this case.

Based on the literature and knowing the migration path of tumor-associated macrophages/microglia, another interesting area for the study is the tumor boundary, as this is where the main forces of the immune system are concentrated. Using the functional coloration in Fig. 4, we obtained a morphological map of the tumor border at various points, from which we can observe the loose structure of a clearly delineated tumor painted in violet, as well as the high concentration of immune cells, which are densely placed just outside the tumor border. We can detect them as bright blue embeddings with rounded and elongated shapes against the background of pink normal tissue. Therefore, we demonstrate the leading role of the immune system in the development of the tumor process (Fig. 4). At the same time, deliberately examining a healthy brain tissue that is not affected by the tumor process provides a normal picture of the distribution of resident microglia in the brain. Figure 5 shows that these are uniformly rare, dark-blue colored, rounded out, and located throughout the brain tissue, although their concentration is much higher in the pathology area.

The main task with the group of animals who were given both 5 ALA and MB was to identify and localize the

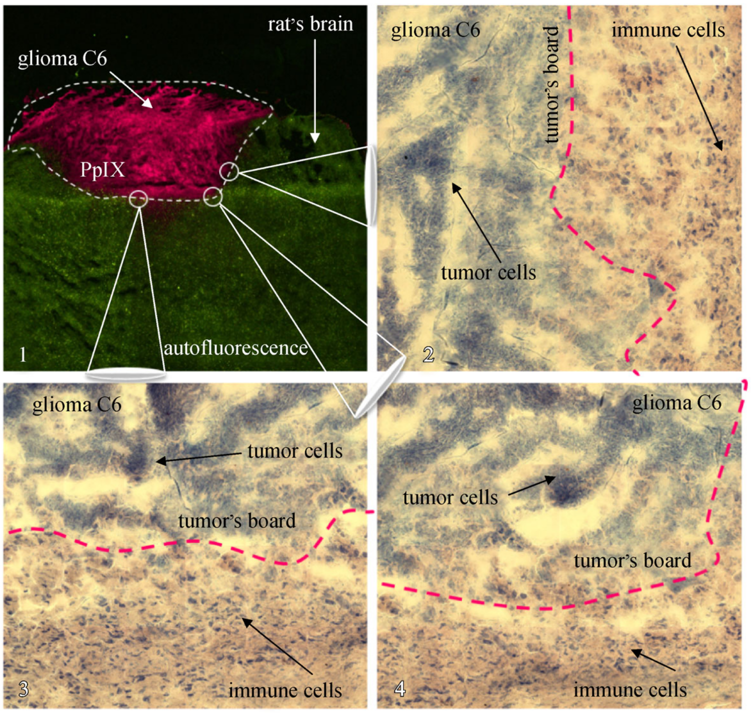


**Fig. 4** Microscopic image of the tumor tissue area around the tumor board in a fluorescent signal (l) and the light after Giemsa stain (2–4). A comparison of the pink fluorescent areas of PpIX accumulation and areas corresponding to the blue light areas: tumor cells; dark-blue areas: immune cells

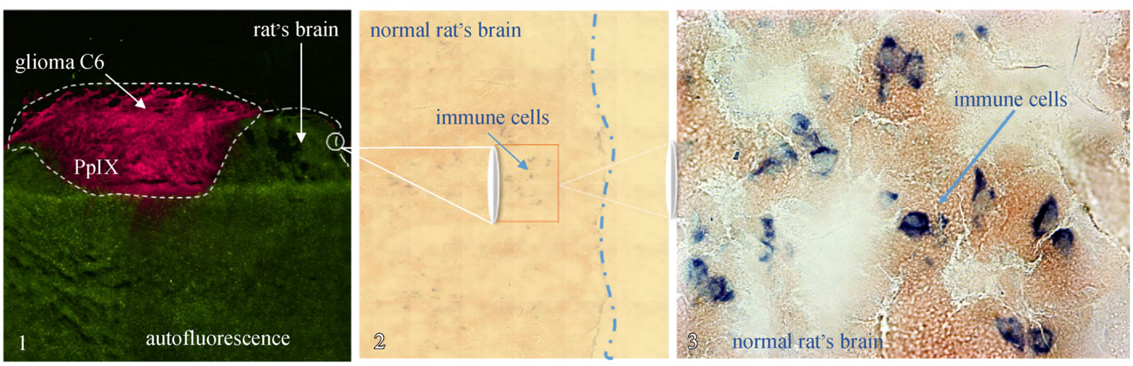


**Fig. 5** Microscopic image of the tumor tissue area around the blood vessel in a fluorescent signal (1) and the light after Giemsa stain with high microscopic magnification (2: 20×; 3: 63×). A comparison of the green fluorescent areas (autofluorescence without PpIX) and areas corresponding to the pink light areas: normal brain cells; dark-blue areas: immune cells

accumulation of the two drugs during their joint administration, with a time difference of around 1.5 h, since the experiment on the animals was concluded at the time of maximum accumulation of both drugs (PpIX, for an average of 2 h; MB, for an average of 30 min depending on the individual). Based on the literature, it was expected that MB, which accumulates selectively in microglia, will characterize the brain map in terms of the location of this cell type, which is particularly interesting and new in the development of the pathological process in the form of glioma. Thus, spectral analysis has shown that when the two drugs are injected together, PpIX accumulates and localizes directly in the tumor tissue regardless of the presence of MB, staining evenly all the cells that are involved in the development of the tumor process (Fig. 6). The reliability of the results has been confirmed by spectral diversity of the analysis area (Figs. 6 and 7): slices were first examined for the localization of PpIX (in the presence of MB) by excitation with 514 nm laser light, with the fluorescence spectrum registered in the range of 600–665 nm. Thereafter, excitation with 418 and 633 nm laser light and fluorescence registration in the range of 617–735 nm facilitated the assessment of the localization of MB (in the presence of PpIX). By superimposing two spectral images and subsequently comparing it with an image of the same slice functionally colored, it was found that MB spreads mainly in the area of the healthy brain, i.e., uniformly colored glial cells, namely microglia, while PpIX, even in the presence of MB, effectively accumulates in the tumor tissue (Fig. 8).

## 4 Conclusions

Herein, it has been shown that the joint administration of 5 ALA and MB allows for the spectral separation of the two drugs, with each having its specificity and localization of accumulation. Thus, note worthily, photoinitiators have been selected for their spectral characteristics, i.e., the ability to be separated by precise spectral equipment, which is an important factor in spectral analysis.

By selective accumulation in tumor cells and tumor-associated macrophages that originated from outside to protect the tumor, 5 ALA will allow fluorescent diagnosis and subsequent photodynamic therapy to destroy the main tumor tissue cell targets. This is important for direct destruction, but also to correct and prevent further development of the tumor process. Moreover, due to the destruction of tumor-associated macrophages, the immune system can disperse normal agents, particularly macrophages, which, without being reprogrammed by the tumor, will facilitate its destruction. In addition, it should be noted that if the normal cells of the immune system successfully migrate to the pathology zone, they are likely to induce conditions similar to inflammation. This is also a favorable feature, as the second photosensitizer chosen, i.e., MB, has a tropical affinity to inflammation areas, which will facilitate the recognition and tracking of the migration of exactly the right cells to combat the remains of tumor tissues after the photodynamic destruction of the bulk of the tumor tissue.

The role of MB in controlling the development of the

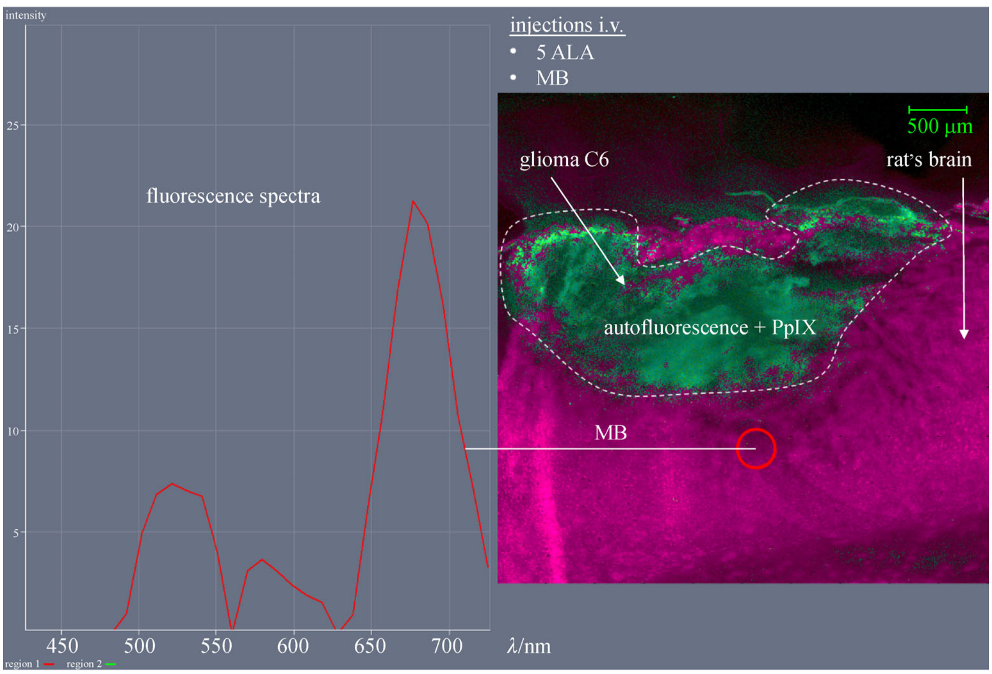


**Fig. 6** Image of a rat brain cryosection obtained with a fluorescence microscope: pink areas indicate fluorescence in the range of 617–735 nm, which corresponds to the accumulation of MB; green areas indicate tumor tissue autofluorescence with PpIX. Spectral characteristics in the marked area are presented according to the area of interest in the cryosection

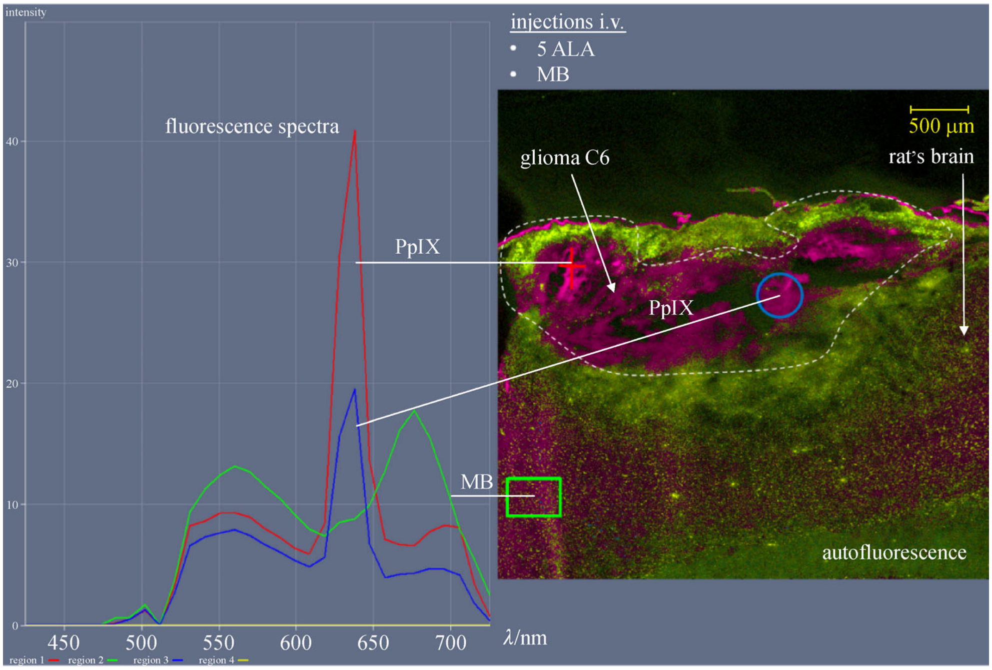


**Fig. 7** Image of a rat brain cryosection obtained with a fluorescence microscope: pink areas showing fluorescence in the range of 617–735 nm, which corresponds to the accumulation of MB; purple areas showing fluorescence in the area of 660–665 nm, which corresponds to the accumulation of PpIX; green areas showing brain tissue autofluorescence. Spectral characteristics in the marked area are presented according to the area of interest in the cryosection

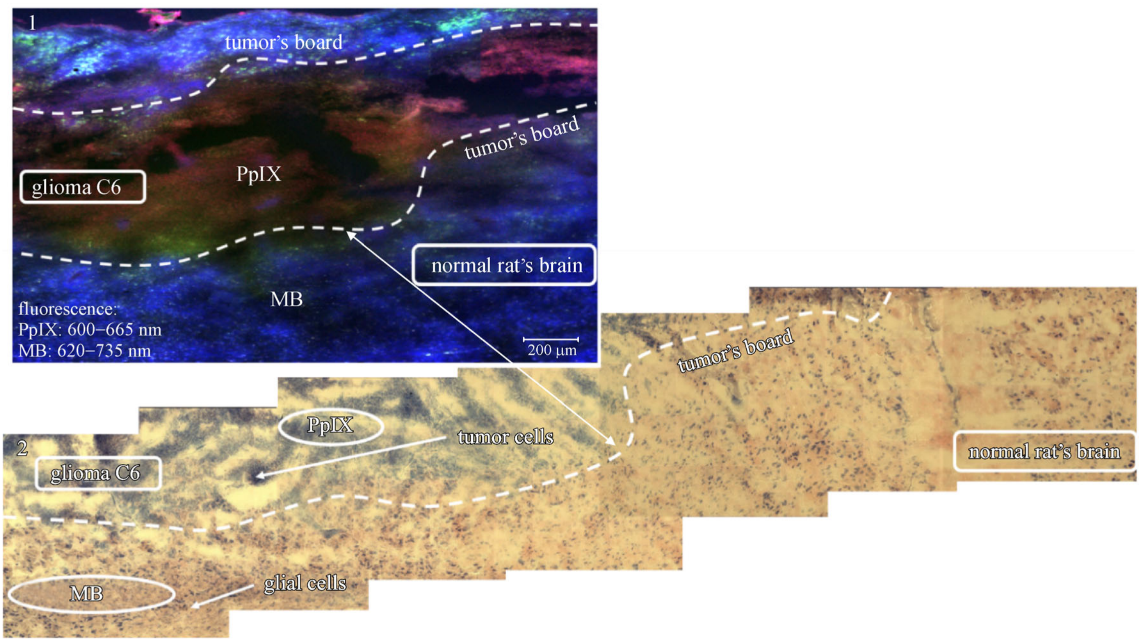


**Fig. 8** Image of a rat brain cryosection obtained with a fluorescence microscope (1): blue areas showing fluorescence in the range of 617–735 nm, which corresponds to the accumulation of MB; purple areas showing fluorescence in the range of 660–665 nm, which corresponds to the accumulation of PpIX. Microscopic image of the cryosection after Giemsa stain (2): light pink areas: normal brain tissue; blue areas: tumor cells; dark-blue areas: immune cells

tumors is also extremely important because the tumor cells can affect the macrophages from outside and reprogram resident microglia. In this case, the reprogramming process is the same microglia activation process that can be controlled by MB, which accumulates in these cells and can activate and deactivate microglia when exposed to laser radiation. Thus, the proposed approach of using two drugs together will promote a multilateral approach to brain tumor control, which is very important, considering the complexity of this process and the lack of effective treatment methods to date. As the pathways for metastasis in the brain are laid by cells of an immunocompetent nature, the proposed multilateral approach to attacking not only tumor cells, but also the activation and correction of the systemic and local immune responses, will significantly reduce the likelihood of metastasis and the rate of growth and development of the tumor.

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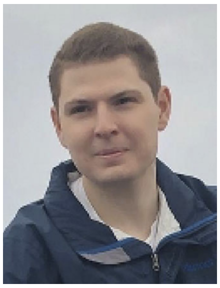

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Now he is professor and head of laboratory at Prokhorov General Physics Institute of the Russian Academy of Sciences, professor at National Research Nuclear University (NRNU) MEPhI, Russia. He is interested in developing the following methods: photodynamic therapy and fluorescent diagnostics of cancer, medical devices, theranostics, and optical spectroscopy. His is the general director of OOO BIOSPEC (Moscow, Russia).