Sub-Cellular Tumor Identification and Markerless Differentiation in the Rat Brain In Vivo by Multiphoton Microscopy

Iris Riemann,[1*] Ronan Le Harzic,[1] Konstantinos Mpoukouvalas,[2] Axel Heimann,[3] Oliver Kempski,[3] and Patra Charalampaki[3,4]

[1]*Fraunhofer Institute for Biomedical Engineering (IBMT), Ensheimer Str. 48, 66386 St. Ingbert, Germany*
[2]*Institut fuer Mikrotechnik Mainz (IMM) GmbH, Carl-Zeiss-Str. 18-20, 55129 Mainz, Germany*
[3]*Institute for Neurosurgical Pathophysiology, Johannes Gutenberg University, Langenbeckstrasse 1, 55131 Mainz, Germany*
[4]*Department of Neurosurgery, Medical University Graz, Auenbruggerplatz 29, 8036 Graz, Austria*

Objective/Background: Aim of the current study was to localize and differentiate between tumor (glioma) and healthy tissue in rat brains on a cellular level. Near-infrared multiphoton microscopy takes advantage of the simultaneous absorption of two or more photons to analyze various materials such as cell and tissue components via the observation of endogenous fluorophores such as NAD(P)H, FAD, porphyrins, melanin, elastin, and collagen, with a very high resolution, without inducing the problems of photo-bleaching on out-of-focus areas.
Methods: In vitro and *in vivo* studies on healthy rat brains as well as C6 glioma cell line allografts have been performed. Near-infrared laser pulses (λ = 690–1060 nm, $\tau \sim$140 fs) generated by an ultrafast Ti:Sapphire tunable laser system (Chameleon, Coherent GmbH, Santa Clara, CA) were coupled into a laser scanning microscope (LSM 510 META, Carl Zeiss, Germany) to observe high quality images.
Results: Several image acquisitions have been performed by varying the zoom scale of the multiphoton microscope, image acquisition time and the wavelength (765, 840 nm) to detect various tissue components. With a penetration depth of $\sim$200 μm *in vitro* and about 30–60 μm *in vivo* into the brain tissue it was possible to differentiate between tumor and healthy brain tissue even through thin layers of blood.
Conclusion: Near-infrared multiphoton microscopy allows the observation and possibly differentiation between tumor (glioma) and healthy tissue in rat brains on a cellular level. Our findings suggest that a further miniaturization of this technology might be very useful for scientific and clinical applications in neurosurgery. Lasers Surg. Med. 44:719–725, 2012.

Key words: multiphoton; brain tumor; in vivo laser diagnostic; autofluorescence

INTRODUCTION

Tumors of the central nervous system (CNS) have an incidence of 10 per 100,000 persons, they represent about 3% of all cancer deaths with a slightly increasing incidence in recent years. About 95% of all patients with malignant glioma die within the first 24 months after a diagnosis [1]. The currently high mortality, largely due to remaining malignant tissue and cells in the brain has to be related to insufficient surgical resection even if the MRI shows a "complete" resection, because remaining tumor cells cannot be identified by a MRI scanner. Those remaining cells show an infiltrative growing pattern into the normal brain tissue and induce recurrent tumor, which is in fact incurable by surgical and oncological methods.

A key factor with patients undergoing neurosurgical intervention due to intracranial neoplasia is the removal in toto of >99% of the tumor volume [2,3], while at the same time minimizing trauma to healthy brain tissue. This plays a crucial role, since increased radical surgery naturally bears a higher risk of damage to important intact cerebral regions and pathways. Still the discussion continues, whether an increased extent of resection (EOR) is beneficial for patients with aggressive brain tumors [4]. Although neurosurgeons have been equipped with new technological features, such as neuro-navigation [5,6], and fluorescence guided surgery [7,8], distinction between healthy and tumor cells remains a challenge.

The only actual treatment for glioblastoma is the complete mechanical resection of the tumor followed by radiation therapy or/and chemotherapy. The main problem with intrinsic growth brain tumors is the diagnostic validity, that is, the precise localization of malignant tissue. Hence preoperative imaging diagnosis using imported

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*Corresponding to: Dr. Iris Riemann, Fraunhofer Institute for Biomedical Engineering (IBMT), Ensheimer Str. 48, 66386 St. Ingbert, Germany. E-mail: iris.riemann@ibmt.fraunhofer.de
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methods (MRI, CT, PET, etc.) is absolutely necessary. In fact very few clinics are able to use these technologies during resections due to high costs. In the past decade, intraoperative detection of 5-aminolevulinic acid induced porphyrins in malignant brain tumor tissue has shown to delineate tumor and normal brain [9]. Recent prospective clinical trials have demonstrated that fluorescence-guided resection of brain tumors increased the EOR and may therefore prolong survival [8]. However the fact that the fluorescence is only visible as long as the surgical field is free of blood remains a problem, because hemoglobin absorbs the fluorescence signal.

It has been shown that multiphoton excited fluorescence of endogenous fluorophores allows structural imaging of brain tumor and CNS at a sub-cellular level [10–14].

Two-photon fluorescence (TPF) imaging is a powerful imaging modality with unique characteristics that can provide information complementary to that from other biological imaging technologies [15–17]. Advantages of TPF microscopy include intrinsic optical sectioning ability (due to the nonlinear two-photon excitation process), deeper penetration depth into tissue (owing to the use of near-infrared excitation light), and reduced photobleaching and phototoxicity in the out-of-focus regions (due to the confinement of fluorescence excitation to the focal region). Furthermore, two-photon imaging requires no additional fluorescent markers and can overcome the problem of blood on tissue surfaces as shown in the present manuscript by deep near-infrared penetration. Here we present results on *in vivo* and *ex vivo* sub-cellular tumor identification and differentiation in the rat brain by TPF and second harmonic generation (SHG) microscopy as well as the possibility of the killing of tumor cells using the same technical set up. Morphological characteristics of individual healthy and diseased cell types can be identified at a single-cell level down to resolution of cellular organelles, which might be beneficial for the tracking and removal of migrating tumor cells after surgery of the main tumor.

MATERIALS AND METHODS

Animal and Surgical Procedures

C6 glioma cells (primary rat glioma) were obtained from the Institute of Neurosurgical Pathophysiology (INcP), Mainz, Germany and cultivated in Dulbecco's modified Eagle medium (DMEM) supplemented with 1% penicillin, 1% streptomycin, 10% fetal calf serum at 37°C, and 5% CO_2 atmosphere. Cells were harvested routinely using trypsin–EDTA (0.05%, 0.02% resp.) and suspended in DMEM.

Prior to injection, viability of the C6 tumor cells were assessed by trypan blue staining. C6 glioma cells (1×10^6/3 animals/group) were implanted stereotactically into the left frontal region of the brain of anesthetized young adult male Wistar rats (Charles River Wiga, Sulzfeld, Germany) as described before [18]. Briefly, burr-hole trephination was carried out and a custom-designed needle was inserted for slow injection of the tumor cell suspension. Tumors were allowed to establish for 10 days on average, and animals were then used for *in vivo* measurements. After this time period, animals implanted with C6 glioma cells began showing signs of apathy and fatigue. Animals were bred and kept at the animal facility in a temperature-controlled environment on a 12-hour light/dark cycle and were fed a regular pelleted rodent maintenance diet and water *ad libitum*. Animal procedures were in accordance with national and international guidelines and were approved by the Governmental Animal Care and Use Committee.

An intraperitoneal catheter was placed for deep anesthesia with chloral hydrate and intravenous catheters were inserted for drug application. Craniotomy was performed after the fixation of the animals on a stereotactic frame and an intracranial window was placed to expose the xenograft tumor, the transition zone and healthy brain tissue, while bleeding was controlled using heat-coagulation, TABOTAMP® and bone wax (Henry Schein VET, Hamburg, Deutschland).

After *in vivo* measurements, the rats were sacrificed and the brain was removed for further *ex vivo* and histopathologic evaluation.

Experimental Setup

Multiphoton-induced autofluorescence and SHG imaging were performed using an inverted Zeiss LSM 510 META NLO femtosecond laser scanning system (Carl Zeiss), coupled to a high repetition rate (80 MHz) tuneable ($\lambda = 690$–1060 nm) Coherent Chameleon titanium: sapphire laser (Coherent Laser Group, Santa Clara, CA). The laser oscillator delivers infrared laser pulses at a maximal average power of 3.5 W with a typical pulse duration of $\tau = 140$ fs. Power is adjustable by means of an acousto-optic modulator (AOM). The non-descanned optical scheme is briefly described in Figure 1. The near infrared laser beam is reflected towards the x, y galvo-scanner by means of a dichroic short pass filter (SP 650). The scanned laser beam passes through an objective inverter (LSM Tech, Etters, PA) to perform upright microscopy for

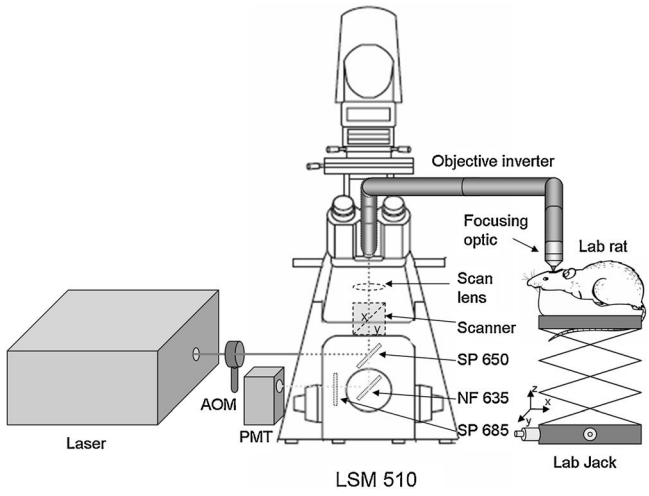

Fig. 1. Global scheme of the experimental setup.

measurements convenience. To generate two photon fluorescence and SHG emission and in order to perform sub-cellular two photon images in air on a relative large theoretical field of view (0.7×0.7 mm^2), a 20×0.75 NA objective (Fluar, Zeiss, Jena, Germany) with a working distance of 0.6 mm has been used. To perform higher resolution images, a 40×1.3 NA, (EC Plan-Neofluar, Zeiss, Jena, Germany) objective under oil confinement has been used. This latter has a shorter field of view (0.35×0.35 mm^2) and working distance (200 μm), and requires the use of a cover slip between the oil medium and tissues. The autofluorescence and SHG signals are recorded on an accurate photo multiplier tube (PMT) detector after they have been transmitted back by the same optical path as for the excitation through the short pass filter 650 nm (SP 650), reflected by a neutral filter 635 nm (NF 635) and through a short pass filter 685 nm (SP 685) which is used to block the eventual residues of the near-infrared excitation. The anesthetized rats as well as *ex vivo* brain tissue samples can be accurately positioned by means of a Lab Jack controllable in x, y, and z. Optimal autofluorescence and SHG signals are generated at an excitation wavelength of 765 and 840 nm, respectively.

RESULTS

Imaging of Tumors In Situ

The brain of previously terminated rats was examined *in situ* through the opened skull and a thin layer of red blood cells. A coverslip was set directly upon the tumor growing in the gray matter of the ipsilateral cortex (Fig. 2) to prevent a drying out of the tissue. The laser wavelength of 765 nm was used to depict cells (Fig. 3A, arrowheads and zigzag arrows) via the autofluorescence of mainly NAD(P)H inside their cytoplasm and endothelial cells of capillaries (Fig. 3A, arrows). Collagen structures of the dura above the tumor were excited at 840 nm as shown in Figure 3B using SHG of collagen. An overlay of images of both wavelengths is illustrated in Figure 3C. Note the different cell types (Fig. 3A, arrowheads and zigzag arrows). The small bright cells (zigzag arrows) bore similarities to the tumor cells (Fig. 4A) whereas the larger cells show a weaker autofluorescence (Fig. 3A: arrowheads; Fig. 4B).

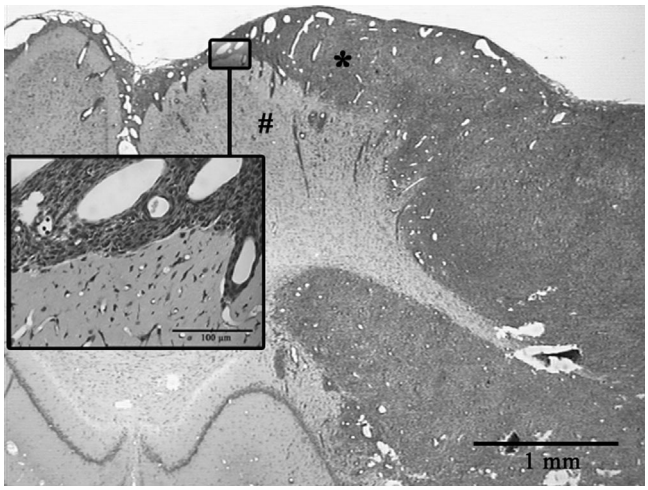

Fig. 2. Micrograph shows a typical section of a rat brain. Cresyl violet staining reveals tumor tissue growing from the cortex surface until the gray matter. The inlet depicts the enlargement of the border zone between tumor (marked by asterix, *) and normal brain tissue (marked by pound key, #).

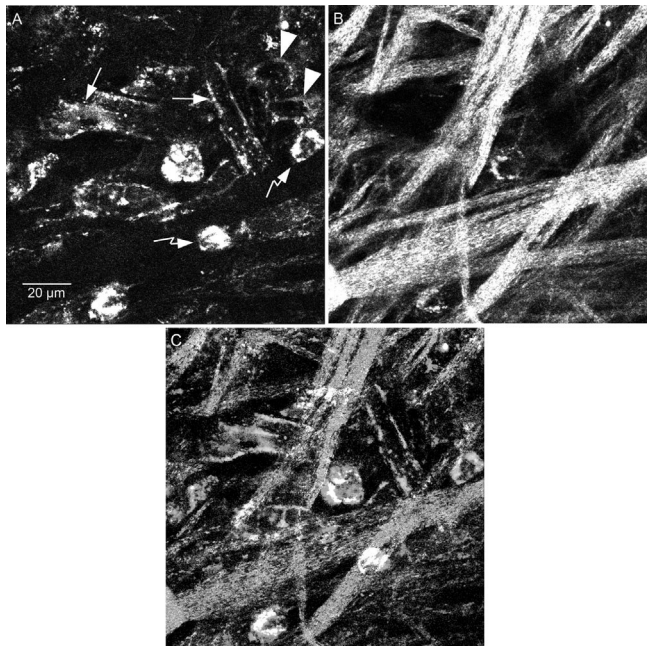

Fig. 3. Imaging of the dura, rat brain. **A**: 765 nm, cells (arrowheads and zigzag arrows), blood capillaries (arrows); (**B**) 840 nm, collagen fibers; (**C**) overlay.

The tumor cells were rather small, round, with small cytoplasm rings around the dominating nucleus and emitted a relatively bright autofluorescence when excited with the laser (Fig. 4A). Fibroblast-like cells of the dura above the tumor are much larger in size and weaker in autofluorescence (Fig. 4B).

To learn more about the structure of the tissue and the pattern of tumor cells inside, 3D scans up to a depth of 120 μm were performed with both 765 and 840 nm. Figure 5 shows cross sections of a tumor region scanned at 765 nm at z-steps of 10 μm each. The stack was performed through a layer of clotted red blood cells (top image $z = 5$ μm, left upper corner) with tissue structures and bright tumor cells clearly visible. The tumor cells were not spread in a regular manner but distributed across the area and cells with lower autofluorescence. These observations were confirmed by 3D stacks through another tumor region by a different rat (Fig. 6). Bright autofluorescent cells were interspersed between larger cells with lower fluorescence signals.

Imaging of Tumors In Situ In Vivo

Imaging of a tumor *in vivo* with somewhat recognizable structures was difficult to accomplish due to the interfering

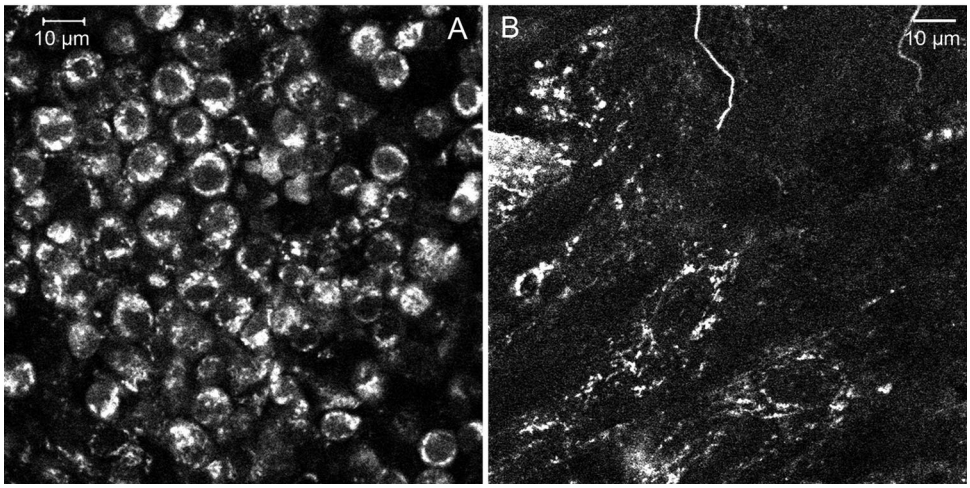

Fig. 4. Imaging of cells inside the tumor (**A**) and of the dura (**B**) at 765 nm.

blood pulse clearly visible at the brain material. The pulsing affected the stability of the image along the *z*-axis and made faster scanning a necessity. The images appeared more blurred and less clear, but single cells or parts of cells were easily distinguishable. Figure 7 shows an *in vivo* image of a tumor region of a rat brain. Due to the faster acquisition time less photons were sampled and the background to noise ratio was higher. The pulse beat appeared as horizontal stripes across the pictures, but using a pixel time of 1.28– 3.8 µs, 512 × 512 pixels, 20×/0.75 objective and 230.3 × 230.3 to 460.7 × 460.7 µm with 8–20 mW at the objective plain lead to a decent imaging of the tissues up to 60 µm in depth.

Laser Destruction of Single Cells Inside a Brain Tumor In Vivo

The next logical step after the establishment of the potential for imaging brain tumors *in vivo* would be the attempt to destroy single cells of interest inside this tissue area *in vivo* as well. Applying the above-mentioned conditions to obtain a decent image the laser power was increased eightfold to 65 mW at the objective plain and a pixel time of 3.8 ms in a defined minuscule line of 6 pixels and targeted it on the cell of interest (Fig. 8A). Due to the blood pulse and the resulting shift of the *z*-plane of the tissue it was a challenging task to hit and image the cell of interest again. Figure 8B shows the resulting increased fluorescence of the destroyed target cell. As shown before [5,19] it is possible to destroy a cell inside a 3-dimensional matrix with high precision and without disturbing neighboring cells or tissue structures above or below the focal plane at all.

DISCUSSION & CONCLUSION

The success of cancer treatment is dependent of the ability to eliminate the tumor as a whole and additionally any stray tumor cells to envision a decrease of metastasis or recurrence incidence. In the last decade in neurosurgery the use of technological achievements, such as HD or 3D- microscopes and endoscopes, navigation modalities, intraoperative MRI scanners, or even the fluorescent staining of the tissue using 5-ALA for better visualization during glioma tumor surgery brought better therapeutic results with higher quality of life and longer survival rates. The biggest disadvantage of all these technical achievements is the fact that they cannot bring the eye of the surgeon to the next level, that is, the cellular/subcellular level. Therefore, the surgeon is still unable to differentiate in real time between normal and tumor cells during surgery. Two-photon microscopy has the potential to overcome this problem [17,20,21] and allows zooming into the focus of interest. It might allow the surgeon to "see" at totally different dimensions, namely on the cellular level as compared to the optical dimensions existing today even in modern operating theatres.

To our knowledge this study is the first one in the literature trying to evaluate the pros and cons such a microscopic technique could have if used during neurosurgical resections of brain tumors.

To examine the potential of two-photon imaging of malignant brain tumors *in situ* and *in vivo* via inducing the natural autofluorescence of the tissues, we used a rat model and a custom microscope with an objective inverter. The first obstacles to overcome were the layer of blood coating the brain surface and the rounded physiognomy of the brain *in situ*. Today there is no safe way to eliminate blood from the optical field in order to recognize the field behind the blood layer—even if the most advanced optical instruments available in the operating theatre are used. Therefore, to see "behind" blood and to differentiate tissue modalities, lying beyond the blood layer, is impossible so far.

The NIR fs-laser beam was easily able to penetrate the blood, but the available area to scan without getting out of focus was rather small, though we were able to attain very precise images and 3D tags with the possibility to discriminate between various cell types. The brighter autofluorescence of possible tumor cells may be attributed

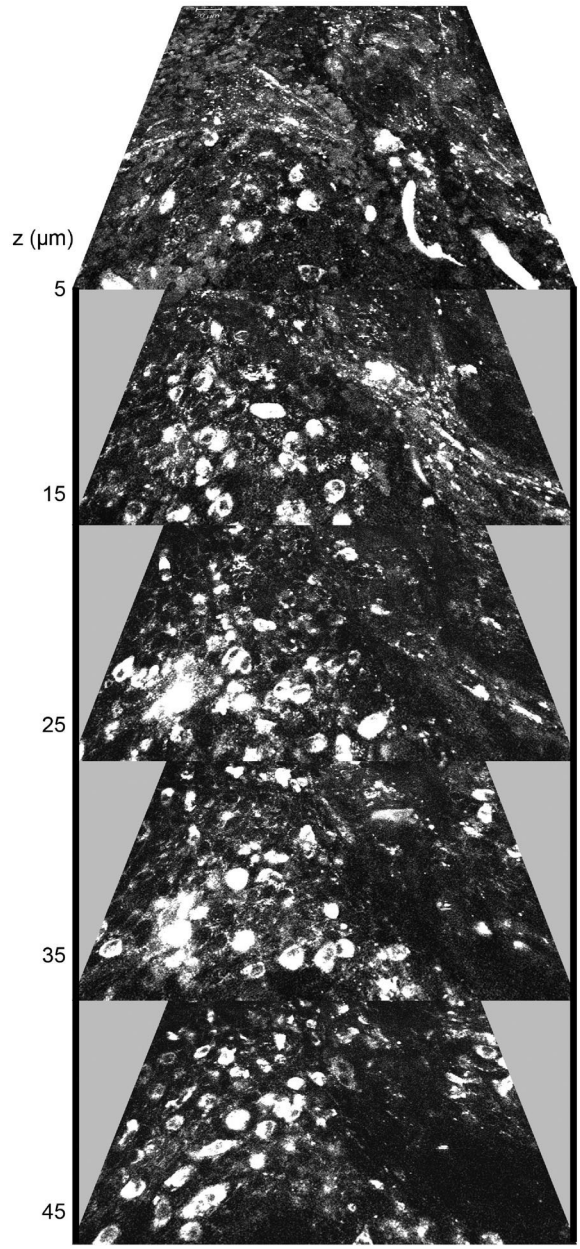

Fig. 5. 3D stack of a tumor region *in situ*, shown are z—sections of every 10 μm, 765 nm.

to an increase of metabolism products such as NAD(P)H, flavins, and degradation products in lysosomes. It should become possible to distinguish between possible tumor cells and endothelial cells due to determination of their different shape, alignment inside the tissue and the nucleus plasma relation. Further work will have to be performed to learn more about possible differentiation of the cell types involved.

In vivo measurements brought on a further problem: blood pulse and respiration which both affect the *z*—stability of the scanned section. A quicker scanning to at least partially compensate this effect led to images of lesser

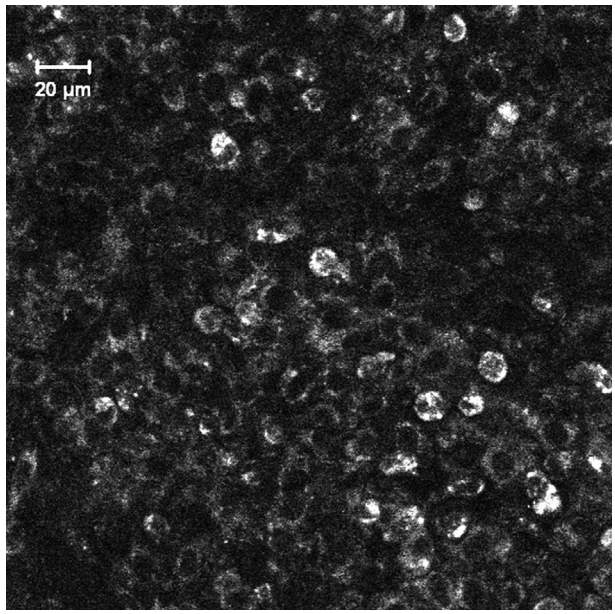

Fig. 6. Autofluorescence image of a tumor *in situ* with bright fluorescent cells interspersed between slightly larger ones with lower autofluorescence signals, 765 nm.

quality, nonetheless tissue structures and different cell types were still visible, distinguishable and destroyable without damaging the neighboring tissue compartments.

The potential of two-photon imaging, however, has to be enhanced to be of practical use for possible intraoperative settings. Due to relative long high quality 3D image acquisition times of common existing systems it will be demanding to scan and evaluate the whole resection area. Therefore, it would be necessary not only to miniaturize the setting and gain more flexibility to measure even at more difficult angles, but to fasten the

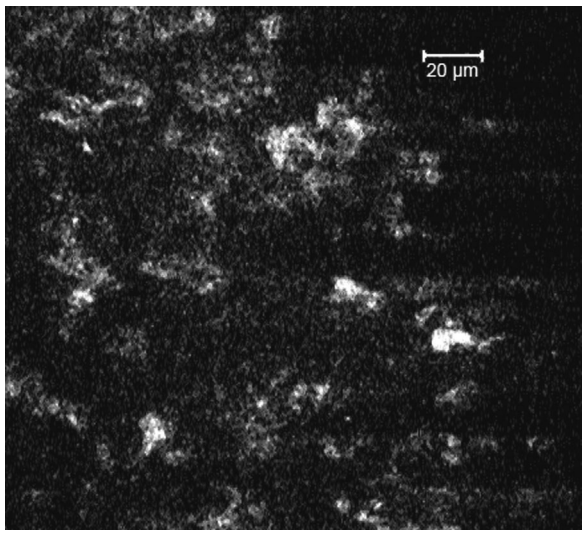

Fig. 7. Image of a tumor region, rat brain *in vivo*, 765 nm, depth: about 30 μm.

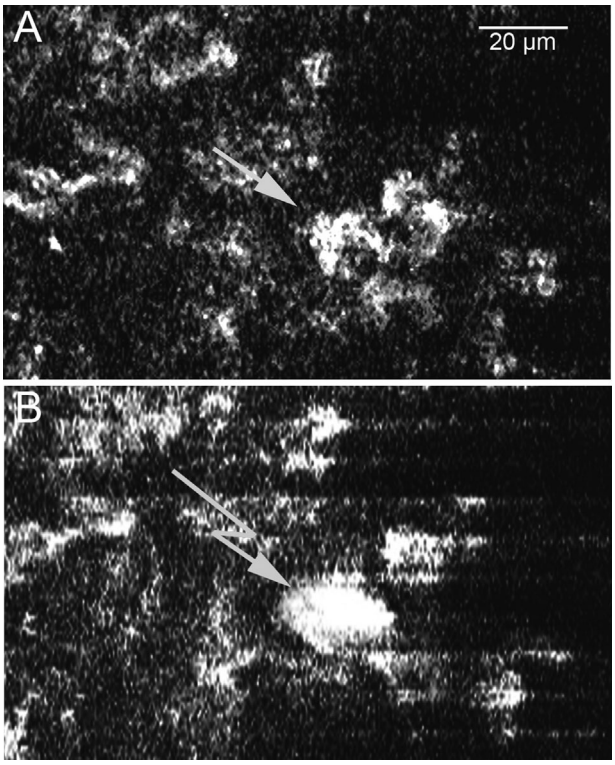

Fig. 8. Destruction of a single tumor cell, rat brain *in vivo*, 765 nm, depth: about 30 µm; (**A**) target cell before laser cut (arrow), (**B**) after laser cut.

process. This can be achieved by development of special software to generate 3D images of tissue areas online, to compensate undesirable side effects such as the unpredictable *z*-plane *in vivo*, enhance the quality of rapidly acquired images and stitch those together in order to get a greater overview over the area, to automate the process in order to accelerate and help the surgeon to analyze and treat areas of interest simultaneously. To accomplish a greater imaging depth or a more rapid scan time, several strategies are conceivable. Increasing incident average laser power is one common means to achieve greater penetration depth of the excitation beam, but it also increases the probability of both excitation saturation and higher-order excitation processes, resulting in decreased optical resolution and more extensive photo damage, respectively. Development and use of dyes having a larger two-photon absorption cross-section to provide better signal, like 5-ALA, which can be easily induced with two-photon excitation [22], would result in the possibility to examine regions of interest more closely and may reduce the need to scan the whole cavitation site. The use of longer wavelengths of the excitation light, and measures to increase collection efficiency of the system (such as better transmittance of filters, beam splitters, etc.) are others possibilities. Concerning the compensation of blood pulsing to obtain good quality and exploitable images, in complementary to fast scanning, other techniques could be used or developed in the future in which signal acquisition is gated to minimize motion or other artefacts caused by great vessel pulsation but also by automatic corrections in the image analysis software.

It is also to be discussed if the two-photon system could be coupled with other techniques. A combination of multiple technologies in one small device like an endoscope, may lead to a new generation of intelligent instruments. Endoscopes are very easy to introduce into brain tumor cavities to look around and identify hidden corners involving remaining tumor tissue after main resection. Creating a multichannel endoscopic device with (i) 3D multispectral view for identifying simultaneously tissue markers, like fluorescein, 5-ALA, or ICG, (ii) one- and two-photon capabilities with tissue scanning modalities for a subcellular view, (iii) tactile sensors for testing tissue stiffness, etc. should be considered. This would lead to up to three or more modalities to differentiate remaining tumor from normal brain after the resection of the main tumor to get the more safety in resection and the higher accuracy in keeping the normal brain intact [23].

The results of the experiments outlined here indicate definitively that the method might obtain a future role in intraoperative treatment of brain tumors. Bringing the eye of the surgeon to a cellular level should allow for a differentiation between remaining tumor cells after extrication of the tumor and healthy tissue in the operation field. Furthermore, it should become possible to eliminate tumor cells by applying more laser energy using the same laser system used to visualize the cells.

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REFERENCES

1. Buckner JC. Factors influencing survival in high-grade gliomas. Semin Oncol 2003;30:10–14.
2. Asthagiri AR, Pouratian N, Sherman J, Ahmed G, Shaffrey ME. Advances in brain tumor surgery. Neurol Clin 2007;25: 975–1003, viii–ix.
3. Lacroix M, Abi-Said D, Fourney DR, Gokaslan ZL, Shi W, DeMonte F, Lang FF, McCutcheon IE, Hassenbusch SJ, Holland E, Hess K, Michael C, Miller D, Sawaya R. A multivariate analysis of 416 patients with glioblastoma multiforme: Prognosis, extent of resection, and survival. J Neurosurg 2001;95:190–198.
4. Sanai N, Berger MS. Glioma extent of resection and its impact on patient outcome. Neurosurgery 2008;62:753–764, discussion 264–756.
5. Tanaka Y, Nariai T, Momose T, Aoyagi M, Maehara T, Tomori T, Yoshino Y, Nagaoka T, Ishiwata K, Ishii K, Ohno K. Glioma surgery using a multimodal navigation system with integrated metabolic images. J Neurosurg 2009;110: 163–172.
6. Hofmann UG, Folkers A, Mosch F, Malina T, Menne KM, Biella G, Fagerstedt P, De Schutter E, Jensen W, Yoshida K, Hoehl D, Thomas U, Kindlundh MG, Norlin P, de Curtis M. A novel high channel-count system for acute multisite neuronal recordings. IEEE Trans Biomed Eng 2006;53:1672–1677.
7. Stummer W, Novotny A, Stepp H, Goetz C, Bise K, Reulen HJ. Fluorescence-guided resection of glioblastoma multiforme by using 5-aminolevulinic acid-induced porphyrins: A

prospective study in 52 consecutive patients. J Neurosurg 2000;93:1003–1013.
8. Stummer W, Pichlmeier U, Meinel T, Wiestler OD, Zanella F, Reulen HJ. Fluorescence-guided surgery with 5-aminolevulinic acid for resection of malignant glioma: A randomised controlled multicentre phase III trial. Lancet Oncol 2006; 7:392–401.
9. Bogaards A, Varma A, Collens SP, Lin A, Giles A, Yang VX, Bilbao JM, Lilge LD, Muller PJ, Wilson BC. Increased brain tumor resection using fluorescence image guidance in a preclinical model. Lasers Surg Med 2004;35:181–190.
10. Kantelhardt SR, Diddens H, Leppert J, Rohde V, Huttmann G, Giese A. Multiphoton excitation fluorescence microscopy of 5-aminolevulinic acid induced fluorescence in experimental gliomas. Lasers Surg Med 2008;40:273–281.
11. Kantelhardt SR, Leppert J, Kantelhardt JW, Reusche E, Huttmann G, Giese A. Multi-photon excitation fluorescence microscopy of brain-tumour tissue and analysis of cell density. Acta Neurochir 2009;151:253–262, discussion 262.
12. Kienast Y, von Baumgarten L, Fuhrmann M, Klinkert WE, Goldbrunner R, Herms J, Winkler F. Real-time imaging reveals the single steps of brain metastasis formation. Nat Med 2010;16:116–122.
13. Leppert J, Krajewski J, Kantelhardt SR, Schlaffer S, Petkus N, Reusche E, Huttmann G, Giese A. Multiphoton excitation of autofluorescence for microscopy of glioma tissue. Neurosurgery 2006;58:759–767, discussion 759–767.
14. Meyer T, Bergner N, Bielecki C, Krafft C, Akimov D, Romeike BF, Reichart R, Kalff R, Dietzek B, Popp J. Nonlinear microscopy, infrared, and Raman microspectroscopy for brain tumor analysis. J Biomed Opt 2011;16:021113.
15. Denk W, Strickler JH, Webb WW. Two-photon laser scanning fluorescence microscopy. Science 1990;248:73–76.
16. Svoboda K, Yasuda R. Principles of two-photon excitation microscopy and its applications to neuroscience. Neuron 2006;50:823–839.
17. Zipfel WR, Williams RM, Webb WW. Nonlinear magic: Multiphoton microscopy in the biosciences. Nat Biotechnol 2003;21:1369–1377.
18. Stummer W, Stocker S, Novotny A, Heimann A, Sauer O, Kempski O, Plesnila N, Wietzorrek J, Reulen HJ. In vitro and in vivo porphyrin accumulation by C6 glioma cells after exposure to 5-aminolevulinic acid. J Photochem Photobiol B 1998;45:160–169.
19. Wang BG, Koenig K, Riemann I, Krieg R, Halbhuber KJ. Intraocular multiphoton microscopy with subcellular spatial resolution by infrared femtosecond lasers. Histochem Cell Biol 2006;126:507–515.
20. König K, Schenke-Layland K, Riemann I, Stock UA. Multiphoton autofluorescence imaging of intratissue elastic fibers. Biomaterials 2005;26:495–500.
21. Schenke-Layland K, Riemann I, Damour O, Stock UA, König K. Two-photon microscopes and in vivo multiphoton tomographs—powerful diagnostic tools for tissue engineering and drug delivery. Adv Drug Deliv Rev 2006;58:878–896.
22. König K, Riemann I, Fischer P. Photodynamic therapy by nonresonant two-photon excitation. Proc SPIE 1999;3592: 43–49.
23. Foersch S, Heimann A, Ayyad A, Spoden GA, Florin L, Kiesslich R, Mpoukouvalas K, Kempski O, Goetz M, Charalampaki P. Confocal laser endomicroscopy for diagnosis and histomorphologic imaging of brain tumors in vivo. PLoS ONE 2012;7:e41760.