Fluorescence-Based Measurement of Real-Time Kinetics of Protoporphyrin IX After 5-Aminolevulinic Acid Administration in Human In Situ Malignant Gliomas

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Received, September 13, 2018.
Accepted, March 31, 2019.
Published Online, May 6, 2019.

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BACKGROUND: Five-aminolevulinic acid (5-ALA) is well established for fluorescence-guided resections of malignant gliomas by eliciting the accumulation of fluorescent protoporphyrin IX (PpIX) in tumors. Because of the assumed time point of peak fluorescence, 5-ALA is recommended to be administered 3 h before surgery. However, the actual time dependency of tumor fluorescence has not yet been evaluated in humans and may have important implications.

OBJECTIVE: To investigate the time dependency of PpIX by measuring fluorescence intensities in tumors at various time points during surgery.

METHODS: Patients received 5-ALA (20 mg/kg b.w.) 3 to 4 h before surgery. Fluorescence intensities (FI) and estimated tumor PpIX concentrations (CPPIX) were measured in the tumors over time with a hyperspectral camera. CPPIX was assessed using hyperspectral imaging and by evaluating fluorescence phantoms with known CPPIX.

RESULTS: A total of 201 samples from 68 patients were included in this study. On average, maximum values of calculated FI and CPPIX were observed between 7 and 8 h after 5-ALA administration. FI and CPPIX both reliably distinguished central strong and marginal weak fluorescence, and grade III compared to grade IV gliomas. Interestingly, marginal (weak) fluorescence was observed to peak later than strong fluorescence (8-9 vs 7-8 h).

CONCLUSION: In human in Situ brain tumor tissue, we determined fluorescence after 5-ALA administration to be maximal later than previously thought. In consequence, 5-ALA should be administered 4 to 5 h before surgery, with timing adjusted to internal logistical circumstances and factors related to approaching the tumor.

KEY WORDS: 5-ALA, Fluorescence guide resection, Malignant glioma, PpIX concentration

Neurosurgery 85:E739–E746, 2019 DOI:10.1093/neuros/nyz129 www.neurosurgery-online.com

Malignant gliomas have a limited prognosis despite modern multimodal therapies.[1,2] Extent of resection as the usual first therapy in a multimodal setting is accepted to significantly prolong patients' overall survival.[2-7] In glioma surgery, discrimination between normal and malignant tissue is often challenging.[8,9] Therefore, to intraoperatively identify glioma tissue more effectively, several surgical adjuncts have emerged, such as intraoperative magnetic resonance imaging,[10-12] ultrasound,[13,14] and fluorescence-guided surgery (FGS).[8,9,15-28]

ABBREVIATIONS: **5-ALA,** Five-aminolevulinic acid; **CPPIX,** PpIX concentration; **FI,** fluorescence intensity; **FGS,** fluorescence-guided surgery; **IDH,** isocitrate dehydrogenase; **LCTF,** liquid crystal tunable filter; **LED,** light-emitting diode; **PpIX,** protoporphyrin IX; **ROI,** region of interest; **sCMOS,** scientific complementary metal-oxide-semiconductor

Five-aminolevulinic acid (5-ALA) induces the selective accumulation of fluorescent protoporphyrin IX (PpIX) in malignant glioma tissue and is used for FGS.[8,9,15-28] This technique has been recently approved by the Food and Drug Administration (https://www.fda.gov/drugs/informationondrugs/approveddrugs/ucm562645.htm). However, the precise mechanisms governing the selective metabolization in tumor

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cells are unclear, as is the exact timeline of accumulation in human brain tumor tissue. The latter factor is essential for determining the optimal timing of initial administration of 5-ALA. A too-small latency after 5-ALA administration could result in fluorescence not yet having reached its maximum during surgery, whereas giving the drug too early might result in inferior fluorescence because of PpIX clearance.

Historically, the time of administration recommended in the summary of the product characteristics (SPC) of Gliolan® was based on animal experiments in which a fluorescence peak was observed 6 h after administration.[29] Therefore, oral administration of 5-ALA was advised for 3 h prior to anesthesia, leaving the remaining 3 h for inducing anesthesia, positioning, draping, craniotomy, and resection of the central tumor core. Almost all clinical trials to date have adopted the 3-h regime (ranging from 2 to 4 h).[9,16-28]

However, the assumption that the animal results can simply be embraced for human brain tumors remains to be tested and is of importance, given the rapid growth in popularity of the method. To our knowledge, no study is available that addresses the timeline of PpIX fluorescence in human brain tumor tissue in Situ. Limited information is available for other organ systems. In a previous report, plasma PpIX concentration (CPPIX) peaked after 8 h,[28] and maxima later than 6 h have also been described for skin procedures, in which they range from 6.5 to 9.8 h after administration of a dose of 40 mg/kg.[30] This suggests that the previously assumed 6-h latency for maximum fluorescence in brain tumor tissue may be too short.

Furthermore, the kinetics of tumor tissue PpIX clearance in human brain tumors are also unknown. Knowledge about PpIX clearance would have further implications for surgery. If slow clearance was observed, this would indicate a longer window in which fluorescence could be helpful for resection. In a practical setting in which surgery is sometimes postponed for logistical reasons, it would be important to know whether unplanned delays would play a significant role for the intensity or quality of fluorescence.

The goal of this study was, therefore, to investigate the time dependence of fluorescence in human brain tumors to determine the best timing for tumor resection after 5-ALA administration, stratifying by glioma grade and also by known fluorescence qualities.[16]

Performing continuous measurements in single patients to determine the patient-specific course of tumor fluorescence did not appear feasible for obtaining useful data because of timing and bleaching issues and an unethical prolongation of surgery. We instead chose a heuristic approach, performing a limited number of measurements per patient in a large number of patients to ultimately generate a profile of PpIX fluorescence in tumors over time. All measurements were made in freshly extracted tissues meticulously protected from light.

METHODS

Patients

We evaluated patients harboring lesions suspicious for malignant glioma, for which surgical treatment was indicated by an interdisciplinary tumor board, independent of this study, at the Department of Neurosurgery, University Hospital of Münster, between December 2017 and May 2018.

Patients received 5-ALA (Gliolan®; Medac, Wedel, Germany) orally at a standard dose of 20 mg/kg b.w., 3 to 4 h before induction of anesthesia, as has been the protocol embraced by our service. The exact time of 5-ALA administration was recorded. Surgery was performed using an OPMI Pentero 900 microscope equipped with the BLUE 400 filter system for PpIX visualization (Carl Zeiss Meditec, Oberkochen, Germany). Frozen sections were obtained prior to tumor resections to confirm the suspected diagnoses of malignant gliomas. When fluorescence was encountered, the surgeon was asked to grade the fluorescence quality of tissue on a three-tiered scale (“strong,” “weak,” or “none”). If feasible, up to 3 biopsies were collected in the course of surgery from each patient: at an early timepoint, an intermediate time point, and a late time point. Spectral characteristics were determined ex Vivo immediately after extraction of tissue samples, as described below. In addition, the elapsed time between 5-ALA administration and tissue biopsy was recorded. All samples were rendered for neuropathological assessment.

Each patient’s written informed consent was obtained. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards. Data collection and scientific use of biopsies had previously been approved by the local ethics committee (University of Münster 2015-632-f-S).

Spectroscopy

Spectroscopy was performed using a continuous hyperspectral imaging system. A light-emitting diode (LED) was implemented as the light source and integrated into an OPMI PICO (Carl Zeiss Meditec, Oberkochen, Germany) system via a liquid light guide. The detected light was then directed to a scientific complementary metal-oxide-semiconductor (sCMOS) camera. The sCMOS camera, which has minimal sensor noise and high quantum efficiency, was employed for highly sensitive detection of fluorescence and for the white light images used to normalize for optical tissue properties. A liquid crystal tunable filter (LCTF) was positioned between an achromatic lens and the sCMOS camera. Remaining light was directed to a color camera with an IMX252 sensor (Sony, Tokyo, Japan), which was used to record a white light image, which could be superimposed with the fluorescence images. A filter swivel with a BLUE 400 observation filter was installed between the 2 beam splitters so that BLUE 400 images could optionally be recorded as well. The individual components (sCMOS camera, color camera [IMX252], LED light source, LCTF, and spectrometer) were controlled with LabVIEW (National Instruments, Inc.; Austin, Texas). Using the sCMOS camera, fluorescence spectra were recorded for the entire wavelength range of the LCTF (from 420 to 730 nm) by taking pictures every 3 nm, starting at the largest wavelength. The same was done for the white light spectra using 5-nm steps, and finally the dark

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TABLE 1. Patients Demography

			Number of patients	Percentage (%)
All			68	
Sex	Male		43	63.2
	Female		25	
Age	Mean (SD)		(59 ± 13.81)	
Histology	Glioblastoma IDH-wild type		57	83.8
	Glioblastoma IDH-mutant		2	2.9
	Anaplastic astrocytoma IDH-wild type		4	5.9
	Anaplastic astrocytoma IDH-mutant		5	7.4
MGMT promoter status	Methylated	Grade IV	40	58.8
		Grade III	9	13.2
	Unmethylated	Grade IV	19	27.9
		Grade III	0	0
KPS score	60		1	1.4
	70		5	7.1
	80		12	17.1
	90		28	41.4
	100		22	31.4

IDH, isocitrate dehydrogenase; KPS, Karnofsky Performance Status; MGMT, O^6-methylguanine DNA methyltransferase; SD, standard deviation; WHO, World Health Organization

spectra, again using 3-nm steps. The result was a fluorescence spectrum and a white light spectrum at every pixel, with background signal.

We measured fluorescence intensity (FI) of tissues in 10 to 30 regions of interest (ROIs) per biopsy, depending on the volume of the biopsy. The intensities were averaged to give a final value for each ROI. The data were standardized by reference values. Reference measurements were made using a nonbleaching reference object (635-nm fluorescence phantom as supplied with microscope; Carl Zeiss, Oberkochen, Germany) that fluoresced in the range of the porphyrin spectrum and were obtained before each tissue measurement to correct for short-term fluctuations in excitation light.

Tissue CPPIX were calculated using MATLAB (The MathWorks, Inc.; Natick, MA), as previously reported.[20,31] First, the fluorescence spectra were normalized using the white light spectra and a dual-band normalization algorithm[32,33] to correct for inhomogeneous tissue absorption and scattering characteristics. This method involves dividing the fluorescence spectrum by a product of the red and blue spectral bands of the white light spectrum and using a system-specific, experimentally derived exponent on the red band. Once normalized, the fact that each spectrum is a linear combination of its constituent basis parts, autofluorescence and PpIX fluorescence, whose spectra were well known, was used to set up a linear system of equations to solve for the proportion of each basis spectrum. The optimal solution was found using a least-squares regression. The measured spectrum was thus normalized and spectrally unmixed, leaving the pure, normalized PpIX spectrum. The magnitude of this spectrum was proportional to the relative CPPIX. To obtain an absolute value, we analyzed fluorescence phantoms with known CPPIX using the same method, thus calibrating the algorithm. In the future, we plan to chemically measure the CPPIX of brain tumor tissue to verify and/or update this absolute calibration.

Histology

All tumor tissues, including the biopsies used for ex Vivo spectroscopy, were assessed histologically according to the World Health Organization classification of 2016.[34] Isocitrate dehydrogenase 1 (IDH1) mutation status was determined using immunohistochemistry.[35] Paraffin-embedded material was examined molecularly for methylation status of the 5′ region of the O-6-methylguanine DNA methyltransferase gene (MGMT) in all tumors. Bisulfite-treated DNA was analyzed by methylation-specific polymerase chain reaction, as described previously.[36]

TABLE 2. Fluorescence Quality of Tissues

		Fluorescence quality		
		Strong	Weak	Tissues
WHO grade	IV	154 (86.6%)	25 (13.4%)	179
	III	20 (90.9%)	2 (9.1%)	22
All		174 (87.1%)	27 (12.9%)	201

WHO, World Health Organization

Statistical Analysis

For statistical purposes, we subdivided the time after administration into 1-h intervals. All measurements occurring within a given hourly interval were averaged, resulting in the time intervals 5 to 6 h, 6 to 7, 7 to 8, 8 to 9, and 9 to 10 h. Dependent and independent samples were not mixed for any of the time points to avoid skewing the data (ie, per time point, only a single sample from 1 patient went into the calculation).

Distribution was evaluated with the F-test between 2 groups and Bartlett's test for average among elapsed time groups. The Kruskal–Wallis test was used for nonparametric testing. Paired data were analyzed by the paired *t*-test. The Bonferroni method was used to adjust for multiple comparisons. Differences were regarded

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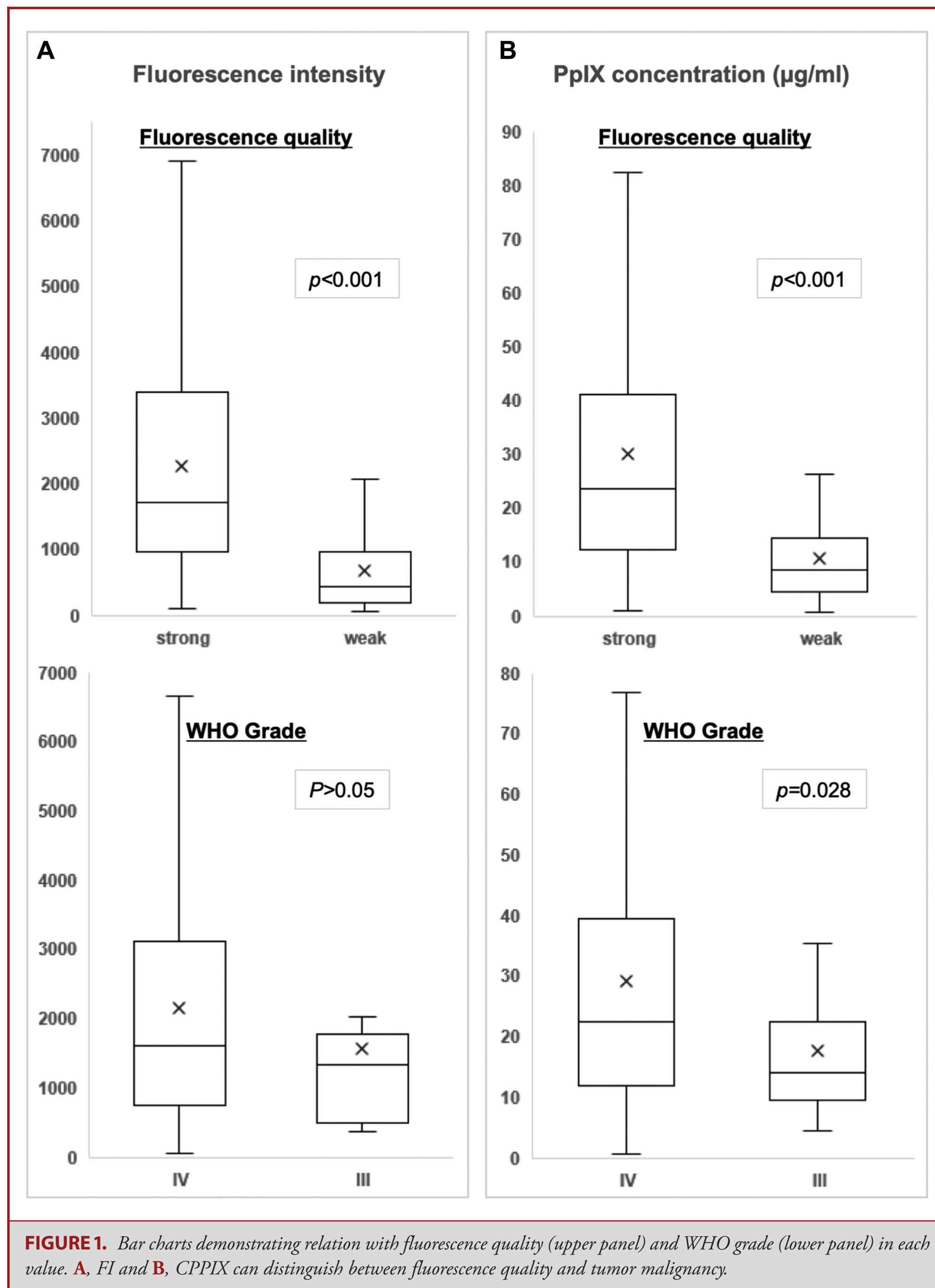

FIGURE 1. *Bar charts demonstrating relation with fluorescence quality (upper panel) and WHO grade (lower panel) in each value.* **A**, *FI and* **B**, *CPPIX can distinguish between fluorescence quality and tumor malignancy.*

as statistically significant if the error probability P was smaller than 5%.

All statistical analyses were performed with EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan), which is a graphical user interface of the R Foundation for Statistical Computing (Vienna, Austria).[34]

RESULTS

Patients and Tissues

Patient demographics are given in Table 1. We included 43 males and 25 females, aged between 23 and 82 yr (mean 59 yr). As

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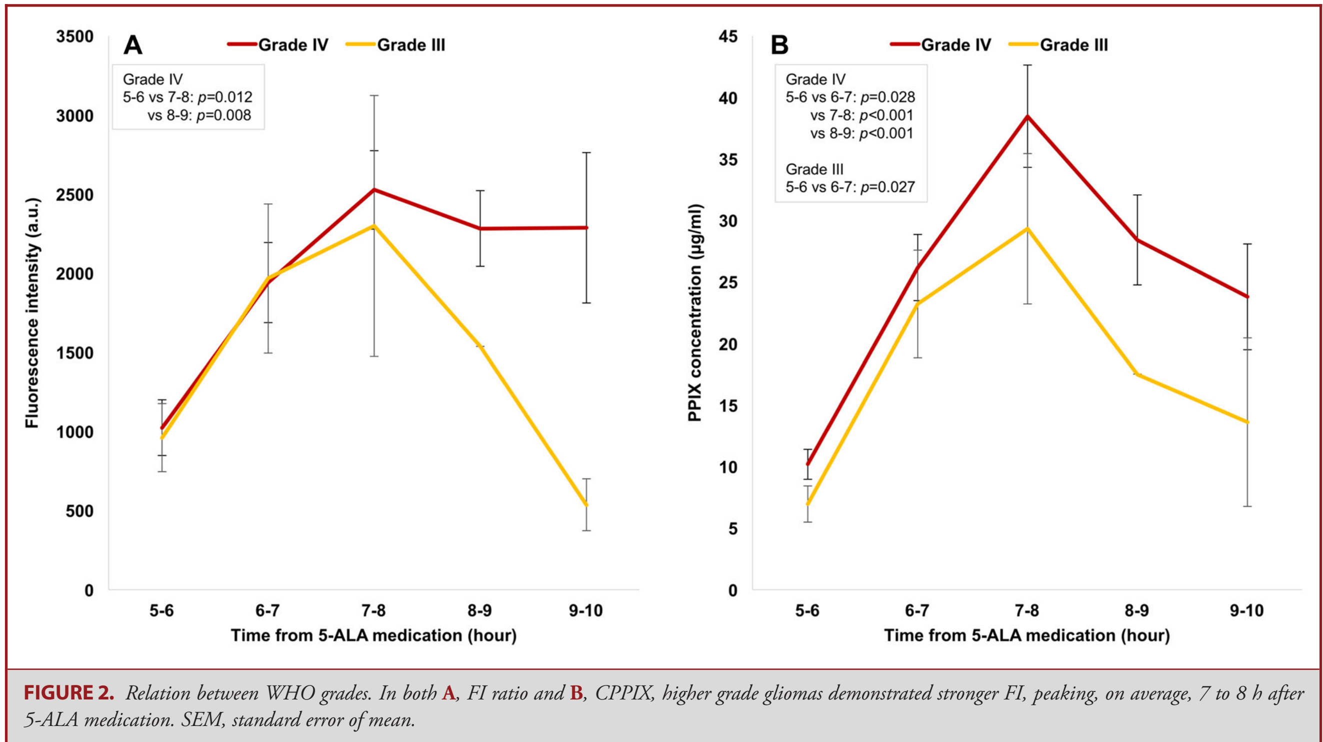

FIGURE 2. *Relation between WHO grades. In both* **A**, *FI ratio and* **B**, *CPPIX, higher grade gliomas demonstrated stronger FI, peaking, on average, 7 to 8 h after 5-ALA medication. SEM, standard error of mean.*

for the etiology, tumors were classified as 59 grade IV glioma and 9 grade III gliomas. A total of 201 tissue samples were collected for the measurement of FI (Table 2).

FI, CPPIX, and Tumor Malignancy

When assessed spectrographically and independently of time, the fluorescence qualities "strong" and "weak," as perceived by surgeons, demonstrated significantly different FI ($P < .001$; "strong vs weak"). When comparing WHO grades III and IV, we could not observe statistically significant difference ($P =$ n.s.) in FI (Figure 1A). The estimated CPPIX likewise significantly differed among observed fluorescence qualities ($P < .001$; "strong vs weak"), and between WHO grades III and IV ($P = .028$; Figure 1B). We could not find a significant difference between FI and the estimated CPPIX in comparison between IDH status, sex, age, and location of the tumor.

Time Dependency After 5-ALA Administration

Overall, the peak of FI appeared, on average, 7 to 8 h after 5-ALA administration. CPPIX demonstrated a similar peak after 7 to 8 h. Figure 2A illustrates the relation between time after oral administration of 5-ALA and FI in analyzed tissue, whereas Figure 2B shows the time relationship with the calculated CPPIX. Interestingly, fluorescence perceived as "weak" by surgeons peaked later than the fluorescence perceived as "strong," namely within the ninth hour after application (Figure 3).

In glioblastomas, we also noted that accumulation occurred more rapidly than clearance after the maximum had been passed (Figure 2).

DISCUSSION

5-ALA is an effective surgical adjunct for identifying tumor tissue in real time during the resections of high-grade glioma[27] and is now being commonly used.[9,15-26,28] Even though 5-ALA has been extensively implemented clinically for more than 10 yr, exact fluorescence kinetics related to 5-ALA preferential metabolism and accumulation of PpIX in human tumor tissue are still unclear. It is, therefore, of utmost interest to characterize the time dependency of 5-ALA-induced fluorescence and to estimate when maximum FI can be expected in malignant gliomas during surgery. Thus, in the present study, we performed a quantification over time of FI to demonstrate fluorescence dynamics of PpIX in malignant glioma tissue. Time fluorescence curves in this ex Situ study revealed the highest FI and CPPIX 7 to 8 h after 5-ALA administration. Early results using an orthotopic brain tumor model[29] had suggested PpIX fluorescence to peak at 6 h. Our observations in human brain tumors using samples collected during surgery and measured immediately ex Situ now show this maximum to be later. This observation is more in line with peak PpIX in plasma (7.8 h)[28] or in skin (6.5-9.8 h).[30]

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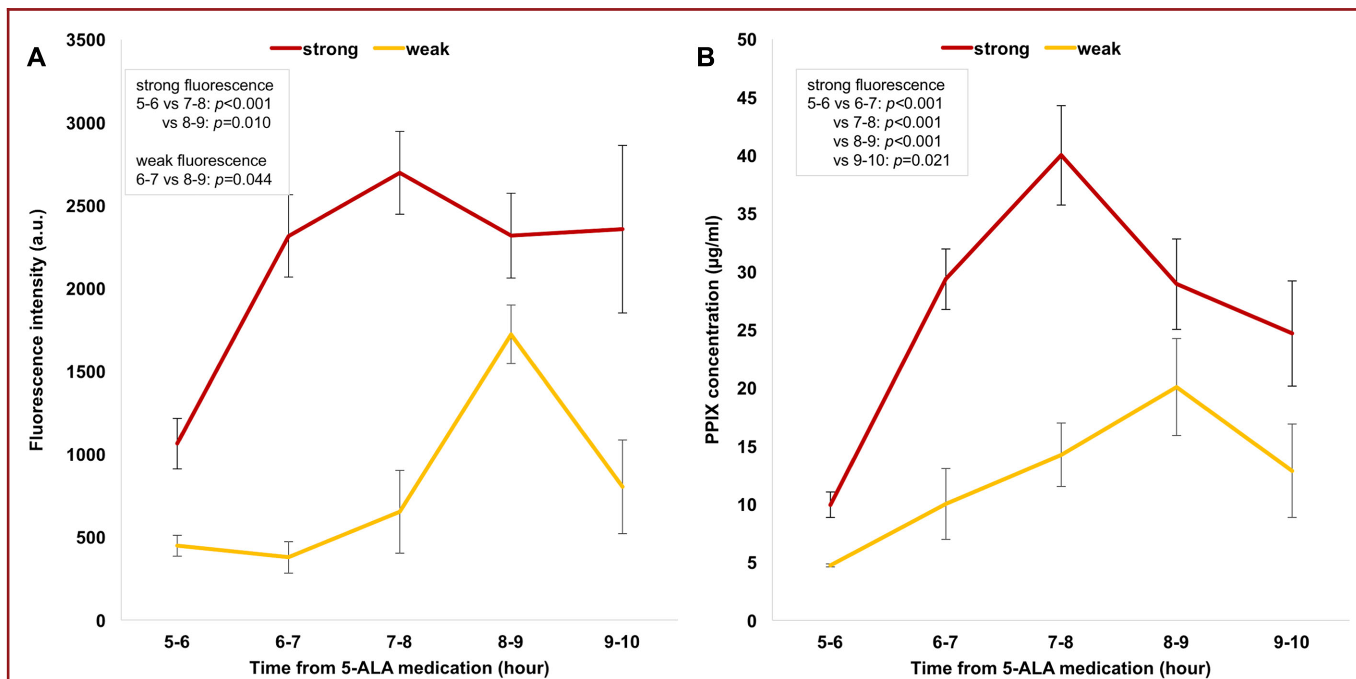

FIGURE 3. **A**, *FI measurements in tissues with "strong" fluorescence delivered highest value 7 to 8 h after 5-ALA administration (averages ± standard error of mean; SEM). Values measured at 5 to 6 h were significantly lower than at 7 to 8 or 8 to 9 h, and values measured at 6 to 7 h were statistically significantly lower than values measured 7 to 8 h after 5-ALA administration. Note the rapid rise and slow clearance of fluorescence. In weakly fluorescing tissue, the maximum was found at 8 to 9 h.* **B**, *Kinetic findings were similar for CPPIX. In both* **A** *and* **B**, *we could not find significant difference changes in time kinetic of "weak" fluorescence tissues. SEM, standard error of mean.*

This observation has implications for the timing of surgery after 5-ALA administration. We now recommend administration not to commence 2.5 to 3.5 h prior to surgery, as specified in the SPC for Gliolan® and as tested in the pivotal randomized approval study,[27] but rather at an earlier timepoint (eg, 4-5 h prior to induction of anesthesia). For the individual institution, logistical aspects should be factored into the optimal time of application, as should aspects related to the patient, such as the time it takes to expose the tumor or to perform mapping.

Interestingly, for glioblastomas, which constituted a majority of samples in this study, fluorescence appears to clear more slowly than it took to accumulate (Figures 2A and 2B), with tissue fluorescence still approximately 65% of maximum even after 9 to 10 h after application. This is higher than 6 to 7 h after application, indicating the window extends beyond our measurement window of 10 h in GBM. Furthermore, samples subjectively rated as having weak fluorescence and predominantly taken from the fluorescing margins showed peak fluorescence after 8 to 9 h, underscoring the necessity of earlier application, because it is this part of the tumor that is reached last, but in which fluorescence-guided resections might play the most important role.[37] Fluorescence qualities have been found to correlate with tumor cellularity and Ki-67/MIB1 index respectively.[16,23,38]

Knowledge of PpIX maxima will also play a role for the timing of application in the emerging field of 5-ALA photodynamic therapy,[39] in which damage to tumor tissue will depend on the availability of PpIX.

We found differences in the kinetics of PpIX when comparing FI and the calculated concentrations in tissue to be negligible. This is fortunate, because a disparity would have had consequences for the timing of fluorescence-guided resections, which rely on the observed fluorescence, whereas PDT would rely on the concentration of cellular PpIX.

In more general terms, our observations stress the time dependency of any method of intraoperative fluorescence, be it for 5-ALA-derived porphyrins or for injected dyes that sequester into the tumor via the breached blood–brain barrier and propagate with edema, such as fluorescein.[40]

Understanding time dependency of FI is essential. For instance, for tumors with minimal fluorescence, for tumor margins or in tumors with difficult visualization geometry (deep-seated lesions), operating at a time point of optimal fluorescence could be important. Furthermore, photodynamic therapy using 5-ALA-derived porphyrins is under scrutiny for high-grade gliomas. The efficacy of this method would also depend on the highest concentrations of PPIX possible.

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CONCLUSION

Our analysis of 201 tissues of 68 patients harboring malignant gliomas demonstrated that fluorescence peaks approximately 7 to 8 h after 5-ALA administration, and even later for marginal, weakly fluorescing malignant glioma tissue. This observation needs to be taken into consideration for the correct timing of 5-ALA administration depending on the surgical approach and logistic scenario of the individual department.

Disclosures

Carl Zeiss Meditec (Oberkochen, Germany) provided the required BLUE 400 filter and the OPMI PICO system. Walter Stummer has received speaker and consultant fees from Medac, Zeiss, Leica, Photonamic, and NXDC and research support from Zeiss.

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Acknowledgment

We would like to thank Carl Zeiss Meditec (Oberkochen, Germany) for providing us with the required BLUE 400 filter and the OPMI PICO system.

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