δ-aminolevulinic acid–induced protoporphyrin IX concentration correlates with histopathologic markers of malignancy in human gliomas: the need for quantitative fluorescence-guided resection to identify regions of increasing malignancy

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Extent of resection is a major goal and prognostic factor in the treatment of gliomas. In this study we evaluate whether quantitative ex vivo tissue measurements of δ-aminolevulinic acid–induced protoporphyrin IX (PpIX) identify regions of increasing malignancy in low- and high-grade gliomas beyond the capabilities of current fluorescence imaging in patients undergoing fluorescence-guided resection (FGR). Surgical specimens were collected from 133 biopsies in 23 patients and processed for ex vivo neuropathological analysis: PpIX fluorimetry to measure PpIX concentrations (CPpIX) and Ki-67 immunohistochemistry to assess tissue proliferation. Samples displaying visible levels of fluorescence showed significantly higher levels of CPpIX and tissue proliferation. CPpIX was strongly correlated with histopathological score (nonparametric) and tissue proliferation (parametric), such that increasing levels of CPpIX were identified with regions of increasing malignancy. Furthermore, a large percentage of tumor-positive biopsy sites (~40%) that were not visibly fluorescent under the operating microscope had levels of CPpIX greater than 0.1 μg/mL, which indicates that significant PpIX accumulation exists below the detection threshold of current fluorescence imaging. Although PpIX fluorescence is recognized as a visual biomarker for neurosurgical resection guidance, these data show that it is quantitatively related at the microscopic level to increasing malignancy in both low- and high-grade gliomas. This work suggests a need for improved PpIX fluorescence detection technologies to achieve better sensitivity and quantification of PpIX in tissue during surgery.

Keywords: δ-aminolevulinic acid, fluorescence-guided resection, malignant glioma, proliferation index, protoporphyrin IX.
Gliomas account for over 70% of all primary brain tumors.1–3 Currently brain tumor research seeks to find diagnostic and prognostic biomarkers for gliomas that would inform both surgical and/or medical treatment. Extent of resection is increasingly accepted as critical to optimal surgical treatment and patient prognosis.4,5 Image guidance facilitates neurosurgical resection but is subject to intraoperative brain shift, which degrades the accuracy of relating navigational information with the surgical field presented to the surgeon.6–12

The use of δ-aminolevulinic acid (ALA)—induced protoporphyrin IX (PpIX) fluorescence-guided resection (FGR) of brain tumors has gained increased clinical interest.13 FGR provides the operating surgeon with an intuitive, real-time surgical guidance tool for delineating tumor tissue that mitigates some of the challenges of image-guided neuro-navigation caused by intraoperative brain shift and deformation. ALA-induced PpIX FGR studies support its utility as a surgical tool for intraoperative delineation of a tumor. For example, the largest study to date, a multicenter, randomized phase III clinical study of ALA-induced PpIX for FGR of malignant gliomas, showed a significant improvement in gross total resection of the contrast-enhancing tumor (65% vs 36%), as well as a higher 6-month progression-free survival (41.0% vs 21.1%), in the FGR group compared with the conventional white-light treatment group.14–16

In a recent study of malignant gliomas, we found that visible levels of ALA-induced PpIX fluorescence correlate with tumor burden and World Health Organization (WHO) histopathological score of resected specimens.17 To date, intraoperative detection of PpIX fluorescence for tumor delineation has largely been subjective (i.e., a modified surgical microscope is used to visualize “pink” fluorescence).13–21 This approach is limited in its sensitivity for identifying low (but significant) levels of accumulated PpIX in a tumor, potentially leaving some amount of resectable tumor unidentified.22 Indeed, we recently reported preliminary in vivo results in human brain tumors23 that indicate PpIX fluorescence may be a tumor-targeting biomarker with a diagnostic performance that exceeds subjective visible assessments when measured quantitatively with a new fiber optic approach.24 Thus, even though ALA-induced PpIX fluorescence has been shown to be a successful biomarker for surgical resection of malignant glioma, the subjective assessments used to date do not appear to be sufficiently sensitive or quantitative to exhaust its full potential. As a result, establishing the underlying relationships between ALA-induced PpIX concentration in brain tumor tissues and their histologically determined malignancy profile is critical to further development and optimization of FGR.

Toward this end, we investigated the hypothesis that quantitative ex vivo measurements of PpIX more sensitively differentiate areas of increasing malignancy in a tumor than do intraoperative visually subjective assessments of the same tissue. We tested our hypothesis by evaluating quantitative ex vivo measurements of PpIX concentration (C_pPpIX) with proliferation indices and neoplasm subtypes on histopathology. We show that ex vivo quantitative measurement of PpIX concentration in tissue is more sensitive at identifying regions of increasing malignancy in both low- and high-grade gliomas than current intraoperative fluorescence imaging. These findings suggest a need for improved intraoperative PpIX fluorescence detection to achieve better sensitivity and quantification of PpIX as a brain tumor biomarker, which in turn could lead to optimal surgical resection.

Materials and Methods

Specimen Acquisition

Tissue specimens evaluated in this study were obtained under informed consent from patients participating in our FGR protocol approved by the Dartmouth-Hitchcock Medical Center Committee for the Protection of Human Subjects. Patients were administered a 20-mg/kg body weight oral dose of ALA (DUSA Pharmaceuticals) dissolved in 100 mL of water approximately 3 hours prior to the induction of anesthesia. The operating room was equipped with a Zeiss OPMI Pentero operating microscope (Carl Zeiss Surgical) enabled with fluorescence imaging and a Medtronic StealthStation Treon (Medtronic) for neurosurgical navigation. The operating microscope was modified for fluorescence imaging of PpIX with a 405-nm light source for excitation and a 620-nm to 710-nm bandpass filter for selectively recording PpIX fluorescence.

The primary guide for resection was conventional neurosurgical technique with white light illumination assisted by neuronavigational guidance. Only tissue judged reasonably part of the planned resection volume, or abnormal tissue by other assessments (i.e., texture, nonfluorescent color, etc.), was included in the resection. In no instance was tissue resected on the basis of fluorescence alone. At different times during resection, the surgeon switched to 405-nm excitation light to visualize fluorescence. Biopsy specimens were collected at the beginning, middle, and end of resection, and digital images in white and blue light modes were recorded concurrently for each biopsy acquisition. Biopsy sites were assigned a fluorescence level by the study surgeon (D.W.R.) of 0 = no fluorescence, 1 = low fluorescence, 2 = moderate fluorescence, or 3 = high fluorescence. For each patient, multiple biopsy specimens were collected in both nonfluorescing and fluorescing (if present) regions within the preoperatively planned resection volume.

Each excised specimen was immediately separated into 3 equal parts for further processing. One part was placed in 10% buffered formalin for hematoxylin and eosin (H&E) and immunohistochemistry (IHC) staining. Another part was placed in optimal cutting temperature compound and frozen in liquid nitrogen. The remaining part was placed in a cryogenic vial and also frozen in...
liquid nitrogen for ex vivo fluorimetric quantification of PpIX.

Histopathology

Neuropathologic analysis was performed on formalin-fixed, paraffin-embedded biopsy tissue processed for H&E staining. A single neuropathologist (B.T.H.) analyzed the H&E tissue slides blinded to the clinical, radiographic, and pathologic diagnoses derived from the main surgical specimen in each case. Each H&E tissue section was assigned a histopathologic score (0-IV) based on the current WHO grading criteria for the particular neoplasms observed. The following histopathologic characteristics were used for each of the biopsies and judged independently regardless of the overall WHO grade assigned: (0) normal or fully necrotic tissue section with nonviable tumor cells observed; (I) tissue section with low number of infiltrating tumor cells (reserved for specific types of glial or glioneuronal neoplasms such as pilocytic astrocytomas, dysplastic neuroepithelial tumors, and gangliogliomas); (II) tissue section with higher number of infiltrating, pleomorphic cells and no observable necrosis, mitotic figures, or endothelial proliferation; (III) tissue section with highly pleomorphic tumor cells with mitotic figures, increased neoplastic cell density and no observable necrosis or endothelial proliferation; (IV) tissue section with highly pleomorphic tumor cells with mitotic figures, increased neoplastic cell density, and either observable necrosis and/or endothelial proliferation). The percentage of area occupied by necrotic tissue compared with nonnecrotic tissue in each slide was determined for each tissue (as estimated by 2 observers: B.T.H. and P.A.V.).

Immunohistochemistry

Formalin-fixed paraffin-embedded tissue sections nearly adjacent to the tissue sections used for H&E histopathologic diagnosis were sectioned into 4-μm-thick sections. To immunostain for the proliferation marker, Ki-67 antigen, tissue sections (n = 133) were first hydrated, followed by antigen retrieval with hydrated autoclaving at 121°C for 10 min. Samples were blocked for endogenous peroxidase activity using 3% H₂O₂ for 10 min followed by a blocking step for nonspecific binding using 10% fetal bovine serum (FBS) in phosphate buffered saline (PBS) for 20 min at room temperature. Tissue sections were incubated overnight with MIB-1 (1:150) anti–Ki-67 primary mouse immunoglobulin G (IgG; Dako) in PBS and 1% FBS at 4°C. Tissue sections were then incubated for 2.5 h with biotinylated (1:200) secondary anti-mouse IgG (Vector Laboratories) in PBS at 37°C. Biopsy sections were then treated with ABC solution (Vector Laboratories) for 30 min and incubated with 3,3’-diaminobenzidine tetrahydrochloride (DAB) substrate for 1 h. Samples were counterstained with hematoxylin (Fisher Scientific) and mounted. Appropriate negative controls were routinely obtained by substitution of the primary antibody with PBS only.

Tissue levels of proliferation were determined by calculating the MIB-1 proliferation index (PI). Briefly, for each tissue section, 3 high-power fields (400×) from nonoverlapping regions in which the tissue displayed the greatest levels of positively stained MIB-1 nuclei (i.e., DAB positivity) were chosen and corresponding images acquired using an Olympus bright field microscope. Manual counting of MIB-1 immunoreactive positive nuclei and nonreactive nuclei visualized by hematoxylin counterstain was performed on each image. For each tissue section, the PI was calculated as the ratio of positive MIB-1 nuclei over the sum of positive MIB-1 and nonreactive nuclei multiplied by 100. Tissues that were mostly necrotic with minimal viable cells (<33%) were excluded from the analysis.

Ex Vivo PpIX Fluorimetry

Tissue samples (n = 133) placed in a cryogenic vial were stored at −80°C prior to fluorimetric quantification of PpIX following a modified protocol from Lilge et al.31 Average tissue weight was 14.7 ± 10.1 mg (range, 1.1–56.1 mg). Briefly, the tissue was combined with 1 mL of Solvable (DuPont Biotechnology Systems) and placed in an undulating water bath at 50°C for 1 h. The tissue/Solvable solution was homogenized with a Tissue Tearor (Fisher Scientific) tool in the original cryogenic vial. The tissue homogenate (0.500 mL) was then combined with 1.125 mL of distilled water and 0.375 mL of Solvable. This solution was incubated in a 50°C water bath for 1 h and then transferred to a quartz cuvette. The solution was tested with a spectrophotometer to ensure an optical density less than 0.1. The cuvette was analyzed with a fluorimeter using an excitation wavelength of 401 nm and a slit width of 5 nm. A standard curve for quantification of PpIX was constructed by serially diluting PpIX by factors of 2 in a solution of distilled water (75% v/v) and Solvable (2.5% v/v). Spectral decomposition was used to separate the contributions of PpIX, its photoproducts, and tissue autofluorescence. Autofluorescence was modeled as a linear combination of the emission spectra of reduced NADH, flavin adenine dinucleotide (FAD), and lipofuscin—components known to exist in brain tissue. Total tissue fluorescence was modeled as a linear combination of all fluorescent species, with a nonnegative least squares algorithm to extract the contribution from each species and determine its PpIX concentration (C_PpIX).

Statistical Analysis

Kolmogorov–Smirnov analyses were used to assess normality of distributions. Wilcoxon rank-sum (Mann–Whitney) analyses were used to compare differences between groups. To further validate the statistical results and to accommodate for the multiple specimens per patient, a robust generalized estimating equation model33 was used to perform a clustered data analysis.
of variance to attest for difference between groups. Medians and interquartile ranges were used to summarize differences in visible fluorescence and CPpIX or PI and across histopathologic scores and qualitative visible fluorescence level. The differences were evaluated with a Kruskal–Wallis test, with a posttest analysis for multiple comparisons using Dunn’s procedure. Spearman’s rank or Pearson’s correlation analyses were used to assess a correlation for nonparametric or parametric variables, respectively. A P-value of <.05 was considered statistically significant for all tests. Data were processed with MATLAB software (version 2010a, Mathworks). Stata 11.0 (Stata Corporation) was used for statistical analyses.

Results

Patient and Data Characteristics

Specimens (n = 133) from a total of 23 patients who underwent fluorescence-guided resection (11 men, 12 women) were collected and evaluated. As summarized in Table 1, the clinical diagnoses of cases studied were as follows: 3 dysembryoplastic neuroepithelial tumors (grade I), 1 ganglioglioma (grade I), 2 mixed oligoastrocytomas (grade II), 1 anaplastic astrocytoma (grade III), 1 anaplastic mixed oligoastrocytoma (grade III), 1 anaplastic oligodendroglialoma (grade III), 1 gliosarcoma (grade IV), 11 glioblastomas multiforme (grade IV), and 2 recurrent gliomas. The mean patient age was 51.6 years (range, 20.0–79.9 years). All high-grade gliomas (n = 17) showed varying levels of visible fluorescence. No WHO grade I glioma (n = 4) showed visible fluorescence. One out of 2 WHO grade II gliomas showed visible levels of fluorescence. Figure 1 provides examples of data collected in the study that illustrate relationships among visual (intraoperative) fluorescence, CPpIX (as measured by ex vivo fluorimetry), and IHC presentation of cellular proliferation (Ki-67 antigen) from specimens acquired during individual low- and high-grade glioma surgeries. Please refer to the Supplementary material for an analysis of the data including only WHO grade II– WHO grade IV tumors. These results corroborate the expectation that visible levels of fluorescence are dependent on the accumulated concentrations of PpIX.

We found a statistically significant difference using Kruskal–Wallis analyses with Dunn’s posttest for multiple comparisons in CPpIX levels between groups classified as nontumor and IV, I and III, I and IV, II and IV (P < .0001) and a statistically significant difference in visible fluorescence levels and CPpIX only between fluorescence scores of 0 and 1, 0 and 2, and 0 and 3 (P < .0001). Furthermore, the corresponding analysis for PI also showed a similar trend, with statistically significant differences in PI between nontumor and IV, I and II, I and III, I and IV, II and IV, and III and IV (P < .0001). We see the same trend of statistically significant differences in visible fluorescence levels and PI only between fluorescence scores of 0 and 1, 0 and 2, and 0 and 3 (P < .0001).

Approximately 40% of the tumor-positive biopsy sites (22 samples), which were nonvisibly fluorescent under the operating microscope, had CPpIX levels greater than 0.1 μg/mL as measured by the fluorimetric assay, whereas only 2 specimens above this threshold were negative for tumor (Fig. 2D). Recent work using the quantitative intraoperative probe23 shows that greater than ~95% of normal tissue contains CPpIX levels below 0.1 μg/mL. Here we chose this concentration as the cutoff value. One of these displayed high vascularity at the choroid plexus and showed no visible fluorescence (− F) but presented with a particularly high level of CPpIX (~0.6 mg/mL). We also found that three-quarters (75%) of the tumor-positive biopsies that did not visually fluoresce during surgery had PIs greater than 1% (Fig. 2F).

Correlation Between Intraoperative Visible Fluorescence, CPpIX and PI

We have previously shown that intraoperative visible PpIX fluorescence strongly correlates with tissue histopathologic score (based on WHO grading criteria).17 We performed a nonparametric Spearman’s rank analysis to test whether a significant correlation also existed with PpIX concentrations (CPpIX) and found a strong correlation between levels of CPpIX in tissue, the levels of intraoperatively visible fluorescence (r = 0.80, P < .0001); (Fig. 2B), and the histopathologic score (r = 0.72, P < .0001); (Fig. 2C). A similarly strong correlation between PI and histopathologic score resulted from the same nonparametric Spearman’s rank analysis (r = 0.61, P < .0001). We also found a statistically significant correlation between the subjective levels of intraoperatively visible fluorescence (scored 0–3) and the PI of tissue (r = 0.54, P < .0001).

Correlation Between CPpIX and Quantitative Histopathologic Parameters of Malignancy

To assess whether CPpIX was able to quantitatively differentiate between discrete levels of malignancy, we
used Pearson’s analysis to test for a significant correlation between \( C_{\text{pIX}} \) and 3 quantitative histopathologic parameters: PI, total number of cells, and total number of proliferating cells. We found the strongest correlations (on a log-log scale, see Fig. 3) between levels of \( C_{\text{pIX}} \) and PI (\( r = 0.70, P < .0001 \)) and total number of proliferating cells (\( r = 0.71, P < .0001 \)) relative to total number of cells (\( r = 0.41, P < .0001 \)). This statistically significant linear correlation between \( C_{\text{pIX}} \) and quantitative histopathologic parameters demonstrate that microscopic levels of malignancy can be differentiated by accumulated tissue levels of \( C_{\text{pIX}} \).

**Discussion**

Extent of resection is a major prognostic factor in brain tumor patients. Furthermore, accurate identification during surgery of the more malignant regions of tumor is important for subsequent neuropathologic diagnosis, which determines the chemo- and radiotherapeutic regime and patient prognosis. Image guidance can help the neurosurgeon to better delineate the tumor margins for surgical removal and subsequent neuropathologic assessment. Nevertheless, image guidance suffers from intraoperative brain shift and deformation, which degrade and limit navigational accuracy.6–12

The major clinical implementations of FGR have used only subjective assessments of intraoperative fluorescence (i.e., negative or positive visible fluorescence). One major limitation of this approach is that subjective FGR suffers from interobserver variability in correctly assessing low levels of fluorescence. A further limitation of subjective ALA-induced PpIX FGR is its inability to detect fluorescence, which may be obscured by physical effects such as variation in tissue optical properties (i.e., optical absorption and transport scattering properties) and camera orientation to the resection cavity. The tissue optical absorption in particular varies among tissue sites and can greatly impact the resultant subjective fluorescence assessment. This can lead to tumor tissue that contains high levels of accumulated PpIX biomarker but is incorrectly identified (i.e., nonvisibly fluorescent); (Fig. 1). In a recent study we used a fiber-optic approach that takes into account tissue optical properties to quantify PpIX concentrations in vivo and showed that significant levels of PpIX concentrations are present in a variety of intracranial tumor histologies below the threshold of visual detection.23

In this study we have shown that \( C_{\text{pIX}} \) evaluated in specimens resected during surgery is identified with regions of increasing malignancy assessed histologically in low- and high-grade gliomas beyond the capabilities of current fluorescence imaging. As expected, a statistically significant difference in \( C_{\text{pIX}} \) occurred between tissues with no visual fluorescence (\(- F\)) intraoperatively and those with positive visual fluorescence (\(+ F\)). A significant trend also occurred between \( C_{\text{pIX}} \) and the

<table>
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<th>Patient No.</th>
<th>Diagnosis</th>
<th>Grade</th>
<th>Gender</th>
<th>Age</th>
<th>Visible Fluorescence</th>
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<tbody>
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<td>1</td>
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\( \text{No} = \) the surgeon did not observe red, visible fluorescence; \( \text{Yes} = \) the surgeon did observe red, visible fluorescence.
subjective fluorescence levels observed intraoperatively, which produced a positive correlation. All but 2 nontumor tissue specimens contained CPpIX levels below 0.1 mg/mL, whereas ~40% of nonvisibly fluorescent tumor tissues were above this threshold (Fig. 2D). These results suggest that improvements in the detection of fluorescence intraoperatively is likely to identify more tumor tissue (especially in low-grade glioma) and if quantitative detection of CPpIX can be achieved during surgery, it may be possible to define a threshold that maximizes the diagnostic performance of PpIX fluorescence for tumor resection.

Although the exact mechanism(s) that leads to preferential accumulation of PpIX in tumor tissue is not well known, cellular proliferation, cell density, mitochondrial content, vascular proliferation, structural changes, and enzymatic up-/downregulation (e.g., ferrochelatase)\(^{34-37}\) have been suggested as contributing factors. Since all of these factors correlate with the degree of tissue malignancy, we also assessed relationships between PpIX fluorescence and measures of tissue malignancy quantified with a MIB-1 PI using Ki-67 antigen as the biomarker. We found a statistically significant difference in PI between nonfluorescent (−F) and positively fluorescent (+F) biopsies (Fig. 2), as well as a strong correlation between PI and subjective levels of fluorescence. Furthermore, the results assessing a difference in CPpIX levels and PI across histopathologic scores and visible fluorescence levels (i.e., Kruskal–Wallis analyses) show that CPpIX levels and PI display similar trends of statistically significant differences between groups. These data support our hypothesis...
that quantitative levels of CPpIX can serve as a useful biomarker to ascertain tumor regions of more proliferative and anaplastic tissue.

In addition, we show that quantitative ex vivo measurements of CPpIX correlate with quantitative histopathologic measures of tissue malignancy by showing a
strong, statistically significant correlation between PI and C_{PpIX} (Fig. 3A). However, “proliferation index” is a relative term quantifying the percentage of abnormal cells over the total number of cells in a given tissue—it does not fully relate measures of tissue malignancy to accumulation of C_{PpIX}. To further elucidate the quantitative relationship between ex vivo measurements of PpIX and tissue malignancy, we determined the correlation between cell density and number of abnormal, proliferating cells as additional factors affecting accumulation of PpIX in tissue. We found a statistically significant correlation (Fig. 3B and C) between C_{PpIX} and total number of cells as well as between C_{PpIX} and total number of abnormal cells. More importantly, these results as well as similarly strong correlations between C_{PpIX} (r = 0.72) and PI (r = 0.61) with tissue histopathologic score indicate the ability of C_{PpIX} to differentiate between varying degrees of tissue malignancy at the microscopic level comparable to the gold standard, i.e., the Ki-67 PI. The predictive value of both qualitative levels of visible fluorescence and C_{PpIX} levels for predicting phenotype (e.g., proliferation, histopathologic grade) is provided by our correlation coefficients, i.e., the r-values, which show a statistically significant, strong correlation between C_{PpIX} and cellular proliferation (r = 0.70) and histopathologic score (r = 0.72). Previous studies have shown that MIB index, although not an absolute marker of tumor malignancy, is positively correlated with WHO grade in gliomas as well as negatively correlated with prognosis.\textsuperscript{38–41} As such, in this study we have focused on gliomas, as we present a quantitative approach to identifying areas of more aggressive, highly proliferative tissue in the resection of gliomas. We have also shown a positive correlation between cell density and C_{PpIX}, but here we show a stronger correlation between C_{PpIX} and cellular proliferation, which argues that the latter is a stronger, more significant predictor of C_{PpIX} levels in glioma tissue.

Our findings also show that a quantitative and more sensitive determination of PpIX concentration can be used to identify microscopic levels of increasing tumor cell proliferation and, thus, regions of increasing malignancy. More importantly, quantitative measurements of C_{PpIX} can be used as tissue biomarkers to accurately identify nonvisibly fluorescent, proliferative tumor tissue. Quantitative assessments open the door for more sensitive intraoperative determination of tumor malignancy in both visibly fluorescent and, perhaps more importantly, nonvisibly fluorescent tissue (e.g., nonfluorescing regions of anaplastic astrocytomas). Such biomarker determinations can guide the neurosurgeon in representative biopsy sampling to achieve a more accurate neuropathological diagnosis and inform patient treatment. For example, it is important to note that all WHO grade III tumors in this study were heterogeneous with anaplastic foci. This point is of importance to our study, since our results show a correlation between increased proliferation and C_{PpIX}, arguing that such anaplastic foci in heterogeneous gliomas could be more easily identified by noting increased levels of C_{PpIX}.

Previous work suggests that blood–brain barrier status is a contributing factor to observable levels of PpIX fluorescence.\textsuperscript{15,18,42} Stummer et al. showed a strong association between postoperative contrast enhancement on MR imaging and levels of red visible fluorescence at the end of surgery. Further, we have recently shown a significant association between intraoperative levels of PpIX fluorescence and 2 MR imaging metrics of contrast enhancement. These studies are inconclusive, since they suffer from brain shift, which degrades the accuracy of associating tissue fluorescence and MR imaging. Furthermore, to our knowledge, no study has undertaken a quantitative assessment unaffected by brain shift (e.g., brain biopsy cases) and correlated histopathologic and imaging findings with quantitative levels of PpIX. It is important to note that the strong positive correlation of r = 0.70, P < .0001 observed in this study for PI and PpIX accumulation argues that (1) PpIX accumulation in gliomas is strongly dependent on cellular proliferation status (i.e., cellular proliferation status in gliomas is a strong predictor of PpIX accumulation); (2) despite the strong correlation observed between C_{PpIX} and cellular proliferation, PpIX accumulation is a multifaceted biological process.

![Fig. 3](https://example.com/fig3.png)

**Fig. 3.** Protoporphyrin IX (PpIX) concentration and quantitative histopathologic parameters of malignancy. Pearson’s correlation analysis (n = 133) of (A) scatter plot of log C_{PpIX} vs log proliferation index (PI) (r = 0.70, P < .0001), (B) scatter plot of log C_{PpIX} vs log of total number of cells (r = 0.41, P < .0001), and (C) scatter plot of log C_{PpIX} vs log of total number of proliferating cells (r = 0.71, P < .0001). PI, total number of cells, and total number of proliferating cells were counted in 3 high power fields from tissues processed for anti–Ki-67 immunohistochemistry.
that cannot be accounted for solely on the basis of cellular proliferation; and (3) the strong correlation presented here suggests the predictive power of \(C_{\text{PpIX}}\) for tissue proliferation. These conclusions are in agreement with previous studies showing that PpIX accumulation is dependent upon factors such as enzymatic up- and downregulation, cellular proliferation, oxygenation status, and mitochondrial content, among others.\(^{42-44}\) The current study investigated the relationship specifically between cellular proliferation and PpIX accumulation in gliomas. The relationship between PpIX accumulation and blood–brain barrier status is of great importance to the field but is beyond the scope of this study.

A limitation of the study is that \(C_{\text{PpIX}}\) measurements were performed ex vivo, which for purposes of intraoperative guidance is impractical. Utsuki et al.\(^{45}\) report on a spectroscopy device for real-time feedback of fluorescence signal that shows improved detection of tumor with no visible fluorescence. Spectroscopic devices are known to improve detection of fluorescence signals from tissue due to geometries optimized for excitation and light collection, which maximize detection of fluorescent light while minimizing bleed-through of extraneous signals.\(^{42}\) Despite improved detection of low levels of fluorescence or fluorescence not visible by a modified surgical microscope, these other fluorescence spectroscopic devices are still limited in their ability to quantitatively determine absolute levels of PpIX. More specifically, the system by Utsuki et al. is limited by its ability to accurately quantify absolute fluorescence that may be obscured by physical effects such as variation in tissue optical properties (i.e., optical absorption and transport scattering properties). The tissue optical absorption in particular varies among tissue sites and can greatly impact the resultant fluorescence assessment.\(^{24, 46-48}\) We have reported on an intraoperative fiber-optic system that measures \(C_{\text{PpIX}}\) levels in vivo and accounts for such variations in tissue optical properties, thus providing significantly improved diagnostic performance compared with other fluorescence spectroscopy. This probe is able to quantify the absolute levels of \(C_{\text{PpIX}}\) in tissue despite variations in tissue optical properties.\(^{23, 24}\) The results presented in this study are a proof-of-principle of the value of \(C_{\text{PpIX}}\) measurements for detection of more malignant, proliferative regions of tissue in gliomas. Since the ability to quantify PpIX levels in tissue for the ex vivo assay correlates with the in vivo quantitative probe, the results from this study are translatable to a real-time in vivo quantification procedure that can be implemented using the quantitative probe. One important observation and possible limitation to this study is the validity of operating on recurrent gliomas with the assumption that \(C_{\text{PpIX}}\) positively correlates with cellular proliferation. Recurrent gliomas following chemotherapy are known to contain significant levels of inflammatory cells, which in turn are known to have increased levels of \(C_{\text{PpIX}}\) fluorescence.\(^{44}\) This fact could be a confounding factor leading to increased \(C_{\text{PpIX}}\) levels in recurrent glioma as a result of inflammatory cell presence and not increased proliferation. In our study, no recurrent glioma specimen contained any significant component of inflammatory cells, which were either diffusely infiltrated with rare tumor cells and had low levels of \(C_{\text{PpIX}}\) or were densely infiltrated by tumor cells with high levels of \(C_{\text{PpIX}}\). Future studies will further inform the surgeon regarding the utility of \(C_{\text{PpIX}}\) determination for the resection of recurrent gliomas. Another limitation of this study is a possible sampling error when dividing the biopsy specimens into 3 parts for histopathologic and biochemical analyses, though generally samples are quite small (less than 0.5 cm in greatest dimension), and the 3 parts should have similar pathology.

The findings reported here provide a rationale for developing the improved PpIX fluorescence detection needed to achieve better sensitivity and quantification of the biomarker in tissue. Quantitative and more sensitive detection of PpIX fluorescence would better enable the neurosurgeon to achieve a more informed real-time assessment of the surgical field, leading to optimal tumor resection.

**Supplementary Material**

Supplementary material is available online at *Neuro-Oncology* (http://neuro-oncology.oxfordjournals.org/).

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