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Real-time spectrum quantification of tumor-related fluorescence during neurosurgery: A preliminary report

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ABSTRACT

Objectives: The fluorescent dye, 5-aminolevulinic acid (5-ALA), is currently applied for fluorescence-guided resections of high-grade gliomas. Present limitations of this technique are qualitative and subjective analyses, which show little of the background structures. This paper describes the intraoperative quantitative analysis of fluorescence intensity, hot-spot enhancement by frame averaging, and observation of surrounding structures by using 1000-nm lighting in real time.

Patients and methods: A sample of diluted protoporphyrin IX (PpIX) in a bottle and 37 samples from nine patients with brain lesions were involved in this study. In this preliminary study, we determined appropriate conditions for image averaging and filters and selected the most sensitive spectrometer. In addition, we utilized a 1000-nm lighting system to visualize surrounding structures with no interference from PpIX fluorescence.

Results: The novel system permitted the real-time quantitative analysis of PpIX fluorescence in operative fields by illuminating structures with 1000-nm-lighting. The real-time quantification provided subjective evaluations for surgical decision-making. We found good correlations between the fluorescence and PpIX contents in brain tissue. Furthermore, 1000-nm lighting visualized the anatomical structures and PpIX fluorescence simultaneously.

Conclusion: The combination of spectroscopy and a 1000-nm lighting system could enable surgeons to create a spectrogram of targets of interest while observing background structures. The spectrometer that we selected is highly sensitive to PpIX fluorescence and enables us to perform intraoperative real-time tissue mapping. By using a real-time system, we can perform quantitative and objective evaluations to achieve maximal tumor resection.

1. Introduction

Glioblastoma (GB), or grade IV astrocytoma, is the most aggressive of primary brain tumors that involve brain tissue invasively [1–3]. There is strong evidence of a correlation between resection and survival rates [1,4]. To achieve maximal resection, preserve essential brain functions, and maintain the patient's performance status, neurosurgeons utilize not only a microscope but also a neuronavigation system and electrophysiological monitoring. However, it remains difficult to identify margins among normal, pathological, and necrotic tissue by visual inspection alone under a surgical microscope.

In the past 20 years, 5-aminolevulinic acid (5-ALA) has become a powerful tool to visualize tissue characteristics by observing specific fluorescence signals [5,6]. It is metabolized to protoporphyrin IX (PpIX) in the patient's body, which emits light at a wavelength of 635 nm after excitation with 400-nm light. A wavelength of 635 nm appears red in color and neurosurgeons can resect a pathological lesion showing red fluorescence under blue excitation light (400 nm). 5-ALA related

Abbreviations: PPIX, protoporphyrin IX; 5-ALA, 5-aminolevulinic acid; MIB-1, methylation-inhibited binding protein 1; HPLC, high performance liquid chromatography

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fluorescence appears not only in glioma, but also meningioma, malignant lymphoma and inflammation. This technique is mainly applied for glioma surgery in clinical practice [7,8]. Although the fluorescence-guided operation is quick and simple [9], several technical issues remain. The first is evaluation depending only on visual inspection and qualitative and subjective analyses. The second is observation of the fluorescence with a dark background, which makes it difficult for surgeons to identify a “red” lesion on anatomical structures and to ensure the preservation of normal and vessel structures.

To perform quantitative and objective analyses and identify background structures intraoperatively, we equipped a neurosurgical microscope with spectroscopy and near-infrared (NIR) lighting systems. This report describes the basics and clinical impact of real-time analyses of 5-ALA PpIX fluorescence when observing lesions and background structures.

2. Material and methods

To select an appropriate spectrometer and determine the contrast balance of the fluorescence and NIR background, we used pure PpIX from Pharmaceuticals, Co., Ltd. (Kobe, Japan). We made dilution of 0.2 mg of pure Protoporphyrin IX powder (Sigma-Aldrich Co., Ltd., Tokyo, Japan) in 2 ml of saline as a "PpIX phantom" to observe the PpIX fluorescence signal and surrounding structures while measuring specific peak areas at 635 nm.

A commercial high-resolution natural-light camera (XCU-CG160C, SONY, Tokyo, Japan) was selected; it weighs 50 g and has a global shutter complementary metal-oxide semiconductor (GS-CMOS) sensitive to wavelengths between 400 and 750 nm with a 1456 × 1088 pixel array. The camera was mounted on the right side of a microscope via C-mount adapters (OH4; Leica Microsystems, Heerbrugg, Switzerland).

The new NIR system weighs 120 g and includes a high-resolution low-light high-definition television camera (KEC-1300MHD, Kantum Ltd., Yokohama, Japan). The sensor consists of a CMOS, which is especially sensitive to longer wavelengths between 770 and 1300 nm, and has a 1384 × 1076 pixel array with a high-pass filter that omits wavelengths below 900 nm. Images recorded by the NIR system were projected onto external monitors via an high-definition multimedia interface (HDMI) cable. A high-intensity xenon 400-W light source and an additional 300-W light (20133120, Karl-Storz SE & Co., Tuttlingen, Germany) with narrow-band filters between 400 and 430 nm were utilized for fluorescence excitation. The luminance of the excitation lights was 1800 lx on a luminance meter (TA415LG, Tasco, Osaka, Japan) at a working distance of 300 mm. For background inspection, we utilized a 300-W halogen light emitting the NIR band between 700 and 1300 nm. Although the lighting system mainly emits NIR components longer than 700 nm, we additionally applied a high-pass filter for wavelengths longer than 1000 nm to totally omit visible light components. In this experiment, we utilized three lighting systems.

We compared three spectrometers from Hamamatsu Photonics, Ltd. (Hamamatsu, Japan) regarding sensitivity, resolution, and appropriate sizing for a surgical microscope. The three models selected were the C10077MA (model A), C10082CAH (model B), and C9405CB (model C). On a catalogue each spectrometer demonstrates the maximum wave height for the same light power emission. Assuming that a spectrometer has higher sensitivity, the score indicates high value. Detail information of each system was follows. Model A showed 10^{-3} of the maximum score between 400 and 700 nm with 9 nm in resolution, while model B demonstrated 10^{-2} of the score between 300 and 900 nm with 1 nm in resolution, Model B is much superior to model A in sensitivity and resolution. Model C, which selected, revealed 10^{5} of sensitivity score between 500 and 1000 nm with 5 nm in resolution. On the catalogue values, model C has the best sensitivity and intermediate wave resolution. From the practical point of view, the sensitivity is the most important for real-time mapping. Each spectrometer was mounted on the eyepiece head box and imported signals from the target point of the microscopic view via a coated glass fiber (Mitsubishi, Ltd., Japan) 1 mm in diameter from a right C-mount adapter. The magnification and working distance were fixed throughout at 5× and 300 mm, respectively, to obtain consistent findings.

To visualize weak PpIX fluorescence signals on the surface, we installed additional functions that averaged 10 of 30 frames/second to increase fluorescence signals and make the red color twice as intense on the recorded fluorescence video in real time.

Nine patients with brain lesions participated in this study; the lesions consisted of six glial-origin tumors, one schwannoma, one metastatic brain tumor due to lung adenocarcinoma, and one meningioma. Each sample was divided into different pieces depending on the fluorescence intensities. Three hours before the operation, the patient ingested 1.5 g of 5-ALA orally with 30 mL of water. After craniotomy, we performed a quantitative analysis of the fluorescence intensities of the lesions using the spectrometer on the microscope (Fig. 2). Recorded digital wave shapes from the spectrum analysis system were immediately transferred to an external computer where software, which we originally developed was running to perform the quantitative spectrum analysis in real time. The software automatically identified the baseline between 500 and 560 nm and integrated a PpIX peak area at 635 nm. In addition, it calculates the ratio, which is the PpIX area divided by the average background intensity, and shows consistent quantitative results.

According to optical science characteristics, the peaks in the region of interest are affected by changing the working distance and magnification. Before we utilized this complex technique in clinical practice, we calibrated the peak values depending on the working distance, magnification, and light power using the PpIX phantom. For spectral analysis, we used wavelengths between 500 and 560 nm, which reflect normal collagen fluorescence as a reference. However, we routinely used 300 mm as the working distance and 5 times-magnification as fixed conditions. Corrections were applied to the measured intensity values according to equations from basic experiments (Fig. 1). The working distance, magnification, and light intensity information were automatically transferred at every 1 Hz from the serial port (RS-232)on the microscope computerand cable of the microscope controller to the external computer via an LAN, which stored the observation conditions. We took the conditions into account during intensity corrections. After craniotomy, we exposed tumor lesions by corticotomy and achieved fluorescence measurements. We resected tumor samples of core lesions with–enhancement, peritumoral edema, and nectosis, which kept homogeneous fluorescence and confirmed the sample locations on a neuronavigation system (Stealth station, Medtronic. USA). The samples were quickly immersed them in liquid nitrogen to cease metabolic processes. This key procedure enabled us to avoid photo-bleaching or metabolic alterations.

The SBI Pharmaceuticals Co., Ltd. laboratory (Kobe, Japan) analyzed the amounts of PpIX, ferrochelatase (FECH), other amino acids, and enzymes in the samples. To measure the PpIX concentration in each sample, the investigators analyzed the supernatant after centrifugal separations by high-performance liquid chromatography (HPLC). The supernatant was analyzed with an HPLC system using a Capcell Pak C18 UG120 column (5 μm, 4.6 × 150 mm, Shiseido, Tokyo, Japan). To investigate relationships between fluorescence intensities and PpIX concentrations, we applied a linear regression analysis.

The exclusion criteria were patients who had an allergic reaction to 5-ALA or porphyrin, those with porphyria, those administered drugs that induce photosensitivity (tetracycline, etc.), and those who were pregnant.

This study was approved by the institutional review board (approval number: 16063). Written informed consent was obtained from each patient before participation in the study.

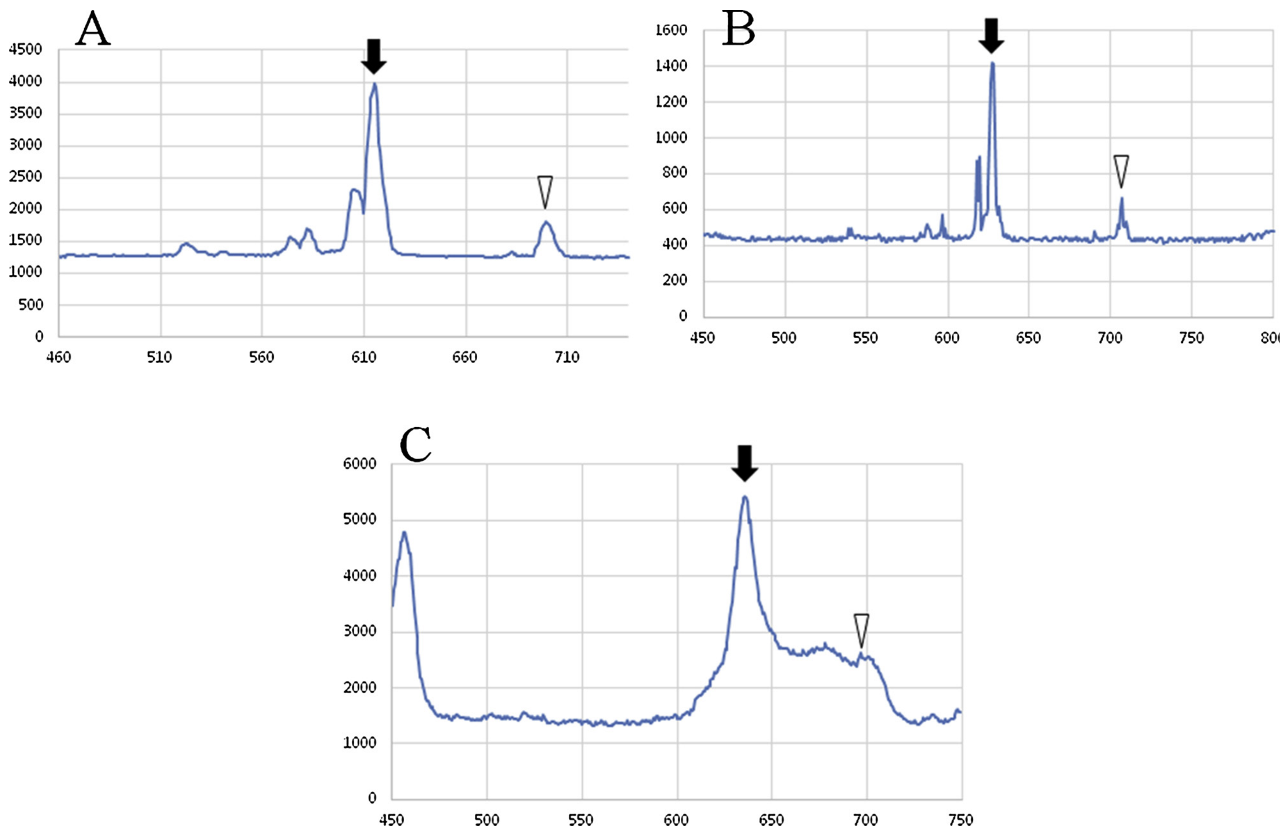

Fig. 1. Comparisons of spectral findings from three spectrometers. **A:** Model A (C10077MA) has the lowest sensitivity and resolution shows broader and unstable waves at 635 (black arrow) and 710 nm (white arrowhead). **B:** Model B (C10082CAH) has lower sensitivity and the highest resolution and demonstrates sharp doublet peaks that are too unstable for evaluation. **C:** Model C (C9405CB) has the highest sensitivity and shows stable wave deflections at 635 and 719 nm despite vibration and light artifacts.

3. Results

3.1. PpIX phantom analysis

In comparison with spectrometer characteristics, we used the PpIX phantom and mounted each of three systems on a c-mount of the microscope. Model A had the worst sensitivity and lowest resolution among the systems, which showed a peak at 630 nm with a doublet shape and a peak at 710 nm. However, they were unstable because of vibration and illumination noise. The second spectrometer (B) with the highest resolution demonstrated the sharpest doublet deflections at similar wavelengths as those of model A. However, they were still unstable because of interference from circumstantial white-light comportments and microscope vibration. Model C had the highest sensitivity and an intermediate resolution. It enabled us to visualize a stable output and two major deflections with few artifacts. Despite the movements of the microscope, model C demonstrated stable and consistent waves from which we calculated tumor-related fluorescence. In this study, we found that sensitivity was the most important factor for establishing a real-time spectral analysis.

3.2. Measurements and analyses in the operating room

The combination of the natural-light camera with a narrow-band filter at 630 nm and 400-nm lighting system evoked various levels of PpIX fluorescence in the suspected lesions. Because of variations, it was difficult to make quick and objective decisions regarding oncological or gliotic tissues based on red color-coded mapping by visual inspection alone. The color enhancement technique in this report enabled surgeons to easily navigate to suspected and pathological lesions. It was typically impossible to observe the fluorescence on raw images under the microscope. Fig. 2B hardly shows the fluorescence; enhancement makes it easier to identify the pathological lesions as red hot spots. After averaging 10 frames of the raw video under 400-nm excitation light, we could identify spots of PpIX fluorescence (Fig. 2A, B, C). A small 13-cm-emitting diode (LED) monitor was mounted on the eyepiece of the head device and showed all the information. Surgeons could view the complex and summarized results by moving the horizontal axis of his or her eye higher or lower (Fig. 2D). The signal corrections were performed according to compensation equations for the working distance, magnification, and light intensity (Fig. 2E, F). By using the same camera, filter, and lighting system, the developed software enables us to perform fluorescence-guided navigation more efficiently.

For the quantitative fluorescence signal analysis, we placed model 3 on the eyepiece head of the microscope (Fig. 2D). In the operating room (OR), we resected core and peripheral parts of malignant lesions with homogeneous fluorescence. Before taking the samples, the neuronavigation indicated resection region in each procedure. Natural light exposed the tumor piece and the baseline drift with an offset from white light (Fig. 3A). Under PpIX observation mode, the target circle of the spectrometer on the resected tumor detected no peak or offset drift. Fig. 3C shows the resected target in the circle and demonstrates obvious deflection at 635 nm, which is typical of PpIX fluorescence suggesting a high-grade glioma. T_1-weighted magnetic resonance imaging (MRI) with gadolinium-diethylenetriamine pentaacetic acid (Gd-DTPA) showed a heterogeneous ring-like enhancing mass in the right temporo-occipital region (Fig. 4A). Fig. 5 consists of natural light and enhanced “red” images with spectrograms. During the operation, the tumor exposed on the brain surface shows an obvious red color and 57.9 on the spectrogram (Fig. 5B). Another circle on the lateral ventricle wall showed strong fluorescence and 76.2 on the spectrogram. Both samples

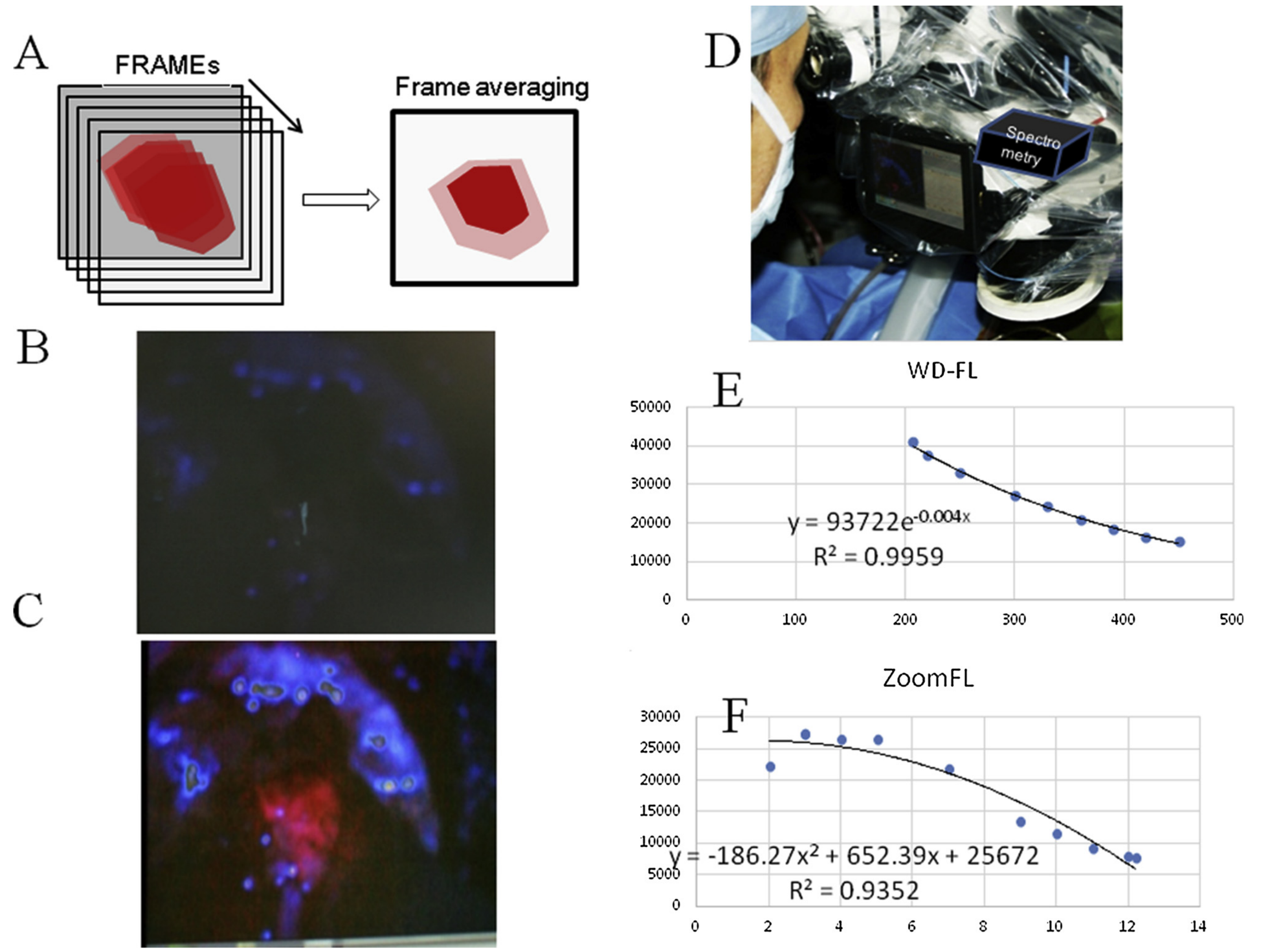

Fig. 2. The basics of the real-time visual enhancement/compensation camera system. **A:** Image averaging 10 of 30 frames enhances PpIX fluorescence. **B:** Pre-averaging the image shows no signal. **C:** Post-averaging enhances 635-nm fluorescence as red. **D:** A small LED monitor is mounted on the headpiece to view different images and the spectrogram. **E:** The correction function for the working distance. **F:** The correction function for magnification.

were glioblastoma from the histopathological diagnosis. The third sample showed little signal on the enhanced image and no peak on the spectrogram, suggesting no tumor invasion (Fig. 5F). The pathological diagnosis was gliosis. The combination of a spectrogram and enhanced views enabled us to make a quick, subjective decision for tumor removal.

3.3. Relationship between fluorescence intensity and the PpIX contents of samples

After we measured the fluorescence intensity with any blood contamination in the region of interest, we resected the measured regions and analyzed the PpIX concentration. Based on the results, we found a positive correlation between the fluorescence intensity and PpIX contents in the resected samples (Fig. 6). Because of the limited number of cases, we do not refer to pathological diagnoses with fluorescence signals in detail in this report.

3.4. Background observation with a 1000-nm lighting system and NIR camera

In the phantom experiment of the PpIX sample, a bottle filled with transparent PpIX solution is observed under natural light (Fig. 7A). Strong PpIX fluorescence is detected by the narrow-band camera after excitation with 400-nm light (Fig. 7B). The 1000-nm light, which does not interfere with wavelength components between 400 and 700 nm, clearly visualizes background anatomical structures. Fig. 7C demonstrates a fused image of PpIX fluorescence and the anatomical structure from the 1000-nm lighting system created with a digital image superimposition software.

During operation, the NL camera under 400-nm of excitation light demonstrated only red brain tissue with no background (Fig. 7D). Fig. E and F showed red fluorescence spots on gray-scale background with light combination of 400 and 1000-nm

4. Discussion

In this report, we propose a combination real-time technique including a spectrometer mounted on a microscope and an additional 1000-nm lighting system to achieve quantitative and objective fluorescence evaluation with visualization of anatomical structures. With this combination, we were able to perform quick tumor resections and fluorescence diagnoses simultaneously. The proposed procedure is based on varied scientific fields including fluorescence, optics, biology, and engineering and requires extensive knowledge of all these fields. However, rapid intraoperative and quantitative tissue mapping have great impacts on neurosurgical procedures. Furthermore, we found a clear correlation between the PpIX contents and fluorescence intensity with anatomical observation using a 1000-nm lighting system.

In previous basic experiments, there have been several papers about the correlation between PpIX contents and fluorescence intensity [10,11]. In this report, we propose a novel technique to analyze

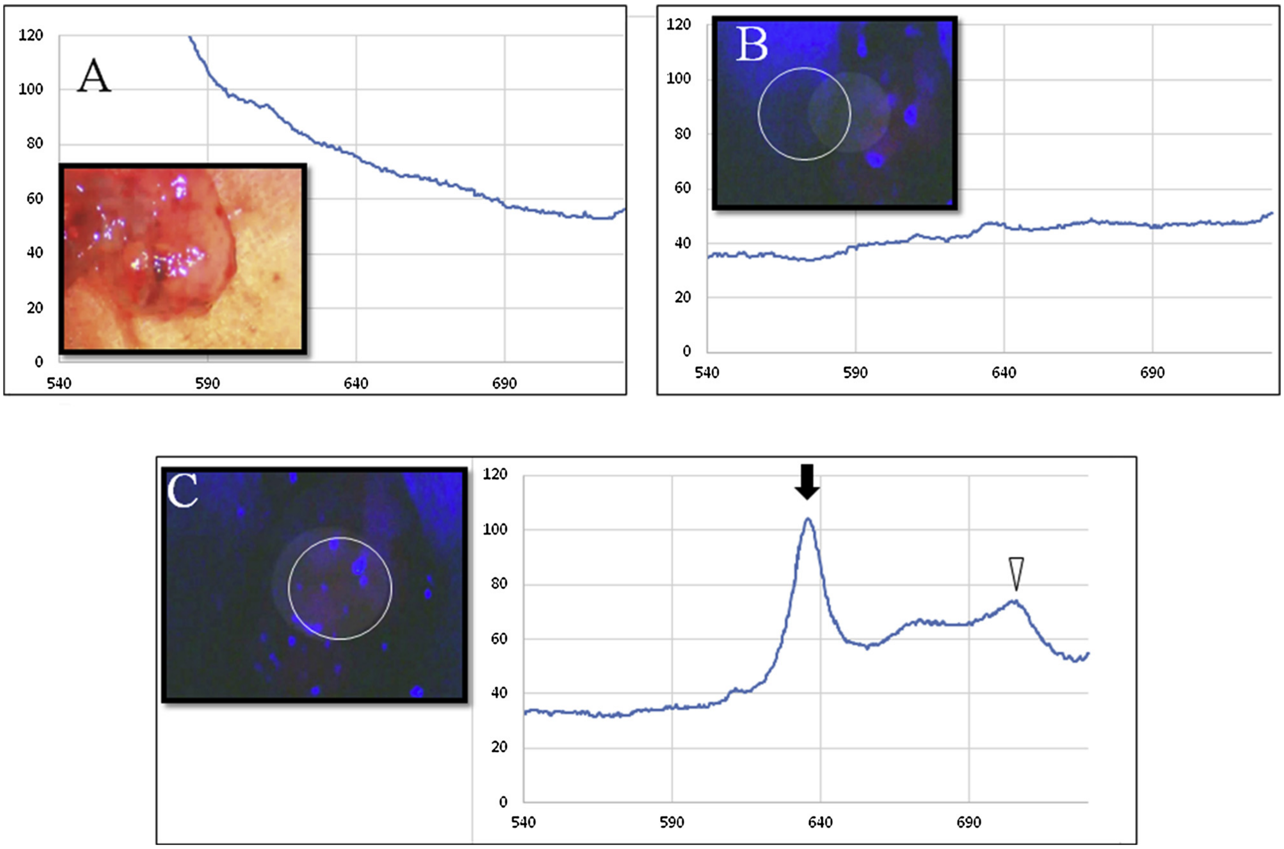

Fig. 3. A spectrogram of a resected tumor piece. **A:** A tumor piece illuminated by natural light with a spectrogram. **B**: A tumor piece under 400-nm excitation light. The spectrum target is out of the "red" tumor. The spectrogram shows no peak related to tumor tissue. **C:** A tumor piece under 400-nm excitation light. The spectrum target is on the "red" tumor. A spectrogram shows two stable deflections at 635 nm (black arrow) and 710 nm (white arrowhead).

fluorescence in real time using a neurosurgical microscope [12]. Although one can expect that there might be a relative correlation between them, even during operations based on past basic studies [13,14], real-time tissue mapping provides feedback to surgeons quickly, enabling them to make critical decisions. In this study, we brought the technique into the OR, which was equipped with a microscope. The intraoperative real-time analysis revealed a good correlation between fluorescence intensity and the PpIX contents in samples.

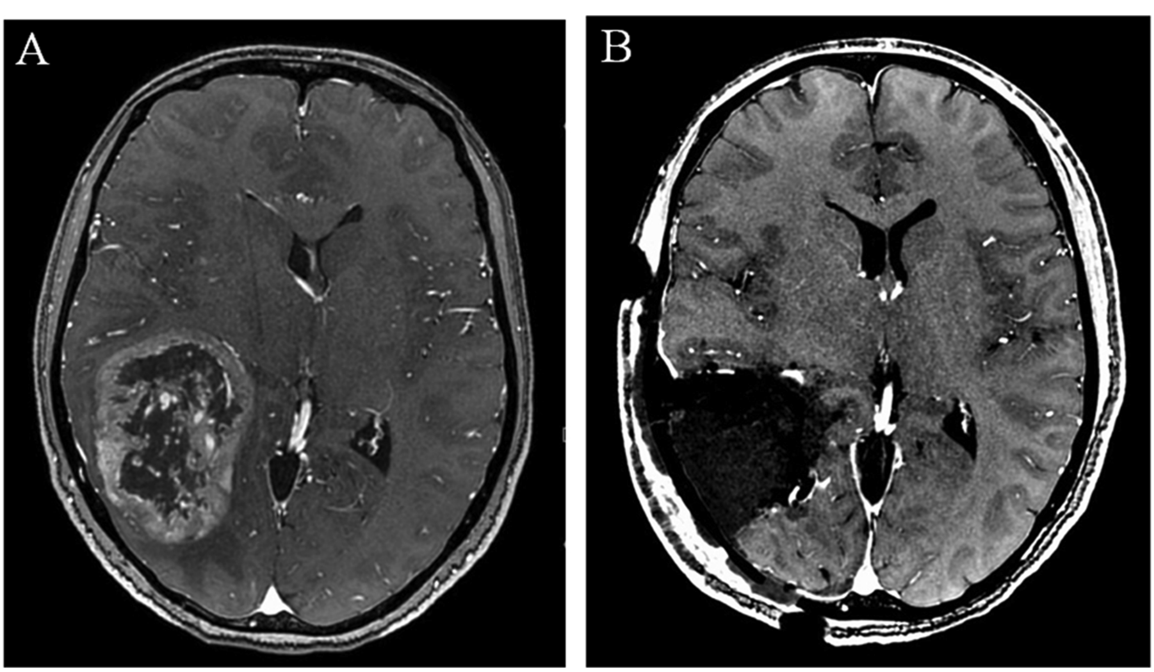

Fig. 4. T_1-weighted MRI with contrast of glioblastoma in the right temporo-occipital region. **A:** Preoperative MRI shows a ring-like heterogeneous lesion. **B:** Postoperative MRI demonstrates total resection of the enhanced lesion.

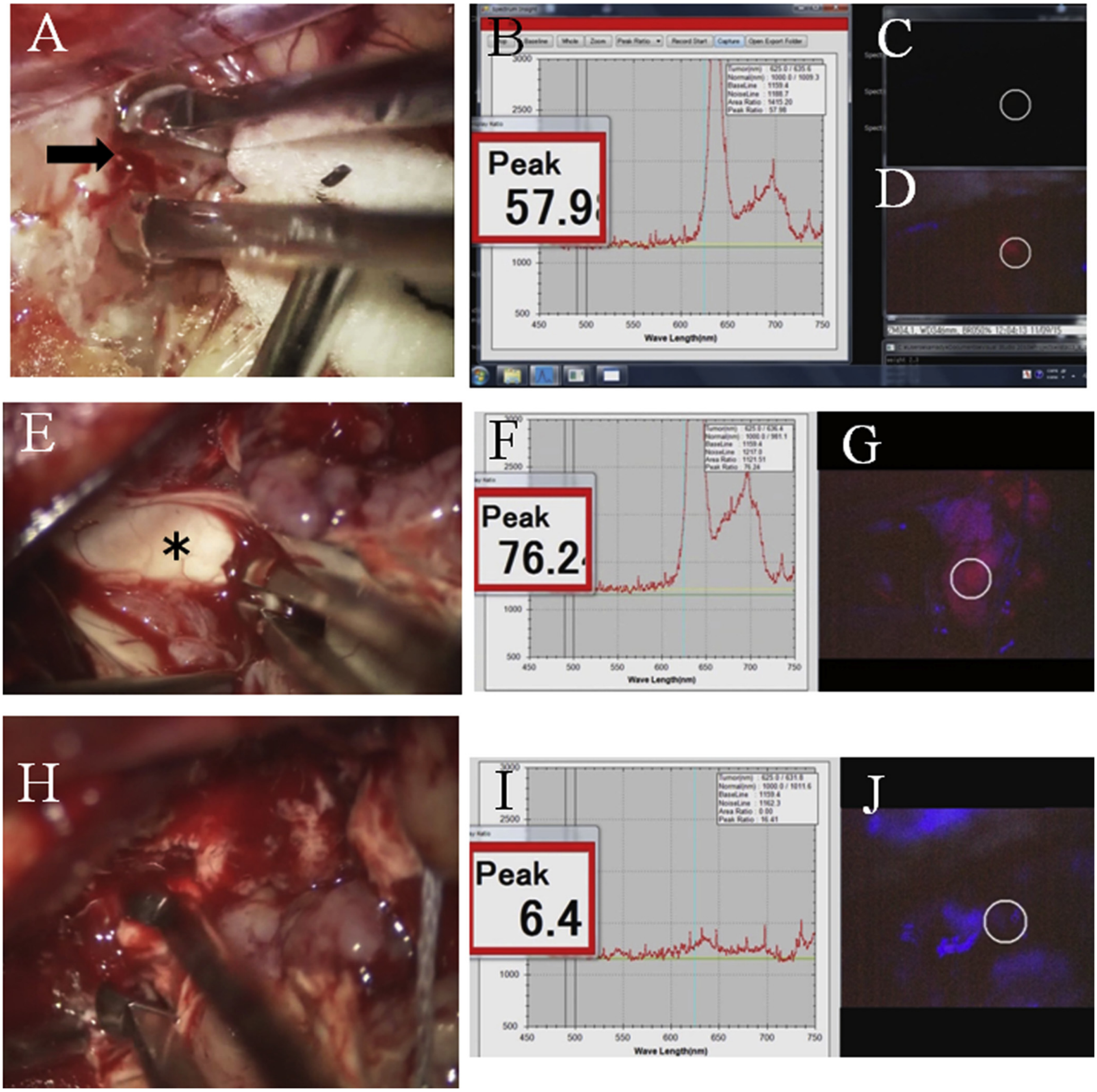

Fig. 5. Intraoperative views and real-time analyses. **A:** A biopsy of a tumor appearing on the brain surface. **B:** An exposed brain tumor shows strong fluorescence of 57.9 on the spectrogram and a red spot (**C** and **D**). **E:** The exposed inferior horn after tumor resection. **F:** The exposed ventricle wall reveals singular fluorescence at 76.2 and a red ventricle wall (**F** and **G**). **H:** The resection cavity. **F:** The resection margin shows no peak or hot spot in the real-time analysis. No signal or fluorescence suggests that the maximal resection was completed.

Another pitfall of 5-ALA-based tumor resection is that it is difficult to obtain anatomical information about the brain surface and resection cavity under 400-nm blue-colored light alone [9,15]. To overcome this issue, we emitted 1000-nm light, which is invisible and did not interfere with the PpIX fluorescence. The 1000-nm component was detectable by the second NIR camera and showed all structures and illuminated the operative fields. In neurosurgical operations, it is important not only to detect fluorescence, but also anatomy, hematomas, and bleeding. NIR light components have the possibility of omitting signals from hemoglobin at 700 nm and can illuminate anatomical structures. In optical science, the wavelength range between 900 and 1400 nm is called the “biological optical window” and is less affected by artifacts and interference from water and hemoglobin. We believe that these techniques and phenomena will contribute to future research topics.

In this report, we do not refer to histopathological diagnoses only by fluorescence intensities because of the limited number of samples. It is generally recommended that samples be categorized as red, vague, or none depending on the fluorescence intensity [9,16] and red suggests the greatest malignancy. Although the color classification is simple, decisions regarding the color-based category are still subjective and differ among surgeons and observation conditions. Therefore, the proposed technique has great potential for more objective evaluations, which minimize the effects of different categorizations among individuals. In addition, this system enables us to perform magnification

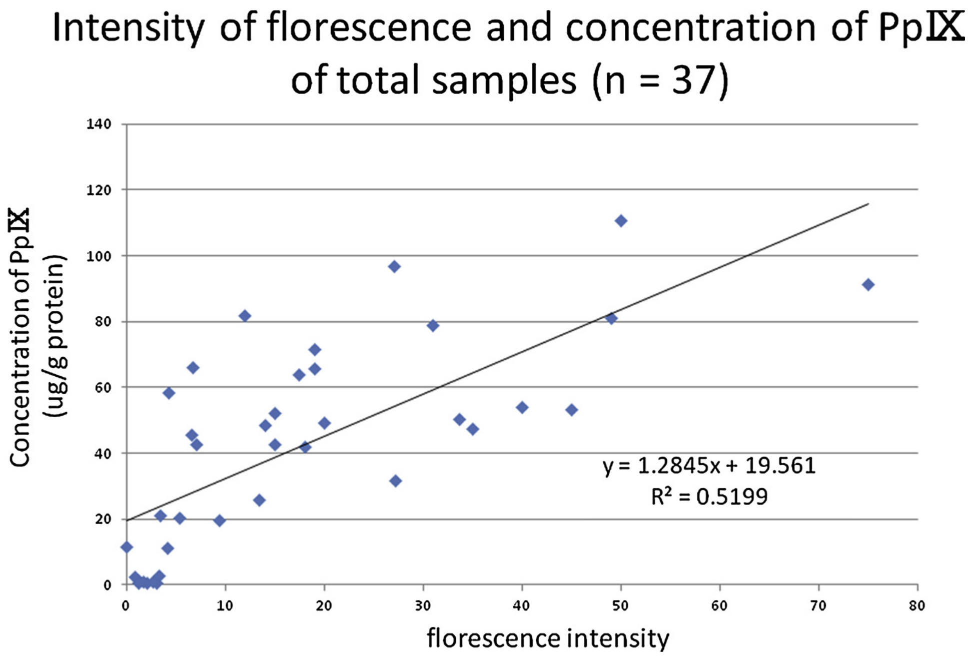

Fig. 6. Scatter plots demonstrate a linear correlation between PpIX fluorescence intensity and PpIX contents in resected samples.

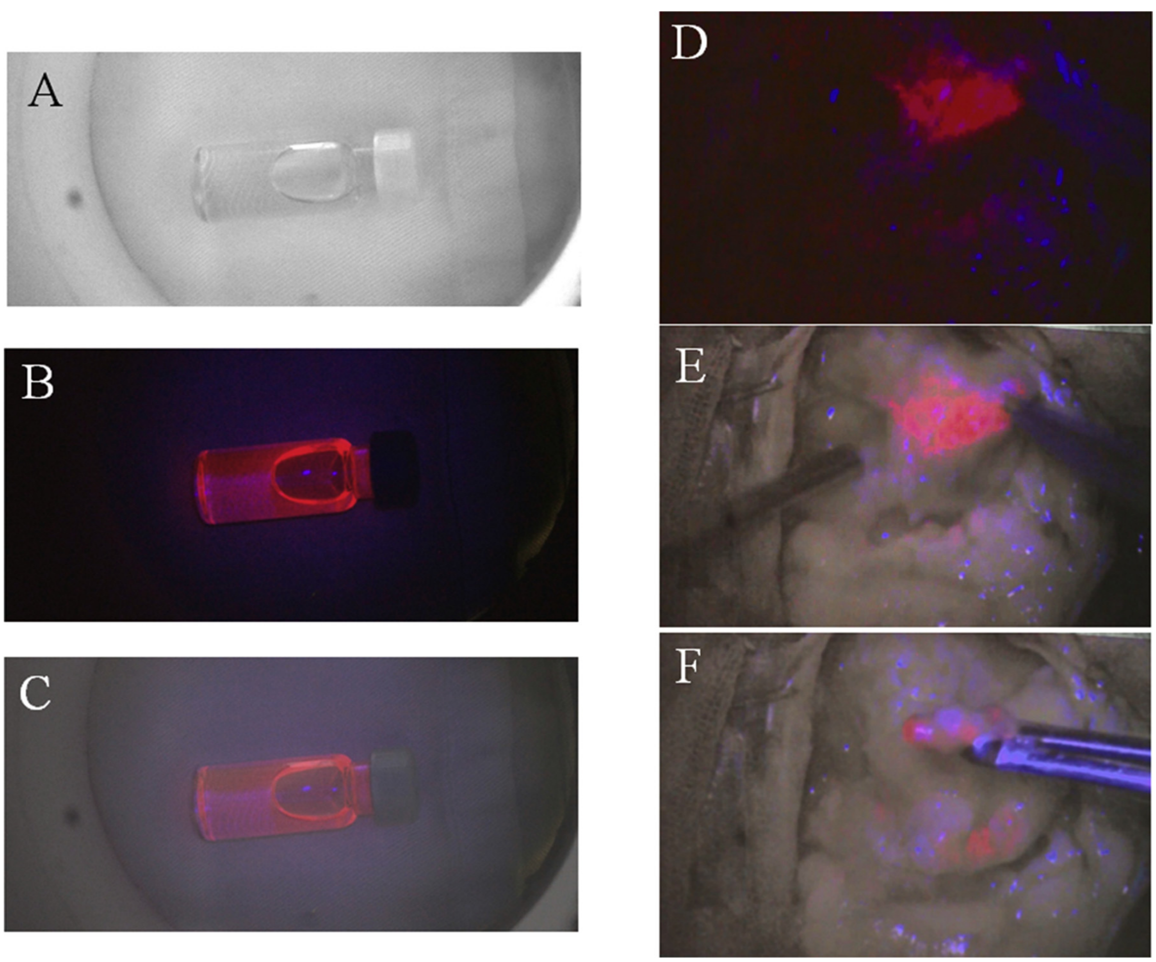

Fig. 7. Fused images from 400-nm (excitation) and 1000-nm (anatomy) lighting systems. **A:** An image of a bottle containing PpIX solution under 1000-nm light. **B:** An image of PpIX solution under 400-nm (excitation) light emitting PpIX fluorescence. **C:** Fused images from 400-nm (excitation) and 1000-nm (anatomy) light show not only fluorescence but also the bottle and surrounding structures. **D**: Operative view only with 400 nm excitation light, **D** and **E:** Fused images from 400-nm (excitation) and 1000-nm (anatomy) light show fluorescence, anatomical structures, and surgical devices simultaneously.

and distance corrections in real time. Quick intraoperative quantification with a microscope is a novel and useful technique, which provides more accurate diagnostic information than is currently available visual inspection. Several papers on basic experiments described how color intensity reflected malignancy and methylation-inhibited binding protein 1 (MIB-1) index in glial-origin tumors. However, this was only from observing PpIX fluorescence, which reflects the PpIX contents in tissues [12,16]. We still require more careful comparisons and interpretations regarding PpIX and histopathology.

As mentioned above, this technique observes PpIX fluorescence, reflecting the contents of PpIX, which is an intermediate product of 5-ALA in hemoglobin metabolism. 5-ALA is converted into PpIX in the mitochondria and FECH metabolizes the eighth and terminal step in the biosynthesis of heme, converting PpIX into heme. The general hypothesis describes that FECH activity might be suppressed in malignant lesions, resulting in the accumulation of PpIX. According to this principle, malignant tumors themselves have increased PpIX, which shows strong fluorescence. However, the hypothesis regarding these findings is still under discussion and we need more detailed analyses of 5-ALA metabolism, enzyme activity, and histopathological diagnoses including the MIB-1 index. In fluorescence studies, one should be careful about photo bleaching [17,18]. Since bleaching interferes with accurate PpIX analyses, it should be carefully addressed in this field.

On the other hand, 1000-nm lighting viewed against background structures avoided interference from the fluorescence and photo bleaching. Since the 1000-nm wavelength is NIR, this component is out of the optical window, but discloses anatomical structures and surgical devices with blood signal suppression. The present system needs not only dual cameras, but also an image mixer. Future iterations of a highly sensitive NIR system would likely be much smaller and able to be used in neuro-endoscopic and robotic surgeries. These novel systems would not only observe PpIX fluorescence but also anatomical structures with blood signal suppression. Especially in the cerebral ventricles, NIR can penetrate bloody cerebrospinal fluid and allow surgeons to perform careful and detailed observations.

5. Conclusion

In conclusion, a combination of a spectrometer and a 1000-nm lighting system would enable surgeons to perform a target spectrogram while observing background structures. The spectrometer we selected is highly sensitive to PpIX fluorescence and has paved the way toward real-time tissue mapping intraoperatively. Based on this real-time system, we can perform quantitative and objective evaluations and achieve maximal tumor resection.

Ethics approval and consent to participate

This study was approved by the institutional review board (approval number: 16063). Written informed consent was obtained from each patient before participation in the study.

Consent for publication

All authors have approved this study for publication. All patients and patients' families accepted to publish the data in this manuscript for publication with the signature on the informed consent sheets.

Availability of data and materials

All analyzed data are stored in SBI Pharmaceuticals Co., Ltd., Kobe. Rest of samples (presented in this manuscript) and new samples from brain tumors are kept in a deep freezer at Department of Neurosurgery in Asahikawa Medical University. This technique is available in the operation room and surgeons obtained feedback of quantitative results for fluorescence signals. "Real-time feedback" (not in labs) greatly contributes to operator's decisions although there were several off-line, extra-operation studies. Main impact is intraoperative real-time quantification.

Competing interest

The authors report no conflicts of interest concerning the materials, methods, or findings of this study.

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Authors' contributions

Kyousuke Kamada designed the study, and wrote the initial draft of the manuscript. Ryogo Anei collected samples by surgery and performed statistical analysis. Satoru Hiroshima and Hiroshi Ogawa and Yukie Tamura performed surgery as an assistant and they did a pathology search of specimens. Fumiya Takeuchi manipulated the instrument during surgery and performed quantitative analysis. Ken Kodama, Yuya Kitajima, Masahiro Ishizuka performed enzyme quantification in the specimen. All authors approved the final version of the manuscript, and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Authors' contributions

Not applicable.

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