

Combined Fluorescence Using 5-Aminolevulinic Acid and Fluorescein Sodium at Glioblastoma Border: Intraoperative Findings and Histopathologic Data About 3 Newly Diagnosed Consecutive Cases

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■ **OBJECTIVE:** Fluorescence-guided glioblastoma surgery is an intraoperative technique developed in recent years. Two main compounds have been used so far: 5-aminolevulinic acid (5-ALA) and fluorescein sodium (FI-Na). Despite a large amount of literature on both techniques, few data are available on the use of both compounds in the same patient.

■ **METHODS:** Three consecutive patients affected by a newly diagnosed glioblastoma underwent surgical resection using both 5-ALA and FI-Na. 5-ALA was orally administered 3 hours before induction of anesthesia at a dosage of 20 mg/kg, whereas fluorescein was intravenously administered at induction of anesthesia at a dosage of 4 mg/kg. Tumor resection was carried out combining these fluorophores. At tumor borders, multiple samples were collected, and fluorescent pattern of each sample was registered. Samples were then analyzed by a neuropathologist blinded for intraoperative fluorescence findings.

■ **RESULTS:** Eighteen samples were analyzed. At tumor margin, bright pink fluorescence was highly indicative of residual tumor (positive predictive value [PPV], 94%), and it was superior to faint pink and fluorescein (PPVs, 89% and 87%, respectively). The gradual reduction of pink fluorescence warned of the risk of gradually entering healthy tissue (specificity of 67% compared with 33% with fluorescein). Using 5-ALA, detecting no fluorescence was highly suggestive of healthy tissue (negative predictive value of 100% compared with 50% with fluorescence).

■ **CONCLUSIONS:** In our experience with 3 patients, the 2 techniques presented different advantages and limitations in specific steps of tumor resection, showing complementary properties. Larger studies are mandatory to investigate the synergistic use of both techniques.

INTRODUCTION

Maximal safe resection is the first step of multimodal therapy needed for high-grade gliomas.¹⁻⁴ Fluorescence-guided surgery is an intraoperative technique developed in recent years. Two main compounds have been used so far in malignant glioma surgery: 5-aminolevulinic acid (5-ALA) and fluorescein sodium.

These 2 compounds are different. 5-ALA is the first metabolite in the heme biosynthesis pathway and is synthesized from succinyl-CoA and alanine. This is the rate-limiting step in the heme biosynthesis pathway. By giving an excess of 5-ALA, the rate-limiting step is circumvented. Protoporphyrin IX (PPIX) is the final metabolite before heme biosynthesis. Epithelia and cancerous lesions, including high-grade gliomas, are capable of accumulating PPIX, which is a molecule strongly fluorescent in the red range.⁵⁻⁸ Fluorescein sodium has been widely used in ophthalmology for a very long time, especially to perform retinal angiography.⁹⁻¹¹ In high-grade gliomas, it preferably accumulates in neoplastic regions because of a leakage from a damaged blood–brain barrier (BBB).¹² Because of the presence of the tumor, the BBB becomes disrupted

Key words

- 5-ALA
- Fluorescein
- Glioblastoma
- Tumor border

Abbreviations and Acronyms

- 5-ALA: 5-Aminolevulinic acid
- BBB: Blood–brain barrier
- GBM: Glioblastoma
- MRI: Magnetic resonance imaging
- NPV: Negative predictive value
- PPV: Positive predictive value

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and more permeable, therefore allowing solutes (e.g., fluorescein) to concentrate at the tumor site. Therefore, compared with 5-ALA, it presents a different mechanism of action. Fluorescein is a result of passive permeability, whereas 5-ALA is a metabolic accumulation.

Each substance requires a modified microscope with a dedicated light source for excitation and an emission filter for optimal fluorescence visualization. Fluorescein is excited by a light wavelength ranging from 460 to 500 nm and emits fluorescent radiation in the wavelength ranging from 540 to 690 nm. Modern microscopes fit the YELLOW 560 system (Carl Zeiss Meditec AG, Jena, Germany) to allow fluorescein fluorescence.¹³ Visualizing PPIX fluorescence, after 5-ALA administration, requires a filtered xenon light to give blue-violet light with a wave of 375–440 nm and an emission filter, allowing visualization of red fluorescence, which has a peak at 635 and 704 nm (visualized by the BLUE 400 system [Carl Zeiss Meditec AG], a dielectric 440-nm short-pass filter).⁷

Regarding administration, oral administration of 5-ALA has been well described. A dosage of 20 mg/kg is orally administered 3 hours before induction of anesthesia.¹⁴ Taking fluorescein into consideration, as recent studies report, administration between 3 and 5 mg/kg at induction of anesthesia allows for best visualization through yellow fluorescence of high-grade gliomas.¹⁵ Recent evidence suggests that giving fluorescein intravenously too late (e.g., during craniotomy or dura opening) can give different results in terms of tissue fluorescence.¹⁶ Acute injection is correlated with a general fluorescence uptake from normal parenchyma as well. This is related to brain perfusion and passage of fluorescein into small capillaries.

Intraoperative findings of the 2 fluorophores are different. Intraoperatively, using BLUE 400, 3 main patterns can be distinguished: necrotic area that usually displays no or only inhomogeneous pink-red fluorescence, solid viable tumor showing bright fluorescence intensity, and transitional area of infiltrating and invasive brain tissue appearing as faint fluorescence. From a pathologic point of view, these 3 different fluorescence patterns have been studied and correlated with tissue specimens.^{17,18} Using fluorescein, a different fluorescence pattern within the tumor is not clear and has never been reported. Furthermore, nonneoplastic structures can be fluorescent, and tissue manipulation, edema bulk flow, dose, and timing administration can play a role in fluorescence distribution because of fluorescein extravagation.¹⁹

Despite the large amount of literature data about the 2 compounds, few data are available in the literature on the surgical

experience using the 2 techniques simultaneously on the same patient. In this study, we retrospectively analyzed intraoperative findings and compared the 2 fluorescence modalities.

METHODS

Patient Enrollment

In this study, we enrolled 3 consecutive patients (Table 1) that underwent surgical resection using both 5-ALA and fluorescein for lesions suggestive of high-grade glioma at preoperative magnetic resonance imaging (MRI) (Figure 1). Patient 1 presented to our attention for aggressive behavior and depressed mood in recent months. An MRI showed a left frontal measuring 43 × 34 × 38 mm. Patient 2 was admitted with a history of headache in the last couple months. An MRI revealed a right insulo-temporal lesion measuring 60 × 45 × 52 mm. Patient 3 was admitted with a history of sudden-onset left hemisindrome. An MRI showed a right fronto-parieto-temporal lesion measuring 69 × 31 × 30 mm. Informed consent concerning surgery and use of 5-ALA and off-label use of fluorescein were obtained for all patients.

5-ALA and Fluorescein Administration and Microscopic Equipment

5-ALA at a dosage of 20 mg/kg was orally administered 3 hours before induction of anesthesia. Fluorescein was administered at a low dose of 4 mg/kg at induction of anesthesia, as recent evidence suggests.²⁰ A Carl Zeiss Kinevo was equipped with both BLUE 400 and YELLOW 560 (Carl Zeiss Meditec AG) and used to detect its respective fluorophore and fluorescence pattern distribution.

Surgical Strategy and Sample Collection

Both fluorophores were used to have the best possible visualization of the lesion and surrounding tissue (Video 1). In all 3 cases, magnetic resonance neuronavigation (Figure 1) and intraoperative neurophysiologic monitoring were used to improve the safety of the procedure. We collected multiple samples of tissue only at the tumor border, where identifying neoplastic tissue under white light was more difficult (Figure 1). Neuronavigation was used for a better localization of craniotomy and corticotomy and not to determine site of biopsies. Our strategy of sample collection was performed as follows: we examined under white light the tumor margins focusing on and sampling small areas suggestive of neoplastic tissue or neoplastic infiltration. Then, we analyzed the fluorescent pattern under blue light and then under yellow light (Video 1). Fluorescence pattern



Table 1. Characteristics of the 3 Patients Who Underwent Surgical Resection of High-Grade Gliomas Using Combined Fluorescence

Patient Number	Sex	Location	Side	Histology	Isocitrate Dehydrogenase-1	GTR
1	M	Frontal	L	GBM	Wild-type	Yes
2	F	Insulo-temporal	R	GBM	Wild-type	No
3	F	Fronto-parieto-temporal	R	GBM	Wild-type	No

GTR, gross total resection; M, male; L, left; GBM, glioblastoma; F, female; R, right.

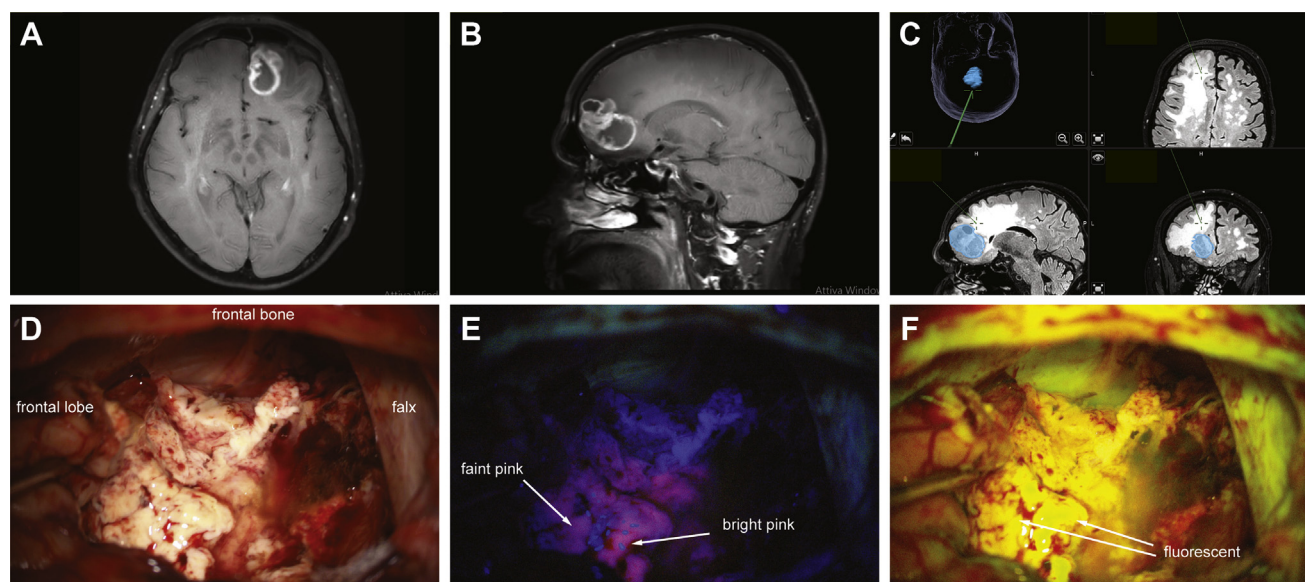


Figure 1. Intraoperative fluorescence pattern at the tumor border. (A and B) Axial and sagittal magnetic resonance imaging scans of patient 1. (C) Neuronavigation is performed to assist surgery for better localization of craniotomy and cortectomy. Morphologic and fluorescence characteristics at the tumor border using white light (D), BLUE 400 (E), and YELLOW 560

(F). Morphologic details (frontal lobe, frontal bone, and falx) (D) are overimpressed for anatomic orientation. Note the heterogeneous fluorescence pattern of 5-aminolevulinic acid (bright pink and faint pink) (E) compared with the homogeneous pattern of fluorescein (F).

of the samples was determined outside the surgical field after each sample was collected and placed on a white gauze to limit fluorescence misinterpretation because of the fluorescent background of the surgical field. Finally, the same area was collected and sent to a pathologist. Each sample was put on record for its specific pattern of fluorescence characteristics, as evident outside the surgical field on white gauze, both for fluorescein and 5-ALA. Fluorescence related to fluorescein was either fluorescent (yellow) or not fluorescent (not yellow), and fluorescence related to 5-ALA was either fluorescent (bright pink or faint pink) or not fluorescent (not pink).

Histopathologic Evaluation

All samples were sent for histopathologic examination. Each sample was stained with hematoxylin and eosin and analyzed and described by an expert neuropathologist blinded for intraoperative fluorescence pattern of samples. Looking at the samples, a diagnosis of solid tumor (glioblastoma [GBM]); infiltrating tumor, where some infiltrating tissue and cells were visible within normal brain parenchyma; or normal tissue was made.

Statistical Analysis

Sensitivity (TP/TP + FN), specificity (TN/TN + FP), positive predictive value (PPV) (TP/TP + FP), negative predictive value (NPV) (TN/TN + FN), and accuracy (TP + TN/TP + TN + FP + FN) were calculated according to the respective statistical formula using 2×2 tables considering test result (fluorescent/nonfluorescent) and disease (tumor/normal parenchyma), where TP is true positive, FN is false negative, FP is false positive, and TN is true negative.

Weaknesses and Strengths of Our Study

The principal points of our work are that we took homogeneous samples, the intraoperative surgeon was always the same operator, and the pathologist was blind to the fluorescence pattern of the collected samples.

Surely, the number of samples per region influenced statistic calculation. A main limitation of the study is that we obtained surgical samples only at the tumor border. In this region, 5-ALA is more effective considering that the necrotic core is not fluorescent. Few true negative (nonfluorescent, nontumor) samples were included in our analysis. This is because under white light we avoided sampling of clearly normal brain parenchyma because it was useless for surgery and for ethical reasons.

RESULTS

Sample Collection

At the tumor border (Figure 1), we collected 3 tissue samples from patient 1, 7 tissue samples from patient 2, and 8 tissue samples from patient 3. In total, 18 samples were analyzed (Table 2). At histopathologic examination, 8 samples were tumor, 7 were infiltration, and 3 were normal. Regarding 5-ALA, 16 samples were fluorescent (7 brightly and 9 lightly) and 2 were not. Regarding fluorescein, 16 samples were fluorescent and 2 were not. Finally, 15 samples showed both fluorescence, 1 sample showed no fluorescence, and 2 samples displayed only one type of fluorescence (1 yellow/not pink and 1 pink/not yellow).

Sample Histopathologic Analysis

We retrospectively looked at sensitivity and specificity of fluorescein and 5-ALA (Table 3). Focusing on fluorescein, from a total of

Table 2. Intraoperative Fluorescence Pattern and Corresponding Histopathologic Characteristics of the 18 Samples Acquired at the Tumor Border

	Yellow Bright Pink	Yellow Faint Pink	Yellow Not Pink	Not Yellow Not Pink	Not Yellow Faint Pink	Not Yellow Bright Pink
Samples	7	8	1	1	1	0
Histology	6 GBM 1 Infiltrating	2 GBM 5 Infiltrating 1 Normal	1 Normal	1 Normal	1 Infiltrating	

GBM, glioblastoma solid tumor; Infiltrating, infiltrating tumor; Normal, normal tissue.

16 fluorescent yellow samples, 14 were registered as tumor, either GBM or infiltrating tumor. The remaining 2 samples were normal tissue. Furthermore, 2 samples were not fluorescent yellow; one was normal and one was diagnosed as infiltrating tumor. This analysis therefore revealed a sensitivity of 93% and a specificity of 33%. The PPV was 87%, accuracy was 83%, and NPV was 50%. When taking into consideration 5-ALA, 15 out of the 16 fluorescent pink samples actually confirmed the presence of tumor, either GBM or infiltrating tissue. The remaining one was normal tissue. Two samples were characterized by no pink fluorescence, and histologic analysis confirmed normal tissue. Therefore, sensitivity was 100% and specificity was 67%. The PPV was 94%, accuracy was 94%, and NPV was 100%. Furthermore, focusing on the bright pink fluorescent samples, all of them were tumor, showing a sensitivity and PPV of 100%. When looking at faint fluorescent samples, from a total number of 9 samples, 8 were tumor and 1 was normal tissue. Two nonfluorescent samples confirmed normal tissue during histologic analysis. Sensitivity was 100%, specificity was 67%, PPV was 89%, and accuracy was 91%.

DISCUSSION

Data from the Literature

Many histologic analyses have analyzed sensitivity and specificity of the 2 compounds. Some authors have shown that pink fluorescence has a PPV for tumor superior to 95%,²¹⁻²⁶ therefore helping the surgeon to locate remnant tumor, particularly at the tumor's borders. As far as fluorescein is concerned, a recent Fluorescein-Guided Surgery for Resection of High-Grade Gliomas: A Multicentric Prospective Phase II Study multicentric controlled prospective phase II trial reported a sensitivity and specificity of

fluorescein in identifying tumor tissue of 80.8% and 79.1%, respectively.²⁰ Even if fluorescence could be a result of false-positive cases (metastases, surgical trauma, etc.), different studies have shown a high reliability in tumor identification.^{13,27-29}

Clinically, a phase 3 randomized trial¹⁴ showed an increase in extent of resection and progression-free survival administering 5-ALA with the aid of a dedicated BLUE 400 filter on the surgical microscope. Using 5-ALA-guided fluorescence, complete resection was achieved in 65% of patients compared with 33% of patients operated under white light. Progression-free survival was shown to improve from 20% to 40%. Regarding fluorescein, a recent multicentric controlled prospective phase II trial has been published where 82.6% of patients underwent complete tumor removal. Progression-free survival at 6 and 12 months was, respectively, 56.6% and 15.2%.²⁰

There are 2 studies that took into consideration simultaneous administration of both 5-ALA and fluorescein. Yano et al.³⁰ were the first to report excisions of GBMs in 8 patients while using both fluorophores simultaneously to investigate staining characteristics. They reported that 5-ALA was more sensitive than fluorescein in detecting tumor cells at the boundary zone. This study however presented 2 main limitations. Fluorescein was administered at a high dosage of 15–20 mg/kg at the dura opening, but the latest studies now suggest a low dose of 3–5 mg/kg at induction of anesthesia. Furthermore, a specific YELLOW 560 filter was not used, and yellow fluorescence was detected through the BLUE 400 filter, which is not specific for fluorescein detection.

Suero Molina et al.³¹ focused as well on simultaneous administration of 5-ALA and fluorescein. However, for simultaneous visualization, an external blue light (375–440 nm)

Table 3. Sensitivity, Specificity, Positive Predictive Value, Negative Predictive Value, and Accuracy for Tumor Detection

Fluorescence Pattern	Sensitivity	Specificity	PPV	NPV	Accuracy
Fluorescein	0.93	0.33	0.875	0.5	0.83
5-ALA (total)	1	0.67	0.94	1	0.94
5-ALA (bright pink)	1	-	1	-	1
5-ALA (faint pink)	1	0.67	0.89	1	0.91

PPV, positive predictive value; NPV, negative predictive value; 5-ALA, 5-aminolevulinic acid.

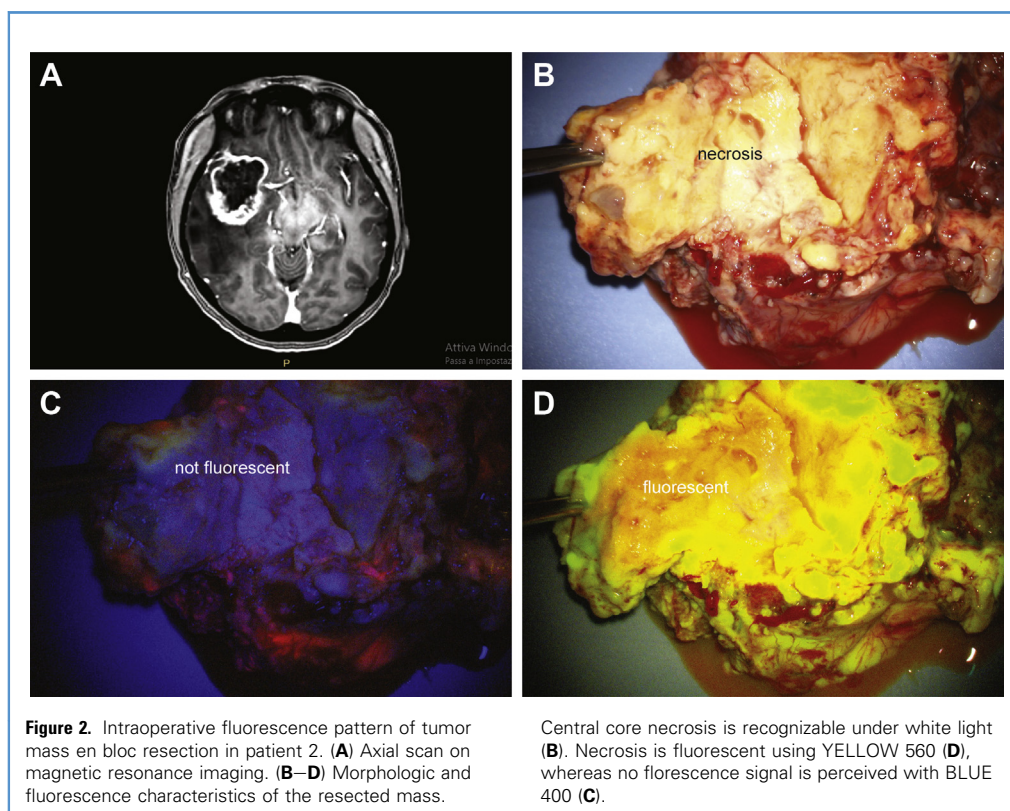
illumination was used to elicit 5-ALA fluorescence, and the YELLOW 560 module alone was used to capture both fluorescein and 5-ALA fluorescence patterns. They reported that, with dual labeling, fluorescein-induced fluorescence created a useful background for 5-ALA fluorescence. They obtained tissue samples at the tumor margin in only 2 out of a total of 6 patients, and there were no reported histologic data to compare the techniques.

Our More Relevant Data

This study presents a main limitation of a very small number of cases. It does not allow us to draw any strong conclusions. However, our study is the first preliminary work, to our knowledge, to compare tissue fluorescence patterns simultaneously using 5-ALA and fluorescein at dosages currently accepted by the scientific community in the same patient.

From our histopathologic data, we appreciated that sensitivity was high both for 5-ALA and fluorescein, even if 5-ALA was a little bit higher than fluorescein (5-ALA was 100%, fluorescein was 93%). The main difference between the 2 fluorescence techniques was about specificity, even if it is important to remember the limited number of nonfluorescent biopsies (1 true negative sample for fluorescein and 2 true negative samples for 5-ALA), considering the surgical uselessness of sampling clearly normal brain parenchyma and the ethical reasons implied. Specificity and PPV of 5-ALA were higher than fluorescein (67% vs. 33% and 94% vs. 87%, respectively). This means that 5-ALA fluorescence can trace GBM tissue better than fluorescein. The NPV was higher for 5-ALA as well when compared with fluorescein (100% vs. 50%, respectively).

Further in detail, in our experience, specificity was 100% and 67% when bright and faint 5-ALA fluorescence was considered, respectively. This can have important implications on surgical strategy. As previous studies report, when 5-ALA fluorescence is used in newly diagnosed GBM, a tumor component that is intraoperatively brightly fluorescent approximately corresponds to ring enhancing tumor at gadolinium MRI with gadolinium, whereas tumor infiltration, which intraoperatively appears as fairly fluorescent, approximately corresponds to infiltrated brain parenchyma surrounding ring enhancement, partially overlapping MRI alterations at fluid attenuation inversion recovery-weighted sequences (Figure 1). Central necrosis is usually not fluorescent under blue light (Figure 2).^{17,32} In our data, this means that with the nonnecrotic component of GBM, bright 5-ALA fluorescence is superior to fluorescein (specificity 100% for 5-ALA vs. 33% for fluorescein) in tracing neoplastic tissue. Alternatively, regarding peritumoral infiltration (faint 5-ALA fluorescence), both 5-ALA and fluorescein present a misleading false-positive rate; however, 5-ALA presents a higher specificity than fluorescein (specificity 67% for 5-ALA vs. 33% for fluorescein). Because of the low specificity of both fluorescence techniques, in both cases, neurophysiologic monitoring is mandatory in this area. A main limitation of our study was that samples were not collected from the intratumoral core because examination was focused on the marginal areas of the tumor. This must be taken into consideration as a potential bias when reading the higher sensitivity of 5-ALA compared with fluorescein as reported by other authors.^{20,33,34}



Combining of the 2 Techniques: Intraoperative Findings and Considerations

Our study cannot draw any definitive conclusions about the comparison between the 2 fluorescence modalities that are based on different fluorescence mechanisms: metabolic for 5-ALA and unspecific (BBB breakdown) for fluorescein. However, we will report some considerations on our small intraoperative experience.

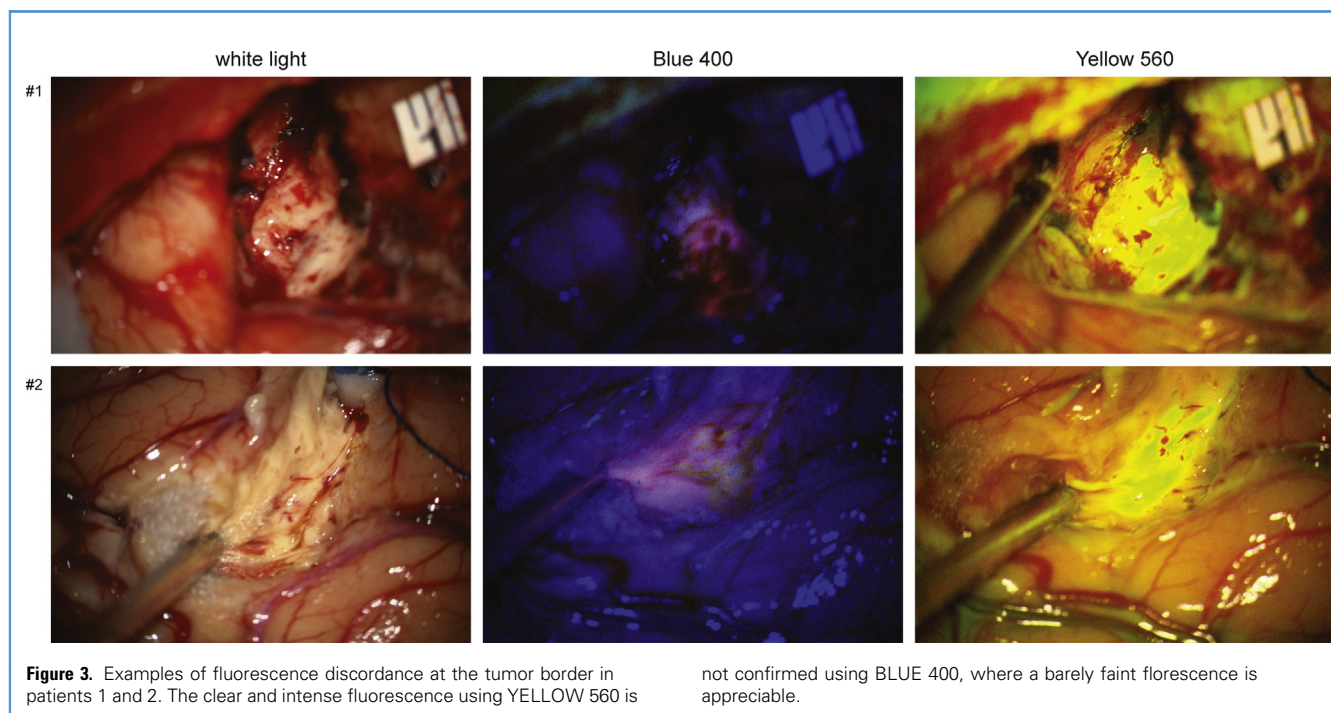
A first consideration must be made on the earliest intraoperative view when combining 2 fluorescence modalities. Our experience was that, at first view, the fluorescent areas were very similar (**Figure 2**). Furthermore, tissue manipulation (e.g., small contusions) can lead to unspecific tissue fluorescence when using YELLOW 560 (**Video 1**); however, in our experience, false-positive areas using YELLOW 560 light were rarely detected far from the tumor (**Figure 3**). Probably, the first view of quite overlapping areas at different fluorescence examination (**Figure 4**) could be explained by similar high sensitivity of the 2 compounds (5-ALA was 100%, fluorescein was 93%).

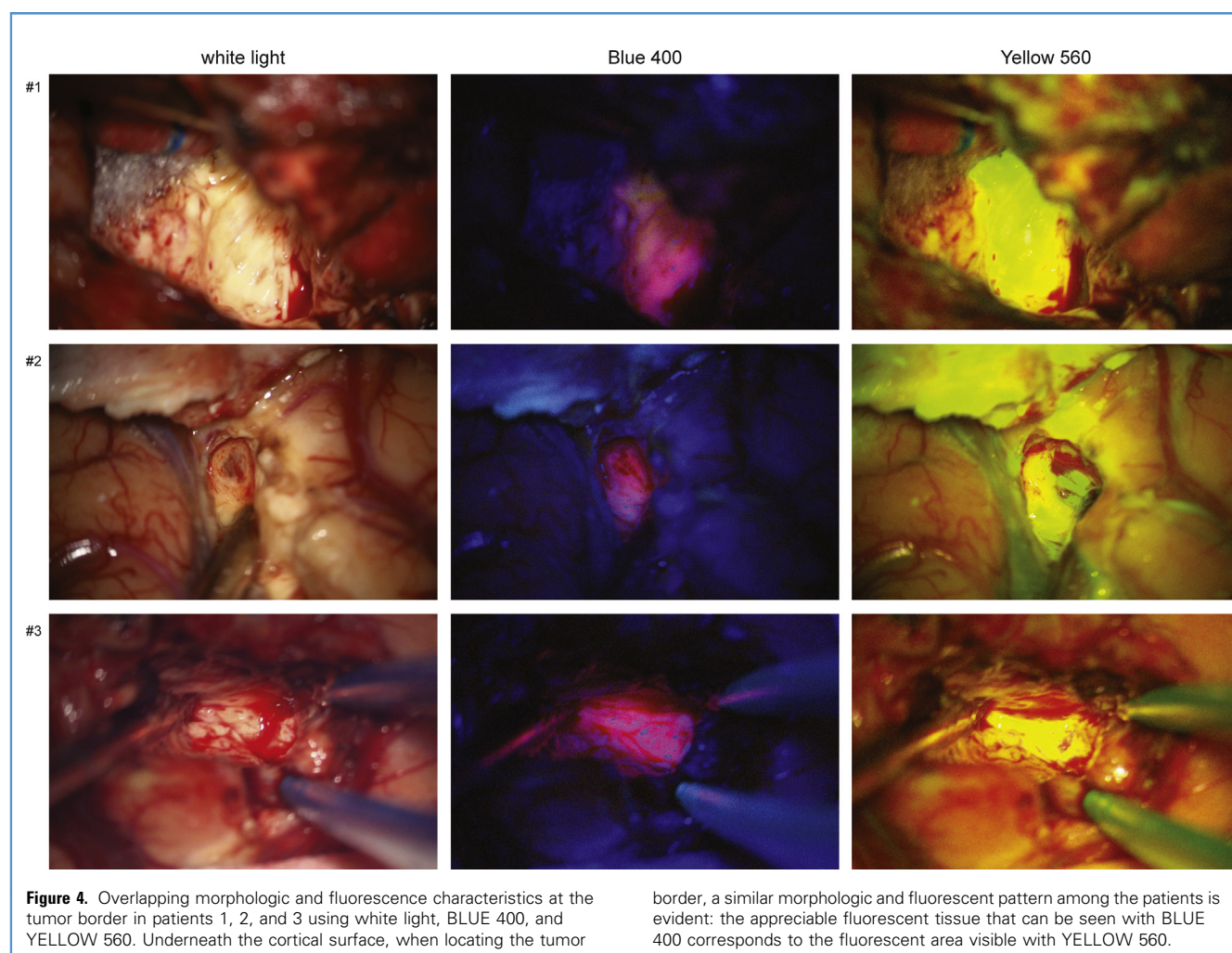
One significant difference in terms of fluorescence between the techniques, as previously mentioned, is that the central necrosis is usually not fluorescent under blue light, whereas it is using YELLOW 560. Therefore, central necrosis is evident as fluorescent using YELLOW 560, whereas it is not using BLUE 400³⁵ (**Figure 2**). It is possible to notice a slight decrease in fluorescence in the most necrotic areas, where fluorescence is more heterogeneous under yellow light (**Figure 2D**). However, it is clear that the same area under blue light is not fluorescent at all (**Figure 2C**). This is important because the nondetection of nonfluorescent necrotic tissue can be a possible

cause of residual tumor left behind³⁶ (Medac/Stummer - Gliolan certification course; pptx Presentation Version 6).

Another difference is that there is a discordance in terms of fluorescence at the tumor border. 5-ALA can show different nuances of fluorescence (bright vs. faint): this allows the surgeon to navigate throughout a newly diagnosed GBM keeping in mind that faint fluorescent tissue is usually surrounding brightly fluorescent tumor. The gradual reduction of fluorescence at the tumor borders (**Figures 2** and **4**) is synonymous with gradual entrance into surrounding healthy tissue. Fluorescein does not present different nuances of fluorescence, and this can make it more difficult to recognize the tumor border (**Figure 4**). Fluorescein specificity at the tumor border was lower (33% compared with 67% with 5-ALA). This aspect and the low specificity under yellow light must be kept in mind by surgeons working only with fluorescein at the tumor borders because of the high risk of entering healthy tissue. Furthermore, considering the low number of nonfluorescent samples, the NPV of 5-ALA was considerably higher than fluorescein (100% vs. 50%, respectively), meaning that, using BLUE 400, nonfluorescent tissue is probably normal parenchyma. This plays a crucial role when working on eloquent areas.

The PPV of bright pink fluorescence (**Table 3**) is superior to faint pink (94% and 89%, respectively), and the PPV of faint pink fluorescence is similar to fluorescein (87%). Therefore, at the tumor margin, bright pink fluorescence is highly indicative of residual tumor and it is probably superior to faint pink and fluorescein, which are similar in identifying tumor tissues at this site.





Another important advantage in our experience with intraoperative use of YELLOW 560 is that nonfluorescent tissue is displayed in a more natural color compared with BLUE 400 (Figure 4). It is brighter and allows a better visualization of anatomic structures. This could allow, when using fluorescein, a reduction in shifting to white light, therefore being less time consuming during the procedure, as already suggested by Suero Molina et al.³¹

CONCLUSIONS

Finally, our preliminary data show the advantages and drawbacks of 5-ALA and fluorescein and their complementary use when intraoperatively dealing with marginal areas of newly diagnosed GBM. Studies designed to address the potential synergy to maximize extent of resection should be considered. Larger studies are mandatory to investigate the synergistic use of both techniques.

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