Dual-labeling with 5–aminolevulinic acid and fluorescein for fluorescence-guided resection of high-grade gliomas: technical note

Eric Suero Molina, MD, MBA,1 Johannes Wölf er, MD,1 Christian Ewelt, MD,1 André Ehrhardt, PhD,2 Benjamin Brokinkel, MD,1 and Walter Stummer, MD1

1Department of Neurosurgery, University Hospital Münster; and 2Karl Storz GmbH & Co., Tuttlingen, Germany

OBJECTIVE Fluorescence guidance with 5–aminolevulinic acid (5-ALA) helps improve resections of malignant gliomas. However, one limitation is the low intensity of blue light for background illumination. Fluorescein has recently been reintroduced into neurosurgery, and novel microscope systems are available for visualizing this fluorochrome, which highlights all perfused tissues but has limited selectivity for tumor detection. Here, the authors investigate a combination of both fluorochromes: 5-ALA for distinguishing tumor and fluorescein for providing tissue fluorescence of adjacent brain tissue.

METHODS The authors evaluated 6 patients who harbored cerebral lesions suggestive of high-grade glioma. Patients received 5-ALA (20 mg/kg) orally 4 hours before induction of anesthesia. Low-dose fluorescein (3 mg/kg intravenous) was injected immediately after anesthesia induction. Pentero microscopes (equipped either with Yellow 560 or Blue 400 filters) were used to visualize fluorescence. To simultaneously visualize both fluorochromes, the Yellow 560 module was combined with external blue light illumination (D-light C System).

RESULTS Fluorescein-induced fluorescence created a useful background for protoporphyrin IX (PPIX) fluorescence, which appeared orange to red, surrounded by greenly fluorescent normal brain and edematous tissue. Green brain-tissue fluorescence was helpful in augmenting background. Levels of blue illumination that were too strong obscured PPIX fluorescence. Unspecific extravasation of fluorescein was noted at resection margins, which did not interfere with PPIX fluorescence detection.

CONCLUSIONS Dual labeling with both PPIX and fluorescein fluorescence is feasible and gives superior background information during fluorescence-guided resections. The authors believe that this technique carries potential as a next step in fluorescence-guided resections if it is completely integrated into the surgical microscope.

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KEY WORDS PPIX; fluorescein; malignant glioma; ALA; fluorescence-guided resection; oncology

MAXIMAL safe resection of malignant gliomas as the first step of multimodal therapy is an accepted goal in malignant glioma surgery.18,23,26,31,38 This aim is not always easily achieved, since the borders of the contrast-enhancing part of the tumor are often difficult to distinguish using conventional white-light microscopy. For this reason, several tools have been introduced, such as intraoperative imaging by ultrasound, MRI, or CT.5,6,19,45,46 The use of 5–aminolevulinic acid (5-ALA) is another well-established method first introduced to neurosurgery in 1998.40,42 Since then, extensive basic and clinical research, including a Phase 3 randomized trial, have been performed.10,12,17,39,43,44 As a nonfluorescent biochemical precursor of hemoglobin, 5-ALA leads to intracellular synthesis and accumulation of fluorescent protoporphyrin IX (PPIX) in malignant neoplasms.15,30,40,42 Commercially available microscopes equipped with blue-violet excitation light are used for visualizing PPIX fluorescence. Such microscopes are available from all major microscope manufacturers. Red fluorescence highlighting viable tumor cells is visualized on a dark blue–green background. The blue-green background is essential for obtaining tissue detail to enable resections under the light conditions needed for fluorescence visualization and is based on emission filter...
characteristics. These filters allow transmission of autofluorescence, and a small part of the remitted excitation light (375–440 nm). Under normal circumstances, this combination of autofluorescence and remitted light is usually regarded as giving sufficient background information. Under certain circumstances, background illumination may be considered too weak, requiring a frequent change of the filters to white light for visualizing anatomy, for re-orientation, or for hemostasis.

Based in part on the acceptance of fluorescence-guided resection using 5-ALA, a second fluorochrome, fluorescein, which is approved for retinal angiography, has recently gained attention in the context of glioma surgery. First described in 1948 by G. E. Moore for finding and resecting intrinsic brain tumors in the age of air ventriculography, Moore discussed possible merits for detecting tumor but already noted fluorescence in edematous brain. After the initial description, and ultimately in the late 20th century, a number of articles discussing the use of fluorescein were published. Fluorescein sodium provides strong fluorescence and, apart from infrequent anaphylactic reactions and seizures, appears to be safe, especially when the applied doses are low. With the introduction of the recent generation of surgical microscopes with new filters, e.g., the Yellow 560 system (Carl Zeiss) or the FL560 System (Leica Microsystems), fluorescein sodium is experiencing renewed attention in the field of fluorescence imaging. In past publications, several advantages of fluorescein over 5-ALA have been pointed out. However, controversy has ensued because of the fact that after intravenous administration of fluorescein, fluorescence can be found in all perfused tissue—being in plasma—and is consequently extravasated whenever the blood-brain barrier is breached, e.g., at resection margins. With this study, we now explore a previously unreported (to our knowledge) different path for utilizing fluorescein fluorescence based on its presence in all perfused tissue. We aimed at combining the selective accumulation of 5-ALA–induced porphyrins for reliable tumor detection, and fluorescein for providing background information by simultaneously visualizing both fluorochromes. This combination would entail a number of evident advantages, provided that the approach is feasible, overcoming some of the limitations of fluorescence-guided resections with fluorescein using blue light incorporated into commercially available, conventional microscopes alone. Yellow-green fluorescein emission light would not interfere with red tumor fluorescence as is the case when trying to illuminate background tissue with additional external light sources. Better background visualization would obviate the requirement of frequently changing between fluorescence and white light modes of the surgical microscope and would be expected to accelerate surgery while maintaining safety and efficacy. Less frequent changes between illumination modalities would reduce the necessity for habituating between high and low illumination conditions by the surgeon, increasing comfort and possibly safety. Bleaching of 5-ALA–induced porphyrins from white light would be reduced, if phases of white light surgery were shortened.

**Methods**

We evaluated 6 patients undergoing microscopic fluorescence-guided resection for cerebral lesions suggestive of high-grade glioma, aiming for better intraoperative visualization of tumor and adjacent tissue (Table 1). By using fluorescein, we hoped for more rapid surgery due to the better visualization of tumor tissue, while operating on a brighter background, as previously reported.

Written informed consent was obtained from all patients included in this series after consultation with the ethics committee regarding compassionate use of a drug in an off-label setting, aiming at improving resection and comparing fluorescein with the gold standard for fluorescence-guided resection, 5-ALA. Approval was granted by the ethics committee for a retrospective compilation of data from this cohort. Prior to surgery, each case was discussed by the tumor board and, in accordance with the interdisciplinary decision, surgery was proposed. Patients received 5-ALA (Gliolan, Medac) at a dose of 20 mg/kg body weight 4 hours before induction of anesthesia; low-dose fluorescein (3 mg/kg intravenous; Fluorescein Alcon 10%, Alcon Pharma GmbH) was injected immediately after anesthesia induction, as previously described. Zeiss Pentero (Carl Zeiss) microscopes equipped with either a Blue 400 or Yellow 560 filter were used during resection.

**Filter Combinations for Dual Visualization**

The excitation maximum of fluorescein, i.e., the wavelength at which the strongest fluorescence is generated, is at 480 nm. In comparison, the excitation maximum of 5-ALA–derived porphyrins is 405 nm, which represents the Soret band of PPIX (Fig. 1). The fluorescence maximum for fluorescein is 525 nm, whereas maximal fluorescence of PPIX is found at 635 nm with a smaller, secondary peak at 704 nm. Thus, using excitation light as

<table>
<thead>
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<th>Case No.</th>
<th>Sex</th>
<th>Histology</th>
<th>Location</th>
<th>Side</th>
<th>MGMT</th>
<th>IDH-1</th>
<th>GTR</th>
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<td>1</td>
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<td>Negative</td>
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</tr>
<tr>
<td>3</td>
<td>F</td>
<td>GBM</td>
<td>Parietal</td>
<td>Rt</td>
<td>Unmethylated</td>
<td>Negative</td>
<td>Yes</td>
</tr>
<tr>
<td>4</td>
<td>F</td>
<td>GBM</td>
<td>Frontal</td>
<td>Lt</td>
<td>Unmethylated</td>
<td>Negative</td>
<td>Yes</td>
</tr>
<tr>
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<td>Occipital</td>
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</tr>
<tr>
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<td>Frontal</td>
<td>Rt</td>
<td>Methylated</td>
<td>Negative</td>
<td>Yes</td>
</tr>
</tbody>
</table>

GBM = glioblastoma; GTR = gross-total resection; — = not performed.
provided by the Zeiss Yellow 560 system (460–500 nm) in combination with specific light for exciting protoporphyrin fluorescence (375–440 nm; D-light C System, Storz, CE approved for patient use) will specifically and optimally elicit the fluorescence of the respective fluorochrome, which can be visualized using the Yellow 560 module alone. As illustrated in Fig. 1, the 530-nm longpass filter optimized for detecting fluorescein fluorescence will also allow visualization of tissue autofluorescence, excited by blue light and PPIX fluorescence. For visualizing fluorescence, the microscope with the activated Yellow 560 filter system was positioned approximately 25 cm away from the surgical field and the light intensity set to 50%, whereas a glass fiber light guide was used to convey violet-blue excitation light (D-light C System) to the surgical field (distance ~ 5 cm, 100% light intensity). Ensuing images were captured using the Zeiss Pentero HD1 Video Camera (Carl Zeiss).

No surgical decisions were made based on these images. Separate biopsy samples (n = 2) were obtained in 2 patients from tissue solely harboring fluorescein and not 5-ALA–derived fluorescence. All samples were stained with H & E and elastica van Gieson. Immunohistochemical and molecular genetic analysis of glial fibrillary acidic protein (GFAP), IDH-1 mutation, Ki-67 MIB1 proliferation index, and MGMT methylation were also performed.

Results

Administering fluorescein created a useful background for PPIX fluorescence, which appears as orange to red, surrounded by intense green fluorescence in normal brain and edematous tissue (Fig. 2). Orange fluorescence resulted from the combination of green fluorescein and red PPIX fluorescence within tumor tissue.

Using the Yellow 560 filter and only illuminating with violet-blue light (375–440 nm) allowed visualization of only isolated red PPIX fluorescence (Fig. 2B). With incremental increases of blue excitation (460–500 nm), the yellow fluorescence of fluorescein in tissue and tumor became visible (Fig. 2C). Co-emitted red and yellow fluorescence gave tumor tissue an orange hue. With further increases of yellow light, PPIX fluorescence was gradually concealed (Fig. 2D). The extent of visible red PPIX fluorescence as depicted using the Yellow 560 filter system was verified by comparing the image with the image obtained by the Carl Zeiss Blue 400 system (Fig. 2E; refer to Video 1 for the entire sequence).

VIDEO 1. Clip showing the dual labeling technique. Copyright Department of Neurosurgery, University Hospital of Münster. Published with permission. Click here to view.

At an intermediate intensity of blue light (460–500 nm) in conjunction with maximal blue-violet light (375–440 nm), green brain-tissue fluorescence appeared helpful for distinguishing background while still allowing specific visualization of the tumor.

Using both methods in conjunction, we found that fluorescein fluorescence was not always able to highlight viable tumor tissue as depicted by PPIX fluorescence. Furthermore, unspecific extravasation of fluorescein was noted at resection margins, likewise differing with PPIX fluorescence, but without actually interfering with PPIX fluorescence detection.

Tissue removed intraoperatively was stained with H & E and elastica van Gieson. Immunohistochemical and molecular genetic analyses of GFAP, IDH-1 mutation, Ki-67 MIB1 proliferation index, and MGMT methylation were also performed. In addition, we obtained samples of tissue harboring fluorescein alone and not 5-ALA–derived
fluorescence from tumor margins in our first 2 patients. However, histopathological evaluation of these tissues did not confirm the presence of tumor cells; thus, we did not remove tissue highlighted by fluorescein alone in the remaining patients. We further observed fluorescein fluorescence in the dura, cortex, and especially in edematous tissue (Fig. 2). In all cases, orange fluorescence could be observed, which implies that tumor cells harboring both PPIX embedded in a tissue matrix containing fluorescein. We did not experience any adverse effects from either fluorescein or of 5-ALA administration.

We did, however, note significant dissimilarities between the images captured by the built-in camera of the microscope and what was visible to the surgeon, with a reduced fidelity in discriminating light intensities and wavelength nuances by the camera. For illustration, we used Zeiss fluorescence phantoms for comparing images captured by the intrinsic camera to images simultaneously captured by a 12-megapixel high-definition camera through the microscope lens (Fig. 3). These images intend to illustrate limitations of the video material generated by the microscope camera, as presented in this study, in contrast to cameras with higher resolution or the surgeon’s optical impression obtained using the microscope lens system.

**Discussion**

**Differential Mechanisms of Fluorescence Accumulation**

5-ALA is a prodrug that elicits the accumulation of endogenous porphyrins in malignant glioma tissue and is used as an intraoperative contrast agent. Technically, surgical microscopes adapted for visualizing fluorescence are constructed not only for visualizing PPIX fluorescence per se, e.g., using a longpass filter for detecting specific 635- and 704-nm emission peaks. Rather, part of the remitted blue excitation is included in the emission light pathway. This light allows discrimination of nonfluorescing tissues and is essential for surgery using the fluorescence mode of the microscope. However, under certain circumstances this remitted light is considered too weak, and surgeons need to shuttle between violet-blue and white light frequently during surgery. This prolongs surgery and requires the surgeon to habituate between illumination conditions, which limits the comfort and ergonomics of this approach. Furthermore, prolonged illumination with strong white light might lead to bleaching of porphyrins.

While PPIX accumulates intracellularly and remains restricted to tumor cells with high selectivity, fluorescein is injected intravenously and circulates with plasma. Fluorescein has a half-life of 23.5 minutes, being converted into fluorescein monoglucoronide, which in turn has a half-life in plasma of 264 minutes. The monoglucoronide has approximately one-third the fluorescence of fluorescein. After intravenous administration, all perfused tissues will show fluorescence for prolonged periods of time, which will slowly subside. In regions of tissue injury or areas of blood-brain barrier breakdown, as in the angiogenic
and enhancing regions of malignant gliomas, fluorescein will be extravasated with edema, initially highlighting the general region of blood-brain barrier perturbation without having any specific affinity to tumor cells. Administering fluorescein alone is used for trying to define marginal tumor in gliomas surgery. Furthermore, surgical injury to brain will also result in fluorescein extravasation in affected tissues. Even though this is the case in high-grade gliomas, it will further leak out during resection, since fluorescein has no specific affinity to tumor cells.

Limitations

We are aware of the technical limitations of our setup, especially regarding the possibility of documenting the surgeon's visual impression through the microscope lens and enhancing regions of malignant gliomas, fluorescein will be extravasated with edema, initially highlighting the general region of blood-brain barrier perturbation without having any specific affinity to tumor cells. Administering fluorescein too soon before surgery leads to high concentrations in vessels and significant leakage from injured tissue with edema and blood.

However, edema spreads into tissue and propagates through peritumoral tissue at a speed exceeding 2 mm per hour in humans and thus raises concerns about selectivity if fluorescein alone is used for trying to define marginal tumor in gliomas surgery. Furthermore, surgical injury to brain will also result in fluorescein extravasation in affected tissues. Even though this is the case in high-grade gliomas, it will further leak out during resection, since fluorescein has no specific affinity to tumor cells.

Varying Fluorescence During Dual Labeling

With our present approach, we have attempted to combine the advantages of both fluorochromes, 5-ALA for selectively marking tumor, and fluorescein for—to an extent—highlighting tumor and providing background fluorescence. Differing excitation wavelengths enable selective elicitation of respective fluorescence, which could be visualized using the same long-pass filter. We found too intense blue excitation light to conceal red-orange PPIX fluorescence, indicating limitations to what might be gained. We made similar observations in all of our 6 patients.

In general, fluorescence is proportional to the amount of excitation light; thus fluorescence emission can be controlled by simply varying excitation light intensity. After oral administration of 5-ALA, tumor cells will begin accumulating PPIX fluorescence, which will peak at 6–8 hours after administration, which gradually decreases thereafter (Fig. 4).

Porphyrin fluorescence is visualized by using as much violet-blue (375–440 nm) excitation light as possible, because PPIX fluorescence is weak compared with fluorescein fluorescence. Thus, there is no reasonable possibility of varying PPIX fluorescence emission by reducing the respective excitation light intensity. Fluorescein fluorescence however, being much stronger, can be directly controlled by simply decreasing or increasing the illumination intensity of blue light (460–500 nm). Because fluorescein is not excited by violet-blue light, fluorescein fluorescence can be regulated as desired by the surgeon independent of PPIX fluorescence. Plasma fluorescein concentrations depend on the amount of fluorescein injected and, in addition, on the time after application; the ability to vary excitation will be a prerequisite and will enable adjusting background fluorescence as needed. Nevertheless, low concentrations injected at an early time point prior to surgery will be more desirable than higher concentrations injected at a short interval prior to resection, because of the much stronger fluorescence compared with porphyrin fluorescence.

Conclusions

Dual labeling with both PPIX and fluorescein fluorescence is feasible as deduced from our experience using both fluorochromes simultaneously. We did not find fluorescein helpful for discriminating additional tumor; rather, we found that fluorescein enhanced the depiction of background during fluorescence-guided resections. We believe that combining both fluorochromes carries potential as a next step in fluorescence-guided resections if completely integrated into the surgical microscope.
References


**Disclosures**

Dr. Stummer reports that he receives speaker fees from Medac and Carl Zeiss.

**Author Contributions**


**Supplemental Information**

**Videos**

*Video 1.* [https://vimeo.com/196291414](https://vimeo.com/196291414)

**Correspondence**

Eric Suero Molina, Department of Neurosurgery, University Hospital of Münster, Albert-Schweitzer-Campus 1, A1, Münster D-48149, Germany. email: eric.suero@ukmuenster.de.