Markers for Identifying and Targeting Glioblastoma Cells during Surgery

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Abstract

Glioblastoma is a highly malignant tumor with a poor prognosis. A factor influencing survival that can be affected by the surgeon is the extent of resection (EOR). Due to the infiltrative nature of the tumor, delineation of tumor from normal brain parenchyma is often challenging. To improve EOR and facilitate tumor visualization, several techniques have been developed over the last few years. This literature review presents an overview of current intraoperative strategies for identifying and targeting glioma cells and discusses the benefits and limitations of each technique. Along with conventional techniques such as neuronavigation and ultrasound, fluorescence-guided surgery with different fluorescent agents such as 5-aminolevulinc acid and fluorescein have been widely used. Recently, newer techniques have emerged and are being translated into the operating room, promising delineation of glioblastoma tissue using targeted approaches or identification on a microscopic level, for instance using Raman spectroscopy or confocal microscopy.

Keywords
- glioma
- interoperative MRI
- 5-ALA
- fluorescein
- indocyanine green

Introduction

Glioblastoma is one of the most malignant tumors of the central nervous system. Despite a better understanding of tumor biology and the development of new treatment approaches during the last decades, the tumor remains incurable with a poor prognosis, characterized by a median survival of 15 months and a 2-year survival rate of 17.4%.[1,2]

Current first-line treatment consists of surgical resection, followed by adjuvant radiation and chemotherapy.[3] Extent of resection (EOR) is an independent predictor of survival, first shown by Lacroix et al, who demonstrated that an EOR $>$ 98% of tumor volume results in a significant survival advantage.[4] Another study indicated that the threshold might even be lower, with an EOR of 78% as a minimum associated with a survival benefit.[5] In addition, data from a large EORTC-NCIC trial, the pivotal trial for approval of temozolomide as first-line therapy for glioblastoma, revealed a greater benefit from adjuvant radiotherapy and chemotherapy in patients with gross total resection (GTR).[6]

Clearly, EOR is a critical driver of outcome in glioblastoma and the only factor that the surgeon can influence directly. Given the infiltrative nature and often eloquent tumor localization, achieving the largest possible EOR is often challenging. Tumor infiltration into the surrounding brain parenchyma is difficult to discriminate with the human eye under standard white light microscopy, and differentiation of tumor margins from normal brain often cannot be performed based on tactile features of tissues. Consequently, several techniques have been developed to improve the ability of the surgeon to identity glioblastoma tissue during surgery.

Several modalities aiming at improving EOR entered the field decades ago, such as neuronavigation, intraoperative ultrasound, and intraoperative magnetic resonance imaging (iMRI). Newer techniques, such as fluorescence-guided surgery

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(FGS) using various fluorescent agents, enables intraoperative real-time imaging of glioblastoma. Recently, more novel techniques have emerged and are being translated into the operating room, promising an even better delineation of glioblastoma tissue, such as Raman spectroscopy, confocal microscopy, or methods of targeted fluorescence.[7–11]

We performed a literature review on currently available techniques that aim at targeting and identifying glioblastoma cells during surgery, discussing their benefits, applicability, and limitations in the field of glioma surgery. This review focuses on FGS and newer techniques; conventional techniques are only mentioned for the sake of completeness but are not reviewed in further detail.

Conventional Techniques for Intraoperative Visualization of Glioblastoma

For most surgical glioblastoma cases, conventional techniques such as neuronavigation and ultrasound are standard of care and have been widely integrated into the operative setting.

Neuronavigation and Ultrasound

Neuronavigation is a basic and ubiquitously available tool for glioblastoma surgery. All relevant preoperative digital scans such as computed tomography (CT), MRI, and positron emission tomography (PET) can be incorporated into the data set for the navigational system and help the surgeon maintain a precise sense of complex three-dimensional anatomical relationships and almost real-time intraoperative localization.[12] Neuronavigation benefits from high surgical accuracy for resection of glioblastomas and can aid in planning the surgical approach.[13,14] A study by Wirtz et al evaluated the effect of neuronavigation on the EOR in glioblastoma compared with standard use of the microscope. They showed that the amount of residual tumor was significant lower in the patients operated on using neuronavigation, without showing a clear difference regarding the number of radical resections.[15] A major limitation using neuronavigation is the loss of accuracy caused by intraoperative brain shift due to application of mannitol, drainage of cerebrospinal fluid, patient positioning, and resection of tissue.[16]

Another uncomplicated and cost-effective method for intraoperative glioblastoma localization is ultrasound. This dynamic method helps identify tumor borders and normal brain structures.[16] A retrospective analysis showed an increase of survival when surgeons used intraoperative ultrasound for identifying residual glioma.[17] However, concerns have been expressed regarding a sometimes poor differentiation of tumor from the zone of peritumoral edema, putting patients at risk for too extensive resections with neurologic sequelae.[18] In addition, ultrasound has its limitations in the delineation of normal brain tissue from high-grade glioma tumor borders after previous irradiation.[19]

Intraoperative acquired data from ultrasound can be used to update the navigation system and help overcome the limitation of brain shift.[20]

Intraoperative MRI

First introduced in the 1990s, iMRI has since undergone further development. It provides almost real-time images during surgery for identification of residual tumor and can also be used to detect possible intraoperative complications such as hematoma.[21]

In addition, the acquired images can be used to update the neuronavigational system to compensate impaired accuracy from brain shift.[22,23] A prospective randomized controlled trial published by Senft et al compared the rates of GTR in glioma patients operated using conventional microsurgery and patients in whom iMRI was used. These authors showed a significant higher frequency of GTR in the iMRI group (96% iMRI group versus 68% control group; $p = 0.023$), providing evidence for the beneficial role of iMRI in glioma surgery.[24] Supporting results were presented by Hatiboglu et al, showing that when used by surgeons, iMRI led to an increase of the median EOR from 84% to 99% ($p < 0.001$) with additional tumor removal after iMRI in contrast-enhancing gliomas.[25]

However, iMRI has distinct disadvantages. This method is expensive, time consuming, and extends the duration of surgery and anesthesia. In addition, repeated application of gadolinium may result in extravasation into the tumor area and resection cavity, leading to false-positive effects.[21,26]

Fluorescence-guided Surgery for Glioblastoma

The application of fluorescing compounds for differentiating tissue during brain surgery was first described in 1947 by George E. Moore, who noted a higher concentration of fluorescein in brain invaded by malignant glioma using a wood lamp.[27] After decades of dormancy, this approach was modernized in 1998 by the senior author of this review, Walter Stummer, by the introduction of 5-aminolevulinic acid (5-ALA) for FGS.[28] FGS is based on the administration of optical imaging agents to patients before surgery, leading to a (selective) accumulation in tumor cells, helpful in intraoperative real-time detection and delineation of tumor tissue. Currently, two agents are being used clinically in the field of glioblastoma surgery: 5-ALA[28] and fluorescein.[27] A third dye, indocyanine green (ICG), is under investigation.[29]

5-aminolevulinc Acid

5-ALA is worldwide the most intensely studied fluorescent agent for brain tumor surgery. It was approved by the European Medicines Agency (EMA) and the U.S. Food and Drug Administration (FDA) for intraoperative visualization of malignant glioma. 5-ALA is a natural metabolite in the hemoglobin pathway and within glioma cells.[30,31] After oral administration of 20 mg/kg body weight (BW) 5-ALA (Gliolan in the European Union [Specialized Therapeutics, Melbourne, Australia]; Gleolan in the United States [NX Development Corp., Lexington, Kentucky, United States) 3 hours before the induction of anesthesia, fluorescence can be visualized by using a surgical microscope with a

xenon light source that can switch between white and violet-blue light (wavelength: 370–440 nm) and is provided with an emission filter for visualization of red tumor fluorescence with peaks at between 635 and 704 nm, thus well in the red range.[30,31] Peak fluorescence can be expected after 6 to 8 hours.[32,33]

It was shown that 5-ALA has a high toxicologic safety with only minor side effects such as a temporary and mild elevation of liver enzymes and transient skin phototoxicity.[34,35]

Visualization of Glioblastomas and Intensity of 5-ALA Fluorescence

The efficacy of 5-ALA for intraoperative visualization of glioblastoma cells was shown by several studies, and investigators uniformly report a high selectivity. In a series of 10 patients, with 89 tissue biopsies, sensitivity of 5-ALA–induced fluorescence for detection of malignant glioma cells was 85% and specificity was 100%.[28] In a meta-analysis, including eight studies on histopathologic analysis and intraoperative 5-ALA fluorescence with $>$ 800 samples from malignant glioma, the specificity for glioblastoma was 88.8% and sensitivity 82.6%.[36]

Using 5-ALA, it is important to differentiate between different qualities of fluorescence because tumor fluorescence is not homogeneous. Two fluorescence qualities can be distinguished: a vivid *solid* red fluorescence, representing viable tumor, and a *vague*, less vivid pink fluorescence, indicating the tumor-infiltrating zone. These findings were supported by histologic and spectroscopic analyses (►**Fig. 1**).[37,38]

Especially in case of solid fluorescence, a positive predictive value (PPV) of 100% was reported. PPV was lower, between 91% and 97%, in tissue with vague fluorescence in invasive areas at the tumor border.[38,39]

Even in recurrent glioblastoma, where tissue scarring and changes induced by previous radiotherapy and chemotherapy are present, 5-ALA–guided resection was still shown to be effective with a PPV of 99.5%.[40]

Similar findings were reported by Lau et al, who analyzed 211 intraoperative high-grade glioma biopsies from different areas of fluorescence intensity graded from 0 to 3. They revealed a PPV of 100% for high-grade gliomas and 97.2% for glioblastomas in case of highest rated amount of fluorescence. However, the negative predictive value was comparably very low with 16.7% for high-grade tumors and 43.9% for glioblastoma, indicating that not all tumor-infiltrated areas may synthesize the dye in concentrations that can be visualized using the surgical microscope.[41] Consequently, 5-ALA is a very useful marker for tumor cellularity, especially in areas with solid and bright fluorescence.

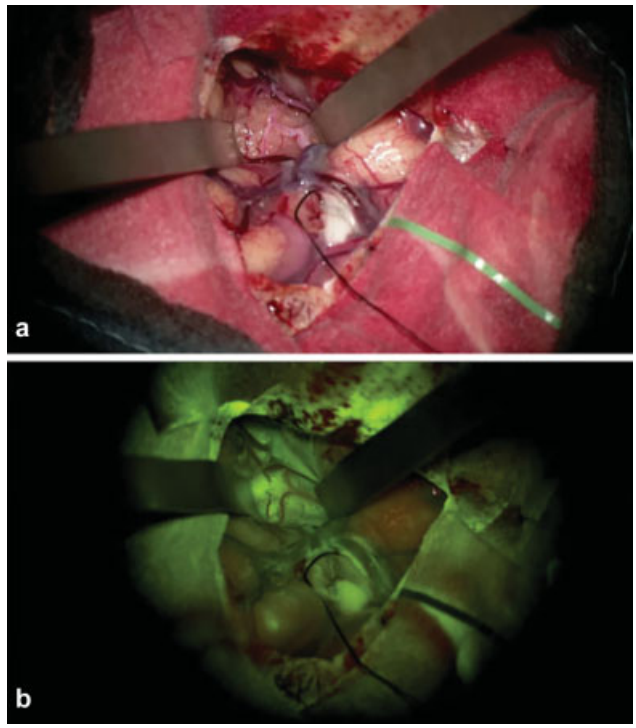

Fig. 2 Use of fluorescein (FL) for resection of glioblastoma. (**a**) After administration of FL under white light: no fluorescent effect. (**b**) After administration of FL under YELLOW 560 nm filter: visible fluorescent effect in the tumor. (Reproduced with permission from Schebesch et al.[54])

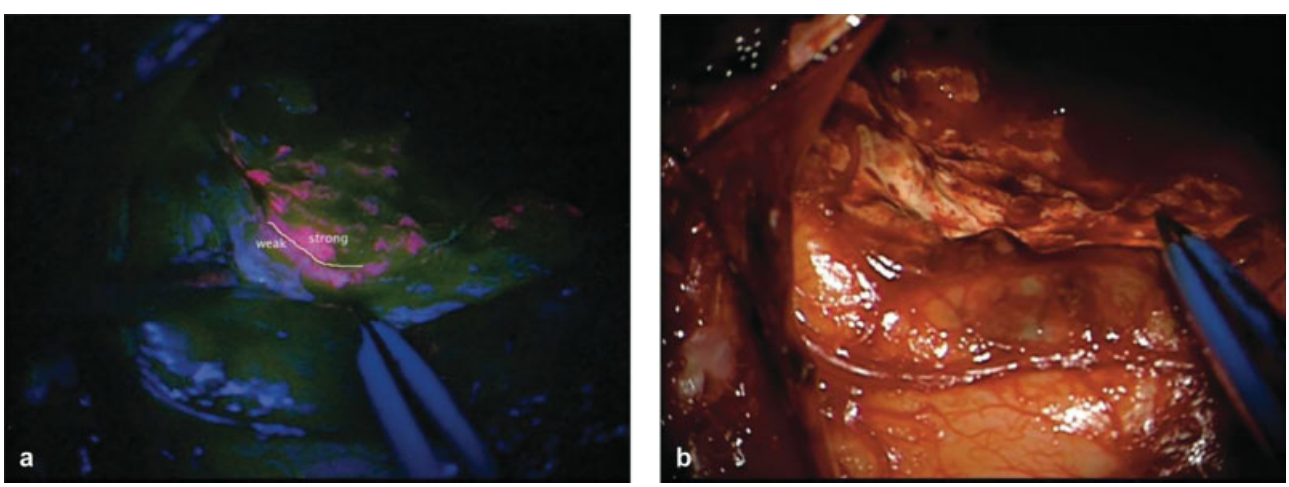

Fig. 1 5-aminolevulinic fluorescence and different fluorescence qualities. (**a**) Cavity with area of strong (red) and weak (pink) and no fluorescence. (**b**) Corresponding white light image. (Reproduced with permission from Stummer et al.[38])

5-ALA has the potential to be used as a tool for detection of solid tumor that can be removed without risk of neurologic deficit but will also help discrimination of infiltrated brain down to a tumor cell density of ~ 10 to 20%, enabling even larger resection volumes in noneloquent regions than those identified by contrast enhancement on MRI.[39]

Influence of 5-ALA on Extent of Resection and Outcome

The first prospective study evaluating the impact of 5-ALA on the EOR was published in 2000 by Stummer et al, showing that complete resection of contrast-enhancing tumor on MRI was archived in 33 (63%) of 52 patients. In most of the remaining patients, complete resection could not be performed due to concerns about neurologic safety. In addition, the improved survival was related to the completeness of resection.[37] A large phase III randomized controlled study included 322 patients with suspected malignant glioma who were randomly assigned to 5-ALA–guided or conventional microsurgical resection. Complete resection of contrast-enhancing tumor was achieved in 90 of 139 (65%) patients in the 5-ALA group compared with 47 of 131 (36%) in the conventional group ($p < 0.001$). Furthermore, patients from the 5-ALA group had a longer 6-month progression-free survival (PFS) (41% versus 21.1% in the control group; $p = 0.0003$) with a median PFS of 5.1 months.[34]

Further studies confirmed the benefit of 5-ALA regarding EOR, and since then 5-ALA has been widely used in the resection of glioblastoma. The initially reported resection rate of 65% that was achieved using 5-ALA FGR was further improved over the last few years due to confidence in the use of the method, as well as advances in intraoperative monitoring and mapping, the latter allowing safe resections in eloquent areas. Díez et al reported GTR rates of 83.3% (30 of 36 glioblastoma patients),[39] whereas a retrospective study by Schucht et al reported a GTR of 96% (51 of 53 patients).[42] In comparison, GTR under white light microscopy is only achieved in 36% of patients,[34] indicating a major benefit of 5-ALA as an intraoperative adjunct for optimizing resection. Even in eloquent areas, the use of 5-ALA FGS, combined with intraoperative mapping or awake surgery, enables GTR rates of up to 76%.[43,44]

A retrospective analysis of 52 glioblastoma patients with optimal resections according to conventional criteria (i.e., complete resection of contrast-enhancing tumors on early postoperative MRI) compared cases with residual fluorescent tissue and complete removal of fluorescent tissue, demonstrating an improved median overall survival (OS) of 27 months (95% confidence interval [CI], 22.4–31.6) in patients without residual fluorescence compared with 17.5 months (95% CI, 12.5–22.5) with residual fluorescence.[45] It is well known that intraoperative fluorescence exceeds the contrast enhancement visible on MRI, by far, marking almost double the resection volume outlined by contrast enhancement on MRI.[46] These data again underline the potential of 5-ALA for greater EOR and increased survival.

Combination of 5-ALA FGS with Intraoperative MRI

Coburger et al evaluated the benefit of the additional use of 5-ALA to iMRI in resection of glioblastoma in a prospective cohort and demonstrated that GTR was achieved significantly ($p < 0.01$) more often using the combined approach of 5-ALA and iMRI (100%) compared with iMRI alone (82%), with higher mean EOR in the combined group (99.7% versus 97.4%, respectively; $p < 0.004$), indicating a synergistic effect of both methods.[47]

Several studies compared the diagnostic accuracy of 5-ALA and iMRI for identifying brain infiltrated by glioma. Coburger et al described a significantly higher sensitivity (91% versus 66%) and specificity (90% versus 60%) for detection of malignant glioma than iMRI at the tumor border.[48] However, so far no clinical evidence has demonstrated the superiority of one method over the other. An ongoing trial (Impact of iMRI on the Extent of Resection in Patients with Newly Diagnosed Glioblastomas: A Prospective Multicenter Parallel Group Clinical Trial [NCT02379572]) aims at providing more data on comparison of both techniques regarding EOR. However, such comparisons may be purely academic because during surgery, technologies should be combined and synergisms utilized in the best interest of patients.

Analyzing the current literature regarding safety and side effects linked to 5-ALA, our data analysis indicates only minor toxicity such as mild and transient erythema or a mild elevation of liver enzymes in single cases without clinically relevant hepatic disorders. Overall, 5-ALA can be considered a very safe and well-tolerated drug.► **Table 1** presents an overview of all included studies.

Fluorescein

Fluorescein sodium, originally and still widely used in ophthalmic surgery for retinal angiography, was introduced into the field of neurosurgery by George E. Moore in 1947, and it was shown to highlight areas of blood-brain barrier (BBB) disruption linked to tumor growth after intravenous application.[27,49] Fluorescein has a characteristic yellow-green fluorescence, with a peak absorption between 465 and 480 nm and an emission peak at 500 to 530 nm (i.e., in the yellow/green range). When administered in high concentrations, fluorescein fluorescence can even be observed under white light.[50] Fluorescein is considered a safe, robust, and inexpensive fluorophore. In some cases, it leads to transient discoloration of urine and skin after administration, and anaphylactic reactions have been described in a few cases.

However, no severe adverse events have been described using the recommended dosage of 3 to 5 mg/kg BW. Fluorescein is administered intravenously just after induction of anesthesia.[51–53] It is distributed via the bloodstream and then extravasates through the disrupted BBB, highlighting regions of the brain with abnormal vasculature, neovascularization, or increased vascular permeability.[27,54] In malignant gliomas that are characterized by a disruption of the BBB, fluorescein accumulates in the extracellular space of the tumor tissue. In

Table 1 Overview of studies analyzing the use of 5-ALA for glioma surgery

Study	Study design	No. of patients	Tumor type	Eloquence	Primary end point	5-ALA dosage	Drug-related side effects	Other intraoperative tools	GTR	Sensitivity	Specificity	PPV	NPV	Impact on survival
Stummer et al, 1998	Prospective, monocentric	10	8 GBMs, 2 AA	Eloquent and noneloquent	First evaluation of 5-ALA in malignant glioma, safety	10 mg/kg BW	None	Neuronavigation (one patient)	70%	85%	100%	90%	NR	NR
Stummer et al, 2000	Prospective, monocentric	52	GBM	Eloquent and noneloquent	GTR, survival, postoperative MRI findings	20 mg/kg BW	One patient with erythema; mild elevation of liver enzymes with no signs of hepatic disorders	None	63%	NR	NR	NR	NR	NR
Stummer et al, 2006	RCT, phase III	139	135 GBMs 4 grade IIIs	Eloquent and noneloquent	GTR, PFS 6 mo, postoperative MRI findings, adverse events	20 mg/kg BW	Liver enzymes were mildly elevated 24 h after surgery	Ultrasound or neuronavigation only for planning of approach	65%	NR	NR	NR	NR	6-mo PFS 5-ALA: 5.1 mo WL: 3.6 mo No difference regarding OS
Nabavi et al, 2009	Prospective, multicentric, single-arm phase II	36	Recurrent HGGs	Eloquent and noneloquent	To assess feasibility of 5-ALA fluorescence guidance for resection of recurrent HGG, determine PPV	20 mg/kg BW	None	NR	19.4%	NR	NR	NR	NR	NR
Díez Valle et al, 2011	Prospective, monocentric	36	28 primary GBMs, 8 recurrent GBMs	Eloquent and noneloquent	GTR; safety, diagnostic accuracy	20 mg/kg BW	None	Neuronavigation, neuromonitoring	83.3%	NR	NR	Solid fl: 100%; vague fl: 97%	66%	NR
Della Puppa et al, 2012	Prospective, monocentric	31	22 primary HGGs, 9 recurrent HGGs	Only eloquent	5-ALA in eloquent areas assisted with functional mapping	20 mg/kg BW	None	IOM, neuronavigation, some cases awake surgery	76% newly diagnosed malignant gliomas; 66% recurrent gliomas	NR	NR	NR	NR	NR
Schucht et al, 2012	Retrospective, monocentric	36	GBM	Eloquent and noneloquent (only if complete resection could be achieved)	CRET and GTR; residual contrast-enhancing tissue	20 mg/kg BW	None	IOM, neuronavigation	96%	NR	NR	NR	NR	NR
Coburger et al, 2014	Prospective. monocentric	34	Primary and recurrent GBMs	Eloquent and noneloquent	Provide a histopathologic correlation of tumor delineation at the border zone of iMRI and 5-ALA	20 mg/kg BW	None	Neuronavigation, iMRI	NR	5-ALA: 85% iMRI: 41%	5-ALA: 80% iMRI: 60%	5-ALA: 69% iMRI: 67%	5-ALA: 43% iMRI: 70%	NR

(*Continued*)

Table 1 (*Continued*)

Study	Study design	No. of patients	Tumor type	Eloquence	Primary end point	5-ALA dosage	Drug-related side effects	Other intraoperative tools	GTR	Sensitivity	Specificity	PPV	NPV	Impact on survival
Stummer et al, 2014	Prospective, monocentric	33	29 GBMs, 4 AAs	Eloquent and noneloquent	Determination of fluorescence quality	20 mg/kg BW	Transient elevation of liver enzymes	Neuronavigation	NR	NR	NR	Strong fl: 96.2%; weak fl: 92.%	39.5%	NR
Schucht et al, 2014	Prospective, monocentric	67	GBM	Eloquent, adjacent to corticospinal tract	Evaluation of mapping and 5-ALA–guided surgery in eloquent regions	20 mg/kg BW	NR	Mapping	57%	NR	NR	NR	NR	NR
Coburger et al, 2015	Prospective, monocentric, retrospective matched pair	33	GBM with intended GTR	Eloquent and noneloquent	To assess impact of additional use of 5-ALA in iMRI-assisted surgery of GBMs on EOR, PFS, OS	20 mg/kg BW	One patient sunburn	NR	5-ALA and iMRI: 100%, iMRI alone: 82%	NR	NR	NR	NR	OS: 5-ALA and iMRI: 18 mo, iMRI: 17 mo; PFS 5-ALA and IMRI: 6 mo, iMRI: 6 mo
Lau et al, 2016	Prospective, monocentric, phase II	59	47 GBMs 12 grade III gliomas primary and recurrent	Eloquent and noneloquent	To examine correlation of intensity of 5-ALA fluorescence with degree of tumor cellularity	20 mg/kg BW	Hypotension (two patients); mild rash (one patient)	None	NR	GBM: 84.2%	GBM 62.1%	GBM: 97.2% HGG: 100%	GBM: 43.9%, HGG: 16.7%	NR
Summary	**Mainly prospective, monocentric cohort**	**Total: 497**	**Primary and recurrent HGGs**	**Eloquent and noneloquent**	**Safety, feasibility, EOR, GTR, PFS, OS, histopathologic correlation, correlation with MRI, combination with mapping and iMRI**	**20 mg/kg BW**	**Rare; erythema, mild elevation of liver enzymes in single cases**	**Neuronavigation, ultrasound, IOM, mapping, awake surgery**	**19.4–100%**	**∼ 85%**	**62–100%**	**> 90%**	**39.5–66%**	**Improved 6-mo PFS**

Abbreviations: 5-ALA, 5-aminolevulinic acid; AA, anaplastic astrocytoma; BW, body weight; CRET, complete resection of enhancing tumor; EOR, extent of resection; fl, fluorescence; GBM, glioblastoma multiforme; GTR, gross total resection; HGG, high-grade glioma; iMRI, intraoperative magnetic resonance imaging; IOM, intraoperative monitoring; MRI, magnetic resonance imaging; NPV, negative predictive value; NR, not recorded; OS, overall survival; PFS, progression-free survival; PPV, positive predictive value; RCT, randomized controlled trial; WL, white light.

1998, Kuroiwa et al introduced an operative microscope equipped with emission filters to visualize fluorescein under yellow-filtered (560 nm) light.[55] Today, various fluorescent filters for visualizing fluorescence are available and incorporated into modern surgical microscopes (e.g., the FL560 System [Leica Microscopes, Wetzlar, Germany] and YELLOW 560 system [Carl Zeiss, Dublin, California, United States]) (►**Fig. 2**).

Encouraged by the success of 5-ALA, several studies analyzed the efficacy and applicability of the comparably less expensive agent fluorescein for resection of malignant gliomas, indicating a propensity for improving EOR (►**Table 2**).[51,54,56–59] However, many of these studies are retrospective, and none of these studies are randomized and may be confounded by case selection.

Several groups reported GTR rates of 80% using the YELLOW 560 filter.[54,56] Diaz et al reported GTR in 100% when using fluorescein in their cohort of 12 glioblastoma patients and demonstrated a good correlation between intraoperative fluorescence and contrast enhancement on MRI.[51] However, the authors emphasized that the accumulation of fluorescein in malignant glioma is related to the passage through the disrupted BBB and cannot be attributed to a specific uptake by the tumor itself, as is the case for 5-ALA.[51,56]

In addition, an analysis of the literature shows no clear consensus about dosage and timing of administration of fluorescein before surgery, although timing seems to be critical because extravasation and distribution of fluorescein follow a certain time course. Intravascular fluorescein will be extravasated after a half-life of 264 minutes and might stain edema in peritumoral normal brain parenchyma as well, increasing the danger of resection of nontumorous tissue.[60] Timing of administration should be planned carefully to minimize these confounders. Furthermore, surgical manipulation of brain tissue will per se disrupt the BBB, leading to unselective extravasation of fluorescein from the bloodstream along the cutting margins, also jeopardizing confident delineation between tumor and normal tissue. Therefore, fluorescein is rather a marker of BBB integrity than a specific tumor-targeting tool.[61] This aspect has to be kept in mind when using this agent.

So far, no studies have revealed reliable data on the effects of fluorescein-guided resection on outcome and survival. Two prospective controlled studies evaluated the effect on survival. One small study described an improved PFS when using fluorescein (7.2 months versus 5.4 months; $p = 0.033$). However, the study lacked randomization and did not use special microscope filters to visualize fluorescence, using only white light.[57,59] A phase II trial (FLUGLIO) evaluated the safety and efficacy of fluorescein in glioma surgery and showed that fluorescein is feasible and safe, allowing complete tumor resection in a high percentage of cases.[62] Nevertheless, further prospective randomized controlled studies are warranted to investigate the benefit of fluorescein for EOR and outcome in glioma patients.

The simultaneous use of 5-ALA and fluorescein was shown to be feasible in glioblastoma surgery. 5-ALA was used to stain the tumor and fluorescein to provide tissue fluorescence of adjacent brain, leading to highly specific tumor visualization as well as enhanced background brightness at the same time.[63]

Indocyanine Green

ICG is a tricarbocyanine with fluorescence in the near-infrared range (NIR) and was approved by the FDA in 1959 for the diagnosis of liver function. ICG has been widely used in ophthalmology. It has a peak emission at 780 nm and excitation at 810 nm.[64,65] ICG is considered safe with a low incidence of adverse side effects such as hypotension, arrhythmia, and anaphylactic shock in 0.05%, and mild symptoms such as nausea or skin eruptions in 0.2%.[29]

The use of ICG in neurosurgery was first described by Raabe et al for visualization of blood flow in cerebral vessels under the surgical microscope, and it is now a frequently used technique in the surgery of aneurysms and other vascular malformations.[29,66,67]

Recently, ICG was used for visualization of malignant gliomas using a technique referred to as second window ICG (SWIG). Twenty-four hours before surgery, 5 mg/kg BW ICG are administered to the patient, leading to the accumulation in tumor tissue mainly due to enhanced permeability and retention effects.[68,69] A NIR camera (NIR light range: 700–850 nm), integrated into the surgical microscope, is used to visualize the tumor at an emission of 780 to 950 nm.

Compared with 5-ALA and fluorescein, which both emit fluorescence within the visible spectrum, ICG has excitation and emission in the NIR region of the spectrum. This advantage enables visualization of ICG fluorescence even in deeper regions, up to 3 cm, and also through the dura. This circumstance helps in planning a precise durotomy and corticotomy.[70]

A pilot study evaluating SWIG in 15 patients with gliomas revealed strong tumor-to-background fluorescence ratios, and a good correlation of contrast enhancement on MRI with intraoperative fluorescence. However, the specificity was very low, 45%, indicating possible illumination of adjacent edema.[68] Up to now, no studies have evaluated the benefit of ICG regarding improvement of EOR or outcome in treatment of gliomas, and further research is warranted to assess the usefulness of ICG.

Novel Techniques for Targeting Glioma Cells

Fluorescence-guided surgery has to date been widely implemented in the daily routine for glioblastoma surgery. However, in its present form there are some limitations regarding the sensitivity for visualization of tumor cells. Consequently, these techniques are being further improved, and other methods, some of them still in the fledgling stages, are undergoing intense research.

Tumor-Targeting Alkylphosphocholine Analogs

Alkylphosphocholine analogs (APCs) are small synthetic phospholipid ether molecules with a purported broad tumor-targeting potential because they are known to be

Table 2 Overview on studies analyzing the use of fluorescein for glioma surgery

Study	Study design	No. of patients	Tumor type	Eloquence	Primary end point	Fluorescein dosage	Drug-relate side effects	Other intraoperative tools	GTR	Sensitivity	Specificity	PPV	NPV	Impact on survival
Koc et al, 2008	Prospective, monocentric, controlled not randomized	47 (control: 33)	GBM	NR	GTR	20 mg/kg BW	NR	NR	Fluorescein: 83% Control group: 55%	NR	NR	NR	NR	Median survival fluorescein: 44 wk Control group: 42 wk (NS)
Chen et al, 2012	Prospective, monocentric, controlled, not randomized	10 (control: 12)	3 GBMs, 3 AAs 4 grade II	Eloquent and noneloquent	Reevaluate the utility and clinical limitations of using fluorescein sodium for treatment and resection of glioma brain tumors	15–20 mg/kg BW	Yellow staining of sclera, skin, and urine disappeared within 24 h	NR	Fluorescein group:80% Control group: 33.3% $p = 0.047$	NR	NR	NR	NR	Fluorescein: PFS 7.2 mo Control: 5.4 mo ($p = 0.033$)
Schebesch et al, 2013	Retrospective, monocentric	26	17 GBMs 5 AAs 3 grade II 1 grade I primary and recurrent	Eloquent and noneloquent	Feasibility and efficacy of fluorescein under YELLOW 560 nm, safety	3–4 mg/kg BW	None	NR	80%	NR	NR	NR	NR	NR
Acerbi et al, 2014	Prospective phase II trial	20	19 GBMs 1 AA all amenable to complete resection	Eloquent and noneloquent	Evaluating the safety of fluorescein-guided surgery for HGGs and obtaining preliminary evidence regarding its efficacy for this purpose	5–10 mg/kg BW	None	Neuronavigation was allowed only for surgical planning, initial tumor localization, and orientation during tumor removal Neurophysiologic monitoring	80%	94%	89.5%	NR	NR	NR
Diaz et al, 2015	Prospective, monocentric	12	9 primary GBMs; 3 recurrent GBMs	Eloquent and noneloquent	Ability of fluorescein to specifically stain glioma cells	3 mg/kg BW	NR	Neuronavigation	100%	82.2%	90.9%	NR	NR	NR
Catapano et al, 2017	Retrospective, monocentric, matched pair analysis	23 (control: 25)	Primary GBM	Eloquent and noneloquent	GTR	5 mg/kg BW	None	Neuronavigation	82.6% (control group: 52%) $p = 0.03$	84%	95%	NR	NR	NR
Francaviglia et al, 2017	Retrospective, monocentric	47	33 GBMs 14 AAs All primary	Eloquent and noneloquent	Safety and EOR	5 mg/kg BW	Yellow staining of sclera, skin, and urine disappeared within 24 h	Neuronavigation	53.2%	NR	NR	NR	NR	NR
Summary	**Both prospective and retrospective cohorts; no RCTs**	**185**	**Primary and recurrent HGGs**	**Eloquent and noneloquent**	**Safety, GTR, feasibility**	**3–20 mg/kg BW, mainly 5 mg/kg BW**	**Only minor, temporary staining of urine and sclera**	**Neuronavigation and IOM**	**53–100%**	**92–94%**	**89–95%**	**NR**	**NR**	**Longer PFS in fluorescein group shown in one study**

Abbreviations: AA, anaplastic astrocytoma; BW, body weight; EOR, extent of resection; fl, fluorescence; GBM, glioblastoma multiforme; GTR, gross total resection; HGG, high-grade glioma; IOM, intraoperative monitoring; NPV, negative predictive value; NR, not recorded; NS, not significant; PFS, progression-free survival; PPV, positive predictive value; RCT, randomized controlled trial.

taken up by malignant cells thorough overexpressed lipid rafts. Due to decreased catabolism in cancer cells, APCs undergo prolonged retention.[71,72] In a glioblastoma xenograft mouse model, Swanson et al showed that two fluorescent APCs (CLR1501 green fluorescence and CLR1502 near-infrared fluorescence) are capable of labeling glioblastoma cells with high tumor-to-normal parenchyma.[69] Further research aims at developing dual-labeled APCs enabling fluorescence-guided visualization and PET imaging with the same agent. Despite still being under investigation in a preclinical status, this technique offers the possibility of targeting and treating glioblastoma at different phases of the disease: resection, staging, and possibly localized radiotherapy.[11,73]

BLZ-100 Fluorescence-Guided Brain Tumor Surgery

BLZ-100 (tozuleristide) consists of the tumor-targeting peptide chlorotoxin, extracted from the venom of scorpions, with assumed specific binding to gliomas, conjugated with the near-infrared fluorophore ICG.[9,74] The agent is administered 24 hours before surgery, and fluorescence is visualized using a NIR camera. Butte et al demonstrated a high affinity of BLZ-100 toward glioblastomas in a mouse model.[75] Further studies are needed to determine the role of this technique as a further step toward using fluorescent-labeled probes with tumor-specific molecular targets to visualize glioma cells with higher accuracy in the clinical setting of glioblastoma surgery, and early-phase clinical studies are underway.

Confocal Endomicroscopy

Major limitations of FGS are the lack of high resolution and the subjective interpretation of fluorescence qualities. Especially at the tumor margin, delineation of tumor tissue from normal brain is often challenging, and prediction of histologic tumor grading from preoperative imaging is often not possible. Intraoperative frozen sections are frequently performed to acquire immediate diagnosis. However, this procedure is time consuming and can be nondiagnostic or even misleading in certain cases.[76] Confocal endomicroscopy is a technique that was recently introduced into the field of neurosurgery. Images are acquired using a handheld probe that has a single optical fiber for illumination and detection. The images are displayed in high resolution in up to 1,000-fold magnification to an LCD workstation. To provide tissue contrast, fluorescent agents like fluorescein are administered.[10,77,78] Confocal endomicroscopy allows real-time visualization of malignant cells and is particularly useful for scanning the tumor margin for residual tumor tissue with high accuracy to enhance EOR and at the same time lower the risk of resection of nontumorous tissue in eloquent areas leading to possible neurologic deficits. For interpretation of acquired images, profound histopathologic knowledge or the presence of a neuropathologist is required.[79]

Raman Spectroscopy

Raman spectroscopy is based on the Raman effect, first described by C.V. Raman in 1928, and refers to the scattering of monochromatic light in tissue. Most photons in the visible spectrum are scattered elastically, implying they have the same level of energy when interacting with a tissue or object. However, some photons transfer or absorb energy to or from the object being imaged, resulting in a transmission of energy. This phenomenon is called inelastic scattering and known as the Raman effect.[80] With the help of a spectrometer (Raman spectroscopy), information regarding the chemical composition of different tissues, for example, the amount and ratios of lipid and protein, can be obtained. These data provide a unique biochemical signature of the tissue and enable delineation between different tissues. In comparison with other techniques, Raman spectroscopy is a label-free visualization method that depends on intrinsic biochemical properties of different tissues to provide image contrast.[81] This technique was shown to be effective in delineation of glioblastoma, necrosis, and normal brain parenchyma as well.[8,82] Normal, necrotic, and glioblastoma tissue was distinguished by Raman spectroscopy in frozen sections with 99.5% accuracy.[8] Jermyn et al used a Raman spectroscopy handheld probe system intraoperatively and found an accuracy of 92% for glioma detection.[83] Similar to confocal endomicroscopy, this technique enables intraoperative tissue analysis before resection and is a promising guide for surgical resection and decision making.[83,84]

Conclusion and Future Perspective

In summary, several intraoperative imaging methods aiming at improvement of intraoperative glioma targeting and visualization are presently available. Neurosurgeons have started to integrate these techniques into their daily routine for glioma surgery. The ultimate purpose of these methods is to increase the EOR while keeping the risk for postoperative neurologic deterioration low.

Still, there are limitations, as discussed earlier and listed in ►**Table 3**, that have to be considered when applying one of these techniques. To overcome these limitations, further research is being performed. One approach is the combination of different techniques, such as neuronavigation and FGS, allowing the generation of comprehensive information on tumor extent, anatomy, and metabolism. Adding newer techniques, like Raman spectroscopy or targeted fluorescence, further information regarding chemical and metabolic composition of the tissue will be provided.

5-ALA appears to be the only available intraoperative tool for direct identification of glioblastoma cells. It has further shown a good correlation with regions of higher metabolic activity in tumor, similar to FET (fluoroethyltyrosine)-PET, although these PET hot spots often cannot be matched on MRI.[85,86] In addition, a higher Ki-67/MIB-1 index and other features of malignancy correlate with the amount of 5-ALA fluorescence observed.[87,88] Most randomized controlled trials are based on the gadolinium-based assessment of

Table 3 Overview of current techniques for intraoperative visualization of glioblastoma cells with their advantages and disadvantages

Technique	Publications	Principle	Application/ development	Advantages	Disadvantages
Neuronavigation	Maciunas et al, 1996 Jung et al, 2006 Wirtz et al, 2000 Orringer et al, 2012	Preoperative images, intraoperative orientation	Widespread use in clinical setting	– Maintaining orientation – Visualization of anatomy – Planning surgical approach – Combination with other tools – Access to various preoperative images including PET, CT, MRI, data for fiber tracking	– Brain shift, loss of accuracy – Relies on preoperative imaging, not real time – Interruption if surgical workflow
Ultrasound	Mercier et al, 2011 Saether et al, 2012	Intraoperative imaging	Widespread use in clinical setting	– Dynamic, cheap, and easy to use – Provides intraoperative real-time images – May be used to update navigation system	– Low resolution
iMRI	Hatiboglu et al, 2009 Senft et al, 2011 Liang et al, 2012 Ozduman et al, 2014 Coburger et al, 2014 Coburger et al, 2015	Intraoperative imaging	Widespread use in clinical setting	– Almost real-time images during surgery – Identification of residual tumor	– Expensive – Time consuming – Extends duration of surgery and anesthesia
5-ALA	Stummer et al, 1998 Stummer et al, 2000 Stummer et al, 2006 Nabavi et al, 2009 Díez Valle et al, 2011 Della Puppa et al, 2012 Schucht et al, 2012 Della Puppa et al, 2012 Stummer et al, 2014 Schucht et al, 2014 Lau et al, 2016	Metabolic	Widespread use in clinical setting, FDA and EMA approval	– Selectively absorbed by tumor cells – Low toxicity, high safety – Intraoperative real-time imaging – Full integration into the surgical microscope and view of full surgical field – Use without interruption to the surgical workflow – Reliable correlation with preoperative contrast enhancement on MRI – Correlation with histopathology – Brain shift is no concern	– Low background illumination o Alternating between white light and fluorescence mode – Imaging surface tool, depth can limit visualization – Requires special microscope – Expensive – Bleaching effect – Time dependency – Subjective interpretation of fluorescence intensities
Fluorescein	Koc et al, 2008 Chen et al, 2012 Schebesch et al, 2013 Acerbi et al, 2014 Diaz et al, 2015 Francaviglia et al, 2017 Catapano et al, 2017	Permeability of BBB	Human use, off-label	– Robust, safe, cheap – Can be visualized under white light (using higher concentrations) – Intraoperative real-time imaging – Full integration into the surgical microscope and view of the full surgical field – Use without interruption to the surgical workflow – Brain shift is no concern	– Not tumor cell specific o Marker of BBB breakdown – Unselective extravasation during surgery – Time dependency – Subjective interpretation of fluorescence intensities
ICG	Lee et al, 2016	Permeability of BBB	Human use, off-label	– Excitation and emission in the near-infrared region O Enables visualization of fluorescence situated deeper in the tissue – Low toxicity, high safety – Intraoperative real-time imaging – Brain shift does not interfere with this technique – Full integration into the surgical microscope and view of the full surgical field – Use without interruption to the surgical workflow – Brain shift is no concern	– Requires special cameras to visualize fluorescence – Not tumor specific – Accumulates due to an enhanced permeability of the BBB – Time dependency – Subjective interpretation of fluorescence intensities
Tumor-targeted alkylphosphocholine analogs	Swanson et al, 2015	Tumor-targeted	Animal model	– Specific detection of tumor cells	
BLZ-100 (tozuleristide)	Butte et al, 2013	Tumor-targeted	Animal model	– Tumor-specific molecular targets	
Confocal endomicroscopy	Hoffman et al, 2006 Foersch et al, 2012	Intraoperative microscopy, fluorescence labeling	Human use, clinical trials	– Intraoperative neuropathologic diagnostic – High resolution in up to 1,000-fold magnification – High accuracy	– Only small field can be analyzed at the same time – Time consuming – Presence of a neuropathologist required to interpret images
Raman spectroscopy	Krafft et al, 2004 Kalkanis et al, 2014 Jermyn et al, 2015	Intrinsic biochemical properties of different tissues	Human use, clinical trials	– Unique biochemical signature – High accuracy	– Only small field can be analyzed at the same time – Time consuming

Abbreviations: 5-ALA, 5-aminolevulinic acid; BBB, blood-brain barrier; CT, computed tomography; EMA, European Medicines Agency; FDA, Food and Drug Administration; ICG, indocyanine green; iMRI, intraoperative magnetic resonance imaging; MRI, magnetic resonance imaging; PET, positron emission tomography.

residual tumor and EOR. For the future, the EOR based on 5-ALA–induced fluorescence might be a more accurate marker.

Currently, the intensity of fluorescence relies on the subjective interpretation of the surgeon. To quantify fluorescence, further attempts have been undertaken, for example, using spectroscopic techniques to determine intraoperative protoporphyrin (Pp) IX concentration in tumor tissue via a handheld device, even in cases with no visible fluorescence under the surgical microscope.[89] For low-grade glioma, where fluorescence is often not visible using standard surgical microscopy, a 100-fold increase in sensitivity of fluorescence detection using handheld spectroscopy can be achieved, resulting in detection of PpIX fluorescence in these slowly growing tumors also.[90]

Targeted fluorescence imaging will soon be available, together with innovations in neurosurgical microscope technology, to help detect optical features in gliomas presently invisible to the human eye.[70] Such technologies will help overcome the limitations of the sensitivity and specificity of the present methods.

Conflict of Interest
None declared.

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