5-ALA in the Management of Malignant Glioma

Herbert Stepp1* and Walter Stummer2
1LIFE Center and Department of Urology, University Hospital of Munich, Feodor-Lynen-Str. 19, 81377 Munich, Germany
2Department of Neurosurgery, University Clinic Münster, Albert-Schweitzer-Campus 1, Gebäude A1, 48149 Münster, Germany

Background: Patients suffering from malignant gliomas have a poor prognosis. For the surgical treatment of these tumors, 5-aminolevulinic acid (5-ALA) has become a new standard.

Aims: This review intends to provide an overview over current status, significance, limitations, and future perspectives of 5-ALA based fluorescence guided surgery and photodynamic therapy for brain tumor patients.

Materials and Methods: From peer reviewed publications on the many aspects connected with this topic, those with potential clinical relevance were selected and put in the context of our own experience.

Results and Discussion: The high tumor selectivity of accumulation of fluorescent protoporphyrin IX (PpIX) after systemic administration of 5-ALA enables intra-operative fluorescence guidance, which is unimpaired by brainshift and does not require expensive equipment. The neurosurgical aim of complete resection of enhancing tumor can now more easily be achieved, which improves prognosis in these patients. Nevertheless, despite better surgery tumors will inevitably recur. In order to further prolong survival, the phototoxic properties of PpIX are presently being exploited in clinical trials of post-operative or interstitial photodynamic therapy (PDT).

Conclusion: 5-ALA based fluorescence guidance and PDT offer an intriguing new option for the management of malignant gliomas. Lasers Surg. Med. 50:399–419, 2018. © 2018 The Authors. Lasers in Surgery and Medicine Published by Wiley Periodicals LLC

Key words: fluorescence guided surgery; malignant glioma; photodynamic therapy (PDT); protoporphyrin IX (PpIX); aminolevulinic acid (5-ALA); stereotactic biopsy radiotherapy. Median overall survival time for GBM is still only ca. 15 months [3,4]. The success of surgical intervention is limited due to the infiltrative growth of the tumor and the impossibility to resect with an ample safety margin [5]. Chemo- and radiotherapy have limited effectivity due to the early buildup of resistance and accumulation of side effects. Therefore, there is urgent need for new modalities, which improve the clinical outcome of malignant glioma treatment.

New treatment modalities, which have shown significant success in other types of cancer have also been or still are under investigation for treatment of GBM. The results are mostly disillusioning: Anti-angiogenic treatment with Bevacizumab, better known under its tradename Avastin®, for example, has shown very significant prolongation of progression free survival in renal cell cancer [6] but did not unequivocally prove to be beneficial in GBM, at least not for overall survival [7]. Currently, immune check point inhibitors are in clinical trials, but appear to be far less effective with GBM than with malignant melanoma [8,9]. Comparably promising initial results have been reported for “tumor treating fields” [10] and a combination therapy using Temozolomide together with lomustin (CCNU) [11]. In Germany, significant public attention has been attained by promising case reports of using methadone along with Temozolomide, but controlled clinical trials are still missing [12,13]. Therapeutic concepts aiming at targeting tumor specific signaling may be promising for the future [14,15].

INTRODUCTION

Malignant Glioma and Treatment Concepts

Among primary brain tumors, malignant gliomas comprise approximately a quarter, with the most malignant one—glioblastoma multiforme (GBM)—accounting for almost half of all malignant brain tumors. In the USA, each year more than 10,000 new cases of GBM are diagnosed [1]. Worldwide, malignant gliomas account for 238,000 new cases of brain tumor and 174,000 deaths annually [2]. Standard treatment consists of surgical debulking—if possible—followed by chemo- and radiotherapy. Median overall survival time for GBM is still only ca. 15 months [3,4]. The success of surgical intervention is limited due to the infiltrative growth of the tumor and the impossibility to resect with an ample safety margin [5]. Chemo- and radiotherapy have limited effectivity due to the early buildup of resistance and accumulation of side effects. Therefore, there is urgent need for new modalities, which improve the clinical outcome of malignant glioma treatment.

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*Correspondence to: Herbert Stepp, LIFE Center and Department of Urology, University Hospital of Munich, Feodor-Lynen-Str. 19, 81377 Munich, Germany.
E-mail: herbert.stepp@med.uni-muenchen.de
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**The Concept of 5-ALA Based Photodynamics for Diagnosis and Treatment of Malignant Glioma**

Five-aminolevulinic acid (5-ALA) is a precursor of fluorescent and phototoxic protoporphyrin IX (PpIX) in the heme biosynthesis pathway. Due to the optical properties of PpIX and the sensitivity of its synthesis to the intracellular metabolic activity, it has potential as a diagnostic (via fluorescence) as well as therapeutic (via phototoxicity) agent. The concept relies on a selective accumulation of PpIX in malignant glioma cells upon a systemic delivery of 5-ALA. The fluorescence of PpIX can easily be imaged intra-operatively and guides surgery in real-time without any concerns about brainshift. 5-ALA has gained clinical approval for fluorescence guided surgery (FGS) of malignant glioma in many countries, most recently (June 2017) in the USA [16]. The corresponding technical implementation in surgical microscopes is intended to be applied for glioma of WHO grades III (mostly anaplastic astrocytoma) and IV (mostly GBM) only. The application in lower grade tumors is not indicated as it leads to a high rate of false negatives, but might identify anaplastic foci as discussed in the clinical application chapter.

The therapeutic effectiveness and safety of 5-ALA induced PpIX has also been investigated in clinical trials on Photodynamic Therapy (PDT) of GBM [17]. The intriguing concept with 5-ALA-based PDT is that only malignant cells are sensitized to a largely undirected irradiation with visible light. PDT might therefore destroy tumor cells within the infiltration zone without harming adjacent functional tissue. PDT could be applied to the resection cavity immediately after having completed fluorescence guided surgery or even completely replace surgery by implanting cylindrical diffuser fibers into the tumor by a stereotactic approach. Both concepts are currently investigated in clinical trials and will be described in more detail below. The technical implementation, however, requires specialized equipment, such as multiport lasers and medical device class III approved light applicators. Therefore, PDT of GBM is still limited to a few neurosurgical centers. However, approval trials are in preparation [18].

The main challenge of any treatment modality for GBM with curative intent is the need of being able to kill the vast majority—if not the entirety—of malignant cells. With mechanical surgical removal it is impossible to reach this aim. Nevertheless, progression free survival (PFS) is clearly prolonged, if all of the tumor tissue is resected that shows uptake of contrast medium (Gd-DTPA) in magnetic resonance imaging (MRI) [19–22]. This so called complete resection of enhancing tumor (CRET) has to be performed with great caution, of course, to not jeopardize functional brain. Therefore, tumor cells will inevitably remain, which have invaded normal brain or reside elsewhere in stem cell niches as tumor initiating cells. Given the heterogeneous genetic profile and the high resistance capacity of GBM-cells [23,24], fighting against any single target (e.g., overexpressed protein) is extremely unlikely to solve the problem. In this context, PDT offers some advantageous properties:

1. PDT causes cell death by very different mechanisms, involving multiple intracellular targets.
2. PDT induced cell death is very immunogenic.
3. PDT induces very low DNA damage, is therefore not negatively influenced by high DNA repair capacity of stem like cells.
4. PDT—depending on the photosensitizer used—provides significant tumor selectivity on a cellular level.
5. 5-ALA based PDT offers additional advantages: no phototoxicity during drug distribution, mitochondrial targeting, photobleaching, fast pharmacokinetics.

**5-ALA—FUNDAMENTALS**

**5-ALA in Heme Biosynthesis**

Heme is well known as part of hemoglobin in the red blood cells (erythrocytes). In the early phase of erythrocyte development, large amounts of heme are produced to be incorporated into globin, where four heme-molecules in each hemoglobin protein accomplish the task of binding— and releasing—oxygen molecules. While heme formation is terminated in mature erythrocytes, all other cells in the body are continuously busy to synthesize heme [25] (Fig. 1). Here, heme molecules mediate the electron transfer in the mitochondrial respiratory chain. In different forms, heme is the functional moiety in the respiratory enzymes of three of the five complexes of the respiratory chain [26]. These complexes use the energy gained by oxidizing hydrogen to water to form ATP from ADP and phosphate as a universal intracellular carrier of energy.

This process starts inside the mitochondria by forming 5-ALA from succinyl coenzyme A and glycine by ALA-synthase (ALA-S). This synthesis step is regulated by heme, which inhibits ongoing synthesis and/or activity of

![Fig. 1. Simplified schematic of the biosynthesis of heme. The numbers indicate potential reasons for a tumorselective accumulation of PpIX (cmp. Table 1). GLY, glycine; SCoA, Succinyl-Coenzyme A; PBG, Porphobilinogen; HMB, Hydroxymethylbilane; UPgen III, Uroporphyrinogen III; CPgen III, Coproporphyrinogen III; PPgen, Protoporphyrinogen. Light gray arrows indicate enzymatic transformations.](image-url)
Tumor Selectivity of PpIX Accumulation

Both, for diagnostic (FGS) as well as for therapeutic (PDT) applications of 5-ALA, selective accumulation of PpIX in GBM cells is mandatory. To avoid side effects (destruction of normal brain), a high positive predictive value of PpIX-staining is required, which means that PpIX must not be found in normal brain (which would be false positives). At the same time, a high negative predictive value is desirable to make sure that all malignant cells have indeed accumulated PpIX. Wherever there is no PpIX, there should not be malignant cells. Of course, this can only be achieved to a certain extent. Unfortunately, the mechanisms leading to the preferential accumulation of PpIX in malignant cells versus normal cells of the same type are not very well understood [27]. It is a multifactorial phenomenon and the key factors depend on the organ, the cell type, and the genetic alterations found in the malignant cells.

In the brain, a first and important mechanism responsible for selectivity is the so-called blood brain barrier (BBB). In normal brain, the wall of the blood vessels is very tight and transport across this barrier is mostly by active transport. In the case of 5-ALA, no active transport was identified so far and the diffusion from blood to normal brain tissue is very low [28]. There is significantly more uptake from blood into the choroid plexus, resulting in some uptake into the cerebrospinal fluid, but still leading to much lower 5-ALA concentrations than circulating in the blood plasma [28,44,45]. Overall, with the exception of the ependymal lining, normal brain is very well protected from 5-ALA and hence photosensitization. In contrast, the growth of neo-vessels induced by gliomas leads to a loss of BBB function and morphology, and molecules may leak through these vessels. This leaky vasculature constitutes the mechanism by which gadolinium-diethylenetriamine-pentaacetic acid (Gd-DTPA) enables contrast-enhanced MRI of brain tumors [46]. It also constitutes the entry port for 5-ALA, from where it can diffuse—unlike Gd-DTPA—with and within perilesional edematous fluid into the infiltration zone.

Unspecific PpIX-accumulation in normal brain may occur in endothelial cells. It has been demonstrated that a transient and localized opening of the BBB can be achieved in normal rat or mouse brains after delivery of 5-ALA followed by a moderate dose of light [47,48]. This indicates a previous accumulation of at least some PpIX in the endothelial cells. This will not hamper the use of 5-ALA for diagnostic purpose, as the volume percentage of endothelial cells within the entire brain tissue is certainly below one percent. For the purpose of PDT, however, one has to consider the formation of edema, if drug or light doses are applied which lead to a significant phototoxicity in this cell fraction [49].

Once 5-ALA has left the deficient blood vessels in GBM tissue and diffuses with edema, cellular mechanisms of a tumor cell selective accumulation of PpIX gain importance. These mechanisms are summarized in Table 1. A review by Collaud et al. [27] provides more details on heme synthesis and on the selectivity of PpIX accumulation in tumor cells.

### TABLE 1. Potential Reasons for a Tumor Selective PpIX Accumulation

| Source of selectivity | Effect | Reference |
|-----------------------|--------|-----------|
| 1 Blood brain barrier leakage in glioma | Normal brain fairly protected from 5-ALA | [28] |
| 2 Increased activity of GABA, pepT1, pepT2 transporters | Uptake of 5-ALA in glioma cells increased | [29–31] |
| 3 Increased activity of ALA-D | More of PpIX-precursor PBG synthesized | [32] |
| 4 Increased activity of PBG-D | More of PpIX-precursor HMB synthesized | [33–36] |
| 5 Reduced ferrochelatase activity | Accumulation of PpIX as one of the substrates of this step | [37–39] |
| 6 Reduced availability of Fe²⁺ | Accumulation of PpIX as the other substrate of this step | [40] |
| 7 ABCB6-transporter | Transport of CPgen III into mitochondria | [41] |
| 8 ABCG2-transporter | Transport of PpIX from mitochondria into the cytosol, but also loss of PpIX through the plasma membrane | [30,42,43] |

1ALA-D, ALA-dehydrogenase; PBG, Porphobilinogen; PBG-D, Porphobilinogen-deaminase; HMB, hydroxymethylbilane; CPgenIII, coproporphyrinogen III. Numbers compare Figure 1.
A more recent review by Yang et al. [50] adds the role of membrane transporters and initial evidence of possible enhancement of PpIX accumulation in a tumor specific manner.

On the level of 5-ALA cellular uptake, it is first important to note that this occurs via active uptake, not just passive diffusion. A close correlation of the expression of the corresponding transporters and PpIX accumulation has been reported [30,39,51–54].

Of special importance may be the finding of a role for the enzyme PBG-D in the cell nucleus, which might indicate a rather direct correlation of cell proliferation with heme synthesis [36]. A tumor selectively upregulated activity of this enzyme has indeed been reported [33,38], but may not be relevant in all cell lines [40]. However, PBG-D is considered rate limiting in heme synthesis upstream of 5-ALA, which means that any modulation of its expression or activity will have a rather direct influence on PpIX accumulation [55].

An important bottleneck in heme synthesis is the step from PpIX to heme. The enzyme ferrochelatase inserts ferrous iron (Fe^{2+}) into the PpIX molecule, eliminating its photophysical properties of fluorescence and phototoxicity. This bottleneck has always been considered a decisive reason for the observed selectivity of PpIX accumulation [33,38–40,56]. Interestingly, human GBM tissue samples showed a reduced expression of ferrochelatase [37]. Similarly, a low availability of iron will increase the PpIX concentration, as demonstrated by co-incubation of cells with ALA and iron-chelators [57–59]. The reason for a tumor-specific down-regulation of ferrochelatase may partly lie in the indirect influence of tumor-suppressor proteins on the ferrochelatase activity, such as has been reported for the p53-dependent activity of mitochondrial frataxin [60].

More recently, the role of membrane transporters has been investigated. Especially the role of ABCG2 is of considerable interest, as this transporter seems to be located not only in the cell membrane but also in the outer mitochondrial membrane. In the cell membrane, it conveys resistance to chemotherapy by pumping the cytostatic drugs out of the cell. PpIX is also a substrate for this transporter, although probably not a very good one [61]. ABCG2 is considered to be upregulated in stemlike cells, which might therefore require higher 5-ALA or light doses to be killed. This is a relevant consideration also in GBM, as ABCG2 upregulation has been found in glioma stem cells [43]. On the other hand, ABCG2 can be effectively blocked by various drugs such as fumitremorgin C, Ko143, specific antibodies, sorafenib, erlotinib, and nicardipine [43,62,63]. At least small molecule inhibitors of ABCG2 will also inhibit this transporter located at the mitochondrial membrane, which is very intriguing, because PpIX accumulation could thus be confined to the mitochondria [64], where it can be expected to be most efficient in cell destruction [65].

An additional factor influencing intracellular PpIX concentration may be the transport of coproporphyrinogen III into the mitochondrion. Recent reports identified ABCB6 as the transport protein for coproporphyrinogen III influx [41,42,66]. Increased expression of ABCB6 in malignant glioma, correlating with intra-operative PpIX-fluorescence, was found in tissue sampled during clinical 5-ALA fluorescence guided resection [41].

Finally, some cytolic factors appear to influence cellular heme synthesis and PpIX accumulation in a tumor selective manner. For instance, cadherin13 expression has an influence on ABCG2 and pepT1 expression. Cadherin13 over-expression was found to be negatively correlated with PpIX accumulation in tissue specimens from glioblastoma patients [51]. When investigating cadherin13 knock-down cells, ABCG2 was found upregulated and pepT1 downregulated, readily explaining the observation with the tissue specimens. Another such factor is vitamin D3. Pretreatment of cells or experimental tumors with vitamin D3 appears to increase PpIX accumulation in a tumor selective manner by upregulation of coproporphyrinogen oxidase [67].

Overall, a conclusive statement on the reasons for and degree of a tumor selective accumulation of PpIX in GBM tissue and cells is not possible. The strongest evidence is probably given for stating that normal brain distant from the GBM is well protected from 5-ALA and PpIX accumulation at the dosage usually applied for FGS and PDT. Within viable GBM tissue, PpIX concentrations can vary broadly both intra- and inter-individually [68–70]. This may reflect the known genetic and morphologic heterogeneity of GBM. At first sight, this seems to rule out any reasonable benefit for FGS or PDT of this highly malignant tumor. However, the cases where PpIX concentrations are really too low to be detected or inefficient for inducing phototoxic cell death are rare, as will be discussed in the clinical chapters.

For practical considerations in clinical application, the correlation of “5-ALA-positivity” with molecular markers used for glioma characterization such as MGMT (O6-methylguanine-DNA methyltransferase) promoter methylation, IDH (isocitrate dehydrogenase) mutation status or Ki67 index may be of interest. Unfortunately, systematic studies are rare. As the vast majority of glioblastomas shows strong PpIX-fluorescence, a dependence on MGMT-status as a determining single factor can be excluded. While an in vitro study found stronger PpIX fluorescence in cells harboring IDH-1 mutation, clinical experience seems to point in the opposite direction, finding non-fluorescent gliomas predominantly in the IDH mutated subgroup [69,71,72]. It may be worth mentioning that the rate of contrast enhancement in MRI among the IDH mutant gliomas is also lower than with IDH wild-type ones [69], suggesting that this effect may be caused by differences in BBB deficiency. A report, which focused on the role of fluorescence in grades II and III gliomas, found a trend, but not a statistically significant positive correlation between fluorescence positivity and IDH-1 wild type [73]. Ki67-index is a proliferation marker and as therefore could be expected, a reasonable correlation with intra-operative PpIX fluorescence has been reported [68,74,75].
Cell Death Mechanisms Following 5-ALA Based PDT (Phototoxic Cell Death)

Dependent on the drug and light dose applied and also dependent on the intracellular localization of the photosensitizer—in our case PpIX—the cell responds very differently. Of course, the obvious intention with PDT of cancer is to induce immediate and complete destruction of all malignant cells. However, with GBM it is unrealistic to achieve this aim. Due to the heterogenic oxygenation of GBM tissue and particularly due to the infiltrative growth pattern of GBM, there will always be GBM cells receiving insufficient photosensitizer and/or light. It is therefore certainly useful to consider the different possibilities [76–79].

At low drug and light doses, some survival pathways such as autophagy [80] may be triggered, which eventually lead to cell survival at very low doses or “autophagic death” or programmed cell death—apoptosis—is initiated at somewhat higher doses. Above a certain threshold dose, equivalent to a minimum of reactive oxygen species (ROS) produced, the survival strategies of the cell will not be successful and post-apoptotic necrosis is the result. At very high doses, a direct rupture of the cell membrane will immediately release the cell contents, modified by ROS attacks, but with little up- or downregulated proteins, as the cell did not have time to change expression profiles. Cell death initiation can occur in a programmed or a more accidental fashion and can lead to production and release of immunogenic signals, such as cytokines or DAMPS (damage associated molecular patterns), but also in a fashion, where anti-inflammatory factors are produced [78]. The underlying pathways involved and their triggering by PDT induced ROS are extremely complex and not understood in every detail. However, the fact that different organelles are involved in different ways should be noted. As a consequence, the type of photosensitizer, its concentration, and intracellular (re-)distribution during exposure of the cell determines the processes triggered upon light irradiation [81]. By designing a protocol of using different photosensitizers targeting different organelles and pathways at different times, the efficacy of cell killing can be much improved [82].

With regard to 5-ALA based PDT, one should point out that in this case the primary location of the photosensitizer is the mitochondria. As the ROS, in this case predominantly singlet oxygen, only travel very short distances inside cells before being quenched by substrate molecules, 5-ALA PDT targets mitochondria more than other organelles [83,84]. With longer incubation times, however, PpIX is released more and more into the cytosol and damage to other organelles may gain relative importance [85].

With regard to 5-ALA based PDT of GBM, it is important to note that PpIX concentration varies broadly within a tumor and also light fluence (the amount of light, which has impinged on a spherical unit surface, also called light dose and measured in J/cm²) will be very different in different parts of the tumor. It is therefore very probable that all different kinds of cell death mechanisms are triggered. Although phototoxicity alone will not be able to destroy all tumor cells, the cocktail of cell death mechanisms obtained may be very effective to stimulate the immune system. PDT in general has been shown to be very immunogenic [86,87] and 5-ALA based PDT in particular has been shown to elicit essential immune stimuli [88–90]. It is therefore fair to speculate that stimulation of immune response may be an important contributor to a favorable clinical response to GBM PDT.

**FLUORESCENCE GUIDED SURGERY**

**Surgical Guidance Tools**

The aim of FGS is to prolong progression free and overall survival safely, that is, not at the expense of functional deficits or compromised quality of life. This aim is achievable if most or all of the MRI-contrast uptaking part of the GBM tissue can be removed [91–94], of course without extending surgery into functional brain. Several surgical guidance tools have therefore been developed as listed in Table 2. A more detailed description and critical comparison of these methods is provided in [95]. Among these methods there are two, which also involve wide field fluorescence imaging. Unlike 5-ALA based FGS, however, these methods rely on i.v. injection of a fluorochrome, Na-fluorescein, or indocyanine green (ICG). To some degree, these fluorochromes will be present in the circulation during surgery and produce unspecific background fluorescence or even leak from injured blood vessels. In addition, there is no tumor cell selective uptake of these fluorochromes. Hence, they may be valuable in roughly outlining the borders of a deficient BBB, but have not proven a degree of selectivity, which is comparable to 5-ALA induced PpIX fluorescence.

As ICG operates in the near infrared (NIR), where tissue autofluorescence is negligible and light penetration maximal, future use of ICG or other NIR fluorochromes attached to tumor specific antibodies or peptides appears promising [96,97].

**5-ALA Based Fluorescence Guided Surgery—Technique**

5-ALA based fluorescence imaging differs from all other fluorescence based methods by the fact that what is applied to the patient is not the fluorochrome but its non-fluorescent precursor. This enables a higher contrast, as there is no unspecific fluorescence background from the circulation or interstitial space. In addition, there is a well-founded tumor selectivity of PpIX accumulation on a cellular level as discussed above. In principle, PpIX fluorescence might be able to identify isolated, single GBM cells surrounded by functional brain. However, surgical removal will not be possible in such a situation, because functional deficits would be unavoidable. As discussed above, surgical guidance can be performed only to a degree, where such side effects can largely be excluded. Within the infiltration zone of GBM, the tumor cell density gradually decreases and surgery has to stop as soon as functional brain is encountered even though it...
may still be infiltrated with malignant cells. It may therefore not be recommendable to drive the sensitivity of PpIX-fluorescence imaging to the technically possible limit. In a routine clinical application it must additionally be considered that room lights might not be dimmed to a degree that it can be excluded that some reflections might be detected and mistaken as PpIX fluorescence. These considerations led to the currently implemented equipment, which is used for PpIX fluorescence imaging in surgical microscopes [127].

Three to four hours before anesthesia, 20 mg/kg body weight of 5-ALA (Gliolan®, ALAGLIO®, medac, Wedel, Germany) are dissolved in 50 ml of drinking water and administered orally [128]. In most cases, this will lead to

### TABLE 2. Surgical Guidance Tools Other Than 5-ALA FGS

| Surgical guidance tool | Principle | Benefit | Comment | References |
|------------------------|-----------|---------|---------|------------|
| Neuronavigation | Correlation of pre-operative imaging with the position of a pointing-pen during surgery | More pronounced resection, but limited information on prognostic impact | Sensitive to brain shift | [98–100] |
| Intra-operative MRI | Intermittent contrast MRI during surgery | Increased rate of CRET (96% vs. 68%) and longer progression free survival (218 vs. 110 days) in Ref. [101] | Cost and complexity of the procedure are limitations | [94,101] |
| Intra-operative ultrasound | Use of small ultrasound probes during resection | Economic, good for low grade gliomas, linear array transducers superior | Easily implemented, but still limited specificity, also limited in delineating small amounts of residual tumor; much experience required | [102–104] |
| Cortical functional mapping | Electrical brain stimulation by electrodes, identification of functional areas | Valuable close to eloquent areas, safety tool, increased rate of CRET possible | Combination with other tools recommended | [105,106] |
| Na-Fluorescein fluorescence | i.v. injection of fluorochrome some hours before surgery, claims to stain GBM | Easy implementation in surgical microscopes | Off-label use, spreads with edema, tumor selectivity questionable | [107–109] |
| Photosensitizer fluorescence | mTHPC, Hypericin, NPe6 | Easy implementation in surgical microscopes | Off-label use, spreads with edema, tumor selectivity questionable | [110–112] |
| Optical coherence tomography | Ultrasound-like images generated by backscattered light | Adaptation to surgical endoscopes available | Experimental, high resolution but small field of view (submillimeter) | [113] |
| Confocal laser endomicroscopy | Confocal imaging with small flexible probes | High resolution structural information, fluorescent staining of specific targets possible | Only very superficial information, small field of view, background staining required | [114–116] |
| ICG fluorescence | Leaks from vessels in deficient BBB | Near infrared fluorochrome, Automated recognition of molecular markers | Experimental, direct contact required | [117,118] |
| Mass spectrometry analysis | Extracting biomolecules from tissue and extract mass fingerprint, statistical classifiers may recognize malignancy | Label-free, CARS for microscopic imaging | No clinical intra-operative experience yet | [119,120] |
| Photoacoustics | Absorption of a light pulse generates an ultrasound signal | Can be labelfree, can sense oxygenation | Tissue contact required, small field of view | [121–123] |
| Raman spectroscopy, CARS | Probing of chemical bonds, e.g., lipid and protein content | Label-free, CARS for microscopic imaging | No clinical intra-operative experience yet | [124–126] |

Ordered with respect to clinical use/experience.
PpIX accumulation in GBM cells. Several manufacturers of surgical microscopes have implemented suitable fluorescence modes (Zeiss, Göttingen, Germany; Leica, Wetzlar, Germany; Möller–Wedel, Wedel, Germany) to image PpIX fluorescence during surgery [129]. They all work in a very similar manner: A broadband light source is filtered to emit violet light to cover the Soret-band absorption of PpIX. The spectral bandwidth of the filter is extended into the blue to emit light at 440–460 nm at the 1%-level [130]. In fluorescence mode, the objective of the microscope is filtered with a long-pass at 440 nm, blocking all of PpIX excitation, but transmitting the low intensity light at around 450 nm. If PpIX is present, its fluorescence with peaks at 635 and 705 nm will be fully transmitted by this filter. What is then observed by the surgeon’s eye or by a sensitive camera is (i) blue light at around 450 nm emitted by the excitation light source and remitted from the tissue surface; (ii) green fluorescence originating from endogenous fluorophores; and (iii) PpIX fluorescence in the red (Fig. 2). The intensity of blue light is scaled in a way to normally be stronger than green autofluorescence and stronger than any unspecific red light. Normal brain tissue therefore always appears blue and PpIX from solid tumor makes the tissue appear red. In the infiltration zone, the intensity of red fluorescence will gradually decrease with the decreasing density of tumor cells. It is then at the discretion of the neurosurgeon to decide how much further tissue can be resected safely. For the resection of GBM it may make little sense to increase the sensitivity of fluorescence imaging, which could easily be done by reducing or eliminating the blue light intensity from the excitation light source. For the detection of low grade gliomas or “non-fluorescent” metastases, however, a more sensitive means of detecting PpIX might be useful. In at least 6 out of 12 low grade tumors, a low but selective PpIX accumulation could indeed be found by Valdes et al. [131] when applying sensitive fluorescence spectroscopy.

5-ALA Based Fluorescence Guided Surgery—clinical Results

**FGS for malignant glioma.** The use of 5-ALA for FGS was first approved in Europe following a multicenter phase III trial conducted by Stummer et al. [20]. With 139 patients having received the study drug, it is still the largest published series assessing the reliability of PpIX imaging for malignant glioma. The study showed that CRET was increased from 36% (white light) to 65% (5-ALA) and progression free survival at 6 months increased from 21% (white light) to 41% (5-ALA). Overall survival was not a primary end point and its prolongation (15.2 vs. 13.5 months) was not statistically significant. Only if all patients with complete resections (disregarding the study arm) were compared with patients with residual contrast enhancement, was the difference significant [21] (17.9 vs. 12.9 months). No significant difference in side-effects was noted for the study arms.

The results of this approval study were confirmed by a series of other studies, summarized by Suero-Molino et al. [132]. From their review of available literature, they conclude that:

1. Normal brain tissue does not appear to induce PpIX expression after ALA administration.
2. 5-ALA based FGS extends beyond the borders of Gd contrast uptake.
3. Apart from rare transient liver enzyme elevation and known light sensitivity of the skin 24 hours after administration, 5-ALA appears to be safe.
4. Unspecific PpIX fluorescence may occur in recurrent tumors and originates from gliosis and reactive astrocytes.

A crucial parameter for the assessment of how accurate and safe FGS may be is the positive predictive value (ppv) of PpIX fluorescence, indicating the potential risk of resecting functional brain if this value is below 100%. Stummer et al. [133] in a study on 33 patients, reported that there was a ppv of 100% for strong fluorescence, and a ppv of 92% (biopsy based) or 83% (patient based) for weak fluorescence. Among primary GBM, similar results were reported in a series of other studies [75,134–137], comprising more than 476 biopsies from tissue with strong fluorescence. Only five of these tissue samples did not contain tumor and only two contained only normal brain tissue. One may thus conclude, that strong PpIX fluorescence with primary GBM patients very reliably predicts tumor. With recurrent tumors, a bit more caution may be

Fig. 2. Intraoperative images during GBM surgery in white light (left) and fluorescence modes (right). Note that no fluorescence is visible in areas covered by extravasated blood.
indicated: in a study with 354 fluorescent biopsies sampled from patients with recurrent GBM, 12 (3.4%) had been judged normal, but were mostly obtained from weakly fluorescent tissue [138]. Rare inflammatory lesions in the brain may also give rise to false positive PpIX fluorescence as shown in a recent case report by Omoto et al. [139].

Optimally, fluorescence guidance during surgery should be able to visualize all tumor cells in order to maximize the extent of resection. In other words, the negative predictive value (npv) should be high. With the current equipment, it is difficult to properly assess the real npv, as the surgical microscopes are (intentionally, as discussed above) far from being sensitive enough to detect PpIX fluorescence, if the tumor cell density in the infiltration zone is below approximately 10%. Of course, the impression through the surgical microscope determines the decision, whether resection might be continued or not. Consequently, clinical studies find non-fluorescent tumor infiltrated tissue in a non-negligible number of cases. Of 36 non-fluorescent biopsies, 9 did contain tumor in the first report on the method by Stummer et al. [140]. However, four of these false negative biopsies were from predominantly necrotic tissue and five showed a low density of infiltrating tumor. Later, these authors [136] reported on a biopsy-based sensitivity of 89%, where 5 non-fluorescent samples out of 237 histologically-positive ones contained solid tumor and another 21 samples showed only diffuse infiltration of tumor. Kiesel et al. [141] presented data on 77 patients, where a biopsy based npv of 49% can be deduced. Solid tumor, however, was only found in 1 of 67 non-fluorescent biopsy samples. In the evaluation of Stummer et al. on 33 patients [133], fluorescence assessment through the surgical microscope was compared with spectroscopic measurements on the same spot at the resection borders.

When judged through the surgical microscope, as many as 60% of biopsies gathered from non-fluorescent tissue demonstrated tumor infiltration. Spectroscopy proved a higher sensitivity and accuracy, which depended on the threshold of PpIX fluorescence detected. A qualitatively equivalent conclusion was drawn by Valdes et al. [142], demonstrating a sensitivity of 95% for high grade glioma, when evaluating according to spectroscopic measurements, while it was only 55% when assessing PpIX fluorescence visually, however based on only 20 biopsies from 3 patients.

In the context of these observations of biopsy samples containing non-fluorescent tumor, it is important to discuss investigations on the absolute concentrations of PpIX found in different parts of GBM tissue or found in different patients. In some cases, it is even reported that the entire tumor did not show PpIX fluorescence. While in such cases one should always question, whether 5-ALA was applied at all or at the right dose, it must of course be considered that, given the known genetic heterogeneity of GBM, very variable PpIX accumulation is indeed rather probable. For instance, Hefti et al. [137] found 1 of 47 patients having a completely non-fluorescent GBM. Three out of 20 GBMs were non-fluorescent in a report of Tsugu et al. [143] and in our own assessment [144], we could not find fluorescence in representative biopsy specimens from 2 of 19 patients. Even in cases where solid GBM tissue shows bright fluorescence, large variations in absolute PpIX concentration were measured within and among patients [68,70]. The PpIX fluorescence observed in non-necrotic, viable and histologically identical tumor tissue from the same patient varied broadly in an investigation by Moiyadi et al. [145]. It was speculated that the PpIX variability may correlate with some biological variability, which is not immediately apparent histologically. Indeed, Piccorillo et al. [146] found that tumor-initiating cells (TICs) within the tumor mass behave differently depending on whether these had been isolated from PpIX-positive or PpIX-negative tumor tissue.

Clinically, the fact that non-fluorescent tumor tissue can be found raises the critical question, whether fluorescence guidance will be useful at all. As currently the accepted best practice in surgery is to aim for a safe CRET, FGS has to demonstrate an increase in CRET over the previous standard and compared to other surgical guidance tools. In fact, this was one of the primary study aims of the approval study [20] and was confirmed many times [147–149], as summarized in the review by Ferraro et al. [150]. Schucht et al. [149] quantitatively assessed how much more of GBM tissue can safely be visualized and resected by FGS in comparison to the pre-operatively determined volume of Gd-contrast uptaking tissue. In 13 patients, the mean resected volume was determined to be 84 cm³, whereas the Gd-contrast tumor volume in the same patients was only 39 cm³. Hence, CRET can not only be achieved in more cases, but a larger volume of—mostly infiltrative—tumor tissue can be removed. This can then be expected to not only prolong progression free survival but also to improve the efficacy of adjuvant therapies [91,92] and to improve quality of life [151].

An interesting, yet unsolved question is, whether the sometimes observed PpIX fluorescence originating from ependyma indicates tumor involvement or not [152]. As mentioned above, 5-ALA may enter the cerebral spinal fluid. The ependymal lining of the ventricles is therefore exposed to 5-ALA and may show unspecific PpIX fluorescence. Nevertheless, tumor cell infiltration into the ependyma was reported in six out of seven cases with fluorescent (but visually normal) ventricle wall [153]. It appears possible that more clarification can only be expected by a more quantitative assessment of the PpIX fluorescence at this location.

**FGS for other brain tumors.** Although 5-ALA is only approved for FGS of WHO grade III and grade IV gliomas, it has also been tested in low grade gliomas, meningiomas, spinal cord tumors, various brain metastases, and occasionally in non-glial primary brain tumors [95,150].

In general, 5-ALA does not lead to visible PpIX fluorescence in low grade gliomas as judged with the surgical microscope. Jaber et al. [73] investigated 82 WHO grade II tumors, of which only 15.9% revealed intra-operative PpIX fluorescence compared to 83.3% of enhancing grade III tumors. They identified a
[18] F-FET-PET uptake ratio of >1.85 as predictive for fluorescence. Low grade tumors, which do not show visible fluorescence under the surgical microscope may still show selective PpIX accumulation when investigated with more sensitive fluorescence spectroscopy [142]. FGS is also a valuable tool to localize anaplastic foci within low grade glioma [154–156].

PpIX accumulation in brain metastases is heterogeneous: only approximately half of them (independent of their histology or origin) show intraoperative PpIX fluorescence—mostly described as patchy within the tumor [157–159]. Of special note is the observation of false positive staining outside the tumor, sometimes even with the tumor itself being non-fluorescent or only weakly fluorescent [158,160]. Keeping this in mind, FGS can still be regarded beneficial, as it provides a chance of detecting infiltrative parts of the metastases.

Meningiomas are much more frequent than high grade gliomas and sometimes also present a challenge during surgery, especially when involving cranial bone invasion. The largest series was published by Millesi et al. [161], stating that 185 of 204 meningiomas showed PpIX fluorescence, which was also helpful in identifying tumor-infiltrated bone flaps in all of 13 cases and satellite lesions in all of seven cases. However, in 25% of cases PpIX fluorescence was also present in the adjacent cortex. Motekallemi et al. [162] in their review conclude that FGS is not as sensitive for meningioma as it is for GBM. In meningiomas, PpIX fluorescence is usually very heterogeneous and false negatives and false positives occur more frequently than with GBM. A meta-analysis of studies with FGS of meningiomas by Foster and Eljamel [163] finds that the surgical plan was “altered intraoperatively in 75% of high-grade and 19% of low-grade meningiomas, improving the extent of surgical excision.” In a recent report by Knipps et al. [164] PpIX spectroscopy proved much more sensitive and only slightly less specific than imaging in detecting the extension of 13 meningiomas.

**5-ALA Based Fluorescence Guided Surgery—Improvements**

Although 5-ALA based FGS has already set a new standard in glioma surgery, improvements are certainly possible and should be developed with high priority. This can be done in two ways: (i) improvement of the reliability of PpIX staining and (ii) improvement of the technical equipment.

**Improvement of the Performance of PpIX Staining**

As discussed above, the current drawbacks with PpIX staining are the variable PpIX concentrations encountered in different GBM and within a tumor and the fact that some tumors or parts of a tumor can even be non-fluorescent, at least using currently available technical equipment. It might therefore be worthwhile to have a closer look at measures for increasing PpIX levels in cancer cells.

One such possibility is to modulate heme synthesis. Successful attempts have been reported in narrowing the bottleneck at the last step to increase PpIX accumulation. Inhibition of ferrochelatase [37] or application of iron chelators [58,165,166] both led to increased intracellular PpIX concentrations.

The hypothesis of stimulating heme synthesis by inducing terminal differentiation as observed in erythropoiesis led to early investigations of co-incubating cells with 5-ALA and differentiation inducers [167]. Indeed, increased PpIX levels and phototoxicity could be observed. Apart from an increased uptake of 5-ALA and a reduced efflux of PpIX, an upregulation of coproporphyrinogen oxidase was found. This was also regarded the main reason for increased PpIX levels found in cells after co-incubation with calcitriol (active form of vitamin D₃), although other mechanisms may also play a role [168]. Chen et al. [169] demonstrated the calcitriol driven PpIX-increase on glioma cells and also indicated that it may occur in a tumor selective-manner.

The clinical application of PpIX enhancers represents a significant challenge, as it would have to be excluded that the attempt to increase PpIX-levels in GBM also produces effective PpIX levels in normal brain. The dosage of 5-ALA might have to be re-adjusted.

A tumor selective increase in PpIX accumulation was also observed, when glioma cells, primary rat embryonic neurons, and astrocytes were exposed to hypothermia during incubation with 5-ALA [170]. When hypothermia was applied in vivo, a neuroprotective effect against PDT was observed, also increasing the selectivity of GBM PDT [171].

As discussed above, the application of ABCG2 blockers, which inhibit PpIX efflux from mitochondria and/or cytosol, may be of practical relevance, especially with regard to PDT. Caution is indicated, however, because ABCG2 is highly expressed in brain endothelial cells, constituting part of the BBB function [172]. Although 5-ALA itself is not a substrate for ABCG2, any PpIX synthesized in endothelial cells might then photosensitize the brain capillaries in a potentially unselective manner.

Finally, a recent report by Yoshioka et al. [173] in *vitro* and *in vivo* demonstrates a tumor selective PpIX enhancement by blocking the oncogenic Ras/MEK pathway.

**Improvement Of The Technical Equipment**

**Minimizing blood absorption.** The currently applied surgical microscopes excite the PpIX fluorescence with violet light, which is strongly absorbed by blood. In fact, a single layer of erythrocytes covering fluorescent tissue completely absorbs the excitation light and PpIX fluorescence is no longer visible. During resection, this is only a minor problem, because the tissue is usually continuously rinsed. At the final inspection of the resection cavity, however, there is a very significant risk of overlooking fluorescent malignant tissue lying underneath a thin layer of dried blood [95] (Fig. 3). Excitation of PpIX fluorescence is not restricted to violet light, it can also be excited with
red light, albeit with a lot lower efficiency [17,174,175]. Red light is much less absorbed by blood and diluted blood covering fluorescent tissue is less likely to obscure PpIX fluorescence [175]. As excitation light and emitted fluorescence have the same color, it is more difficult to visualize PpIX fluorescence with surgical microscopes, but certainly possible with appropriate optical filtering. Corresponding modifications of a surgical microscope have been demonstrated by Roberts et al. [176], where the resulting PpIX fluorescence image is displayed as a pseudo color overlay to the white light image.

**Increasing optical accessibility.** With large deep seated tumors, a surgical microscope is not the ideal instrument to visualize the entire surgical cavity. PpIX fluorescence imaging requires rather perpendicular access to the inspected tissue surface by both, illumination with excitation light and imaging of PpIX distribution with the objective lenses. The problem may be solved by employing side-view endoscopes equipped with fluorescence technology, as suggested by different groups [177–180].

**Improving sensitivity.** As already mentioned above, the current implementation of PpIX fluorescence imaging has intentionally not been designed to perform with highest possible sensitivity, mainly in order to limit resection into the infiltration zone to a degree, which can still be considered safe and to minimize mistaking any ambient light reflections as PpIX fluorescence. In some cases, a more sensitive imaging of PpIX fluorescence may however be reasonable, for example, when investigating the suitability of 5-ALA for visualizing low grade gliomas or brain metastases.

All measures aiming at increasing sensitivity of PpIX detection inherently carry the risk of reducing specificity. Inside the brain, this is of special importance and danger, because any false positives may lead to removal of uninfiltrated brain. Any changes to the approved equipment should be thoroughly investigated to allow for a safe clinical implementation.

The easiest way to increase sensitivity is to block blue light illumination completely or to remove the blue channel of the digital image [130] and/or to use a more sensitive camera [181]. A more reliable way, however, is to record fluorescence spectra, where PpIX fluorescence is very characteristic with two peaks at 635 and 705 nm, whereas tissue autofluorescence shows an uncharacteristic decreasing intensity with longer wavelengths in this wavelength range. The possibility of detecting the presence of PpIX in such spectra increases sensitivity very reliably. However, specificity starts to decrease at a certain point, as expected [133,142]. For the purpose of detecting low grade gliomas, spectroscopy appears promising, showing specific PpIX accumulation in 6 out of 12 lesions investigated by Valdes et al. [142].

**Avoiding ambient light interference.** Fluorescence imaging usually requires highly sensitive light detection of the fluorophore emission. This is also the case with FGS. Room lights in the OR must therefore be dimmed to not interfere with fluorescence imaging. By using pulsed excitation light sources and appropriately synchronized gated cameras, this problem can easily be overcome. This approach had been suggested by Sexton et al. [182]. It also allows for a simultaneous display of white light and pseudo-color fluorescence images.

**Quantitative fluorescence measurement.** The current technical implementation allows for characterization of fluorescence intensity only rather than for
quantification. Neurosurgeons usually discriminate three levels of fluorescence “qualities”: strong, vague (or weak), and no fluorescence, indicating solid tumor, infiltration zone, and normal brain, respectively [183]. For FGS this may be regarded as sufficient. For scientific reasons, it would be desirable to have a more quantitative measure of the PpIX content in the tissue observed. Extracting information from detected fluorescence spectra allows at least for providing an objective absolute value of PpIX fluorescence intensity at a certain spot [184]. However, although fluorescence intensity is proportional to PpIX concentration, it also depends on absorption and scattering of the tissue. These optical tissue parameters need to be considered carefully, before the resulting measured intensity can be taken to represent PpIX concentration as an absolute value. Methods to correct for optical tissue parameters have for example, been proposed by a group in Rotterdam [185–187] and suggestions for implementation into surgical microscopes have been made by Valdes et al. [131,188] and Xie et al. [189]. An intriguing technology to obtain quantitative fluorescence imaging is using spatially modulated light at the excitation and emission wavelengths to retrieve absorption and scattering parameters to further correct fluorescence images [190,191] and even to reconstruct 3D distributions of PpIX [192].

5-ALA for guided brain biopsy. Stereotactic brain tumor biopsies are indicated to confirm suspected GBM from preoperative imaging and especially, if characterization with respect to tumor markers such as MGMT appears necessary to decide on a personalized treatment strategy [193]. However, the procedure carries non-negligible risks of inducing hemorrhage on the one hand and falsely collecting only non-diagnostic tissue (mainly necrosis) on the other hand [194,195].

5-ALA induced PpIX exactly stains the type of tissue needed for a reliable histopathological diagnosis: viable (non-necrotic) solid tumor [195]. PpIX accumulates in solid GBM with a positive predictive value close to 100%, while not staining the necrotic core of GBM, because PpIX accumulation only takes place in live cells. Hence, taking brain biopsies with fluorescence guidance might be a good idea [196]. The current procedure is quite cumbersome, involving on-site pathology and multiple biopsies through the entire tumor without definite guarantee of having acquired properly evaluable tissue. With fluorescence guidance integrated in the biopsy needle, there is only need for a single biopsy at the site where PpIX fluorescence is first detected. The pathologist will not need to be in the OR, but will definitely be presented with tissue well-suited for diagnosis. The proof of principle was demonstrated by investigating the fluorescence of biopsy specimens ex situ with the surgical microscope [197–200]. The first clinical case with intraoperative PpIX fluorescence guidance, demonstrating suitable equipment was reported by Eigenbrod et al. [193] and Göbel et al. [201]. When taking fluorescence spectra with a fiber optic integrated in a biopsy needle, the presence of extravasated blood cannot be easily circumvented. The excitation light for PpIX with the usual wavelength of 405 nm is readily absorbed by blood. Hence—even more relevant than for PpIX imaging (see above)—excitation with red light might be preferable. Markwardt et al. [175] found that excitation of PpIX fluorescence (the longer wavelength peak at 705 nm) with red light at 635 nm instead of violet light at 405 nm (the shorter wavelength emission at 635 nm) is 50-fold less sensitive, but still sufficient and much less sensitive to blood absorption: a layer of whole blood with a thickness of more than 200 μm was needed to reduce PpIX fluorescence to 10%, whereas the same reduction was observed with less than 20 μm when excited at 405 nm.

Not only is the accuracy of stereotactic brain biopsies increased by PpIX fluorescence guidance, but also safety, because only a single biopsy is needed instead of multiple biopsies. Safety can be increased further, if an injury of blood vessels can be avoided. One of the possibilities for detecting blood vessels with fiber-optic technology is to measure light transmission through a small tissue volume at two wavelengths with much different blood absorption, for example, 650 and 578 nm as demonstrated by Markwardt et al. [202]. Figure 4 shows a putative prototype of a fiberoptic brain biopsy needle capable of detecting the presence of PpIX as well as larger blood vessels during insertion of the needle and of the tissue sample intended for removal.

5-ALA BASED PHOTODYNAMIC THERAPY OF MALIGNANT GLIOMA

History of GBM-PDT

The poor prognosis for patients with malignant glioma has very early on stimulated clinical trials to evaluate, whether PDT might offer longer survival at the same or even better quality of life compared to other treatment options [203–205]. The photosensitizer used was mostly Photofrin® [206–208]. The overall conclusion is that PDT may offer a significant survival advantage with occasional long-term survivors [209], but that there is also a considerable risk of treatment related side effects [207]. This is probably due to the limited tumor selectivity of photosensitizer accumulation. In any case, PDT is today not a broadly available treatment option, although the chlorin-type photosensitizer NP6 (Laserphyrin®) has obtained clinical approval for PDT of malignant glioma in Japan [210]. Recent experience with 5-ALA based PDT of GBM as well as with the use of other photosensitizers in Russia, Belarus and Ukraine is summarized by Zavadskaya [211].

Advanced Photobleaching of PpIX Enables Highly Selective GBM Destruction

The higher tumor selectivity of PpIX accumulation compared to other photosensitizers, a fact which has been proven in thousands of diagnostic applications worldwide.
has now led to the initiation of approval studies for PDT [18]. One of the advantages of PDT with 5-ALA based PpIX is the fast photobleaching of PpIX. This may appear implausible at first sight, because photobleaching implies destruction of the photosensitizer and thus limits the achievable amount of reactive oxygen species. Prolonged irradiation will not continue to produce phototoxic effects. As a consequence, cells which do not contain enough photosensitizer at the start of irradiation cannot be killed even with extremely high doses of light, provided the intensity is low enough to avoid thermal damage. This consequence of photobleaching can now be taken advantage of [212,213]: Because normal brain has been shown to accumulate minute amounts of PpIX only, if any, there is no risk of a phototoxic damage to normal brain even if it is exposed to high doses of light. Then, there is a realistic chance to eradicate tumor cells even among normal functional brain without overly ambitious light dosimetry. The light dose requirements are restricted to make sure that the entire tumor receives a minimum dose of light. As argued above, an overdose with detrimental side effects is not possible. One can even increase the depth of selective necrosis by one or two light penetration depths as illustrated and described in Figure 5 and by Potter et al. [213].

**PDT of the Resection Cavity**

An obvious concept for implementing PDT in the treatment scheme for GBM is applying it to the resection cavity. If PDT is performed immediately following FGS, there would be no need for an additional drug application, as it has been shown in clinical pilot trials that timing and diagnostic dose of 5-ALA (20 mg/kg bodyweight 4 to 6 hours prior to surgery) are sufficient and efficient for PDT in humans [17,214–216]. A clinical pilot trial with this concept has been initiated in France [217]. Irradiation is performed with a balloon diffuser, which is inserted into the surgical cavity.

**Stereotactic Interstitial PDT**

Another concept is replacing surgery by interstitial PDT (iPDT). "Interstitial" means that the light irradiation has to be delivered inside the tumor tissue, usually with multiple bare fibers or cylindrical diffusers [218]. Experience with iPDT with bare fibers has been obtained for the treatment of head and neck tumors [219] and prostate cancer [220]. Cylindrical diffusers have been used for PDT of prostate cancer [221] and malignant glioma [17,214–216]. For brain tumor iPDT, it is certainly recommendable to use cylindrical diffusers, as the emitting surface area is a lot larger and the fluence rate (light intensity, measured in mW/cm²) at the tissue/light emitter interface correspondingly lower. This improves light distribution and makes it less sensitive to local variations of absorption with a risk of blood coagulation in the worst case. Furthermore, it is easier to achieve a sufficient overlap of target light fluence with the tumor volume [222]. Interstitial PDT in the brain is also subject to more pronounced clinical restrictions than elsewhere in the body: the inevitable invasiveness of inserting fibers must be kept at the absolute minimum and the precision of fiber placement at the maximum. Unwanted side effects caused...
which was determined to 1080 J/cm² [144]. With the above light dose which leads to at least 95% of photobleaching, “advanced photobleaching,” the target light dose is the parameters found in the literature. In the approach of average parameters, which are in the lower range of are not feasible, one will have to rely on the mentioned individual measurements of optical tissue parameters have certainly to be considered as well. As long as experimental errors, but strong individual differences parameters was high. This may have been partly due to penetration depth of approximately 3 mm [223]. It should be freely chosen as required to cover the diameter of the tumor along each trajectory.

The positioning of the fibers requires a careful treatment planning. An important input parameter for the treatment planning is the expected depth of necrosis, which in turn depends on the optical properties of tumor and normal brain. Corresponding measurements during open surgery resulted in absorption and effective scattering parameters of 0.02 and 2 mm⁻¹ at 635 nm, equivalent of an optical penetration depth of approximately 3 mm [223]. It should be mentioned that the standard deviation of these parameters was high. This may have been partly due to experimental errors, but strong individual differences have certainly to be considered as well. As long as individual measurements of optical tissue parameters are not feasible, one will have to rely on the mentioned average parameters, which are in the lower range of parameters found in the literature. In the approach of “advanced photobleaching,” the target light dose is the light dose which leads to at least 95% of photobleaching, which was determined to 1080 J/cm² [144]. With the above mentioned optical tissue parameters, this is achieved at approximately 4 mm distance from the surface of a cylindrical diffuser, which emits a light power of 200 mW per centimeter of diffuser length for 1 hour. The light power of 200 mW/cm should not be exceeded. Higher powers would run the risk of inducing thermal side effects by increasing tissue temperature by more than 4°C [214]. From these calculations, the recommended interfiber distance is given as 10 mm. If a sufficient treatment penetration into the infiltration zone should be guaranteed, the outermost applicators should be as close as 3–4 mm to the borders of Gd-contrast in MRI. It can be expected that the depth of necrosis is normally considerably larger than the optical penetration depth under the condition of advanced photobleaching, which tries to handle the worst case scenario of minimal PpIX accumulation.

Treatment planning of iPDT is still a work in progress [218,224]. It uses pre-operative Gd-contrast MRI images, fused with CT images containing 3D markers and appropriate software allows for virtual positioning of applicators. In the published clinical pilot trials, software for radioactive seed implantation was used with parameters for an X-ray isodose ranging 3.8 mm around the diffuser [214]. The aim of the neurosurgeon is to virtually place the appropriate number of diffusers in a way that (i) injury of vessels and critical structures is avoided and (ii) the treatment volume, defined as the tissue volume receiving at least the target light dose overlap as much and as precisely as possible with the tumor volume. The output parameters of this procedure are (i) the dose volume histogram and (ii) the 3D positioning parameters to be reproduced with the mechanical diffuser insertion device, be it a stereotactic frame or a robot.

An intriguing alternative to light application via optical fibers is to make the cells emit the light themselves via bioluminescence. This can either be induced by first transfecting the cells with plasmids to produce luciferase and apply luciferin to initiate chemiluminescence or rely on ROS-induced activation of luminol [225]. Whether such systems will emit enough light to reliably kill photosensitized cells is controversial [226]. A European research project is currently exploring the feasibility of triggering PDT by mitochondria-powered chemiluminescence to non-invasively treat inaccessible tumors such as GBM [227]. Even if these preclinical investigations should show very promising results, the additional pharmaceutical drug needed will pose a considerable obstacle to the initiation of clinical pilot trials.

One of the safety issues in iPDT is the possible induction of edema, which, if severe, can lead to neurological deficits when edema reaches functionally intact brain. With drug and light doses which were found to effectively and selectively treat C6 rat brain tumors [228], some edema formation was indeed observed, but considered to be in an asymptomatic range [49]. Other photosensitizers, such as Photofrin® or phthalocyanine lead to a much greater edema formation [229,230], probably because the photosensitization of endothelial cells is stronger than with 5-ALA. Side effects observed with 5-ALA based iPDT of GBM in humans may partly be due to edema formation, but resolve within a few days [215]. Edema formation can normally easily be managed by application of steroids. As

![Diagram showing photobleaching and light dose distribution](image_url)
there are theoretical concerns of a possible interference with 5-ALA penetration into the tumor tissue, the steroid dosage prior to iPDT was not increased. Edema induced by iPDT can be significant at times. Especially if tumors are located in so called eloquent locations this might lead to temporary neurological deterioration (unpublished observations). It is important to counsel patients and families on this possibility. Patients with malignant glioma usually have a varying degree of steroids. After iPDT treatment we temporarily increase this dose to 3 × 8 mg dexamethasone to counteract edema.

The clinical experience with 5-ALA based iPDT reported so far is indeed promising [17, 214–216]. The most recent report [215] summarizes 15 patients with de novo tumors: Compared to a group of GBM-patients with optimal surgery (“complete tumor resection”), iPDT proved significantly better with a median progression free survival of 16 versus 10.2 months, P < 0.001 and a 3-year survival of 56% versus 21%, P < 0.01. Six out of 15 patients of the iPDT group experienced long-term progression free survival of more than 30 months (range: 32–68 months). Transient morbidity was seen in seven patients (transient aphasia, pulmonary embolism).

One of the open questions with iPDT is, whether individual differences in drug and light distribution need to be considered to guarantee maximal treatment success for every patient. Indeed, especially light absorption has a great influence on the calculated treatment time needed to achieve the aim of “advanced photobleaching,” easily leading to clinically intolerably long irradiation times of several hours [231]. This could, however, be prevented by reducing the interfiber distance during treatment planning. As more fibers are then required, this would only be done if really necessary. Hence, assessment of the individual conditions appears recommendable, but should not increase the invasiveness of the procedure. One such approach might be realized by using the implanted cylindrical light diffusers as detectors. It has been demonstrated that at least the presence or absence of PpIX can be measured by having one of the light diffusers switched on, while a light diffuser next to this one is connected to a spectrometer, which then simultaneously records PpIX fluorescence at 705 nm as well as transmitted laser light at 635 nm [17]. In this report, the absence of recorded PpIX fluorescence correlated with unfavorable clinical outcome. From the laser light transmission between fibers, one can calculate optical tissue parameters as demonstrated for iPDT of the prostate by Liang et al. [232].

How much improvement can be expected from iPDT of GBM? Long-term survival or even cures would require the destruction of virtually all tumor cells. But will all GBM cells be loaded with sufficient PpIX and will treatment light reach out to all GBM cells in the infiltration zone? The answer is: probably not. Madsen et al. [233] have shown in an experimental model that the existence of non-sensitized infiltrating tumor cell nests must be taken into account. This had to be expected, as the BBB deficiency is restricted to the solid part of the tumor, whereas the infiltration zone can extend centimeters away from this border. Still, very long-term survivals have been reported [216], even for Photofrin® based PDT [209]. This may be attributable to the efficient induction of an immune response. Initial investigations indeed demonstrate that PDT, including 5-ALA based PDT causes a very immunogenic type of cell death as already mentioned in the introduction [86–90]. It may be of advantage that in iPDT the treated tumor mass inevitably has to remain inside the brain, giving the immune system the chance to see all possible “damage associated molecular patterns” presented by this genetically heterogeneous type of tumor. If this can be substantiated in ongoing clinical trials, it may be a very promising concept to try to exploit this immunogenic cell death and support its efficiency by for example applying immune checkpoint inhibitors or delaying the appearance of regulatory T-cells. Further approaches for improvement could exploit the possibilities of enhancing PpIX accumulation as described in the introduction, where rather instantaneous clinical applicability might be the delivery of vitamin D3. Also, the application of brain hypothermia is an established procedure in neurology [234]. Apart from applying 100% oxygen breathing during PDT, it may be important to exploit preclinical observations of an improved tumor tissue response to PDT by fractionated irradiation [235]. How tumor tissue phototoxicity might be enhanced by simply interrupting irradiation a few times for a few minutes only can be understood when looking at oxygen diffusion between capillaries during irradiation, resulting in a relative depletion of oxygen available for the cells furthest away from the next capillary [236].

Other potential improvement concepts are in an earlier stage of preclinical proof of concept. Bioluminescence induced PDT may eventually overcome the challenge of a homogenous light delivery and dosimetry. The generation of a PDT-induced anti-tumor vaccine may support the immune stimulatory effect generated by iPDT. This approach has been discussed for a long time [237, 238] but is still in the stage of preclinical investigations [239].

CONCLUSIONS

5-ALA offers valuable diagnostic and therapeutic options in the management of malignant glioma. 5-ALA based fluorescence guidance has already become a standard adjunct for GBM surgery in many European countries and in Japan. The FDA approval obtained in 2017 will certainly further boost its use to achieve extended resection of malignant glioma safely. Multiple clinical studies have confirmed that PpIX fluorescence extends beyond the borders of Gd-contrast in MRI with a very high positive predictive value for strong fluorescence. The current technical implementation is cost-effective, simple to use and free of brainshift problems. Future technical improvements should first of all focus on adding red light excitation to render the judgement of the resection cavity at the end of surgery more reliable. In addition, pulsed excitation and spectral imaging should be considered to increase sensitivity and reduce the influence of artifacts.
caused by ambient light. Quantification of PpIX concentration is certainly desirable, but is difficult to obtain as long as the strong influence of extravasated blood cannot be eliminated. It might be more valuable to also consider biological boosts of a selective PpIX accumulation, such as vitamin D₃, iron chelators, or ABCG2 blockers.

Such biological boosters of selective PpIX accumulation are even more interesting for the therapeutic use of 5-ALA. The clinical experience reported so far is already quite promising and includes some intriguing long-term survivors. This may largely be attributable to the immunogenic nature of PDT-induced cell death and may also indicate the efficient destruction of stem-like tumor cells by PDT. PDT of the resection cavity or by stereotactic interstitial approaches is certainly warranted with higher patient numbers. Accordingly, trials are in preparation, including the development of the necessary equipment and dosimetry software. These trials should also generate data to identify immune stimulation parameters and outcome predictors, which may lead to improvement strategies.

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