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**Clinical Applications of Contact Spectroscopy in the
Intra-operative Detection of Fluorescence in High
Grade Glioma**

Thesis presented by

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for the degree of

Doctor of Medicine

University College Cork

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Declaration

This thesis is submitted by the undersigned to University College Cork for examination for the degree of Doctor of Medicine.

This thesis is the result of my own work. The material contained herein has not been presented, nor is currently being presented, either wholly or in part for any other degree or qualification, either at University College Cork or elsewhere.

All external references and sources are clearly acknowledged and identified within the contents. I have read and understood the regulations of University College Cork concerning plagiarism and Intellectual Property.

The research was carried out under the direction and supervision of Professor H. Paul Redmond, Department of Surgery, Cork University Hospital and Professor Michael G.J. O'Sullivan, Department of Neurosurgery, Cork University Hospital.

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Abbreviations

2-HG	2-hydroxyglutarate
5-ALA	five-aminolevulinic acid
ABCB6	ATP binding cassette subfamily B member 6
ABCG2	ATP binding cassette subfamily G member 2
ALAD	aminolevulinic acid dehydratase
ATRX	alpha thalassaemia/mental retardation X-linked
α -KG	alpha-ketoglutarate
BBB	blood-brain barrier
BRAF	V-raf murine sarcoma viral oncogene homolog B1
CPOX	coproporphyrinogen oxidase
CSF	cerebrospinal fluid
EGFR	epidermal growth factor receptor
EMA	European Medicines Agency
FDA	Food and Drug Administration
FGS	fluorescence-guided surgery
FECH	ferrochelatase
Gy	gray
HGG	high grade glioma
HIF-1 α	hypoxia-inducible factor-1 α
ICG	indocyanine green

IDH	isocitrate dehydrogenase
iMRI	intraoperative magnetic resonance imaging
LGG	low grade glioma
m ²	metre squared
MAPK	mitogen-activated protein kinase
mg	milligrams
MGMT	O ⁶ -methylguanine-DNA methyl-transferase
NADPH	nicotinamide adenine dinucleotide phosphate
NADH	nicotinamide adenine dinucleotide
nm	nanometers
PBGD	porphobilinogen deaminase
PpIX	protoporphyrin IX
PPV	positive predictive value
TME	tumour microenvironment
UROD	uroporphyrinogen decarboxylase
US	ultrasonography
VEGF	vascular endothelial growth factor
WHO	World Health Organisation

Presentations/Publications

PRESENTATIONS

“Intraoperative Optical Spectroscopy for the Detection of Tumour Fluorescence”

American Association of Neurological Surgeons Annual Scientific Meeting

2nd – 6th May 2024

Chicago, USA

PUBLICATIONS

Use of a Single-Fibre Optical Probe for the Detection of Tumour Fluorescence in High Grade Glioma.

Operative Neurosurgery 29(1):p 34-40, July 2025.. doi:
10.1227/ons.0000000000001397.

Abstract

Fluorescence-guided surgery is known to improve rates of complete resection in high grade glioma, translating to an improvement in progression-free survival. The conventional technique which utilises microscope widefield blue-light fluorescence, although beneficial, is associated with some drawbacks which may be addressed through utilisation of alternative contact-based techniques. Contact-based spectroscopy is helpful for detecting protoporphyrin IX (PpIX) fluorescence, but this technique has not been validated for clinical use.

In this work, we describe a series of clinical research experiments whereby the use of an optical fibre probe for the detection of PpIX is investigated. In chapter 2, a prospective comparative study demonstrates that the microscope-based technique identified PpIX fluorescence with an accuracy of 61%, while the optical probe was 74% accurate.

In Chapter 3, the characteristics of the probe are investigated in a cadaveric animal model, followed by in vivo use to demonstrate the practicality of the probe. Here, we demonstrate that use of the probe is not associated with an increase in post-operative complications. Finally, chapter 4 describes the design of a machine-learning algorithm which aims to determine IDH status and MGMT methylation status based on spectroscopic features. Here, we describe how a prediction model may be able to determine IDH status but is unreliable for predicting MGMT methylation status.

In conclusion, optical fibre spectroscopy demonstrates an improved level of accuracy compared to the conventional microscope-based technique, is not associated with intra- or post-operative complications, and may offer the ability to determine certain molecular characteristics of tumour tissue in real time.

Chapter 1: Introduction

1.1 Glioma

Gliomas are the commonest primary brain tumours in adults, with an incidence of approximately 6 per 100,000 per year(1). These tumours can originate from different cells of the central nervous system including astrocytes, oligodendrocytes and ependymal cells. Gliomas therefore comprise a heterogenous group of lesions with numerous histological subtypes and molecular profiles, and thus the approach to their management can be varied. Adult gliomas are often diffusely infiltrating, whereas in the paediatric population tumours may be infiltrating or circumscribed. The 2021 World Health Organisation (WHO) Classification of Tumours of the Central Nervous System(2) groups diffusely infiltrating gliomas according to their immunohistochemical, ultrastructural and molecular features (figure 1.1).

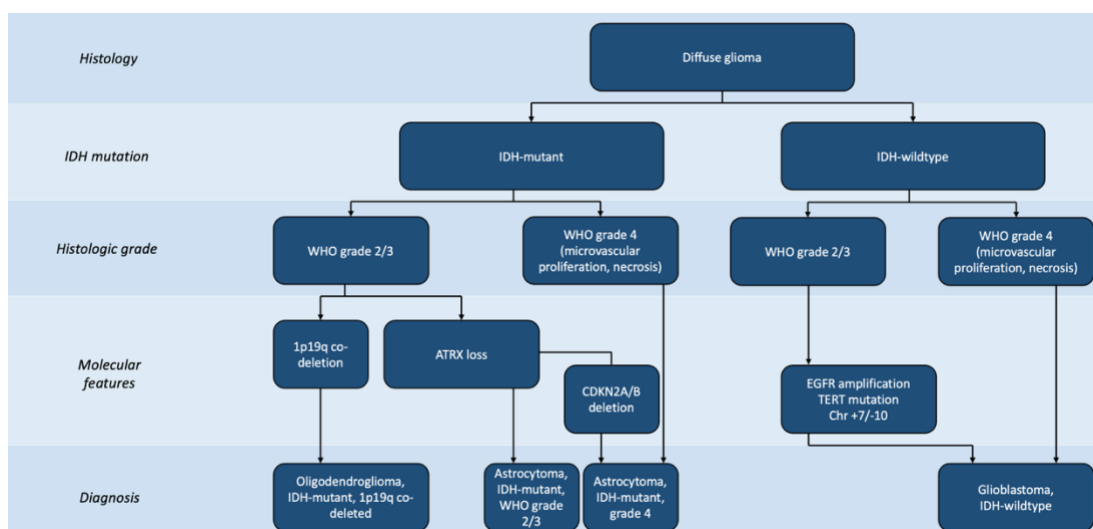


Figure 1.1 Diagnostic algorithm for adult diffuse-type gliomas (based on 2021 WHO CNS tumour classification). This figure demonstrates the importance of molecular features for providing an integrated final diagnosis.

In Ireland, over 300 adults are diagnosed with diffuse gliomas per year, of whom over 70% will have a diffuse high grade glioma (HGG). Most arise sporadically, while approximately 5% are associated with genetic cancer syndromes(3). Common presenting symptoms include seizures, focal neurological deficit and cognitive disturbances. Tumour grade, as well as molecular and immunohistochemical features, is related to the behaviour and prognosis associated with these tumours. The overall median survival for diffuse HGG remains between only 14 and 16 months,

without significant improvements since the introduction of the Stupp protocol of radio-chemotherapy almost two decades ago(4).

HGG cells are known to spread through the brain by a variety of routes, including perineural and perivascular spread, as well as along axons and white matter fibres. The management of patients with diffuse high grade glioma is multifaceted. Standard treatment consists of surgery for maximal safe debulking, followed by the so-called Stupp protocol. This treatment protocol consists of an initial phase of oral temozolomide administration at a dose of 75mg/m² on days 1 through 42, with concurrent radiotherapy at a total dose of 60Gy. This is followed by 6 consecutive 4-week cycles of temozolomide alone at a dose of 150-200mg/m².

Additional therapies which have been investigated include tumour-treating fields(5), intra-cavitary chemotherapy wafers(6), photodynamic therapy(7), and immunotherapy(8). These therapies have offered varying degrees of success in the treatment of patients with high grade glioma, although their adoption into the treatment algorithm for these patients varies widely.

In general, the effects of surgery, radiotherapy and chemotherapy in combination offer the best chance of control in HGG. Nonetheless, the importance of surgical resection cannot be understated. Lacroix et al.(9) identified that a resection threshold of 98% significantly improved median survival from 8.8 months to 13 months. In comparison, earlier work investigated the utility of radiotherapy alone, without surgical debulking, and identified a very modest improvement in survival to 35 weeks from 14 weeks(10).

1.2 Molecular biology of glioma

1.2.1 IDH

Isocitrate dehydrogenase (IDH) is an enzyme which plays a key role in energy metabolism, where it acts to catalyse the oxidative decarboxylation of isocitrate to alpha-ketoglutarate (α -KG). It is also involved in the reduction of NADP⁺ to nicotinamide adenine dinucleotide phosphate (NADPH) (figure 1.2). IDH exists in

three forms – IDH 1 which is primarily located in peroxisomes and cytoplasm, and IDH 2 and 3 which are present in mitochondria(11).

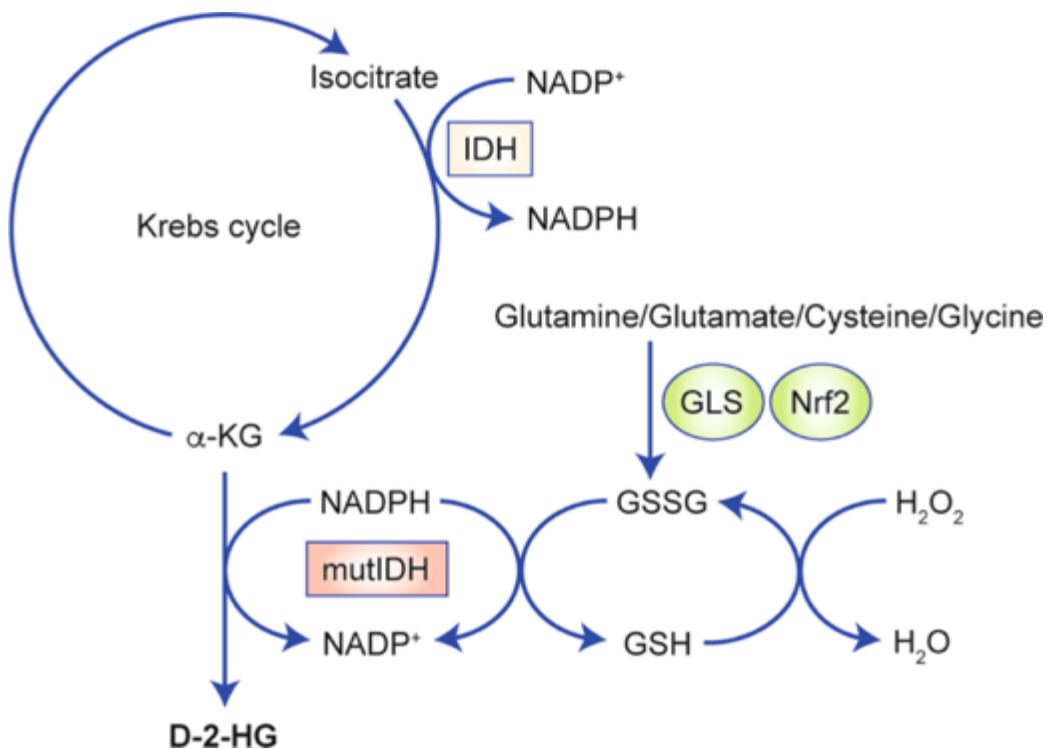


Figure 1.2 Role of IDH (adapted from Han et al, 2020(12)). IDH is involved in the reduction of NADP⁺ to NADPH and the oxidative decarboxylation of isocitrate to alpha-ketoglutarate.

Several IDH mutations are associated with adult diffuse gliomas, the most common among them being the IDH1 R132H mutation(13). These mutations contribute to glioma development by producing high levels of 2-hydroxyglutaric acid (2-HG) which acts to inhibit glioma stem cell differentiation, upregulate vascular endothelial growth factor (VEGF) expression, and increase the levels of hypoxia-inducible factor-1α (HIF-1α).

In addition to their involvement in the pathogenesis of glioma, IDH mutations are involved in the tumour microenvironment (TME) of gliomas and in their invasion into the adjacent brain. The invasive nature of glioma is the primary surgical challenge in the management of this disease and is the main reason for the recurrence and local aggressiveness with which it is associated. Recently, vaccines and immune therapies targeting the IDH gene mutations have been investigated and developed with the aim of delaying recurrence and improving survival(14).

Adult diffuse gliomas are classified by their IDH status, i.e. IDH-wildtype glioblastoma is differentiated from IDH-mutant diffuse astrocytoma by the absence of an IDH gene mutation(2). Low grade (grade 2) diffuse adult glioma is commonly associated with an IDH mutation, whereas high grade (grade 3 and 4) tumours are classified into IDH-mutant astrocytoma grades 3 or 4, or IDH-wildtype glioblastoma. Generally, tumours harbouring an IDH mutation are associated with a better prognosis(15).

Immunohistochemistry detects the IDH1 R132H mutation, the most frequent variant which accounts for up to 90% of IDH-mutated cases(2,16). Other less common mutations such as the IDH1 R132C, IDH1 R132G and IDH2 R172K mutations are identified using next-generation sequencing techniques. This process increases the length of time taken to achieve a molecular diagnosis.

1.2.2 1p19q

Combined loss of the short arm of chromosome 1 and the long arm of chromosome 19 is associated with tumours of oligodendroglial cell origin, and its presence is necessary for the diagnosis of oligodendroglioma(2). Additionally, this finding is recognised as a predictive marker of therapeutic response to chemotherapy(17–19).

1.2.3 ATRX

ATRX is a histone chaperone protein which plays a key role in brain development(20). Inherited mutations result in alpha thalassaemia/mental retardation X-linked syndrome(21), while acquired mutations have been reported in several human cancers, including pancreatic neuroendocrine tumours(22) and osteosarcoma(23). In glioma, ATRX mutations and IDH mutations are strongly correlated(24), while ATRX mutations and 1p19q codeletions rarely (if ever) co-exist(25). In brief, therefore, ATRX mutations can be correlated with astrocytic cell lineage, while 1p19q co-deletion suggests a tumour of oligodendroglial origin(26).

1.2.4 MGMT

O⁶-methylguanine-DNA methyl-transferase (MGMT) promoter methylation status is another important prognostic biomarker in glioblastoma. MGMT functions to

remove alkyl groups from the O-6 guanine position, repairing the DNA damage that occurs as an effect of alkylating chemotherapies(27) such as temozolomide.

There exist various methods of determining MGMT status(28), with some authors suggesting discrepancies between MGMT promoter methylation status and MGMT protein/gene expression and/or patient outcome(29–31). Nonetheless, there is a significant association between MGMT hypermethylation and efficacy of temozolomide(32).

1.2.5 EGFR

Epidermal growth factor receptor (EGFR) is a transmembrane protein involved in cellular signalling pathways relating to tumour metabolism, invasion and proliferation(33). Its presence in glioblastoma is associated with more aggressive behaviour and it has also been implicated in the development of resistance to radio-chemotherapy(34). The commonest EGFR mutation in glioblastoma is the in-frame deletion of exon 2–7, termed EGFRvIII, and occurs in approximately 50% of cases of glioblastoma where EGFR is amplified(35).

1.2.6 BRAF

V-raf murine sarcoma viral oncogene homolog B1 (BRAF) encodes the B-raf protein which plays a crucial role in the MAPK pathway. BRAF mutations are associated with a variety of human cancers, and in tumours of the central nervous system they contribute to both tumour development and progression(36). While these mutations are more frequently encountered in paediatric brain tumours, they can be seen rarely in glioblastoma, particularly the epithelioid subtype(37). Data investigating the prognostic role of BRAF mutations in glioblastoma are lacking. BRAF mutations can offer a role as targets for immune therapies in the treatment of certain other tumour types.

1.3 Surgical management of glioma

Although complete surgical clearance is impossible, surgery remains an important aspect of the management of patients with high-grade glioma. Maximal surgical

resection, although not curative, is strongly associated with improved progression-free and overall survival(38–40). The extent of resection must however be balanced against the risk of incurring a neurological deficit – therefore safe and accurate methods of detecting tumour tissue are essential.

Several adjuncts exist to assist the neurosurgeon with the intra-operative identification of tumour tissue. Imaging techniques such as intraoperative magnetic resonance imaging (iMRI) and ultrasonography (US) have emerged as methods of addressing this. iMRI has been shown to increase rates of gross total resection (GTR)(41), however it prolongs the length of surgery and adds to the complexity of the surgical workflow(42).

Intraoperative US is less time-consuming and does not significantly alter the surgical workflow, while providing benefit in relation to tumour localisation and delineation from surrounding neurovascular structures(43,44). It has also been shown to improve the extent of resection(45). However, it can be limited by the tumour type and consistency, and previous treatment(44).

Fluorescence-guided surgery (FGS) improves the visualisation of tumour margins and improves the extent of surgical resection(46). Its use has been demonstrated to improve progression-free survival for patients with high-grade glioma(47). It demonstrates ease of integration into the surgical workflow and the skills required to perform FGS are readily acquired without a steep learning curve. Additionally, FGS has been shown to be non-inferior to iMRI and is associated with a shorter duration of surgery(48).

1.4 Fluorophores and their neurosurgical applications

Fluorescence is defined as the process in which a material absorbs light at one wavelength and emits light at a different wavelength. The discovery of exogenous fluorophores dates to the late 19th century with the synthesis of fluorescein in 1871(49). Neurosurgical applications of fluorescence began with fluorescein in 1948, which continues to be of use in the resection of brain and spinal tumours(50), and in the treatment of cerebrospinal fluid (CSF) fistulas. Indocyanine green (ICG) was

discovered later and was first utilised in cerebrovascular neurosurgery in the early 21st century(51) and continues to be useful not only for vascular indications, but also in oncology and endoscopic surgery.

Five-aminolevulinic acid (5-ALA) was described by David Shemin in 1953(52) and was initially used in the diagnosis and management of skin, gastrointestinal and bladder cancers. Its use in glioma surgery was first described by Walter Stummer in 1998(53,54), and following a randomised, controlled trial in 2006 which demonstrated improved rates of complete resection of high grade glioma and improvements in progression-free survival(47), it was licensed for use in high grade glioma surgery by the European Medicines Agency (EMA) in 2007. The United States Food and Drug Administration (FDA) followed suit several years later, approving its use in 2017.

5-ALA has several attractive features when compared to other, exogenous fluorophores; firstly, it is an endogenous human metabolite that is synthesised naturally from succinyl-coenzyme A and glycine in the mitochondria and therefore its use is not associated with side effects that are often seen from administration of foreign agents(55). Secondly, it is rapidly metabolised in the liver, with up to 80% of it being excreted within 24 hours(56).

In high grade glioma, 5-ALA is preferentially metabolised towards protoporphyrin IX (PpIX), which has interesting fluorescent properties (figure 1.3). PpIX fluorescence is of great clinical value as it can provide immediate intraoperative feedback, correlates reliably with imaging features, and demonstrates ease of integration with the surgical workflow.

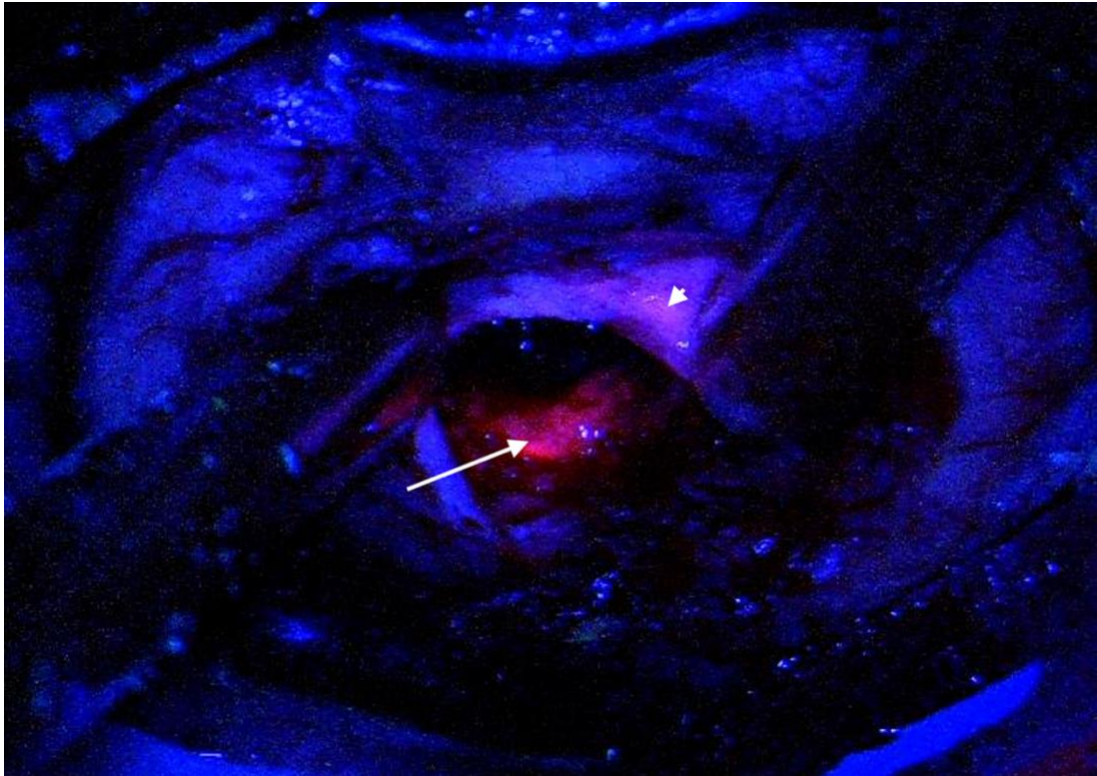


Figure 1.3 PpIX fluorescence in a left parietal glioblastoma; "solid" (arrow) and "vague" (arrowhead) fluorescence is demonstrated. Solid fluorescence is typically associated with the enhancing portion of the tumour, while vague fluorescence is typically seen in the peripheral tumour-infiltrating zone.

1.5 5-ALA induced fluorescence in glioma

PpIX can accumulate in cells due to several reasons, including increased 5-ALA levels, 5-ALA synthase hyperactivity, or dysfunction of the ferrochelatase (FECH) enzyme (which produces heme from PpIX). Changes in numerous other enzymes involved in the heme synthesis pathway, such as porphobilinogen deaminase (PBGD), ALA dehydratase (ALAD), uroporphyrinogen decarboxylase (UROD) and coproporphyrinogen oxidase (CPOX), have been associated with altered levels of PpIX in not only human glioma but also other cancer tissues(55). Although the precise mechanisms for PpIX accumulation in glioma cells are not fully understood, several contributing factors have been described (figure 1.4).

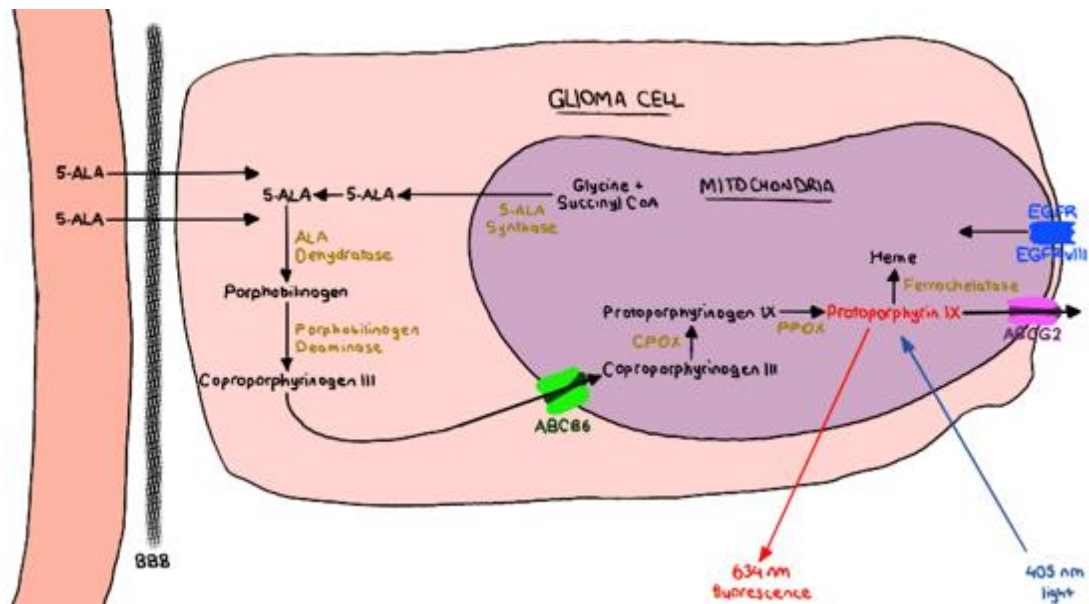


Figure 1.4 Molecular mechanisms underlying PpIX accumulation in glioma cells. The role of BBB breakdown, enzyme dysfunction, and cell membrane proteins is demonstrated.

Blood-brain barrier dysfunction is thought to play a role in the accumulation of PpIX in high grade glioma tissue(57). PpIX has been seen to accumulate in tumour tissues and in other areas without a defined blood-brain barrier(58). The intact blood-brain barrier is relatively impermeable to 5-ALA, although liposomal encapsulation has been shown to facilitate transport and increase porphyrin concentrations in the brain(59,60).

Cellular transport mechanisms (e.g. ABCB6 and ABCG2 proteins) and enzyme expression may also play a role in PpIX concentrations in glioma cells(61–63). Additionally, environmental parameters which may be altered in the tumour microenvironment may also affect the levels of PpIX. Examples include changes in oxygen levels, temperature, and pH(64).

During glioma surgery, PpIX fluorescence can be visually observed in tissue where the cell density is at least 20-30%. With current techniques, fluorescence in low-grade gliomas and in the so-called “low-density infiltration zone” at the periphery of high-grade gliomas cannot be reliably detected. Many recurrences in high grade glioma occur near the resection margins(65), however, and therefore reliable methods of detecting tumour tissue in this marginal zone represent an unfulfilled need.

1.6 Fluorescence-guided surgery for low grade glioma

While the use of 5-ALA fluorescence is well-established in high grade glioma surgery, this is not the case in low grade glioma (LGG). Maximal safe resection of LGG leads to a significant improvement in prognosis(66–68) and in some cases complete resection may potentially be curative. However, the utility of FGS is limited given that visible fluorescence is only seen in 0-19%(69–74) of cases. Resection is made difficult by the fact that LGG tissue can be exceedingly difficult to differentiate from normal white matter. Therefore, there is a need for improved visualisation, either through improvement of existing methods of visualisation or the development of new techniques.

While 5-ALA fluorescence may be visible in a small proportion of LGGs, assessment of fluorescence by quantitative means leads to improvements in sensitivity from 21-30% to up to 58%(74,75). Work by Widhalm et al(75) and Valdes et al(74) has described the use of quantitative measurements of PpIX to improve the sensitivity of this technology in surgery for LGG.

Notably, most studies investigating the role of PpIX fluorescence in LGG were described prior to the introduction of the 2021 WHO classification of CNS tumours(2), which places particular emphasis on molecular characteristics. These cohorts(73,75–77) included patients with IDH-wildtype tumours which would now be classified by molecular features as grade 4 tumours. Further work into quantitative assessments of PpIX fluorescence in “true” low grade tumours is therefore warranted.

1.7 Intra-operative assessment of fluorescence

Various methods of fluorescence detection have been described(54,78,79). The most common technique in clinical practice is the use of a neurosurgical microscope with a blue-light filter that can deliver light at 405nm wavelength into the surgical cavity. The current microscope-based technique demonstrates sensitivities of 20-30%, specificities above 90% and overall accuracies between 70 to 90% in high grade glioma(80,81). The limitations in sensitivity can partly be attributed to the geometric

constraints of light collection (for example deep surgical cavities, or areas that are hidden by more superficial brain tissue).

Contact probe technologies may provide improved results as they circumvent the problems of suboptimal light excitation and collection that are an inherent limitation of microscope-based tools. Additionally, tissue fluorescence is affected by the absorption and scattering of light that occurs both at excitation and emission wavelengths, and therefore visual assessments of fluorescence are inherently subjective in nature as a result of tissue optical properties(82,83). Quantitative assessments using optical technology can alleviate these factors.

Several works have attempted to characterise the spectral properties of gliomas by investigating a variety of contributing biomarkers. Although PpIX is known to be involved in the visualisation of fluorescing tissue, the sensitivity is not high enough to allow the detection of high grade glioma tissue in the low density infiltrative zone. Additionally, there are other endogenous fluorophores which fluoresce in the same spectral region as PpIX and can mask the fluorescence signal, including nicotinamide adenine dinucleotide (NADH)(84), lipofuscin(85) and flavin(86). It has been suggested that the use of these autofluorescence spectra in addition to PpIX may improve the accuracy of the technique in the delineation of tumour margins(87).

1.8 Contact spectroscopy

Contact fluorescence spectroscopy is a promising technique where a probe excites molecules in a material with light and collects emitted fluorescence directly at the point of contact. This technique possesses several advantages over the microscope-based technique(88). It affords improved light excitation and collection geometries due to the contact-based nature of the technology. It offers improved spatial resolution at the millimetre level. It can also provide full fluorescence spectra which has potential benefits in terms of extraction of specific spectral features and parameters.

Intraoperative contact spectroscopy requires an excitation source, usually a laser emitting diode (LED) emitting blue light, in addition to a fiber-optic probe and a

spectrometer which receives emitted light via this fibre. Computer software can then record the spectra in real time and compare the emission to known profiles in order to detect the presence of PpIX.

Despite the aforementioned benefits of contact spectroscopy, no studies have validated the use of this technology in large patient cohorts and therefore the use of this information to guide decision-making may not yet be justified. This thesis aims to establish the accuracy of contact spectroscopy compared to widefield blue-light microscopy, determine the ease of integration into existing workflows, and explore the relationship between spectral features and the genetic makeup of glioma.

Chapter 2: How Accurate is Contact Spectroscopy for PpIX Identification?

2.1 Abstract

Background: High grade glioma is the most common primary brain tumour in adults. The overall median survival is between 14-16 months. Fluorescence guided surgery by detection of PpIX fluorescence has been shown to improve extent of resection, translating to improved progression-free survival. PpIX is also known to accumulate at lower concentrations in low grade glioma. Microscope-based fluorescence detection techniques are associated with several pain-points, some of which may be addressed by using contact-based spectroscopy.

Aims/objectives: We aimed to investigate the accuracy of an optical fibre at detecting PpIX fluorescence by performing real-time spectroscopy in patients with high grade glioma.

Methods: Adult patients undergoing fluorescence-guided surgery for high grade glioma were recruited prospectively. Intra-operatively, samples from cortex, white matter and tumour were taken. These samples were interrogated using standard white and blue light microscopy, and the optical fibre. The specimens were then assessed by neuropathology to determine whether tumour tissue was present within them.

Results: We collected 80 samples from 25 patients. The median age was 66 years and 56% of patients were female. The accuracy of the probe for detecting PpIX fluorescence was 74.3%, compared with 60.8% for the operating microscope.

Conclusion: The optical fibre probe was more accurate than the operating microscope for detecting PpIX fluorescence. This technology has potential value in improving the accuracy of fluorescence-guided surgery and has potential practical benefits relating to surgical workflow.

2.2 Introduction

High grade glioma (HGG) is the most common primary brain tumour in adults, with an incidence between 3.2 to 4.4 per 100,000 per year(89). The overall median survival remains between 14-16 months, without significant improvements since the introduction of the Stupp protocol of radio-chemotherapy almost two decades ago(4).

HGG cells are known to spread through the brain by a variety of routes, including perineural and perivascular spread, as well as along axons and white matter fibres. Although complete surgical clearance is impossible, surgery remains an important aspect of the management of these patients, and maximal surgical resection, although not curative, is strongly associated with improved progression-free and overall survival(38).

Given the established correlation between extent of resection and oncological outcomes(9,38,39,68), technologies which can improve the extent of resection are of great interest. Among these surgical adjuncts, FGS emerged in the late 1990s(53,54) and has been shown to improve extent of resection, translating to improved progression-free survival[44]. 5-aminolevulinic acid (5-ALA) is a prodrug which can be administered orally, and results in accumulation of higher levels of protoporphyrin IX (PpIX) in tumour cells as compared to normal healthy brain tissue(55,57,64). PpIX absorbs blue light and emits it as red light which allows for it to be used as a fluorescent marker of abnormal tumour cells. The landmark paper on 5-ALA guided surgery identified an improvement in rates of complete resection from 36% to 65%, translating to a higher 6-month progression-free survival(47).

Although its use has been most robustly described in HGG, elevated levels of PpIX are found in other pathologies including low grade glioma(90), meningioma(91) and some metastatic tumours(92). In low grade glioma, the rates of visible fluorescence have been reported to be in the range of 0 to 19%(69–74). However, when quantitative measurements of PpIX are performed, the rate of positivity rises to close to 70%(74).

Different methods of fluorescence visualisation have been described. The most common technique involves the use of a neurosurgical microscope with a blue-light filter that can deliver light at 405nm wavelength into the surgical cavity. Using this technique, abnormal tissue is identified under blue light, and the surgeon then switches back to white light to continue the resection. This process is repeated until the resection has been completed to his/her satisfaction.

The current microscope-based technique demonstrates sensitivities of 20-30%, specificities > 90% and overall accuracies between 70 to 90% in high grade glioma(80,81). The limitations in sensitivity can partly be attributed to the geometric constraints of light collection (for example deep surgical cavities, or areas that are hidden by more superficial brain tissue), but also to the natural sensitivity of the human eye.

Some “pain-points” with this current technique include the significant cost of high-specification microscopes, subjectivity in assessments of vague fluorescence between surgeons, and the aforementioned limitations in sensitivity (due, for example, to the particulars of light delivery and collection in the context of glioma surgery)(82,83).

Contact spectroscopy is a promising technique which has several advantages over the microscope-based technique(88). It affords improved light excitation and collection geometries, and improved spatial resolution at the millimetre level. It can also generate full fluorescence spectra which allows the extraction of multiple spectral parameters. The use of contact optical fibre technology for FGS has been suggested previously as a potential solution to some of the previously mentioned pain-points(88), but a robust fibre-optic solution has not been validated to date. In this work, we describe a novel device that delivers blue light at 405nm and detects PpIX fluorescence spectra through a single optical fibre (figure 2.1).

The objective of this study was to determine the accuracy of an optical fibre for detecting PpIX fluorescence in patients with high grade glioma and compare it to the standard microscope-based technique.

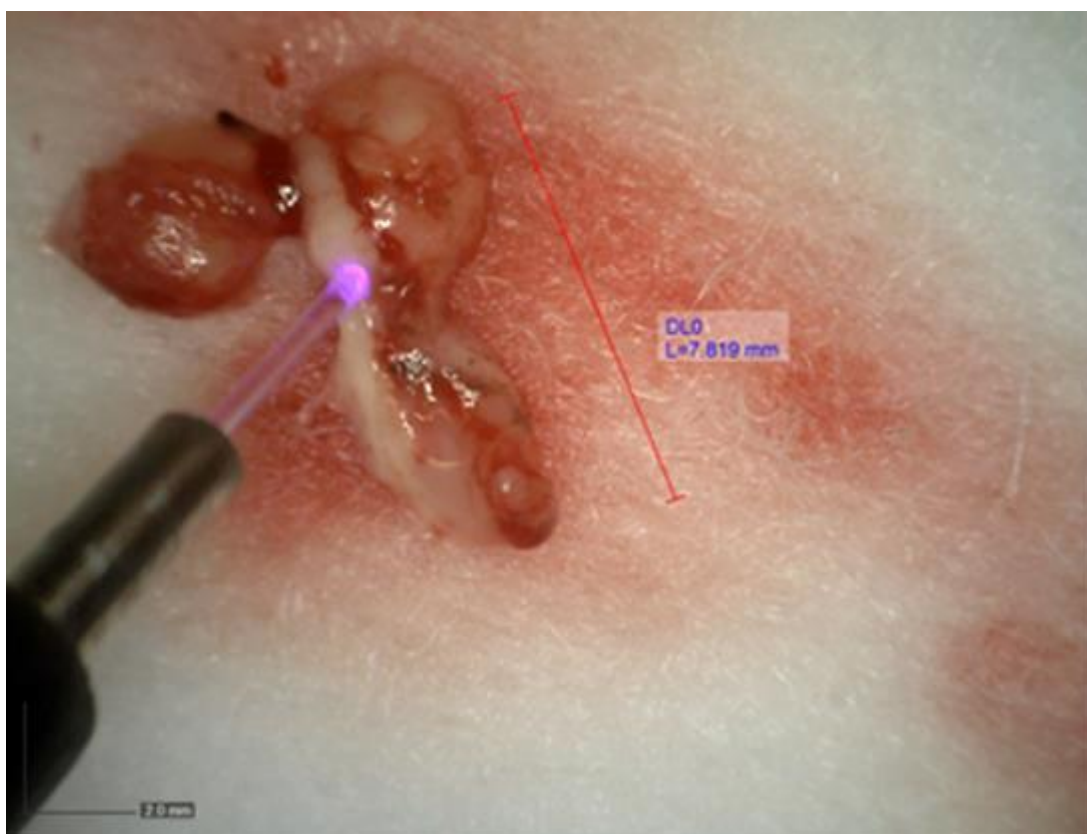


Figure 2.1 Optical fibre being used to assess a piece of tissue. The fibre probe interrogates an area of tissue measuring fractions of a millimetre.

2.3 Methods

The optical fibre device (figure 2.2) transmits 405nm wavelength blue light to the tip of the optical fibre. Light at this wavelength has a penetration depth of the order 500 microns in brain tissue. When touched to a piece of tissue, the 405nm light interacts with any PpIX that is present and any fluorescence that is generated can travel back up the same fibre to a spectrometer. These spectra can be saved as raw data, or they can be processed by a computer and have features extracted from them. A probe reading was considered positive when PpIX fluorescence was detected from a piece of tissue. PpIX fluorescence was defined to be detected when the height of the 635nm emission band was greater than 200 counts (six times the standard deviation of the spectrometer noise) above the surrounding baseline in at least three spectra (figure 2.3). The assessment of the spectra was carried out on blinded and randomised data and had to be agreed by two investigators.

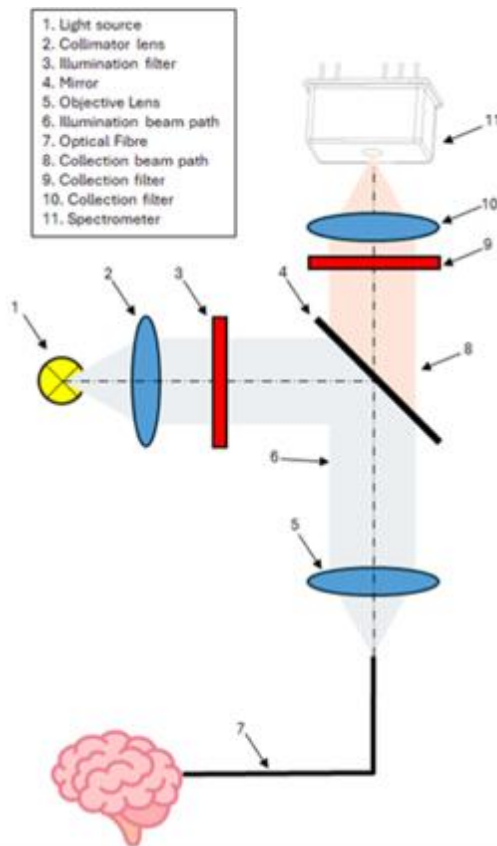


Figure 2.2. Spectroscopy hardware setup. The probe fibre (labelled 7) is connected to a fluorescence detection system comprising the blue LED excitation, fluorescence microscope optics (lenses, mirrors and filters) and a spectrometer.

Ethical approval for the study was obtained from the local Clinical Research Ethics Committee. We recruited adult patients who presented to our regional neurosurgical unit with suspected HGG (based on imaging characteristics) for inclusion in this study. In addition, patients with a previous diagnosis of HGG who presented with recurrent tumours were also considered for inclusion. We included patients who were listed for craniotomy for 5-ALA guided resection, or for 5-ALA guided biopsy, of their tumours. Those undergoing surgery without the use of 5-

ALA and those listed for stereotactic needle biopsy were excluded. Data were collected between January 2022 and May 2023. Demographic characteristics were captured for each case.

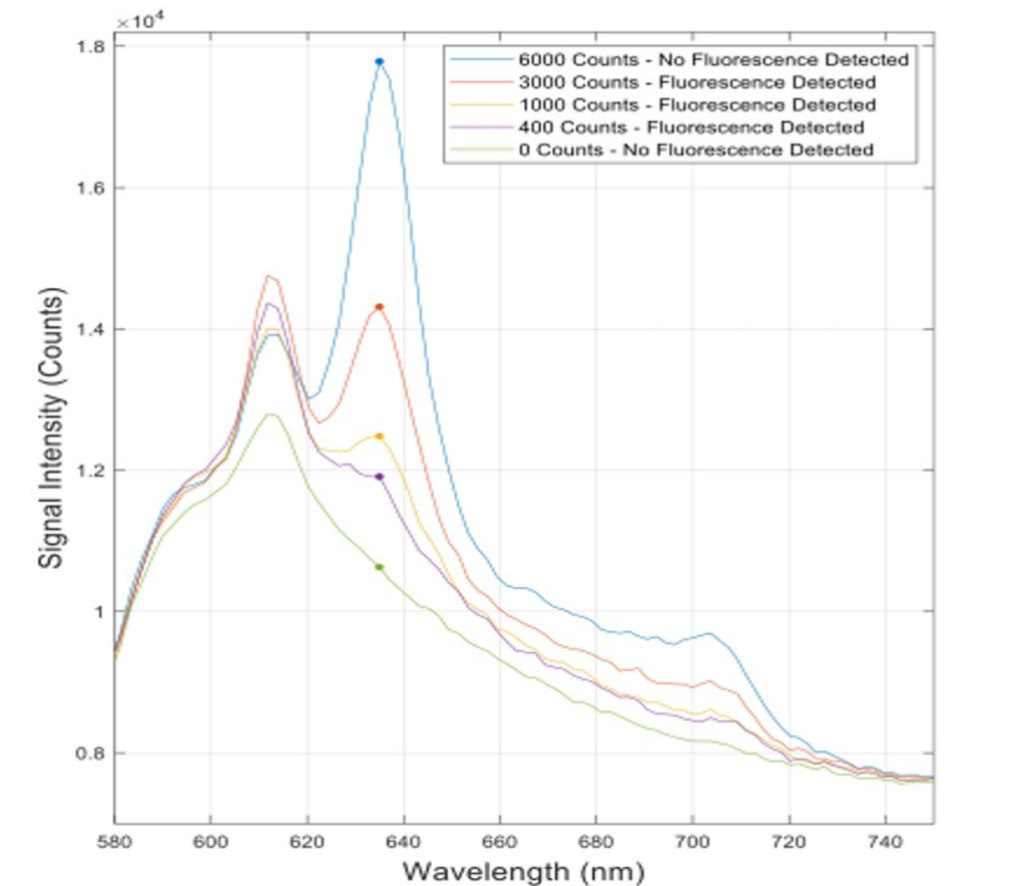


Figure 2.3. Protoporphyrin IX fluorescence spectra. Spectra were adjudged to show PpIX fluorescence where there was a band at 635nm that had an intensity greater than 200 counts. The 200 counts was measured from the local baseline and was 6 times the standard deviation of the spectrometer noise.

Eligible patients were provided with a Patient Information Leaflet, and those who consented to inclusion in the study received oral 5-ALA at a dose of 20mg/kg (Gliolan; medac GmbH, Wedel, Germany) approximately 2-4 hours prior to induction of anaesthesia and proceeded to surgery as per the standard local protocol. At surgery several specimens were collected from cerebral cortex, white matter and tumour. These tissue samples were viewed under the white light and blue light of the neurosurgical microscope (Zeiss Kinevo 900; Carl Zeiss AG, Oberkochen, Germany) prior to removal and their appearances were recorded. Once the specimens were taken, they were then interrogated with the fibre probe and fluorescence spectra

were recorded. Finally, the specimens were delivered to the neuropathology laboratory where they were assessed by two neuropathologists for the presence or absence of tumour material. This procedure did not interfere with or delay the standard workup for delivering a tissue diagnosis.

Following investigation with the probe, the tissues samples (i.e. cortex, white matter and tumour) were fixed in buffered formalin and then using standard laboratory protocols processed to infiltrate with histological wax prior to cutting and staining of tissue sections on a microtome. The sections were stained with haematoxylin and eosin and reviewed by the reporting neuropathologist. They were assessed for the presence or absence of tumour. Tissue showing areas suspicious for tumour infiltration were described as such.

After the fluorescence spectra were downloaded from the device, the data were blinded and randomised, and each spectrum was assessed for the presence or absence of a PpIX emission band. Results were then tabulated by matching each spectrum/specimen with its corresponding histopathological evaluation, appearance under white light, and visual (microscope) fluorescence. We created 4x4 confusion matrices comparing both the microscope appearances and the probe measurements against the histopathology results. Descriptive statistical analysis was performed in IBM SPSS v28.0. McNemar's test was performed to investigate the difference between the sensitivity of the optical fibre and that of the microscope.

2.4 Results

Twenty-five patients were recruited during the study period (table 2.1). A total of 80 tissue samples were obtained, with an average of 3.2 specimens per patient (range 2-6, SD 0.818). Due to a technical error resulting from inadvertent disconnection of the probe's optical components, measurements were not obtained from one patient (6 samples) and therefore the results of 74 samples are included in our analysis. Fourteen patients (56%) were female. The median age was 66 years (range 34-86 years). Four patients (16%) were undergoing repeat surgery for recurrent tumours.

Nineteen patients had a final integrated diagnosis of IDH-wildtype CNS WHO grade 4 glioblastoma, 3 had IDH-mutant CNS WHO grade 4 astrocytoma, 2 had IDH- wildtype CNS WHO grade 3 astrocytoma, and 1 had IDH-mutant CNS WHO grade 3

N = 25		
Age (median)		66 years (range 34-86)
Sex		
	Female	14
	Male	11
Tumour location		
	Frontal	12
	Temporal	9
	Parietal	2
	Occipital	1
	Thalamic	1
Pathological diagnosis		
	Glioblastoma, IDH-wildtype	19
	Astrocytoma grade 4, IDH-mutant	3
	Astrocytoma grade 3, IDH-wildtype	2
	Oligodendroglioma grade 3, IDH-mutant	1

Table 2.1 Patient demographics.

oligodendroglioma. The most common tumour locations were the frontal lobe (48%) and temporal lobe (36%). The most common presenting symptoms were focal neurological deficit (n=10), cognitive impairment (n=7) and seizure (n=7).

The presence of fluorescence under the neurosurgical microscope correlated with histopathology with an accuracy of 60.8% (sensitivity 56.7%, specificity 100%). The accuracy of the fibre probe was 74.3% (sensitivity 71.6%, specificity 100%). There was a significant difference between the sensitivity of the optical fibre and that of the microscope ($\chi^2 = 11.84$, $p < .001$). The confusion matrices representing these findings are demonstrated in tables 2.2 and 2.3.

		Histology	
		Positive	Negative
Microscope	Positive	38	0
	Negative	29	7

Table 2.2. Confusion matrix demonstrating the microscope results compared to the histological gold standard. Sensitivity and accuracy results are calculated from these figures.

		Histology	
		Positive	Negative
Fibre probe	Positive	48	0
	Negative	19	7

Table 2.3. Confusion matrix demonstrating the fibre probe results compared to the histological gold standard. Sensitivity and accuracy results are calculated from these figures.

2.5 Discussion

Our data suggest that this optical fibre probe is promising for use in the identification of PpIX fluorescence in high-grade glioma. Our preliminary results demonstrate a statistically significant improvement in sensitivity in comparison to the neurosurgical microscope, resulting in an improvement in overall accuracy from 61% to 74%.

When relying on visual inspection, the reporting of PpIX fluorescence can vary due to differences in interpretation by different observers[90]. Stummer et al described differing intensities of fluorescence in early work on FGS(93), and while “solid” fluorescence is quite easily visible, the “vague” fluorescence which can often be seen at the so-called edge of a tumour or in areas with less cell density can be described differently by different surgeons. Therefore, the ability of this probe to detect PpIX and report a binary “yes” or “no” result when fluorescence is, or is not detected, would be of value.

Furthermore, there are suggestions that PpIX fluorescence is present in low grade gliomas at concentrations that cannot be detected by visualisation(74). Previous work by Valdés et al described concentrations of PpIX in normal tissue, non-visibly fluorescing low grade glioma tissue, and visibly fluorescing low grade glioma tissue(74). In theory the sensitivity of the optical-fibre probe could be adjusted to detect lower levels of PpIX and therefore there is the potential for this technology to be useful in low grade glioma surgery, although further dedicated research into this area is required.

Additionally, there are several potential practical benefits. The visibility of PpIX fluorescence is reduced as the distance between the blue light source and the tissue increases, limiting the use of the technology in its current form in deep resection cavities(94). The use of a contact device such as the one described in this work reduces this effect. Furthermore, the probe can be used to touch tissue which may not be directly in the surgeon's line of sight and may therefore be hidden from the blue light of the operating microscope. This may be useful in identifying areas of tumour hidden around the edges of the resection cavity and under more superficial folds of brain tissue.

Limitations of this study include the relatively small sample size. Based on the catchment area of our department we anticipated approximately 40-45 cases of glioblastoma per year, not all of whom were suitable for fluorescence-guided surgery. Additionally, several patients did not consent to inclusion in the study, resulting in our current study size.

The variation in numbers of specimens per patient resulted from differences in tumour location and suitable regions of cortex and white matter available for sampling. Future research could consider these limitations by prolonging the study duration and attempting to further standardise the sampling protocol.

2.6 Conclusions

This work demonstrates that this optical fibre technology appears to have higher sensitivity when compared against the current microsurgical method, without compromising specificity. Additional research utilising more advanced methods of tissue analysis may be of benefit.

The utility of this technology in HGG will likely stem from the objective nature of the results delivered and from practical applications of the probe. In the area of low-grade glioma surgery, further research is needed to examine the potential utility of this probe in reliably detecting the lower concentrations of PpIX often seen in these tumours.

Chapter 3: Practical Applications of the Optical Fibre

3.1 Abstract

Background: Fluorescence-guided surgery improves the extent of resection and progression-free survival in high grade glioma surgery. Contact optical spectroscopy provides an alternative method of fluorescence detection to the standard microscope-based technique, and has potential practical benefits.

Aims/objectives: To evaluate the safety and accuracy of contact optical spectroscopy in glioma surgery.

Methods: A pre-clinical study was performed in a cadaveric animal 5-ALA enriched tumour model, followed by subsequent in vivo testing of the optical probe in patients undergoing fluorescence-guided glioma resection surgery.

Results: In one cadaveric animal model and 3 humans subjects (intraoperative data collection), the optical probe demonstrated no cortical/white matter injury and no increase in post-operative complications. The optical probe was 88.9% accurate for protoporphyrin IX detection, compared to 33.3% with the operative microscope.

Conclusions: Contact optical spectroscopy is a safe, efficient and accurate technique in fluorescence-guided glioma surgery.

3.2 Introduction

The introduction of fluorescence-based techniques in glioma surgery has led to improvements in extent of resection and progression-free survival in high grade glioma (HGG)[44]. The standard microscope technique has been in widespread use since the mid-2000s, and relies upon the surgeons ability to visualise the contrast between normal tissue and fluorescing tumour tissue. Other technologies have emerged which aim to improve the detection of fluorescence, such as loupe-based illumination and visualisation tools[76], and contact-based techniques.

Contact-based spectroscopy affords improved spatial resolution, and in addition to reliably detecting protoporphyrin IX (PpIX) it can also facilitate the extraction of

specific spectral features of the tissue in question[85]. However it alters the surgical workflow to a greater extent as it introduces new hardware to the procedure, and therefore practical considerations regarding the efficacy and safety of its use warrant exploration.

We aimed to evaluate the feasibility and safety of introducing the use of an optical probe to the workflow in glioma resection surgery.

3.3 Methods

We created a series of experiments, beginning with a cadaveric animal model and progressing to use of the optical probe intraoperatively/in vivo. Ethical approval was obtained from the local Clinical Research Ethics Committee.

The fibre probe used in this study was a standard hard-clad silica core 400µm diameter multimode optical fibre, with a tip diameter of 750µm. The fluorescence probe system comprises a blue LED light-source and standard epi-illumination microscope optics, but has a spectrometer in place of eye-pieces or a camera. Put simply, the device transmits 405nm wavelength blue light to the tip of the optical fibre. When touched to a piece of tissue, the 405nm light interacts with any PpIX that is present and any fluorescence that is generated can travel back up the same fibre to a spectrometer. These spectra can be saved as raw data, or they can be processed by a computer and have features extracted from them.

3.3.1 Animal study

Cadaveric surgery was performed on sheep brain which was prepared by a method described by Buckley et al(95) in 2023. Gelatine “tumour” material was made which contained the same concentration of PpIX as is found in human brain tissue during 5-ALA fluorescence-guided surgery. A burrhole was made in the calvarium and the “tumour” was injected into the brain using a hypodermic needle.

The sheep head was subsequently placed in a standard Mayfield three-pin head clamp. Surgery was carried out by one neurosurgeon using standard neurosurgical instruments. The effects of the probe on the cortex and white matter were

evaluated. Additionally, the handling properties of the probe in the surgeon's hands were noted.

3.3.2 Human Study

We recruited adult patients who presented to our regional neurosurgical unit with suspected HGG based on imaging characteristics. We included patients who were listed for craniotomy for 5-ALA guided resection of their tumours. Those undergoing surgery without the use of 5-ALA and those listed for stereotactic needle biopsy were excluded. Data were collected between January 2023 and July 2023.

Eligible patients were provided with a Patient Information Leaflet, and those who consented to inclusion in the study received oral 5-ALA at a dose of 20mg/kg (Gliolan; medac GmbH, Wedel, Germany) approximately 2-4 hours prior to induction of anaesthesia and proceeded to surgery as per the standard local protocol.

Once the craniotomy and durotomy were performed, the optical probe was used to collect measurements from the cortical surface under the white light and blue light of the operating microscope (Zeiss Kinevo 900; Carl Zeiss AG, Oberkochen, Germany). The operating surgeon was blinded to the results of the spectral analysis. This procedure was repeated in the white matter and the tumour tissue.

The operating surgeon's description of the white-light and blue-light appearance of each area was recorded (i.e. "normal cortex/white matter" vs "infiltrated white matter" vs "tumour"). Spectral measurements were then taken using the optical fibre. Specimens were taken from each interrogated area for later analysis by neuropathology. Fluorescence-guided surgery then proceeded in the standard fashion until a satisfactory resection was achieved.

The effects of the probe on the cortical and white matter tissues were noted. Each patient underwent neurological examination pre- and post-operatively and new neurological deficits were recorded. Patients were followed up for 6 months to assess for any surgical complications. The time taken to interrogate the tissue and provide a positive or negative result was also noted.

The acquired spectra were analysed post-operatively, and the presence or absence of PpIX fluorescence was recorded for each specimen. Histopathological examination confirmed the presence or absence of tumour tissue. 4x4 confusion matrices were constructed to calculate the accuracy of the microscope appearances and the optical fibre findings when compared to histopathology.

3.4 Results

3.4.1 Animal study

The probe was used in two forms. Firstly, the bare optical fibre was used to probe the tissue (figure 3.1). Secondly, the probe fibre was used in conjunction with a suction handle which provided visual feedback to the surgeon in the form of a light situated at the tip of the suction instrument – an orange light indicated that the device was switched on and functioning, and its colour changed to green once PpIX fluorescence was detected (figure 3.2). Use of the optical fibre probe in this model did not induce any physical damage to cortical or white matter tissue. The integrated suction device handled similarly to a standard neurosurgical suction instrument.

It should be noted that although no tissue damage was observed, there was no systematic measurement and this determination was made on the basis of gross observation of the interrogated tissues. This experiment served primarily as a trial study to assess the setup and exclude potential tissue handling problems for the subsequent human study.

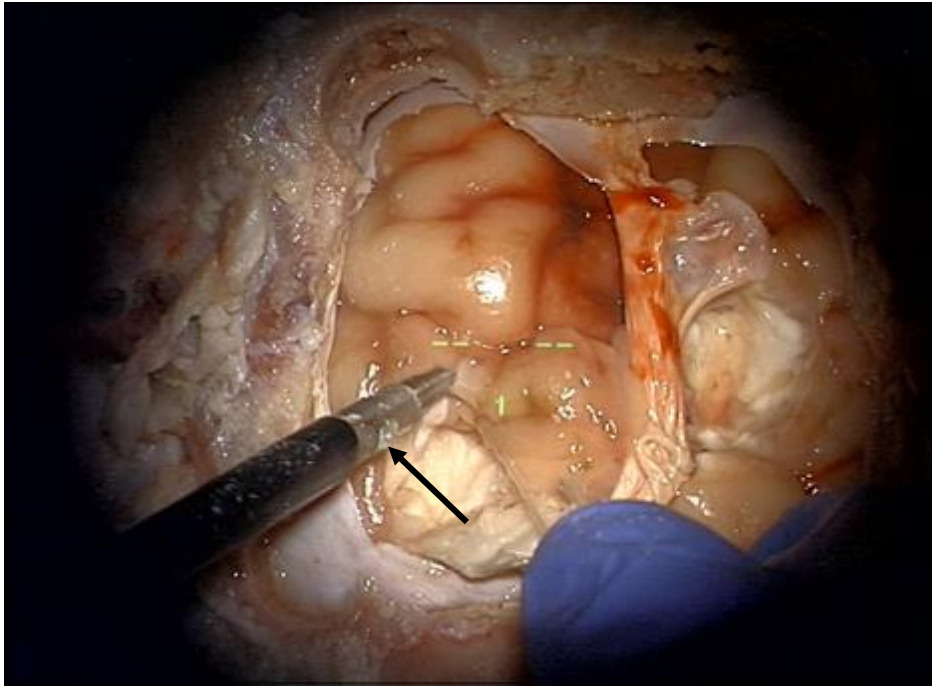


Figure 3.1. Interrogation of the animal model (in the right hand is the bare optical fibre, while the left hand demonstrates the suction handle in which another optical fibre is implanted. The light indicator is denoted by the black arrow).



Figure 3.2. Green light indicator indicating positive PpIX fluorescence.

3.4.2 Human study

Three patients were recruited for the in vivo study, aged 42, 67 and 74 years. All 3 were male and had tumours which were found to be IDH-wildtype glioblastoma. Two

tumours were located in the temporal lobe and one in the frontal lobe. The clinical characteristics are demonstrated in table 3.1.

	Age (years)	Sex	Tumour location	Presenting symptom	Diagnosis
Patient 1	42	Male	Left frontal	Headache	IDH-wt GBM
Patient 2	74	Male	Right temporal	Cognitive impairment	IDH-wt GBM
Patient 3	67	Male	Left temporal	Focal deficit	IDH-wt GBM

Table 3.1. Clinical characteristics (wt = wildtype; GBM = glioblastoma).

Figure 3.3 demonstrates the imaging findings and intraoperative appearances for patient 1. Figure 3.4 demonstrates the findings for patient 2, and figure 3.5 demonstrates those for patient 3.

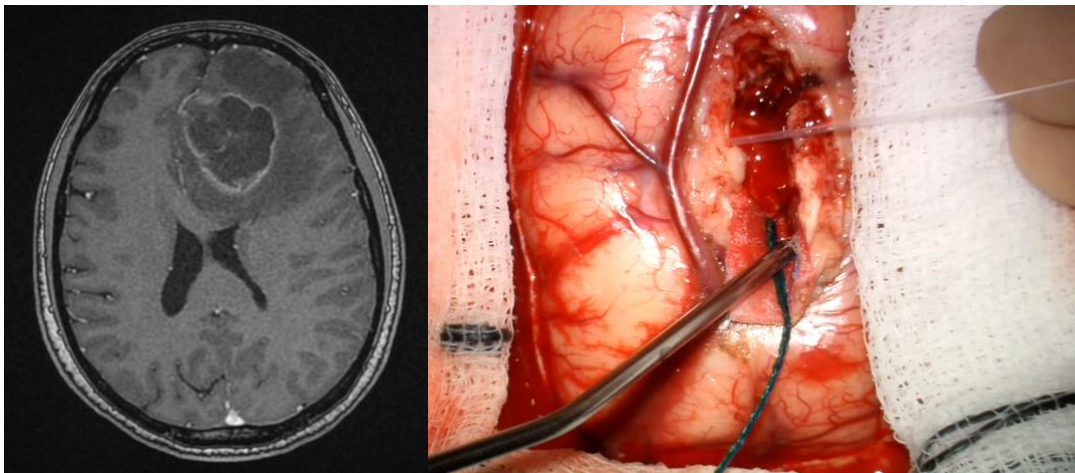


Figure 3.3. Patient 1 (left - axial contrast-enhanced MRI demonstrating left frontal high grade glioma; right - intraoperative probing of infiltrated white matter).

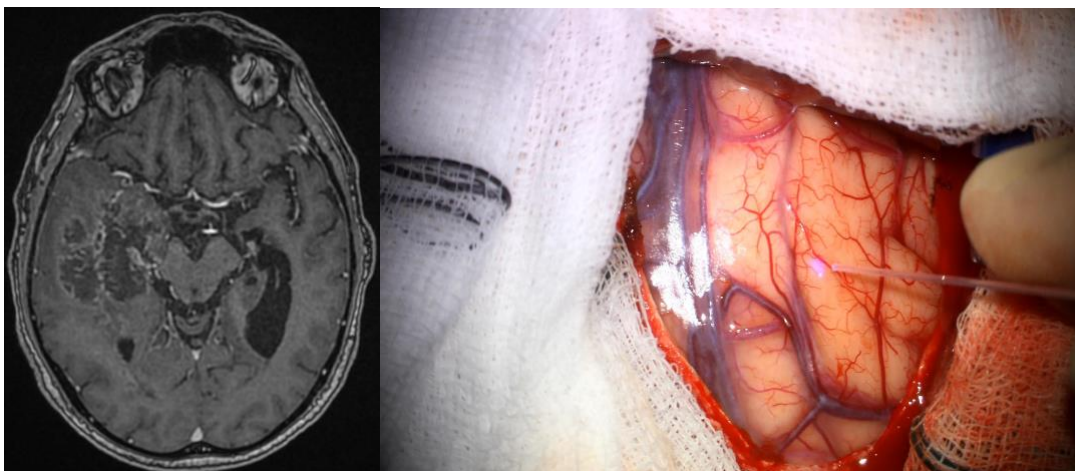


Figure 3.4. Patient 2 (left - axial contrast-enhanced MRI demonstrating right temporal high grade glioma; right - intraoperative probing of cortex).

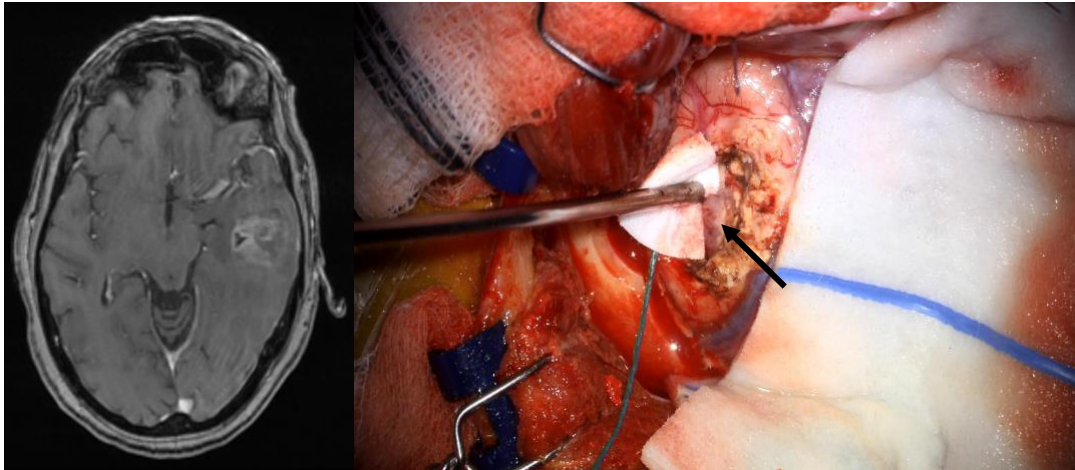


Figure 3.5. Patient 3 (left - axial contrast-enhanced MRI demonstrating left temporal high grade glioma; right - intraoperative view of tumour (black arrow)).

There were no instances of fibre-induced injury to cortical tissue or white matter. Post-operative imaging did not reveal the presence of significant volume haematoma in any of the 3 patients. There were no new post-operative neurological deficits. There were no instances of post-operative infection up to 6 months follow-up.

The accuracy of the microscope appearances was 33.3%, with a sensitivity of 25% and specificity of 100%. The optical fibre readings were 88.9% accurate, with a sensitivity of 100%, and one “false positive” result being identified (i.e. in one sample where the optical fibre detected PpIX fluorescence spectra, pathological examination did not reveal evidence of tumour). The optical probe was able to measure PpIX spectra under the microscope white light up to a light intensity of 30%.

3.5 Discussion

This work demonstrates that contact spectroscopy using an optical fibre is a safe technique which does not induce mechanical injury to the brain and is not associated with an increase in post-operative haemorrhagic or infective complications. Additionally, the optical fibre was able to identify PpIX fluorescence with an accuracy of 88.9% compared to the microscope’s 33.3% accuracy.

There were limitations with our study around the matching of the site of the optical measurement (typically volumes with a diameter measuring hundreds of microns) and the final neuropathological result (which can involve sections of tissue that are

millimetres across). This may have contributed to the one instance of “false positive” fluorescence detection by the optical fibre.

So-called “false positive” results have been described elsewhere with PpIX fluorescence in high grade glioma. Utsuki et al(96) described false positive fluorescence in 4 of 28 glioblastoma samples; when looking closely at these samples, they were associated with neutrophil infiltration as well as reactive astrocytes and macrophages. It is now understood that 5-ALA labels the tumour microenvironment (TME) of glioblastoma(97–99), and therefore fluorescence from tissue that shows an absence of tumour cells does not equate to an error.

In this study, we compared microscope and probe measurements with findings on corresponding haematoxylin-and-eosin stained sections from the tissue interrogated by the probe. In future, it would be of interest to interrogate the specimens using more advanced methods such as immunohistochemistry as well as molecular techniques including nanodrop PCR and single cell RNA sequencing(100). This would increase the sensitivity of true tumour detection, provide more reliable assessments, and may also generate valuable information on the relationship between fluorescence spectra and different areas within the tumour, which may be useful in future research.

The blue excitation light used in 5-ALA fluorescence-guided surgery only penetrates several hundred microns into tissue before being lost to scattering. This can be a limitation (if tumour is buried beneath a layer of tissue, the light will not get to it) or a strength (surgery can be very precise, with fluorescence only being detected where the probe is brought into contact with the tissue). In standard fluorescence-guided surgery using the microscope technique, a layer of blood/irrigation fluid over the tissue can block/absorb excitation light and affect the fluorescence, whereas when using the contact technique the probe can be placed through the fluid and detect fluorescence from the tissue underneath.

The inherent sensitivity and specificity of a spectrometer compared to the human eye is also advantageous. The eye can be “tricked” when stray ambient light scatters

from the surgical site, is filtered by the microscope filters and thus appears as a faint red background “fluorescence”. When areas of PpIX fluorescence in the tumour margin are faint or vague, or become photobleached, the ambiguity of faint red tissue can become a challenge for the surgeon. These issues are addressed by the use of a spectrometer which can tell different types of “red light” apart, and can separate weak optical signals that are hidden in larger backgrounds.

The spectroscopic system described here collects and analyses spectra, and reports the presence or absence of PpIX fluorescence emission in those spectra. It carries out this process multiple times a second, and therefore does not introduce any delay to the procedure (i.e. it operates in “real time”). The means by which the system analyses the spectra and the precise technical characteristics are outside the scope of this chapter but will be described in future work.

3.6 Conclusion

Contact optical fibre spectroscopy is a safe technique which can provide rapid and accurate spectral information. Its use for the detection of PpIX fluorescence in high grade glioma surgery provides higher accuracy than the standard microscope-based technique.

Practical benefits of this technique include the rapid “real-time” information provided, and the ability to interrogate tissue through blood/fluid present in the operative field. Furthermore, this study identified that the optical fibre may be able to function under microscope white light conditions, albeit at low intensities.

Further research into the effects of ambient light and the white light conditions under which glioma surgery is carried out would be of benefit in order to identify potential factors which may impact the accuracy of the technique.

Chapter 4: Spectroscopic Characterisation of Tumour Subtypes

4.1 Abstract

Background: Molecular features play a significant role in both the diagnosis and prognosis of gliomas. Molecular information is generally unavailable at the time of index surgery, however upfront knowledge of this information may provide valuable information with which the surgeon can tailor the surgical approach.

Aims/objectives: To develop and validate a machine learning algorithm which can determine IDH status and MGMT methylation status based on spectral features.

Methods: Samples of cortex, white matter and tumour were collected from patients with high grade glioma, and the samples were interrogated with a fibre probe which recorded fluorescence spectra. The fluorescence data were blinded and randomised, and the spectral data were trimmed to include only relevant wavelengths. Molecular data including IDH status and MGMT methylation status were recorded for each tumour specimen and incorporated into a machine learning algorithm. The algorithm was trained on half the patients exhibiting each label and the model was then tested on the other half.

Results: The algorithm was unable to determine MGMT methylation status. The results were more promising for IDH mutation status, however reliable classification was not consistently achieved.

Conclusions: Accurate real-time classification of molecular characteristics of glioma would be useful in tailoring the surgical approach. Further studies incorporating IDH and 1p19q status would be beneficial – these studies would benefit from larger sample sizes in order to bypass some of the limitations of this smaller study.

4.2 Introduction

Gliomas represent a heterogenous group of tumours. Molecular information has become integral for accurate diagnosis of these tumours, particularly since the introduction of the 2021 WHO classification of central nervous system tumours(2).

The molecular profile is helpful not only for tumour classification, but it also provides valuable information regarding response to adjuvant therapies as well as prognosis.

Adult diffuse glioma can be broadly classified based on the presence or absence of an IDH mutation, and by the 1p19q co-deletion which differentiates oligodendroglioma from astrocytoma. This distinction is relevant for surgical decision-making in cases where residual disease remains after surgery; survival in IDH-mutant astrocytoma has been demonstrated to correlate strongly with extent of resection(39), while in patients with 1p19q co-deleted tumours the effect of supramaximal resection, although present, is not as profound(101).

The molecular status of a tumour is not known until a full diagnostic workup has been performed on the resected tissue by the neuropathologist. Therefore, although it can help to guide treatment of patients with residual disease after initial surgery, molecular information cannot be used to guide surgical decision-making at the time of the initial surgery.

In glioblastoma, 5-ALA uptake and subsequent induction of heme biosynthesis has been shown to result in altered spectral features within different areas of tumour tissue. In addition to the canonical PpIX peak which is present at 635nm, a second peak at 620nm has been noted in tissues and cell cultures. The origin of this peak in vivo is not fully elucidated, and has been suggested either to be a different aggregate of PpIX or a wholly different porphyrin(84). Some groups have described that the $PpIX_{620/634}$ ratio is decreased at the core of the tumour and increases towards the surrounding infiltrative zone, suggesting that it may be used to increase the sensitivity of tumour detection in low density margins(85).

Furthermore, Suero Molina et al(102) identified that this ratio was much higher in tumours harbouring an IDH mutation than IDH-wildtype tumours, suggesting that the biochemical constitution of a tumour can differ by molecular subtype and that these biochemical differences may be identifiable by certain spectral features. There are several potential challenges in identifying these spectral features, including the complexity of spectral analysis, the contribution of multiple different fluorophores

to the spectral signature, and the risk of drawing incorrect conclusions on the identity of fluorophores(102).

This study aimed to identify spectral features that can help to identify IDH status and MGMT methylation status, and to generate a machine learning algorithm to identify these characteristics from spectral parameters.

4.3 Methods

We recruited adult patients who presented to our regional neurosurgical unit with suspected high grade glioma (HGG) for inclusion in this study. We included patients who were listed for craniotomy for 5-ALA guided resection, or for 5-ALA guided biopsy, of their tumours. Those undergoing surgery without the use of 5-ALA and those listed for stereotactic needle biopsy were excluded. Data were collected between January 2022 and July 2023. Ethical approval was obtained from the local Clinical Research Ethics Committee.

Patients who consented to inclusion in the study received oral 5-ALA at a dose of 20mg/kg (Gliolan; medac GmbH, Wedel, Germany) approximately 2-4 hours prior to induction of anaesthesia and proceeded to surgery as per the standard local protocol. At surgery, samples of cortex, white matter and tumour were collected. Once the specimens were taken, they were then interrogated with the fibre probe and fluorescence spectra were recorded.

Finally, the specimens were delivered to the neuropathology laboratory where they were processed to infiltrate with histological wax prior to cutting and staining of tissue sections on a microtome. The sections were stained with haematoxylin and eosin and reviewed by the reporting neuropathologist. Standard diagnostic molecular analysis was performed for each tumour included in the study, including immunohistochemistry for IDH 1 mutation, followed by subsequent molecular sequencing in IDH-wildtype tumours from patients aged 55 years or below. MGMT methylation and 1p19q co-deletion status were also routinely tested.

After the fluorescence spectra were downloaded from the device, the data were blinded and randomised, and each spectrum was assessed for the presence or absence of a PpIX emission band. The spectral data were trimmed to include only relevant wavelengths, in this case 560nm to 750nm, excluding wavelengths that could be affected by incident LED light and including wavelengths that are theorised to potentially exhibit relevant information.

Each of these trimmed spectrum bands is normalised by subtracting its mean and dividing by its standard deviation. This is to ensure that overall light levels are not used for differentiation as they may be artefacts not arising from tissue/light interaction, but from the general environment conditions. This normalisation process ensures that only differentiating characteristics in the relative shape of the spectra are used to predict IDH or MGMT status.

Molecular data including IDH status and MGMT methylation status were recorded for each tumour specimen and incorporated into a machine learning algorithm. Only one patient harboured a 1p19q-codeleted tumour and therefore an algorithm for 1p19q status was not included. These labels were used as targets in training a machine learning model to determine if, using the underlying trimmed and normalised spectrum as input features, the model could predict IDH and MGMT status. IDH and MGMT status were treated as two separate, binary classification problems.

The IDH data collected in this patient cohort exhibits class imbalance – that is the data set has an unequal distribution of classes which may lead to bias in the trained model. This issue is present with many machine learning applications. In this instance, the IDH status for all the recorded data is shown in table 4.1, demonstrating that 85% of the data is IDH wildtype.

While the MGMT label data does not suffer from the same bias (table 4.1), there are some cases in which methylation data is undefined and the data from these patients is therefore excluded from this analysis.

Molecular status	n	%
IDH		
Wildtype	23	85.2
Mutant	4	14.8
MGMT		
Methylated	11	40.7
Unmethylated	13	48.1
Undefined	3	11.1

Table 4.1. IDH and MGMT methylation status of cohort included in the study. Class imbalance can be seen in the IDH mutation status, with only 14.8% of tumours harbouring an IDH mutation. Additionally, in 11% of tumours MGMT methylation status could not be defined.

Any one patient exhibits only one IDH and one MGMT status. To avoid patient-specific spectral information being used by the model to determine IDH or MGMT status, the training is carried out on half the patients exhibiting each label and the model is tested on the other half. In the training routine, five-fold cross validation is used to avoid overfitting. This process is performed in stages, with the data being randomly divided into 5 parts – 80% of the data is used to train the model while the remaining 20% is used as test data. The complete training and testing cycle is shown in figure 4.1. Note that the process is repeated five times so that all the data is used to test the model. An overall confusion matrix is accumulated over all the patients to provide the results.



Figure 4.1. Model training and testing process. The data were separated into training and testing groups, with no patient's data being included in both groups simultaneously to avoid overfitting. Please note that this process was repeated 5 times, so that all spectra were included as training data at least once.

4.4 Results

In total, 83 specimens were collected from 27 patients. Over 8000 spectra were collected and analysed. Twenty-one patients had a final integrated diagnosis(2) of IDH-wildtype CNS WHO grade 4 glioblastoma, 3 had IDH-mutant CNS WHO grade 4 astrocytoma, 2 had IDH-wildtype CNS WHO grade 3 astrocytoma, and 1 had IDH-mutant CNS WHO grade 3 oligodendroglioma.

In the following confusion matrices, the results are presented in percentages of the true class. That is, each row (representing the true class label) sums to 100%. The upper-left and upper-right quadrants demonstrate the percentage of samples that were classified correctly and incorrectly, respectively. The lower-left and lower-right quadrants show the percentage of samples classified incorrectly and correctly, respectively.

IDH status

The IDH status prediction model results are demonstrated in figure 4.2. 73% of IDH wildtype samples and 87% of IDH mutant samples were correctly classified ($\chi^2 = 4.9$, $p = .027$).

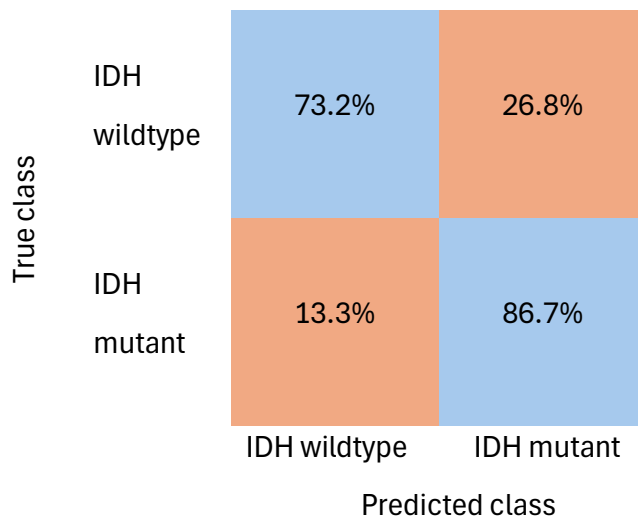


Figure 4.2. IDH status prediction on unseen data. The model correctly predicted IDH wildtype and IDH mutant specimens in 73.2% and 86.7% of cases, respectively.

MGMT methylation

The confusion matrices for the MGMT methylation prediction model are shown in figure 4.3. These demonstrate that the MGMT classification model performed poorly. The classification of unmethylated samples was accurate in fewer than half of cases, while the methylated samples were more often misclassified (67%) than correctly classified ($\chi^2 = 1.4$, $p = .24$).

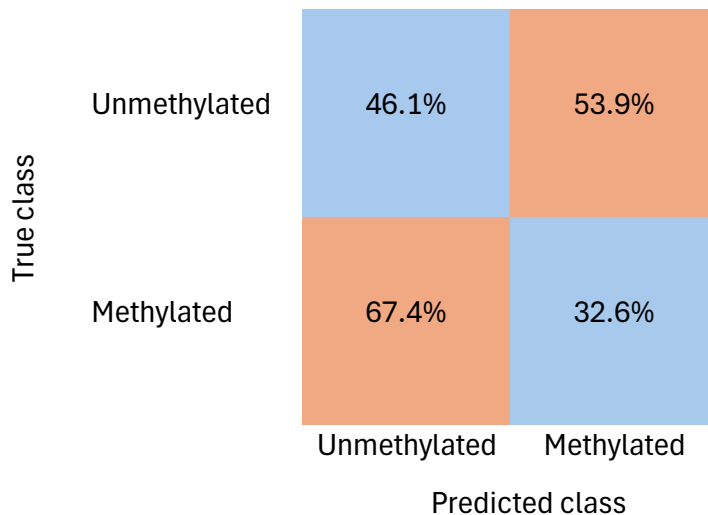


Figure 4.3. MGMT status classification results on unseen patient data. The model was unable to reliably predict MGMT methylation status – 46.1% accuracy for unmethylated specimens and 32.6% for methylated specimens.

4.5 Discussion

Molecular features play a substantial role in the workup of patients with glioma, for both diagnostic and prognostic purposes. In adult-type diffuse glioma, 1p19q status differentiates oligodendroglioma from astrocytoma(2), and IDH status carries implications regarding treatment(14) and prognosis. Additionally, surgical decision-making may be impacted by the molecular characteristics of the tumour.

For IDH-wildtype glioblastoma, maximising resection is known to prolong progression-free survival(47,66). In IDH-mutant tumours, overall survival is improved by extensive resection compared to subtotal resection, and so-called “supratotal” resection provides the greatest benefit in patients with astrocytoma compared to oligodendroglioma(101). Therefore, upfront knowledge of molecular characteristics may provide valuable information to which the surgeon can tailor the surgical approach.

This study suggests that a machine learning algorithm may be able to classify tumours by IDH mutation status, however the accuracy of such an algorithm is not to a reliable standard. It does suggest however that this technique may be a viable one and is certainly an area for future research.

With regards to MGMT methylation status the results were wholly unreliable. Unfortunately, our patient population did not allow for meaningful modelling of 1p19q codeletion status, as only one patient harboured a 1p19q-codeleted tumour. Future work would benefit from including 1p19q status among the machine learning programmes.

In this study, only 15% of patients had tumours harbouring an IDH mutation. This imbalance in the target classes is a known challenge for classifier models whereby the classifier is biased towards the larger class. We attempted to address this bias in several ways. Firstly, an ensemble classifier was used – the outputs of multiple trained models were combined to create a stronger overall classifier. Secondly, the confusion matrices are provided in the results to provide a better insight into the performance. Finally, undersampling was employed to balance the number of training labels in each class.

4.6 Conclusion

Our machine learning algorithm showed promise for differentiating IDH-mutant tumours from those without an IDH mutation. The results for MGMT methylation classification were unreliable.

Future research would benefit from including tissue from IDH-mutant, IDH-wildtype and 1p19q-codeleted tumours. Brain tumour biobanks may also prove beneficial as these provide large quantities of tissue with which these machine learning algorithms can be developed and adequately validated.

Chapter 5: Conclusions

Conclusion

The management of high grade glioma remains a significant challenge. The current standard of care mandates input from the neurosurgeon, oncologist and radiation oncologist. Surgery represents the first step in the patient's journey, and the surgical paradigm is maximal safe resection with removal of as much enhancing tumour as possible while maintaining neurological function.

The development of different surgical adjuncts has improved our intraoperative visualisation of tumour tissue. Fluorescence-guided surgery using widefield blue-light microscopy has been demonstrated to improve rates of resection and progression-free survival. Although effective, the technique is limited by the particulars of light delivery and subjective assessments of fluorescence.

The work described in chapter 2 establishes that contact spectroscopy is an accurate technique which provides improved tumour detection at the marginal zone of high grade glioma. Fluorescence-guided surgery has been demonstrated robustly to improve progression-free survival(47), and the ability of contact spectroscopy to provide better margin control will likely contribute further to this improvement in oncological outcomes.

Chapter 3 demonstrates the safety of this technique while also identifying several potential practical benefits, including the ability of the probe to function under white light and through blood/CSF which may be present in the field. 5-ALA guided surgery has already been demonstrated to be more efficient than intraoperative MRI while providing similar extent of resection(48); further improvements in the efficiency of the technique can translate to reduced operative time with subsequent benefits relating to post-operative complications due to prolonged immobility, as well as economic benefits.

Chapter 4 explores applications of this technology in the growing area of intraoperative molecular diagnostics. There is growing evidence for an individualised

molecular-based approach to the surgical treatment of glioma. In IDH-mutant tumours, for example, the role of supramaximal resection, beyond the anatomical margin and to functional limits, has been suggested to provide oncological benefit(39,101). Other technologies such as hyperspectral imaging and Raman spectroscopy have endeavoured to provide this information, however these methods involve complex data interpretation and take time. The results from this chapter suggest that machine learning models may be able to identify IDH status in real-time based on fluorescence spectra, providing rapid and actionable results, thus allowing surgeons to tailor their approach and improve outcomes in this group of patients.

Future directions

Future research into other potential uses of this technology should focus on several areas. First, the ability to extract specific spectral features could potentially aid in identifying fluorescence signatures which act as surrogates of the biochemical and/or molecular features of these tumours. As noted in chapter 4, this could potentially have significant implications in the peri-operative management of these patients. Furthermore, research into this area may further advance our understanding of the pathophysiology of glioma.

Secondly, its use in other types of tumours warrants exploration – in particular, low grade glioma surgery remains challenging due to the difficulty in distinguishing normal tissue from tumour tissue under white light alone. There is evidence to suggest that quantitative measurements of PpIX are possible in low grade glioma, and further research may identify another potential use of contact spectroscopy in this domain.

Finally, correlation of spectral characteristics with more advanced methods of tissue analysis such as nanodrop PCR and single cell RNA sequencing may provide useful avenues for future research.

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