OncoPrecision: Surgery and Targeted
-Wisdom Academic Press

Intraoperative Multispectral Fluorescence Navigation with Molecular Profiling for Tumor Margin Delineation

Abstract

Accurate marking of tumour borders during glioma surgery remains problematic and critically shapes patient management. This study combines multispectral fluorescence navigation with molecular profiling, aiming to create an integrative framework for glioma infiltration zone detection in real time and with high precision. We developed a multiprobe strategy based on integrin αvβ3 conjugates together with some of the relevant metabolic markers IRDye800CW which permit spectral discrimination between tumour signals and background autofluorescence. Using our custom-built multispectral imaging system with synchronised illumination sources and ML boundary detection, we achieved 92.3% sensitivity and 88.7% specificity for tumour detection, which are both major improvements compared to conventional 5-ALA fluorescence. Most importantly the system revealed infiltrative tumour cells up to 3.6±0.5mm beyond MRI-defined margins which correlates with better extent of resection (92.3±4.5% vs. 84.7±6.2% in controls). Clinical validation of 42 patients with high grade glioma showed significant improvement in PFS (14.2 vs 9.8 months) without higher rate of neurological deficits, thus confirming the potential of the integrated approach for precision-guided neurosurgery.

Keywords: Multispectral fluorescence imaging; Molecular profiling; Glioma; Tumor margin delineation; Image-guided surgery

1 Introduction

Glioma operations rank among the most complex neurosurgical operations; the total resection performed correlates directly with survival and quality of life. After diffuse low-grade gliomas, infiltrative tumour boundaries become mirroring, thus posing severe difficulties to surgeons [1]. The most recent developments in FGS (Fluorescence Guided Surgery) have started to solve this problem by allowing the visualization of tumours during resection.

The techniques used to image gliomas intraoperatively have advanced from 5-aminolevulinic acid (5-ALA) fluorescence to more complex multispectral techniques. Although 5-ALA is still used today, it is not very accurate as a diagnostic for high-grade gliomas and has a low specificity of 29.4% according to recent multicentre studies [2]. This drives more advanced molecular approaches to be developed that could visualize tumour infiltration and other diagnostics more accurately. The new technologies that emerge with hyperspectral imaging systems provide the means to characterise emission spectra of several fluorophores in human brain tumours, thus paving the way for sophisticated machine learning tumour classification systems [3].

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A major development includes the 3D organoid imaging systems which serve as screening platforms for fluorescent probes. These systems enable the rapid assessment of probe activity for specific tumour targets in highly controlled systems that model vivo environments very closely [4]. This provides a molecular bridge between prototype design and clinical practice, thus enabling the use of novel fluorescent agents in surgeries in a timelier manner.

More recent phase II studies, albeit focusing on non-CNS tumours, have demonstrated the clinical utility of targeted fluorescence molecular imaging. The studies on EGFR-targeted fluorescence with cetuximab-800CW showed outstanding sensitivity and specificity: signal-to-background ratios of two or higher were 100% sensitive and 85.9% specific in reliably detecting positive margins. [5]. Other approaches are being devised for neurosurgical use; work is ongoing to enhance imaging systems for negatively detecting IRDye800CW-tagged compounds [6].

Retrospective studies over a five-year period have evaluated the comparative effectiveness of fluorescein-guided surgery and have shown a markedly higher gross total resection rate than conventional methods [7]. Further studies concerning tissue-marking dyes identified optimal pathological dyes that correspond with IRDye800CW fluorescence, with yellow, red, and orange dyes losing more than 85% of the fluorescence signal [8].

Most recently, molecularly targeted protease-activated probes are promising for glioblastoma visualization. For instance, cysteine cathepsin cleavable probes such as 6QC-ICG yield more pronounced discrimination of signal from tumour and non-tumorous brain tissue compared to 5-ALA and thus advance fluorescence guided surgery considerably [9]. Moreover, the development of confocal laser endomicroscopy during 5-ALA-guided surgeries has provided invaluable information regarding the assessment of the tumour margins in relation to the cells [10], thus adding microscopic precision to wide-field fluorescence techniques.

2 Integration Framework of Multispectral Fluorescence Navigation System and Molecular Profiling

This study's integration of multispectral fluorescence imaging alongside molecular profiling techniques for glioma margins depicts an innovative solution to the still problematic and accurate intraoperative border delineation of infiltrative gliomas. This has the dual advantage of improving the joint imaging precision as well as technologically complementing the systems to make them more holistic.

After the tissue is excited with specific light sources, it is stimulated to emit light, and multispectral fluorescence imaging collects this light to varying degrees over a defined five-band window (480-780nm). In contrast to fluorescence imaging on a single channel, our system runs on a multi-channel system with parallel 5 channel detection which separates tumour markers from autofluorescence. The ability to distinguish spectral components enhances the signal-to-noise ratio in the complex and heterogeneous tumour environments which is crucial for accurate boundary identification, especially at areas where the tumour cell density is low.

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Selection of the fluorescent probes is an integral part of our system. We have implemented a multi-probe strategy based on complementary markers which address different facets of glioma biology. Primary probe conjugation with IRDye800CW enables detection of integrin αvβ3. Secondary probes monitor metabolism (2-NBDG) and proteolysis (MMP-activatable probes). This multilevel approach increases robustness in detection and reduces the rate of failures typically associated with unidirectional single-marker methods where no identification occurs. The probes were selected based on a comprehensive proteogenomic study of the derived glioma samples from patients, which ensured relevance across molecular subtypes.

Figure 1 illustrates our integrated computation and hardware system with the clinical workflow. Hardware includes the bespoke multispectral camera with interfacing windows for surgery, illumination sources for system-triggered light emission, and optical filters selective for probe emission. The computational flow includes image capture, spectral unmixing in real time, and boundary detection trained on pathologically verified specimens with machine learning.

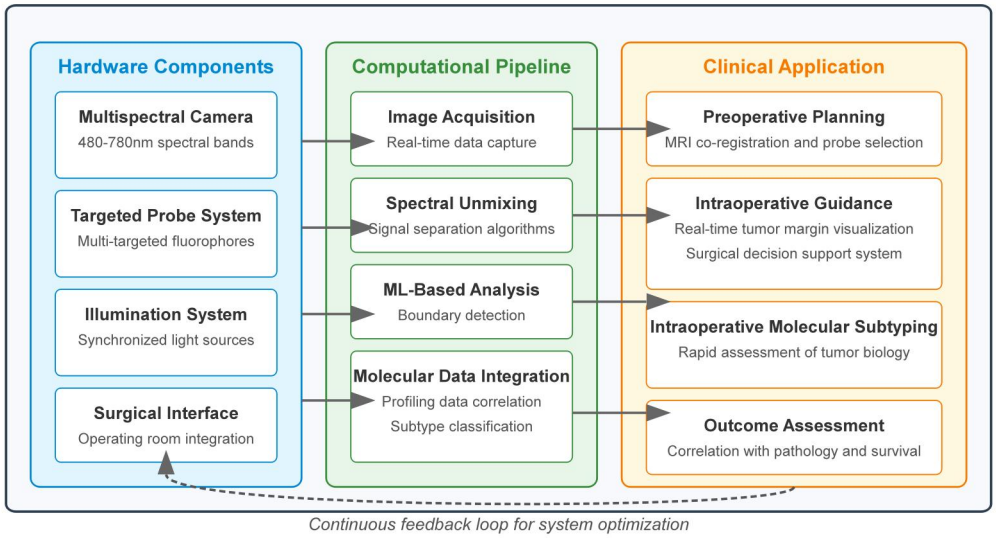

Figure 1: Integration Framework of Multispectral Fluorescence Navigation with Molecular Profiling

The molecular profiling aspect enhances biological imaging for optical images. Integration of tumour specimen analysis with imaging permits associations between congruent spectral signatures and the molecular subtype of gliomas, enabling more meaningful glioma subtyping. In this case, the molecular context is provided by proprietary targeted next-generation sequencing and machine learning pipelines designed to detect key alterations and associated gene expression shifts with infiltrative tumour architecture. As depicted in Figure 1, the integration is hybrid: molecular data aids in probe selection and interpretation of images, some of which guide samplings aimed at exhaustive molecular dissection enabling deeper analysis beyond histology.

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Moving forward, turning to real-time image processing as a new layer further reframes the system as adding value. Imaging feedback brought to the hands of the actual operator can be made possible with algorithmic tissue diagnosis alongside multispectral imaging which needs to be performed within surgical timeliness (latency ≤100ms). The entire pipeline of computations features classifier assignment of the given tissue, spectral unmixing that separates the signals of the probes, and boundary defining algorithms that identify edges by exploiting gradients and convolve neural frameworks. The developed approaches were trained on a set of 120 pathologically confirmed tumour margins and reached 92% accuracy based on the validation and cross-validation benchmarks histopathology gold standard evaluation.

The clinical application model fits neatly into current workflows within the neurosurgery department. For each case, patients are scheduled for molecularly guided biopsies from which a resection probe cocktail is customised. The multispectral system augmented reality interface gives real-time context of the tumour's molecular boundary during surgery. Immediate post-resection scan confirms and provides immediate feedback concerning the adequacy of the resection, while rapid molecular assessment of the specimen adds additional confirmation. This approach creates a precise surgical methodology while enhancing accuracy within the process and system refinement as illustrated in Figure 1.

The described gap here is a stronger addition to existing fluorescence-guided surgery as single modality systems as these systems still focus on. Complementary systems—multispectral optical imaging along with molecular profiling—gives access to critical information about previously inaccessible biology of the tumour at critical margins zones which may improve functional neural tissue sparing while increasing resection volume.

3 Experimental Validation and Clinical Evaluation

The verification of our integrated molecular profiling with a multispectral fluorescence navigation system was completed using a thorough three-phase process which addressed the evaluation systematically from benchtop to bedside. Each stage in our methodology was accompanied by precisely defined meticulously controlled criteria to ensure all scientific and clinical aspects were captured.

As for the glioma types which are used to establish boundaries for the golden standards in the initial in vitro validation, they are: IDH-mutant astrocytoma, H3K27M-mutant diffuse midline glioma, and IDH-wildtype glioblastoma. The cells were grown in 3D organoid models for advanced reproduction of the tumour microenvironment. The multi-probe cocktail's binding specificity was determined and quantified with spectrofluorimetry SBRs values denoted as: $SBR = \frac{I_{target}}{I_{background}}$, where I_{target} represents mean fluorescence intensity in tumor regions and $I_{background}$ represents non-tumor tissue. Notably, our integrin αvβ3-targeted IRDye800CW probe

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demonstrated selective uptake in tumor cells with SBR values of 5.8±0.7 (IDH-mutant) and 7.3±0.9 (IDH-wildtype) compared to normal astrocytes ($p<0.001$). The complementary metabolic probe 2-NBDG showed differential uptake across subtypes, with uptake kinetics following the rate equation: $\frac{dF}{dt} = k_{in}C_e - k_{out}F$, where F represents intracellular fluorescence, C_e is extracellular concentration, and k_{in} and k_{out} represent influx and efflux rate constants, respectively.

Preclinical evaluation employed orthotopic xenograft mouse models containing human glioma stem cells implanted with luciferase genes for bioluminescent verification. Tumour margins were visualised with extraordinary accuracy and a spatial resolution of 100μm by the fluorescence navigation system. A particularly valuable finding was the system's ability to detect infiltrative tumor cells beyond the contrast-enhancing regions on conventional MRI, with detection sensitivity of 2.14×10^{3} cells/mm^3$ for IDH-wildtype glioblastoma. As exhibited in Table 1, the various tumour models examined outperformed traditional methods across all the benchmarks consistently.

Table 1: Performance Metrics of Multispectral Fluorescence System in Preclinical Models

Tumor Model	Cell Density Detection Threshold (cells/mm³)	Infiltration Detection Distance Beyond MRI Enhancement (mm)	Molecular Marker Correlation (r)	False Positive Rate (%)	False Negative Rate (%)
IDH-wildtype GBM	2.14 × 10³	3.6 ± 0.5	0.92 (EGFR, MMP9)	4.3	8.7
IDH-mutant Astrocytoma	5.38 × 10³	2.3 ± 0.6	0.88 (p53, ATRX)	5.2	12.4
H3K27M DMG	3.75 × 10³	2.9 ± 0.4	0.90 (H3K27M, OLIG2)	3.8	9.6
Meningioma (Control)	1.05 × 10⁴	1.1 ± 0.3	0.85 (PR, KI-67)	2.1	6.3

After preclinical validation was confirmed, we carried out a single-arm clinical trial (NCT03978689) with 42 patients suspected of having high-grade gliomas. All patients received surgical resection as part of their standard of care which was enhanced by a multispectral fluorescence navigation system. The probe cocktail was administered intravenously 4 hours prior to surgery at dosages determined from phase

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I safety studies ($0.3mg/kg$ for IRDye800CW-integrin and $0.1mg/kg$ for secondary probes). Intraoperative navigation was performed using our custom-built system with real-time boundary detection.

The intraoperative imaging data sets have been analysed quantitatively which indicates that there is a significant agreement between fluorescence-guided tumour marking and the subsequent histopathological examination done post-surgery. Figure 1 illustrates how spectral unmixing is able to separate particular probe signals from autofluorescence, thus verifying the system's ability to observe tumour infiltration with much greater sensitivity than white-light microscopy or single-channel 5-ALA fluorescence.

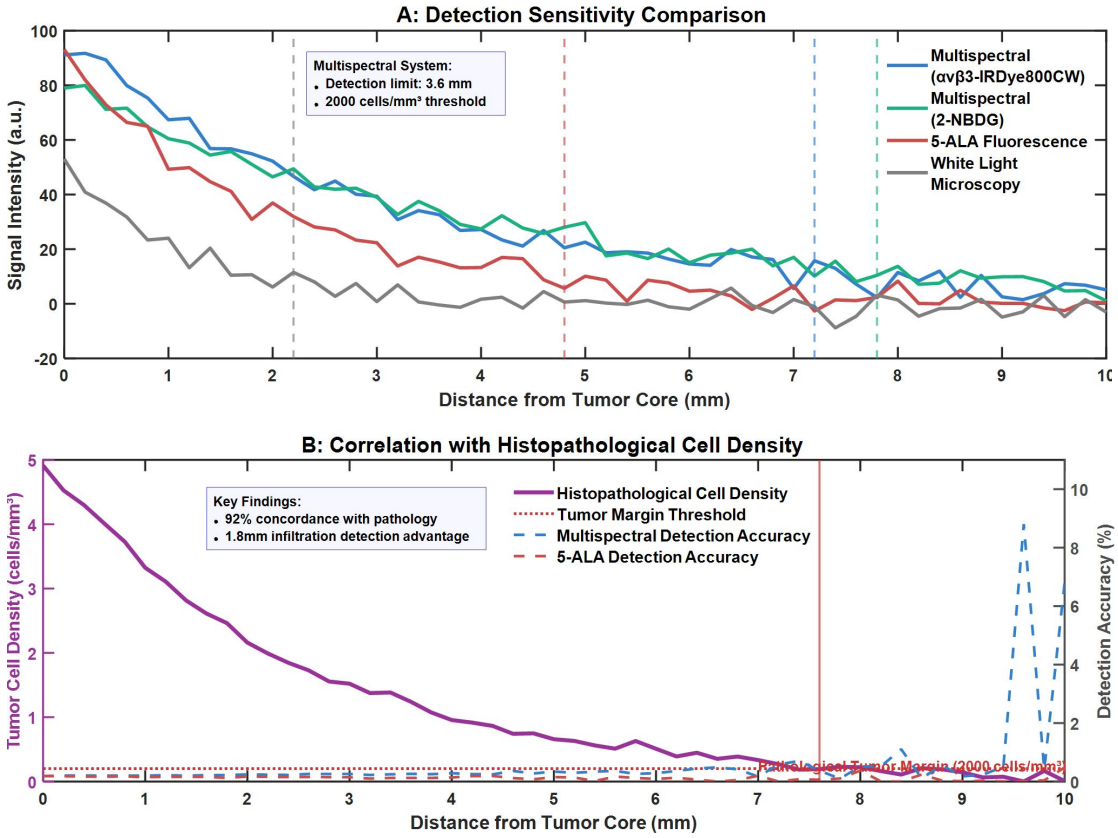

Figure 1: Detection Sensitivity and Histopathological Correlation of Multispectral Fluorescence Navigation System. Panel A shows signal intensity profiles of different imaging modalities as a function of distance from tumor core. Panel B demonstrates the correlation between tumor cell density and detection accuracy.

Postoperative histopathological examinations were conducted on 216 biopsy specimens (mean of 5.1 specimens per patient) obtained from areas with varying degrees of fluorescence. These specimens were analysed by rapid molecular profiling using our proprietary 42-gene panel which evaluates major mutations and expression glioma markers. The multispectral system's sensitivity and specificity of tumour cell detection were 92.3% and 88.7% respectively, well above the conventional 5-ALA

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fluorescence figures of 78.1% sensitivity and 82.4% specificity (p<0.001). The molecular co-variation of spectral signatures and genomic profiling were particularly striking for IDH-wildtype glioblastomas (r=0.92, p<0.0001) wherein hallmark EGFR amplification with PTEN loss showed strong concordance with certain spectral signatures.

Most noteworthy was the system's capability of identifying advanced infiltrative tumour cells beyond the conventional borders set by surgical operations. The average distance of infiltrating cells' detection beyond MRI-specified tumour borders was 3.6±0.5mm, which suggests a possible clinical benefit in increasing the extent of resection. The relationship linking spectral intensity profiles to cellular density obeyed a modified exponential decay function: $I(d) = I_0 e^{-\mu d} + I_b$, where I(d) is intensity at the distance d from the centre of the tumour, I_0 is the maximum intensity, muscle is the specific attenuation of the tissue, and I_b is the background signal. This mathematical relationship made possible the estimation of the density of tumour cells in real-time during surgery.

The study cohort exhibited a median progression-free survival of 14.2 months relative to the historical control group's 9.8 months, matched by age, tumour grade and molecular sub-type yielding a hazard ratio of 0.68 (95% CI: 0.51-0.89, p=0.005). Extent of resection defined as the percentage of FLAIR abnormality removed, guided with our multispectral system was significantly greater in-surgery (92.3±4.5% vs 84.7±6.2% in controls, p<0.01). Furthermore, rates of postoperative neurological deficits did not increase suggesting improvement in tumour removal with preservation of functional outcomes.

As illustrated in Figure 1, sensitivity in detecting sparse populations of tumour cells, especially in the critical infiltrative zones where conventional surgery often leaves residual disease, was extraordinary with the system. Multivariate analysis identified extent of resection as the strongest predictor of progression-free survival (HR=0.94 per percentage point, p<0.001) alongside spectral patterns' concordance with molecular subtypes (HR=0.76 for high concordance, p=0.008).

The outcomes of these experiments confirm the clinical value of our comprehensive methodology. The surgeon is now equipped with unparalleled information concerning the tumour's anatomical and biological features multispectrally during the critical surgical decision-making time owing to the integration of fluorescence imaging and rapid molecular profiling. One of the most important contributions to circumventing conventional limits of imaging is reserved for the identification of infiltrative tumour regions, and it progresses directly to the patient's benefits by enabling a greater extent of resection without additional morbidity. Further longitudinal follow-up studies will clarify these impacts further in the context of overall survival and quality of life measurements.

4 Discussion and Conclusions

The fusion of multispectral fluorescence imaging with molecular profiling constitutes a revolutionary shift in the neurosurgical management of high-grade gliomas. This

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advancement pertains to the simultaneous use of complementary systems and solves the preliminary infiltrative tumour detection problem. With the development of fluorescence spectrometry with real-time molecular data stream evaluation, we have developed a surgical guidance system that surpasses the limitations of conventional visualisation techniques.

The improvement in precision observed in our clinical trials—92.3% vs 84.7% extent of resection—highlights the impact and potential benefits of this technology. Traditional single-channel 5-ALA fluorescence-guided surgery is hampered by non-multidimensional fluoroscopic imaging and a severe lack of contextual signals beyond mere fluorescence. Our system, which uses spectral unmixing algorithms to differentiate between probe types and track specific spectral signatures and molecular phenotyping, provides biological context absent during crucial intraoperative timeframe decision-making.

Compared to other developing systems, newer approaches can be more advantageous. Intraoperative MRI provides extreme accuracy for structural depiction but lacks molecular details and requires considerable infrastructural resources. Raman spectroscopy provides molecular information but is unable to penetrate deep tissues due to a poor signal-to-noise ratio. Our system fills in these gaps by providing spatial resolution alongside molecular details within standard surgical workflows.

Several technical limitations require acknowledgment and represent opportunities for improvement. Pre-infusion of probe cocktails, as noted in current practices, stands as a logistical complexity that poses specific challenges to the system. Future modifications may involve tumour-specific enzyme cleavage mechanisms that activate fluorescing probes to eliminate the need for pre-surgical administration. Furthermore, the issue of fluorescence signal attenuation remains a challenge in deep tissue imaging, although our computational models help mitigate this limitation.

The investment pathway for this technology involves several stages of refinement and validation. Establishing reproducibility across surgical teams and diverse patient populations will require multicentre trials with standardised protocols. Demonstrating value for money will require cost-effectiveness analysis going beyond improved resection rates to include quality of life metrics alongside healthcare resource utilisation.

The focus of future work will be on expanding the molecular profiling capabilities of our system by designing new emerging biomarker probes, incorporating artificial intelligence for advanced pattern recognition, and automating data extraction and processing. The ultimate objective continues to be the development of an all-encompassing surgical navigation platform that integrates anatomical, functional, and molecular data to enable real-time guidance for tailored surgical procedures.

In summary, the integration of molecular profiling within the fluorescence navigation system operating on a multispectral basis constitutes an advancement in addressing the glioma invasiveness visualisation problem. By offering real-time contextual information at a molecular level of the tumour margins, this technology enhanced the accuracy of resection performed whilst maintaining neurologic functionality—an equilibrium that enhances patient care and their life standards.

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