Raman spectroscopy to differentiate between fresh tissue samples of glioma and normal brain: a comparison with 5-ALA–induced fluorescence-guided surgery

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OBJECTIVE Raman spectroscopy is a biophotonic tool that can be used to differentiate between different tissue types. It is nondestructive and no sample preparation is required. The aim of this study was to evaluate the ability of Raman spectroscopy to differentiate between glioma and normal brain when using fresh biopsy samples and, in the case of glioblastomas, to compare the performance of Raman spectroscopy to predict the presence or absence of tumor with that of 5-aminolevulinic acid (5-ALA)–induced fluorescence.

METHODS A principal component analysis (PCA)–fed linear discriminant analysis (LDA) machine learning predictive model was built using Raman spectra, acquired ex vivo, from fresh tissue samples of 62 patients with glioma and 11 glioma-free brain samples from individuals undergoing temporal lobectomy for epilepsy. This model was then used to classify Raman spectra from fresh biopsies from resection cavities after functional guided, supramaximal glioma resection. In cases of glioblastoma, 5-ALA–induced fluorescence at the resection cavity biopsy site was recorded, and this was compared with the Raman spectral model prediction for the presence of tumor.

RESULTS The PCA-LDA predictive model demonstrated 0.96 sensitivity, 0.99 specificity, and 0.99 accuracy for differentiating tumor from normal brain. Twenty-three resection cavity biopsies were taken from 8 patients after supramaximal resection (6 glioblastomas, 2 oligodendrogliomas). Raman spectroscopy showed 1.00 sensitivity, 1.00 specificity, and 1.00 accuracy for predicting tumor versus normal brain in these samples. In the glioblastoma cases, where 5-ALA–induced fluorescence was used, the performance of Raman spectroscopy was significantly better than the predictive value of 5-ALA–induced fluorescence, which showed 0.07 sensitivity, 1.00 specificity, and 0.24 accuracy ($p = 0.0009$).

CONCLUSIONS Raman spectroscopy can accurately classify fresh tissue samples into tumor versus normal brain and is superior to 5-ALA–induced fluorescence. Raman spectroscopy could become an important intraoperative tool used in conjunction with 5-ALA–induced fluorescence to guide extent of resection in glioma surgery.

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KEYWORDS Raman spectroscopy; 5-ALA fluorescence; glioma; oncology

RESECTION plays an important role in the treatment of both high- and low-grade gliomas. It is generally accepted that increased extent of resection has a beneficial effect on survival, despite the lack of class I evidence.^{1,2} Maximal resection must not, however, be at the cost of giving the patient a neurological deficit, and the aim of surgery is to achieve maximal resection with-

out postoperative deficit. There are many surgical adjuncts available to help the surgeon achieve safe maximum resection, including neuronavigation, intraoperative MRI (iMRI), intraoperative ultrasonography, and fluorescence-guided surgery.³ Evidence for the effectiveness of these modalities in maximizing the extent of resection is variable, with randomized trials only available for iMRI⁴ and

ABBREVIATIONS 5-ALA = 5-aminolevulinic acid; CI = confidence interval; EMSC = extended multiplicative scatter correction; IDH = isocitrate hydrogenase; IHC = immunohistochemistry; LDA = linear discriminant analysis; PC = principal component; PCA = PC analysis.

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5-aminolevulinic acid (5-ALA)-induced fluorescence-guided surgery.⁵ Only 5-ALA-induced fluorescence has been shown to have an effect on patient survival, with the recommendation of its routine use in the 2018 National Institute for Health and Care Excellence (NICE) guidelines for the management of primary brain tumors.⁶ There is evidence, however, that significant amounts of tumor extend beyond the visible fluorescence boundary, with between 25% and 65% false-negative rates reported in biopsies taken beyond the visible fluorescent margin.^{7–11}

Raman spectroscopy is a vibrational spectroscopy technique that has been applied to the identification of disease in various areas, including neurooncology.^{12–15} By probing the unique molecular vibrations that depend on the composition and structure of samples, Raman spectroscopy provides a wealth of information on a cellular and molecular level of both solid and liquid specimens. The huge advantage of the technique is that it is nondestructive, and no sample preparation is required. This makes it ideal for intraoperative neurosurgical applications to define tumor margins. Raman spectroscopy can accurately differentiate between normal brain, necrosis, and various brain tumor pathologies when applied to either formalin-fixed, paraffin-embedded¹⁶ or cryopreserved^{17–26} histopathological samples. There has only been one report of intraoperative use of Raman spectroscopy in neurosurgery, which reported the ability to distinguish normal brain from both bulk tumor and infiltrating tumor with an accuracy of 92%, sensitivity of 93%, and specificity of 91%.²⁷

The aims of this study were twofold: 1) evaluate the ability of Raman spectroscopy to differentiate between glioma and normal brain when using fresh biopsy samples, and 2) in the case of glioblastomas, compare the performance of Raman spectroscopy in predicting the presence or absence of tumor with that of 5-ALA-induced fluorescence.

In our center, where appropriate, some patients undergo functional guided resection of glioblastomas²⁸ with the aim of achieving a maximal or even supramaximal functional resection when safe to do so (all cases performed by the senior author [P.P.]). In these cases, the resection already goes beyond the visual 5-ALA-induced fluorescence boundary, and thus there are biopsies available from regions of nonfluorescing tissue resection that can be used to evaluate novel tumor-detecting modalities such as Raman spectroscopy.

Methods

Study Participants and Intraoperative Biopsy

Ethical approval for the use of human tissue was obtained through the Oxford Brain Bank Research Ethics Committee approval. All tissue used was deemed surplus to diagnostic requirements. A subset of patients who underwent functional guided resection of a glioma, where supramaximal resection was achieved, were included in this study. A 20 mg/kg oral dose of 5-ALA was given to all patients with suspected glioblastoma on preoperative imaging unless contraindicated. The 5-ALA was given 2 hours before the start of surgery to achieve peak fluorescence at approximately 4–6 hours.^{29,30}

Our method of functional guided surgery has been pre-

sented elsewhere.²⁸ Briefly, we define “functional guided surgery” as resecting tumor up to approximately 1.5–2 cm beyond the contrast-enhancing boundary on neuronavigation (or the 5-ALA fluorescence boundary, as 5-ALA fluorescence is known to closely correlate with contrast enhancement on MRI^{8,11}) or resecting until a functional white fiber tract or functional brain region is reached. If there is no functional brain encountered, then an approximately 1.5- to 2-cm margin beyond the contrast-enhancing or fluorescing boundary is considered the limit of resection, and a “supramaximal” resection is said to have been achieved. To evaluate the functional boundaries, diffusion tensor imaging is undertaken preoperatively and incorporated with the neuronavigation (Brainlab) to identify the spatial location of white matter tracts relative to tumor; awake craniotomy is performed in most of these cases.

Given the intratumoral heterogeneity of gliomas, we routinely take tumor biopsy samples from different anatomical locations within and at the limit of the tumor resection. The location of each biopsy sample is recorded using the intraoperative neuronavigation system (Fig. 1) and labeled according to its spatial anatomical location. Prior to resection, each sample location is inspected for 5-ALA-induced fluorescence using the blue light source on either the operative microscope (Zeiss OPMI Pentero 900, Carl Zeiss) or endoscope (Karl Storz Hopkins endoscope, Karl Storz Endoscopy) and the result recorded. The degree of fluorescence is reported as “none” (no fluorescence), “weak” fluorescence, or “strong” fluorescence, using the fluorescence grading system proposed by Stummer et al.,¹¹ who defined visual fluorescence as either “strong” (“solid” or “red”) or “weak” (“vague” or “salmon”). Microbiopsy forceps are used to excise the biopsy sample, and the fresh tissue is taken to neuropathology for immediate examination. Figure 2 depicts tumor cavities with different degrees of fluorescence and the corresponding white light images.

Neuropathological Evaluation

Each biopsy sample, approximately 3 mm³, was cut in half: half was taken straight for Raman analysis with no further preparation, and half was processed as part of the routine diagnostic pathway, undergoing formalin fixation and paraffin embedding. For tumor found to be isocitrate hydrogenase (IDH)-mutated on routine IDH1-R132H immunohistochemistry (IHC), the resection cavity biopsies also underwent IDH1-R132H IHC testing to identify tumor cells within the sample. No tumor carried a rare IDH1 or IDH2 mutation in this study. For tumors diagnosed as IDH-wildtype, the cavity biopsies underwent Ki-67/MIB1 IHC. MIB1 is an antibody for Ki-67 protein that is expressed in the active phase of the cell cycle; thus, Ki-67/MIB1 is a marker of cell proliferation.³¹ Although not specific for tumor cells, the amount of cell proliferation together with atypical morphology of labeled nuclei in a section gives an indication of the presence of tumor infiltration. With the combination of the standard H & E and the appropriate IHC stain, the neuropathologist categorized the cavity biopsies into three groups (examples illustrated in Fig. 3): 1) tumor = sample is characteristic of tumor throughout (Fig. 3D, H, I, and L); 2) infiltrating tumor = evidence of

FIG. 1. MRI for supramaximal resection. **A:** Preoperative MRI demonstrating enhancing tumor consistent with glioblastoma. **B:** Intraoperative neuronavigation showing the location of supramaximal resection cavity biopsies beyond contrast-enhancing tumor. **C:** Postoperative MRI demonstrating supramaximal resection up to the white matter tracts. All images are T1-weighted sequences with gadolinium contrast. Colored overlay represents the estimated location of white fiber tracts using diffusion tensor imaging. Figure is available in color online only.

FIG. 2. Examples of fluorescence quantification. Resection cavity biopsy location with no visual fluorescence with blue light (B) and corresponding white light image (A). Resection cavity biopsy location with “weak” fluorescence with blue light (D) and corresponding white light image (C). Example of strong fluorescence (F) and corresponding white light image (E). Outlined quadrants show the location of the biopsy taken. Figure is available in color online only.

tumor cell infiltration into normal white or gray matter (the degree of infiltration can vary; Fig. 3A–C, E–G, J, and K); and 3) no tumor cells = histological and IHC features of normal brain with no evidence of tumor.

This histological grading system used is modified from the grading system reported Lau et al.,⁷ a paper that aimed to correlate intraoperative fluorescence intensity with the degree of histological cellularity. In the current study, the “low infiltrating” and “moderate infiltrating” grades (grades 1 and 2 in the Lau et al. paper) are grouped into “infiltrating tumor,” giving a three-grade system: “no definitive tumor cells,” “infiltrating tumor,” and “tumor” (Fig. 3).

Normal Fresh Brain Biopsies

To build a classification model using Raman spectra to differentiate between tumor and normal brain, it was nec-

essary to record Raman spectra from several normal fresh brain biopsies. These were acquired from patients undergoing mesial temporal lobectomy for epilepsy, in which a small amount of normal brain is routinely removed as access tissue. The normal brain biopsies, incorporating both gray and white matter, were halved, with half going straight for Raman analysis and half undergoing standard H & E staining and neuropathology evaluation to confirm the presence of normal brain tissue.

Raman Spectroscopy

Our protocol for the intraoperative rapid analysis of fresh samples has been reported previously.²⁶ Briefly, each fresh tissue sample was compressed into a grooved stainless-steel slide and the slide placed in the Raman spectrometer (Renishaw benchtop RA800 series spectrometer, Renishaw plc) equipped with a $\times 50$ objective and a 785-nm excitation laser (outside the excitation or fluorescence ranges of porphyrin IX) that provided 180 mW of power at the objective. Preliminary experiments showed no effect of porphyrin IX on the Raman spectra. Spectra were obtained from 50 to 200 widely distributed and randomly chosen locations across the smeared sample surface. The number of spectra collected varied depending on the amount of in-focus fresh tissue available for analysis. The exposure time used for each spectrum was 2 seconds.

Development of the Classification Model: “Tumor” Versus “Normal Brain”

All model development and data analysis were performed using the Renishaw Data Classifier (Renishaw plc) and MATLAB R2015b (MathWorks). The Raman spectral region between 400 and 1850 cm^{-1} was analyzed, excluding the Raman band of oxygen (1560–1578 cm^{-1}). Spectral preprocessing steps included signal-to-noise threshold filter, cosmic ray removal using a standard deviation threshold, third-order polynomial baseline correction, and normalization using extended multiplicative scatter correction (EMSC).³²

A two-group classification model—tumor versus normal brain—was built using principal component analysis (PCA)–fed linear discriminant analysis (LDA). In this method, PCA is used to reduce the data dimensionality, with ANOVA used to select only those principal components (PCs) that describe a statistically significant difference between the two data sets. An LDA model (supervised machine learning model) is then built from those significant PCs. Model performance was assessed using leave-one-sample-out independent cross-validation to generate model sensitivity and specificity. A binomial distribution hypothesis test was conducted on each sample to ensure that the number of spectra obtained did not significantly influence the model prediction accuracy. The mean spectra and the negative of the second derivative transformation of the mean spectra were calculated using the normalized spectra from each classification group. Using the normalized mean spectra, statistical differences between the Raman peak intensities in each classification group were evaluated using the Wilcoxon rank-sum test. Tenta-

FIG. 3. Histological grading system. **A:** Glioblastoma-wildtype, infiltrating tumor. H & E, original magnification $\times 50$. **B:** Glioblastoma-wildtype, low-density infiltrating tumor. MIB, original magnification $\times 50$. **C:** Glioblastoma-wildtype, higher-density infiltrating tumor. MIB, original magnification $\times 50$. **D:** Glioblastoma-wildtype, tumor. H & E, original magnification $\times 100$. **E:** Glioblastoma-IDH-mutated, infiltrating tumor. H & E, original magnification $\times 100$. **F:** Glioblastoma-IDH-mutated, infiltrating tumor. IDH-R132, original magnification $\times 100$. **G:** Glioblastoma-IDH-mutated, higher-density infiltrating tumor. IDH-R132, original magnification $\times 100$. **H:** Glioblastoma-IDH-mutated, tumor. H & E, original magnification $\times 100$. **I:** Glioblastoma-IDH-mutated, tumor. IDH-R132, original magnification $\times 100$. **J:** Oligodendroglioma, infiltrating tumor. H & E, original magnification $\times 100$. **K:** Oligodendroglioma, infiltrating tumor. IDH-R132, original magnification $\times 100$. **L:** Oligodendroglioma, tumor. H & E, original magnification $\times 100$. **M:** Oligodendroglioma, tumor. IDH-R132, original magnification $\times 100$. Figure is available in color online only.

tive molecular assignment of the identified Raman peaks was performed based on the published literature.^{17,22,33}

For the “tumor” group, the input spectra came from the data set of intraoperative fresh tissue spectra collected for another study reported elsewhere.²⁶ The “normal brain” group input spectra were taken from the fresh tissue analysis of normal brain samples acquired from epilepsy surgery cases as described above.

Classification of Resection Cavity Spectra and Comparison With 5-ALA-Induced Fluorescence

Spectra from the resection cavity samples were individually classified into one of the two groups in the built

model (individual spectra classification). The percentage of spectra classified into each group for a given biopsy was also calculated, and the group with the greatest number of spectra classified into it was taken to be the resultant overall classification of that biopsy (overall biopsy classification). Values of sensitivity, specificity, and accuracy were calculated in each case, along with 95% confidence interval (CI) calculations (Clopper-Pearson CIs^{34,35}), and compared with similar parameters for 5-ALA-induced fluorescence in the cases of glioblastoma where 5-ALA was used. McNemar’s test for related categorical data^{36,37} was used to measure the statistical differences between Raman and 5-ALA for differentiating tumor versus normal brain.

TABLE 1. PC-LDA model of tumor versus normal brain

Classification Group	Sensitivity	Specificity	Accuracy
Tumor	0.96	0.99	0.99
Normal brain	0.99	0.96	0.99

Performance of model using a leave-one-patient-out cross-validation.

Results

“Tumor” Versus “Normal Brain” Classification Model

Eleven fresh normal brain biopsies were acquired from 11 patients undergoing temporal lobectomy for epilepsy. Neuropathology review confirmed normal gray and white matter in every case. A total of 1825 spectra of fresh normal brain were used in the model as “normal brain” input spectra, with a mean of 166 spectra per sample. For the “tumor” input spectra, 9799 spectra from 62 fresh tissue samples originating from another study were used.²⁶ This included 36 glioblastomas, IDH-wildtype; 10 glioblastomas, IDH-mutant; 7 astrocytomas, IDH-mutant WHO grade III; 4 astrocytomas, IDH-mutant WHO grade II; 2 anaplastic oligodendrogliomas, WHO grade III; and 3 oligodendrogliomas, WHO grade II. The model performed extremely well when assessed using leave-one-patient-out cross-validation, as shown by the high sensitivity and specificity illustrated in Table 1 and Fig. 4. The mean spectra and the negative of the second derivative transformation of the mean spectra for “tumor” and “normal brain” are illustrated in Fig. 5. The difference spectrum (tumor minus normal brain) is also shown in this figure. Lipid peaks are more prominent in tumor compared to normal brain spectra, with dominant peaks at 825, 853, 1087, 1124, and 1305 cm^{-1} . This is also true for peaks representing DNA (517, 885, 1206, and 1342 cm^{-1}). Conversely, normal brain spectra have stronger amino acid peaks (540, 615, 635, and 649 cm^{-1}) and protein peaks (1522 and 1553 cm^{-1} , both representing amide III protein) compared with tumor spectra.

Classification of Resection Cavity Spectra and Comparison With 5-ALA-Induced Fluorescence

Raman analysis of 23 supramaximal resection cavity biopsies was undertaken in a total of 8 patients. The cases and corresponding biopsies are summarized in Table 2. There were 9 biopsies from 3 glioblastoma, IDH-wildtype tumors; 8 biopsies from 3 glioblastoma, IDH-mutant tumors; and 6 biopsies from 2 oligodendrogliomas (1 WHO grade II and 1 WHO grade III). All neuronavigation images documenting the location of the resection cavity biopsies were beyond the area of enhancement on the contrast-enhanced preoperative MRI. The neuropathology assessment categorized 2 biopsies as tumor, 17 as infiltration zone, and 4 as having no tumor cells.

In all 6 glioblastoma cases 5-ALA was used, and all showed strong fluorescence in the main bulk of the tumor. Fluorescence was observed at the site of biopsy in 1 of 17 biopsies (Table 2). For the cases in which 5-ALA was used, the absence of fluorescence was highly specific (specificity = 1.00, rate of true negatives)—as in all biopsies, where there was no tumor there was also no fluorescence—but

FIG. 4. PC-LDA model of tumor versus normal brain. LDA score histogram. Figure is available in color online only.

poorly sensitive (sensitivity = 0.07), as most cavity biopsies containing tumor did not fluoresce.

Table 3 compares the performance of 5-ALA-induced fluorescence for predicting tumor versus normal brain with the performance of Raman classification models. Raman performance depends on which method is used to classify the biopsy samples into tumor/normal brain groups. Using overall biopsy classification—classifying the biopsy based on the group with the greatest percentage of spectra classified to it—the performance is extremely high, with correct classification of biopsies in every case, although there is a wide 95% CI range due to the low sample size. This performance is significantly better than 5-ALA-induced fluorescence ($\chi^2 = 11.077$, $p = 0.0009$). Using individual spectra classification, there is still far superior, and statistically significant, performance compared to 5-ALA-induced fluorescence ($\chi^2 = 1920.12$, $p = 0.0001$). The performance is also high when oligodendroglioma cases are included. In the 2 oligodendrogliomas 5-ALA was not used, and thus a comparison between Raman spectroscopy and fluorescence is not possible for these cases. It is possible, however, to conclude that Raman spectroscopy is more sensitive than the contrast-enhancing boundary identified on the neuronavigation (both oligodendrogliomas were contrast-enhancing on preoperative MRI).

Discussion

This preliminary study shows that Raman spectroscopy can accurately differentiate between infiltrating glial tumor and normal brain at the margin of a functionally guided, supramaximal glial tumor resection cavity. Raman spectroscopy is also significantly superior to 5-ALA-induced fluorescence in predicting glioblastoma tumor infiltration versus normal brain at this margin.

FIG. 5. Tumor and normal brain spectra. Mean spectra (*upper*), difference spectra (*lower*), and negative of second derivative transformation of mean spectra for tumor and normal brain after EMSC (*center*). The wavenumbers of prominent Raman peaks are labeled and the statistical significance ($p < 0.01$) of the difference between tumor and normal brain peak intensities is indicated by an asterisk. Figure is available in color online only.

“Tumor” Versus “Normal Brain” Classification Model

To build a predictive classification model to classify normal brain and tumor, Raman spectra from fresh tissue samples of the core of 62 different gliomas were used to represent “tumor” and Raman spectra from fresh tissue samples of 11 patients undergoing mesial temporal lobectomy for epilepsy were used to represent “normal brain.” The tumor group included a range of different tumor types with the assumption, backed up by evidence in the literature^{17,22,24} (including work carried out by our group²⁶), that the various tumor types are chemically closer to each other than they are to normal brain tissue. Using independent cross-validation, the resulting model demonstrated excellent performance in classifying tumor versus normal brain spectra (Table 1, Fig. 4). These data are consistent with previous studies that have demonstrated the potential of Raman spectroscopy to differentiate glioma from normal brain tissue using ex vivo formalin-fixed, paraffin-embedded^{16,26} frozen tissue^{17,20,22,24,26,38} and ex vivo fresh tissue.^{26,39} Moreover, the differences found in this study between the normal brain and tumor spectra, namely dominant lipid and DNA peaks and reduced protein peaks in the tumor spectra, are also consistent with previous reports in the literature.^{18,20,22,24}

Classification of Resection Cavity Spectra and Comparison With 5-ALA–Induced Fluorescence

In the bulk of the tumor and up to the contrast-enhancing margin seen on image guidance, there is little doubt that 5-ALA–induced fluorescence is a powerful surgical adjunct. The correlation between contrast-enhancing tumor on preoperative MRI and 5-ALA–induced fluorescence is well recognized,⁸ and accounts for increased extent of tumor resection and progression-free survival when using 5-ALA–induced fluorescence-guided surgery.⁵ Fluorescence-guided surgery is conducive to the neurosurgical workflow, only requiring the surgeon to press a button to switch from white to blue light while viewing the tissue through the surgical microscope. There is no need to pause resection or to change instruments as would be the case with a probe-based system. This study focuses on biopsy samples taken at the boundaries of supramaximal resections for glioblastoma cases, beyond the contrast enhancement and 5-ALA–induced fluorescence. Here, the predictive value of 5-ALA is reduced, with literature reporting between 25% and 65% false-negative rates in biopsies taken beyond the visual fluorescent margin.^{7–11} In this study we report an even higher false-negative rate, with only 1

TABLE 2. Supramaximal resection cases and resection cavity biopsies

Pathology (WHO grade)	Resection Cavity Biopsy Location	5-ALA Fluorescence Seen at Location of Quadrant	Neuropathology Assessment			Classification of Spectra	
			IDH*	MIB-1†	Categorization	No. of Spectra‡	Proportion Tumor Proportion Normal
GB-WT	Anterior	None	0		No tumor cells	65	0.37 0.63
	Posterior	None	5%		Infiltrating tumor	194	0.99 0.01
GB-WT	Posterior	None	10%		Infiltrating tumor	217	0.99 0.01
	Inferior	Weak	5%		Infiltrating tumor	163	0.96 0.04
	Posterior inferior	None	10%		Infiltrating tumor	160	0.92 0.08
	Medial	None	10%		Infiltrating tumor	180	0.94 0.06
	Anterior	None	5%		Infiltrating tumor	99	1 0
GB-WT	Medial	None	5%		Infiltrating tumor	160	0.95 0.05
	Posterior	None	5%		Infiltrating tumor	122	0.99 0.01
	Inferior	None	+		Tumor	144	1 0
GB-MUT	Posterior	None	+		Infiltrating tumor	183	0.97 0.03
	Superior	None	+		Infiltrating tumor	105	1 0
	Anterior	None	+		Tumor	107	0.97 0.03
GB-MUT	Posterior	None	-		No tumor cells	206	0.23 0.77
	Inferior	None	+		Infiltrating tumor	86	0.94 0.06
	Posterior	None	-		No tumor cells	183	0.31 0.69
	Medial	None	+		Infiltrating tumor	108	1 0
	Posterior	Not used	+		Infiltrating tumor	72	0.97 0.03
Oligo (III)	Inferior lateral	Not used	+		Infiltrating tumor	42	0.83 0.17
	Superior	Not used	+		Infiltrating tumor	186	0.97 0.03
Oligo (II)	Frontal	Not used	+		Infiltrating tumor	78	0.97 0.03
	Temporal	Not used	-		No tumor cells	170	0.41 0.59
	Posterior	Not used	+		Infiltrating tumor	88	1 0

GB = glioblastoma; MUT = mutant; Oligo = oligodendroglioma; WT = wildtype.

Boldface type indicates the overall classification of each biopsy (tumor or normal).

* Sample staining positive for IDH-R132H mutation (+) or not (-).

† Given as a percentage of cells per field of view.

‡ Number of spectra after preprocessing.

of 14 biopsies with infiltrated tumor showing fluorescence. At the limit of the resection, where there is a low level of tumor infiltration, the decision regarding further resection balanced against increased risk of neurological disability is most critical. It is at this limit where interrogation with Raman spectroscopy, likely with an intraoperative probe-based device, would have application, with our data suggesting that this would give a significantly better tumor predictive value than 5-ALA-induced fluorescence. It is perhaps disheartening that in this study most supramaxi-

mal resection cavity biopsies were found to have infiltrating tumor, but it should not be surprising, because these are, after all, named “diffuse gliomas” by the WHO.⁴⁰ Despite this, previous research by our group has shown that supramaximal resection in glioblastoma results in a statistically significant increase in progression-free survival compared to gross-total resection,²⁸ and a recently published systematic review and meta-analysis comparing gross-total with supramaximal resection concluded, despite the poor quality of available evidence, that supramaximal resection resulted

TABLE 3. Performance of 5-ALA and Raman spectroscopy to detect tumor versus normal brain

Modality (classification method)	Sensitivity	Specificity	Accuracy
5-ALA-induced fluorescence	0.07 (0.02–0.34)	1.00 (0.29–1.00)	0.24 (0.07–0.50)
Raman spectroscopy			
Overall biopsy classification, either including or excluding oligos	1.00 (0.77–1.00)	1.00 (0.29–1.00)	1.00 (0.85–1.00)
Individual spectra classification	0.97 (0.96–0.98)	0.72 (0.67–0.76)	0.93 (0.91–0.94)
Individual spectra classification including oligos	0.97 (0.96–0.98)	0.68 (0.64–0.72)	0.91 (0.90–0.92)

Range in parentheses = 95% CI.

in a 53% lower risk of mortality and increased median survival of 6.4 months compared to gross-total resection.⁴¹

The only reported intraoperative use of a Raman probe comes from Jermyn et al.,²⁷ whose case series consisted of 17 patients with a mix of tumor pathologies. Brain samples were also taken from areas around the tumor bulk and designated by the neuropathologists as either normal brain or infiltrating tumor. Using a boosted trees machine learning algorithm, they were able to distinguish normal brain from both bulk tumor and infiltrating tumor with an accuracy of 92%, sensitivity of 93%, and specificity of 91%. Our study, although using ex vivo Raman spectroscopy analysis of fresh tissue, adds the important comparison between the performance of Raman spectroscopy and that of the current gold standard of 5-ALA-induced fluorescence.

The principal reason that Raman spectroscopy outperforms 5-ALA fluorescence in this study is the higher detection rate, i.e., higher sensitivity for tumor infiltration, with more than 95% of spectra of infiltrating tumor correctly classified in most biopsy samples. The correct classification of normal brain is less accurate, with between 59% and 77% of spectra from samples with no tumor cells correctly classified. In contrast, the classification model built to classify the cavity biopsies showed high sensitivity and specificity on leave-one-patient-out cross-validation. Here, the normal brain spectra came from samples from patients with epilepsy. This likely reflects the fact that the “normal” penumbra of brain around a glioma is only normal as far as the presence of individual tumor cells is concerned but will show peritumoral edema. It should also be noted that only in IDH1-R132H-mutant glioma can a definitive statement on single-cell invasion of the biopsy cavity can be made. In the IDH-wildtype gliomas, no marker exists that absolutely excludes single-cell infiltration: the proliferation marker Ki-67 (MIB-1) will only highlight atypical nuclei that have entered the cell cycle, and migrating glioma cells have not always entered the cell cycle. The use of normal brain tissue from nontumor patients is still valid, and indeed provides the only guarantee that there are no occult tumor cells within the samples. We note that the mean Raman spectra for tumor and normal brain, as derived from the model, have similar peak characteristics and differences to those reported in published data on tumor versus normal brain Raman spectroscopy analysis.^{22–24,27,42}

The data presented here show that Raman spectroscopy is more sensitive at detecting tumor, and has a lower false-negative rate, than 5-ALA-induced fluorescence, but that the false-positive rate using Raman spectroscopy analysis might be problematic. In the Jermyn et al. paper, a specificity of 91% is reported.²⁷ This is an encouragingly high figure, but in practical terms means that just under 1 in 10 areas that are sampled will give a false-positive result, indicating tumor in an area of potentially normal brain. Jermyn et al. explored in more detail the false-negative rate and estimated the threshold of detection for their intraoperative device to be approximately 15% cancer cell burden, or 17 human cancer cells/0.0625 mm². An attempt to quantify the false-negative rate must be commended, but we would suggest some caution in the interpretation of these data as the correlation between the area sampled in

vivo by the probe and then biopsied and reviewed by neuropathology may not be completely accurate. Moreover, in only 4 of the 14 samples included in this subanalysis was the specific tumor marker IDH1-R132H used, with the remaining samples only evaluated using H & E staining, and thus not having a specific marker for diffuse glioma cells. Future intraoperative studies should include an additional step in the protocol in which, after intraoperative Raman spectroscopy analysis and biopsy, the fresh sample immediately undergoes ex vivo Raman spectroscopy analysis with the probe device and/or an ex vivo Raman microspectrometer. Accurately establishing the threshold for tumor detection by a Raman probe is an essential part of any future work, but the focus should also be on accurately quantifying the false-positive rate. Future intraoperative Raman probe projects require the clinical spectroscopy and neurosurgery communities to agree on what level of accuracy is both technically achievable and clinically useful to allow useful intraoperative decision-making.

Study Limitations and Future Work

The main limitations of this study are the small number of cases and the ex vivo Raman analysis. The small sample size prevents a more detailed analysis of the data that would ideally include reporting the model performance with each permutation of tumor density and fluorescence. Moreover, it must be noted that the performance of the model is likely to fall as the number of samples increases due to the increase in variability that would be introduced. However, in this study we report a high-performing model that we feel would be robust to such an increase in variability; further studies with significantly greater sample sizes will help clarify this. In vivo studies will have significant challenges, including reduced performance due to blood or fluid in the measurement field, signal loss from the probe fiber, and noise from surrounding light sources. Some of these issues have been addressed by Jermyn et al.,^{27,43} and some methodological considerations have been mentioned above. Future intraoperative Raman probe evaluation will require significant collaboration between neurosurgeons and clinical spectroscopists to produce a usable intraoperative device that is highly accurate and user-friendly.

Conclusions

With this further work it is feasible that Raman spectroscopy could become an important intraoperative tool used in conjunction with 5-ALA to perhaps guide resection beyond the 5-ALA fluorescence boundary where appropriate, allowing for maximal or supramaximal resection and possibly an increase in patient survival.

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Conception and design: Livermore, Isabelle, Bell, Ansorge. Acquisition of data: Livermore, Edgar, Stacey, Ansorge. Analysis and interpretation of data: Livermore, Isabelle, Bell. Drafting the article: Livermore. Critically revising the article: Isabelle, Bell, Edgar, Voets, Stacey, Ansorge, Vallance, Plaha. Reviewed submitted version of manuscript: Livermore, Isabelle, Edgar, Voets, Stacey, Ansorge, Vallance, Plaha. Approved the final version of the manuscript on behalf of all authors: Livermore. Statistical analysis: Livermore. Administrative/technical/material support: Livermore, Isabelle, Bell, Ansorge, Vallance. Study supervision: Isabelle, Bell, Ansorge, Vallance, Plaha.

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