SUPPLEMENTARY MATERIALS

METHODS

Image segmentation

SRS microscopy images were segmented and analyzed using custom software developed in the MATLAB Image Processing Toolbox (The MathWorks, Inc.). The segmentation program has three modules. The first imports and preprocesses the image via histogram truncation, removal of blood vessels and artifacts, division of each image into 300 × 300 μm^2 FOVs, and homomorphic filtering of each FOV. The second segments axons from the 2845 cm^{-1} channel as follows. First, each FOV is converted to a binary image using a threshold derived from the mean intensity of that FOV. The image is de-noised via image opening, and remaining objects are dilated, creating a "rough mask" of candidate objects. In parallel, the FOV is also sharpened and converted to binary using a threshold defined by Otsu's method (*43*). Image opening is performed and the result is convolved with a Sobel edge kernel, converted to binary using Otsu's thresholding, and opened again. All remaining objects are then sorted by eccentricity (defined as the ratio of the major and minor axes lengths of the bounding ellipse) and area. Those having eccentricity less than 0.85 and size less than 600 pixels are removed. Pixels included in both this mask and the rough intensity mask are counted as axons. The number of perimeter pixels belonging to the resultant objects is summed as the axonal length.

Nuclear segmentation begins with subtraction of the 2845 cm^{-1} channel from the 2930 cm^{-1} channel, and setting all pixels with intensities less than the FOV mean plus 1.5 standard deviations to zero. Image opening is then conducted with a 5 × 5 square mask, and the image is converted to binary via Otsu's thresholding. All objects smaller than one half the size of a user-

selected “smallest nucleus” are then removed. A watershed algorithm is then used to separate contiguous objects containing multiple local maxima. Objects are then thickened with maintained connectedness, and holes within objects are filled in.

Fresh human brain tumor specimens

Tissues were transferred to 4°C saline, shipped same-day to the laboratory (X.S.X.), sectioned to a thickness of 1 mm, placed in a glass slide with a central cavity sealed with two cover slips, and imaged in transmission mode, as described above. The number of biopsies per patient varied (range: 1-10; average: 2.9) depending on the size of the tumor and availability of tissue for admission to the brain tumor tissue bank. The number of FOVs imaged per specimen (on average 29 FOV per specimen and 67 FOV per patient) varied depending on the size and quality of the specimen. A materials transfer agreement for sharing of specimens from the University of Michigan with X.S.X. at Harvard University (A14238) was in place.

H&E microscopy and immunohistochemistry

SRS microscopic images were compared with corresponding specimens that were formalin-fixed, paraffin-embedded, sectioned at 6-μm thickness, H&E stained, and mounted on glass slides.

Immunohistochemistry was performed on formalin-fixed, paraffin-embedded, 6-μm tissue sections, using antibodies against neurofilament (clone 2F11; Ventana Medical Systems), CD68 (clone KP-1; Ventana Medical Systems and Roche Diagnostics), and EGFR (clone 5B7;

Ventana Medical Systems and Roche Diagnostics). All immunohistochemistry was performed on an automated Ventana platform using the Ultraview DAB detection system.

Survey methodology

A web-based survey was created by randomly selecting 75 SRS and 75 corresponding H&E 300x300 μm FOVs (including 25 FOVs representing normal to minimally hypercellular brain tissue, 25 FOVs representing infiltrating tumor, and 25 FOVs representing dense tumor) from 6 patients. The selected FOVs were approved by two neuropathologists (S.C.P and A.F.). Three blinded neuropathologists (S.R., M.S., and S.V.) were asked to rate the individual FOVs as normal to minimally hypercellular, infiltrating tumor, or dense tumor. Survey responses were recorded and the correlation between the two modalities was analyzed using the κ statistic and its two-sided 95% CI, based on the asymptotic variance of the κ statistic and the standard normal distribution (*45*). Analyses were performed using SAS software version 9.2 (SAS Institute Inc.).

SUPPLEMENTARY DISCUSSION

Optical techniques such as confocal fluorescence microscopy and photoacoustic imaging have also been explored for imaging brain tumor histology (*30, 31*). However, these methods rely upon the uptake of dyes, which are unevenly distributed within tumors (*32, 33*) and do not allow direct visualization of normal brain tissue that should be preserved. Consequently, label-free techniques have also been proposed for imaging during brain surgery. Notably, spontaneous Raman spectroscopy has been successfully applied to the detection of tumor infiltration in a neurosurgical setting. However, to acquire spontaneous Raman spectra during surgery, ambient lighting must be turned off and custom lighting sources would be needed to illuminate the operating room (*17*). In addition, point spectroscopy techniques, such as spontaneous Raman spectroscopy, lack the high spatial resolution of SRS microscopy and rely exclusively on spectroscopic analysis for diagnosis.

Optical coherence tomography (*34*), confocal reflection (*35*), and third harmonic generation (*36*), reveal some morphologic features of brain tissue but lack the chemical contrast that helps to distinguish tumor-infiltrated and non-infiltrated brain. Second harmonic generation and two-photon excited fluorescence microscopy primarily highlight collagen and auto-fluorescent species, respectively. Compared to other organs, we have found that both signals are weak in brain tissue. SRS microscopy is unique because it achieves high sensitivity and specificity for detecting brain tumor infiltration by combining chemical specificity (protein:lipid ratio) and high-resolution morphological information (cellularity and axonal density).

Prior to the demonstration of SRS in 2008 (*16*), coherent anti-Stokes Raman scattering (CARS) microscopy was proposed for brain tumor imaging (*37*) because of its ability to detect tumor infiltration based on alterations in the CH range of the Raman spectrum and tissue architecture in glioma models (*38*). SRS microscopy was later shown to have superior nuclear contrast in comparison to CARS microscopy (*19*). Background signals in SRS microscopy (*39*), such as cross-phase modulation, and

thermal effects are much weaker than the CH region SRS signals and do not degrade image quality. Two-color two-photon absorption provides an additional color that can highlight erythrocytes. Combined with lock-in detection, SRS imaging can be carried out under standard ambient lighting conditions as would occur in a typical operating room. A broadband CARS method incorporating the fingerprint region of the Raman spectrum promises to further increase the chemical specificity of the technique (*40*). In this report, we have chosen to focus on high-speed imaging based on narrowband SRS and strong CH region signal such that we can rapidly acquire high-resolution histologic images.

SUPPLEMENTARY FIGURES

Figure S1. SRS microscopy of pediatric medulloblastoma. (**A**) A pediatric medulloblastoma (arrowhead) is composed of (**B**) highly cellular tumor with small round blue cells that may organize into (**C**) pseudorosettes (arrowheads) and often contain (**D**) apoptosis (arrowheads) and (**E**) mitotic figures (arrowheads). Both SRS microscopy (left) and H&E-stained sections (right) are shown in (B to E).

Figure S2. SRS microscopy in a previously irradiated recurrent oligodendroglioma. (**A**) An oligodendroglioma (arrowhead) previously treated with surgical resection and radiation locally recurred and was imaged with SRS microscopy during a second resection. Treatment-related changes included: (**B**) hemosiderin deposits, (**C**) hypocellular areas with disrupted brain parenchyma, and (**D**) hyalinized vessels, all secondary to radiation necrosis. Both SRS microscopy (left) and H&E-stained sections (right) are shown in (B to D).

Figure S3. SRS microscopy of minimally hypercellular gliomas. SRS microscopy depicts slight increases in cellularity in fresh surgical specimens from Patients 16 (**A**), 13 (**B**), and 14 (**C**) with diffuse infiltrating gliomas. Increased cellularity, with little disruption of the normal axonal architecture, is most clear in areas of cortical infiltration (A and B) but is also apparent within white matter (C). Mean classifier value for each of the specimens was 0.99 (A), 0.99 (B), and 0.968 (C).

Figure S4. SRS and traditional microscopy of extrinsic brain tumors. SRS imaging of Patient 7 (**A** to **D**) and Patient 8 (**E** to **H**). (A) A contrast-enhancing meningioma (arrowhead) contains spindled meningothelial cells growing in a fascicular pattern (B) with focal whorling (dotted lines, C) and calcification (arrowhead, D). (E) A left parietal non-small cell lung carcinoma (arrowhead) reveals hypercellular epithelioid tissue (F) with scattered multinucleated tumor cells (arrowheads, G) and alternating areas of viable tumor (arrowheads, H) and necrosis (asterisks, H). Both SRS microscopy (left) and H&E-stained sections (right) accompany respective MRIs.

Figure S5. SRS microscopy of spinal schwannoma. (**A**) A spinal schwannoma at the conus medullaris (arrowhead) in Patient 9 was composed of (**B**) sheets of fusiform cells featuring (**C**) elongated nuclei admixed with (**D**) hemosiderin-laden macrophages. SRS microscopy images (top) and H&E-stained sections (bottom) are shown in (B and C); SRS microscopy (left) compared to CD68 immunostaining (right) is shown in (D).

Figure S6. Validation of SRS image segmentation. (**A**) Segmentation of SRS microscopy FOVs (left) showing nuclei detected (right) in dense tumor (top), infiltrating tumor (middle), and normal tissue (bottom). The number of nuclei detected is compared to a manual count of the same FOV. (**B**) Segmentation of SRS FOV images (left) showing axons detected (right) in dense tumor (top), infiltrating tumor (middle), and normal tissue (bottom). Axon perimeter length was re-normalized to a 10-point scale based on our full SRS image library and compared to manual rating on a 10-point scale (0 = no axons, 10 = maximum axonal density). (**C**) Mean number of nuclei per FOV detected by manual count and by image segmentation for representative normal brain (n = 2), infiltrating tumor (n = 2), and dense tumor (n = 2) biopsies. (**D**) Mean axonal density of FOVs manually rated and segmented as above for representative normal brain (n = 2), infiltrating tumor (n = 2), and dense tumor (n = 2) biopsies.

Figure S7. Quantitative analysis of a normal specimen imaged with SRS microscopy. From left to right, SRS microscopy lipid channel, SRS microscopy protein channel, overlay of SRS microscopy lipid and protein images, heat map of calculated axon densities (arbitrary units) for all FOVs, heat map of nuclei per FOV, heat map of calculated protein:lipid ratio for all FOVs, and classifier values for all FOVs. Insets show FOVs with high (red), average (yellow), and low (blue) classifier values. Note that while the mean classifier value is greater in cortex (top of biopsy), all FOVs have classifier values well below the cutoff of 0.5.)

Figure S8. Quantification FOVs used to create the classifier. Scatterplot of quantified parameters of individual 300×300 μm^2 FOVs (n = 1477) comprising biopsies plotted in Fig. 4 (n = 51).

Figure S9. Planned workflow for ex vivo SRS-guided brain tumor resection. The proposed workflow for *ex vivo* SRS microscopy is a three-step process: registered biopsy collection, image acquisition and co-registration, and SRS-enhanced navigational guidance. Specimens are collected with instruments that are trackable by the navigation system and positional coordinates at the biopsy site (red circle) are recorded. Excised tissue is imaged via an SRS microscope and co-registered into the MRI-based navigational dataset. Finally, a merged dataset with MRI and SRS information can be used to navigate within the operative field based on both structural data based on MRI and histologic data based on SRS imaging.

Figure S10. Planned workflow for in vivo SRS-guided brain tumor resection. The proposed workflow for *in vivo* SRS microscopy enables navigation based on MRI and SRS data simultaneously. A handheld SRS probe, tracked by the navigation system, collects histologic images, which are co-registered to the MRI-based navigational dataset in real time. A merged dataset with MRI and SRS information can be used to navigate within the operative field based on both structural data based on MRI and histologic data based on SRS imaging. The handheld probe would be used to scan the cavity in regions where tumor infiltration is suspected to ensure that densely infiltrated tissue is removed and non-infiltrated regions are preserved.

SUPPLEMENTARY TABLES

Table S1. Descriptive statistics of the test case series.

Patient No.	Age (yrs)	Tumor location	Diagnosis
1	40	Right temporal	Histologically normal cortex*
2	26	Right temporal	Histologically normal cortex*
3	22	Right temporal	Histologically normal cortex*
4	55	Right frontoparietal	Glioblastoma†
5	63	Right temporal	Oligodendroglioma†
6	13	Posterior fossa	Medulloblastoma
7	62	Left parietal	Meningioma
8	59	Left occipital	Metastatic non-small cell lung carcinoma
9	62	Left cerebellar	Metastatic breast carcinoma†
10	68	Conus medullaris	Cystic schwannoma
11	51	Left temporal	Glioblastoma
12	70	Left parietal	Metastatic non-small cell lung carcinoma
13	54	Right parietal	Oligodendroglioma
14	39	Right frontal	Oligodendroglioma
15	29	Left temporal	Anaplastic oligoastrocytoma
16	50	Right parietal	Glioblastoma
17	67	Right frontal	Oligodendroglioma
18	66	Right parietal	Glioblastoma
19	67	Left frontal	Glioblastoma
20	59	Left frontal	Glioblastoma
21	66	Right occipital	Glioblastoma†
22	30	Left frontal	Oligodendroglioma

*Tissue obtained from patients undergoing anterior temporal lobectomy for intractable epilepsy.

†Tissue obtained from patients was not incorporated into the quasi-likelihood GAM.

Table S2. In-depth verification of automated method for cellular density quantification. Ten representative FOVs from controls (Patients 1 and 2), infiltrating glioma patients (Patients 17 and 22), and dense glioma patients (Patients 16 and 21) are presented to demonstrate the close correlation between manual (MCC) and automated, MATLAB-generated cell counts (ACC). FOV, field of view.

Patient No.	Diagnosis	FOV	MCC	ACC
1	Control	1	18	15
		2	19	18
		3	21	20
		4	14	17
		5	18	18
		6	21	21
		7	22	21
		8	23	19
		9	12	16
		10	16	16
2	Control	1	8	9
		2	19	19
		3	24	24
		4	20	21
		5	16	17
		6	20	24
		7	24	24
		8	37	43
		9	17	19
		10	20	23
16	Infiltrating glioma	1	29	30
		2	22	24
		3	45	50
		4	31	32
		5	24	27

		6	36	38
		7	29	31
		8	39	42
		9	43	47
		10	28	32
21	Infiltrating glioma	1	59	60
		2	69	71
		3	52	51
		4	70	71
		5	59	56
		6	58	53
		7	66	61
		8	64	63
		9	43	40
		10	56	60
17	Dense glioma	1	165	155
		2	143	154
		3	157	160
		4	143	134
		5	136	128
		6	145	149
		7	139	130
		8	137	139
		9	152	146
		10	148	141
22	Dense glioma	1	181	188
		2	194	191
		3	202	205
		4	215	217

	5	203	209
	6	183	188
	7	192	181
	8	197	190
	9	188	178
	10	189	178

Table S3. Test characteristics of independent biopsy parameters and the classifier as predictors of the presence of tumor infiltration.

Classification condition	Area under curve	Sensitivity (%)	Specificity (%)
Normal vs. abnormal, axons only	0.913	82.0	89.0
Normal vs. infiltrating, axons only	0.827	58.0	97.0
Normal vs. dense, axons only	0.982	94.2	93.4
Normal vs. abnormal, nuclei only	0.9744	87.5	96.4
Normal vs. infiltrating, nuclei only	0.973	84.0	96.6
Normal vs. dense, nuclei only	0.976	86.6	99.2
Normal vs. abnormal, protein:lipid ratio only	0.81	71.8	83.0
Normal vs. infiltrating, protein:lipid ratio only	0.636	40.6	86.8
Normal vs. dense, protein:lipid ratio only	0.948	89.0	86.7