Quantitative, spectrally-resolved intraoperative fluorescence imaging

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Intraoperative visual fluorescence imaging (vFI) has emerged as a promising aid to surgical guidance, but does not fully exploit the potential of the fluorescent agents that are currently available. Here, we introduce a quantitative fluorescence imaging (qFI) approach that converts spectrally-resolved data into images of absolute fluorophore concentration pixel-by-pixel across the surgical field of view (FOV). The resulting estimates are linear, accurate, and precise relative to true values, and spectral decomposition of multiple fluorophores is also achieved. Experiments with protoporphyrin IX in a glioma rodent model demonstrate in vivo quantitative and spectrally-resolved fluorescence imaging of infiltrating tumor margins for the first time. Moreover, we present images from human surgery which detect residual tumor not evident with state-of-the-art vFI. The wide-field qFI technique has broad implications for intraoperative surgical guidance because it provides near real-time quantitative assessment of multiple fluorescent biomarkers across the operative field.

The clinical foundation of surgical oncology is underpinned by the simple but universal premise that for most solid tumors achieving complete resection improves patient outcomes. Unfortunately, despite significant advances in surgical technology and technique3–4, rates of complete resection remain surprisingly low and/or the need for re-excision persists in many primary cancers (e.g., in brain5–6, breast7, and prostate8, among others). Intraoperative tissue characterization also enables conservation of normal tissue, which is critical to functional preservation or patient satisfaction and quality of life. For example, re-excision rates in breast lumpectomy have been reported to be as high as 40% in some studies9, largely because of residual disease found at the margin of the resected specimen that was not apparent to the surgeon at time of procedure. Glandular preservation in prostate cancer surgery is desirable clinically10, but has not been widely adopted because surgical margins have been difficult to identify intraoperatively without histologically assessing the excised tissue in the operating room (OR)11.

Nowhere are the principles of complete resection and the application of technology to meet the concomitant procedural challenges more evident than in neurosurgical oncology, where the highly invasive and infiltrative nature of malignant glioma typically leads to recurrence12–13. Major technologies and procedural trends that apply across surgical specialties (i.e., wide-field, white-light visualization of the surgical field through the operating microscope (OM)14, navigational tracking15, image-guidance16, intraoperative imaging17, etc.) were born in neurosurgery ORs, which have become a clinical platform for technology innovation and translation across all of surgery.

The most recent advances, now found in many modern OMIs, involve use of light at specific wavelength(s) which stimulates fluorescent emissions from biomarkers that can be associated with specific tissues in the field-of-view (FOV), and offer the hope of identifying disease, intraoperatively, at the cellular and/or molecular levels18–20. Accordingly, visualization of protoporphyrin IX (PpIX) red fluorescence induced by oral administration of 5-aminolevulinic acid (ALA) has become standard-of-care for resection of high-grade glioma in Germany based on outcomes from a randomized multi-center Phase III trial comparing progression free survival in patients undergoing either fluorescence-guided resection or conventional resection under white light visualization18.

While these OMIs have allowed surgeons wide-spread access to visual fluorescence imaging (vFI) in the OR, the information is not quantitative and resection decisions have been reached based on subjective assessments of the visible fluorescence (e.g., red fluorescence from PpIX) from the surgical surface. Current intraoperative vFI does not correct for the distorting, non-linear effects of tissue optical properties21 which reduces the correlation...
between perceived fluorescence and actual biomarker concentration. Indeed, vFI leaves significant tumor undetected\(^2\)\(^3\)\(^4\)\(^5\)\(^6\)\(^7\)\(^8\)\(^9\)\(^10\)\(^11\)\(^12\); it does not take full advantage of the specificity of fluorescent biomarkers (e.g., because diagnostic concentrations exist which are not visible\(^2\)\(^3\)\(^4\)\(^5\)\(^6\)\(^7\), or their spectral signatures (e.g., because single bandpass or longpass filters have typically been used to collect the fluorescence signal\(^1\)\(^2\)\(^3\)). Spectrally-resolved detection, i.e., detection of the full spectrum, enables decomposition of non-specific autofluorescence in tissue, which improves image contrast and detection accuracy of cancerspecific biomarkers\(^2\)\(^3\)\(^4\)\(^5\). With the advent of intraoperative fluorescence imaging and accompanying fluorophores for surgical guidance (e.g., PpIX\(^1\)\(^8\), folate receptor targets\(^2\), fluorescent peptides\(^3\), blood pooling agents such as fluorescein and indocyanine green\(^4\)\(^5\)\(^6\)\(^7\), etc.), decomposition of overlapping fluorescent agents from the same emission signal becomes possible with spectrally-resolved approaches, thus allowing simultaneous, spatially-coregistered in vivo imaging of multiple reporters\(^1\)\(^2\)\(^3\)\(^4\).

In this paper, we present the first demonstration of wide-field quantitative fluorescence imaging (qFI) in vivo for intraoperative surgical guidance. Critical to this success is spectrally-resolved image capture with concomitant correction of the emitted fluorescence for tissue optical property variations and fast (near real-time) data processing to estimate absolute fluorophore concentrations pixel-by-pixel across the FOV. Quantitative results are reported for both animal and human intracranial tumors in the intraoperative setting. Here, we show pre-clinical imaging of PpIX and fluorescein in tissue, resulting from simultaneous detection of full visible spectrum fluorescence in the same animal tumors. Quantitative identification of more than one fluorophore within the same FOV offers new possibilities for simultaneously delineating multiple tissue characteristics of critical importance to the surgeon. We also show the first human results of qFI as a novel imaging technique to improve tumor detection during the resection of glioblastoma multiforme (GBM). While we present these results in the setting of neurosurgery, the data acquisition and processing methods reported for intraoperative wide-field qFI of absolute concentrations of multiple fluorophores are readily applied to other fluorescent reporters and/or surgical oncology procedures.

**Results**

**Phantom validation.** Quantification of fluorophore concentration. The quantitative performance of our wide-field qFI instrumentation in terms of estimating fluorophore concentration was evaluated and validated in phantoms. A spectrally-constrained dual band normalization\(^1\)\(^2\)\(^3\) was used to correct the raw fluorescence signal for the nonlinear distortions caused by tissue optical properties at both the excitation and fluorescence emission wavelengths (Methods). The approach compensates for varying optical attenuation in tissue which enables quantification of absolute fluorophore concentrations. Previously-validated, tissue-simulating liquid phantoms fabricated with varying optical absorption (\(\mu_a\)) and reduced scattering (\(\mu_s'\)) properties at the excitation and main emission peak of PpIX (\(\lambda = 405\) nm and \(\lambda = 635\) nm, respectively) were formed with increasing PpIX concentrations from 0.02 to 5 \(\mu\)g/ml, commensurate with the range commonly found in normal and pathological tissues. Table 1 summarizes the 9 combinations of absorption and scattering optical properties that were mixed with each of 9 concentrations of PpIX (total of 81 phantoms) (Methods).

We acquired spectrally-resolved, wide-field image data with our qFI system to calculate pixel-specific PpIX concentrations, \(C_{\text{PpIX}}\), across the FOV (Methods) and compared these values to the actual PpIX concentrations shown in Figure 1. The linearity of the qFI estimate with increasing \(C_{\text{PpIX}}\) averaged over pixels for all 9 combinations of phantom optical properties resulted in a coefficient of determination, \(R^2 = 0.88\). The mean percentage error (mPE) in the imaged \(C_{\text{PpIX}}\) estimates was 24% (PE range: 0.3 – 71.4%). The mean coefficient of variation (pixel standard deviation divided by pixel mean), mCOV, is also shown in Figure 1 as a function of true \(C_{\text{PpIX}}\) for each optical phantom combination and indicates a mean of 2.7% with a range from 0.1% to less than 45% (at the lowest PpIX concentration of 0.02 \(\mu\)g/ml and highest optical absorption).

To relate these results to state-of-the-art vFI, we integrated the area under the curve of the raw fluorescence signal, \(F_{\text{RAW}}\), over the range \(\lambda = 610 – 720\) nm in Figure 1. In this case, not only is the linearity of the response to increasing \(C_{\text{PpIX}}\) significantly degraded (\(R^2 = 0.27\) for \(C_{\text{PpIX}} > 0.30\) \(\mu\)g/ml; \(R^2 < 0.1\) for \(C_{\text{PpIX}} > 0.02\) \(\mu\)g/ml), but the \(C_{\text{PpIX}}\) estimates are also less accurate (mPE = 55%) when the phantom optical properties were changed but \(C_{\text{PpIX}}\) remained the same.

**Spectral decomposition of multiple fluorophores.** We evaluated the ability of our qFI system to spectrally resolve multiple fluorophores by adding concentrations of fluorescein to the homogeneously distributed PpIX phantoms of increasing \(C_{\text{PpIX}}\). Figure 2 shows qFI estimates as a function of increasing (true) \(C_{\text{PpIX}}\) for 4 different concentrations of fluorescein. Here, we considered \(C_{\text{PpIX}}\) ranging from 0.156 \(\mu\)g/ml to 1.25 \(\mu\)g/ml combined with fluorescein concentrations from 1.25 to 10 \(\mu\)g/ml which are representative of clinically relevant concentrations\(^2\). The strongly linear and accurate relationship between wide-field qFI-derived \(C_{\text{PpIX}}\) estimates and the actual PpIX concentrations was preserved (\(R^2 = 0.99\), mPE = 13%) across all FOVs and phantom concentrations (PE range: 0.5 and 56.3%). COV response is also shown in Figure 2 and mCOV was 1.2% (mCOV range: 0.2 – 9.2%).

**Animal studies.** In vivo qFI was evaluated in an established rodent model of GBM, which displays the histopathological hallmarks of the disease, including invasion of parenchyma and periventricular spread with single cell infiltration. Animals implanted with CNS-1 tumors were given 100 mg/kg of ALA 2 hrs prior to surgery. At the start of surgery, four (4) of these animals also received i.v. injection of 100 mg/kg of 10% fluorescein sodium solution. Exogenous ALA administration leads to selective accumulation of PpIX in tumors whereas fluorescein functions as a blood pooling agent, preferentially accumulating in regions of leaky tumor vasculature.

**Quantification of fluorophore concentration.** In 4 of the animals receiving only ALA, we compared wide-field qFI estimates of \(C_{\text{PpIX}}\) with in vivo spectroscopy probe recordings in both tumor and normal brain in multiple locations in each animal (for a total of \(n = 41\) samples). In previous work\(^6\), the accuracy of the probe was evaluated in detail and the instrument was found to reproduce known phantom and in vivo values of \(C_{\text{PpIX}}\) with an error of less than 10%. Here, we considered the probe readings to represent the gold standard for \(C_{\text{PpIX}}\). In paired t-tests, no statistically significant differences were found between the two data sets. Supplementary Figure 2 presents a
scatter plot of \( C_{\text{PpIX}} \) estimates from the wide-field qFI image locations coregistered with the position of the probe against the probe values. A linear relationship is evident with a statistically-significant \((p < 0.0001)\) correlation coefficient of \( R = 0.79 \). When probe \( C_{\text{PpIX}} \) was greater than 40 ng/ml, mPE for the qFI estimates of \( C_{\text{PpIX}} \) was less than 35%.

Figure 3 shows a representative set of images from a typical animal in the ALA-only group which illustrates white-light, vFI, and qFI views of the same surgical field. A red PpIX fluorescence is observed in the bulk tumor under vFI. Congruence between the vFI and white-light indications of tumor is evident. Corresponding qFI maps of PpIX concentrations of the same FOV reveal \( C_{\text{PpIX}} > 150 \) ng/ml in the tumor bulk. Interestingly, infiltrating tumor margins (\( C_{\text{PpIX}} \sim 100 \) ng/ml) outside the tumor bulk are evident on qFI, but not vFI (Fig. 3c, white arrow), demonstrating improved margin delineation with qFI at PpIX concentrations which were below the visual threshold for detection.

Spectral decomposition of multiple fluorophores. Four (4) animals receiving doses of both ALA and fluorescein were imaged after surgical exposure of the tumor to evaluate the spectral separation of the contributions of these two fluorophores. Figure 4 shows representative results from a typical animal where white-light, vFI and qFI views of the same surgical field are displayed. The vFI presentation in the bulk tumor is visually dominated by the green fluorescein color. The wide-field \( C_{\text{PpIX}} \) image and the normalized fluorescein map indicate localization of both molecules with greatest concentrations in similar regions of the tumor bulk, but with some distributed heterogeneity (including in the immediately adjacent and surrounding margin). Again, qFI detected significant levels of PpIX in the infiltration zone which would have otherwise gone undetected with vFI (see arrows in Fig. 4C vs. Fig 4B). Sample spectra are also shown which illustrate the decomposition of the relative contributions of each fluorophore (and tissue autofluorescence) despite the lack of visual discrimination.

Human studies. To date, surgical fields in 12 brain tumor patients receiving 20 mg/kg of ALA approximately 3 hours prior to the procedure have been assessed with the qFI system. Figure 5 shows a comparison of vFI and qFI in a representative case. At the start of this surgery, vFI indicates a heterogeneous distribution of PpIX fluorescence whereas the corresponding qFI view demonstrates the
presence of more significant, previously undetected tumor biomarker. Normal brain similarly does not show spectral evidence of tumor based on the qFI view which was confirmed by probe measurement. A similar series of image acquisitions near the end of surgery in the same patient illustrates no evidence of disease in the vFI view but histologically-confirmed residual tumor in the qFI images. Corresponding spectra from histologically-confirmed tumor-positive (near the end of surgery) and tumor-negative portions (at the end of surgery) of the qFI-derived C_{PpIX} map are shown which demonstrate the presence and absence of PpIX signal, respectively (Fig. 5L).

**Discussion**

In this paper, we describe the implementation and evaluation of a fast (near real-time), quantitative and spectrally-resolved wide-field fluorescence imaging approach for intraoperative identification and delineation of tumor during surgery. Critical to the success of the technique is acquisition of white-light reflectance spectra to correct the detected fluorescence for attenuation caused by tissue optical absorption and scattering in the fluorescence excitation and emission wavelength bands. The method produces quantitative estimates of fluorophore concentration, but also enables spectral decomposition of contributions from multiple fluorophores in the FOV.

Phantom results demonstrate the sensitivity and linearity of the approach to PpIX fluorescence. Specifically, we detected PpIX concentrations as low as 20 ng/ml, independently of phantom optical properties, with linear changes in the imaged signal to increases in the true PpIX concentration up to 5000 ng/ml. The absolute accuracy in the wide-field C_{PpIX} image estimates was better than 25% and their precision resulted in a coefficient of variation across the image of less than 10% for all optical property combinations provided C_{PpIX} was greater than 40 ng/ml. Our in vivo human data indicate that a detection threshold of ~100 ng/ml will provide positive predictive values in excess of 90%22,23. Importantly, qFI was at least an order of magnitude more sensitive to PpIX concentration than vFI (~40 ng/ml vs. ~600 ng/ml), and generated improved contrast (~10:1) at concentrations at least one order of magnitude lower (~40 ng/ml vs. ~600 ng/ml for qFI compared to vFI, respectively).

![Figure 3](image1.png) **Figure 3** | Wide-field qFI estimates of C_{PpIX} in a rodent model of glioma. (A) White light FOV. (B) Corresponding vFI demonstrating red PpIX fluorescence in the tumor bulk. (C) Map of qFI estimates of C_{PpIX}. (D) Corresponding image overlay demonstrating C_{PpIX} >150 ng/ml in the tumor bulk, as well as significant (C_{PpIX} ~100 ng/ml) PpIX in the infiltrating margin (white arrow). Color bar values are in ng/ml.

![Figure 4](image2.png) **Figure 4** | Wide-field qFI in the presence of two fluorophores in a rodent model of glioma. (A) White light FOV. (B) Corresponding vFI demonstrates green fluorescein fluorescence in the tumor bulk, and no visible PpIX red fluorescence. (C) Map of qFI estimates of C_{PpIX}. (D) Corresponding image overlay shows C_{PpIX} >200 ng/ml in the tumor bulk. (E) Map of normalized fluorescein levels. (F) Corresponding image overlay demonstrates fluorescein levels in the tumor bulk as well as diffusely scattered through normal brain. Significant PpIX concentration (C_{PpIX} ~100 ng/ml) was found in the infiltrating margin (white arrow in C) with minimal fluorescein present. Spectral decomposition (far right) indicates the varying contributions (at arrow locations in B marked +, top spectral plot, and * in bottom spectral plot) from each fluorophore in the FOV. Color bar values for C and D are in ng/ml and for E and F in normalized arbitrary units.
Pre-clinical results in a rodent glioma model indicated that the qFl-derived $C_{PpIX}$ was also linear with the response of our quantitative, direct-contact optical spectroscopy probe and yielded a highly statistically significant ($P < 0.001$) correlation coefficient of 0.79 based on 41 measurements obtained from multiple animals. When $C_{PpIX}$ measured with the probe was greater than 40 ng/ml, the mean percentage error (assuming the probe is the gold standard) in the in-vivo qFl-derived $C_{PpIX}$ estimates was 30% whereas the errors exceeded 100% when all probe data were included. These results are specific to this tumor biomarker and suggest that the in-vivo sensitivity of the qFl technique is presently about 40 ng/ml which is below the intraoperative threshold of 100 ng/ml based on our clinical experience with PpIX to date. In these animals, we observed significant heterogeneity in the tumor bulk (with values ranging from 50 ng/ml to 500 ng/ml), and $C_{PpIX}$ levels up to 100 ng/ml in the infiltrating margins that were not visibly apparent with vFl. These data indicate that our qFl technique can not only detect the more infiltrating margins of tumor, but also yields absolute concentrations with reasonable accuracy.

The importance of qFl relative to state-of-the-art vFl was particularly evident, and equally convincing, in the animals receiving both ALA and fluorescein as shown in Figure 4 where the vFl view is substantially altered by the presence of fluorescein. Again, substantial PpIX concentration ($C_{PpIX} > 200$ ng/ml) is recovered in the tumor bulk that was not visually evident, and weaker but still accurate, PpIX concentrations ($C_{PpIX} \sim 100$ ng/ml) are found in the infiltrating tumor margins, providing evidence of improved detection of invasive disease. An important caveat to these results is that the fluorescein images are not quantitative (rather normalized) because we did not determine the qFl calibration factors or investigate the optimal excitation/emission normalization bands to correct for tissue optical property distortions of the fluorescein fluorescence signal. While this optimization and calibration can be done, the non-quantitative nature of the fluorescein images does not diminish the significance of the PpIX results nor the ability to decompose measured spectra into estimates of absolute concentrations of multiple fluorophores. Quantitative, multiple reporter imaging opens the door to novel fluorescence detection approaches in the intraoperative setting (e.g., measurement of reporter kinetics).

The human data near the end of surgical resection was even more compelling in terms of the clinical importance of wide-field qFl surveillance. When tumor appeared to be completely removed by vFl, previously unidentified infiltrative tumor was evident with qFl enabling further resection (see Figure 5). In addition, wide-field qFl provided efficient detection compared with impractical, point-by-point application of a quantitative probe. These results suggest

Figure 5 | Wide-field qFl during a human GBM surgery. Images at the beginning (A–C), near the end (E–G), and at the end of the case (I–K) show vFl, white light, and white light+qFl views, respectively. High levels of PpIX are evident at the start of surgery (in B), whereas near the end of surgery, no PpIX is noted on vFl (in F), but significant PpIX was found in qFl near the center of the FOV (in G) with histological corroboration as tumor (in L). At the end of surgery, no significant PpIX levels remained in the qFl acquisition (in K). Spectra obtained at the beginning of surgery (in D) from three locations (1 = vFl positive tumor, red mark in A; 2 = qFl positive/vFl negative tumor, blue mark in A; 3 = normal brain, green mark in A), and spectrum acquired near the end of surgery (in H) from one location (1 = qFl positive/vFl negative tumor, blue mark in E) are shown.
improved infiltrative tumor margin detection will be possible with our quantitative qFI technique.

While the qFI results presented in this paper are impressive (particularly the data in phantoms where the true C_{PpIX} Values are known and can be accurately quantified), further advances are likely to improve the wide-field qFI accuracy, especially in vivo at concentrations below 40 ng/ml. For example, better instrumentation fidelity (a more sensitive camera and powerful light source) should increase the accuracy of the technique. Further, the approach described here relies on normalization with respect to pre-selected excitation and fluorescence emission wavelengths which have been optimized to correct for the attenuation effects of tissue optical properties. A model-based strategy that compensates the fluorescence signal by explicitly estimating tissue optical absorption and scattering across the full spectrum, is anticipated to be more accurate and reduce dependencies on instrumentation and measurement calibration/scaling factors to convert the raw signal acquisitions into concentration estimates^{22,27}. Based on our experience during the development of the quantitative optical probe (which applies full-spectrum analyses), in vivo errors below 20% appear possible, although at the expense of considerably more computational effort that may be difficult to execute in near real-time in the OR.

In conclusion, we present a novel wide-field imaging technique, qFI, which provides quantitative and spectrally resolved images of fluorophore concentrations in (residual) tumor tissue, and readily integrates into the surgical workflow. Our approach demonstrates improved detection of residual disease compared to state-of-the-art VFI that could increase the completeness of tumor resection with preservation of normal function, which is known to correlate with improved patient outcomes. This method has potential for broad impact across other surgical oncology applications and in the detection of other fluorescent biomarkers of disease, including those that may be present simultaneously.

Methods
Quantitative fluorescence imaging (qFI) system design. The quantitative fluorescence imaging (qFI) system used in this study (dimensions: 22.0 cm x 5.0 cm x 8.5 cm, weight = 900 g) consisted of three main components: a custom optical adapter, a liquid crystal tunable filter (LCTF) (Cambridge Research Instruments), and a charged-coupled device (CCD) camera (PCO.Pixelfly, Cooke Corp.) connected to a computer control system as shown schematically and physically in Supplementary Figure 1. The custom adapter attached these components (LCTF and camera) to an optical port on a Zeiss Pentero OM, whereas commercially available components and optics (Edmund Optics) connected the LCTF and CCD camera. The LCTF performed fast (50 ns) single-band filtering (7 nm full-width at half-maximum) of incoming light in the visible range, λ = 400 – 720 nm, and displayed a wavelength specific response with a maximum transmittance of 64% at 710 nm. The CCD camera (1396 x 1024 pixels; 2 x 2 binning; 62% quantum efficiency at 580 nm) sensed incoming light and transmitted the digital data to a computer control system. It had an enhanced red-near-infrared mode which improved sensitivity in that spectral region. Custom LabView® software (National Instruments, Austin, TX) integrated control of the data acquisition, processing, and image display.

The surgeon’s FOV through the OM was co-registered with the qFI data, providing one-to-one comparisons. At standard working distances between the OM and surgical field, the maximum and minimum FOVs were 50 mm x 40 mm and 10 mm x 7.5 mm, respectively. Spectrally-resolved acquisition ranged from 2 to 8 seconds per white light and fluorescence image capture. Here, we operated in the visible portion of the spectrum to image two clinically-approved fluorophores dyes (i.e., PpIX and fluorescein). With the advent of imaging agents in the near infrared (NIR), the design could accommodate either an LCTF operating in the NIR or a dual-visible-NIR LCTF.

Spectrally-resolved quantitative fluorescence imaging data. Each qFI acquisition captured a 3D image cube at 5 nm spectral resolution (λ = 450 – 720 nm) under white light exposure followed by a 3D image cube at 3 nm spectral resolution (e.g., for λ = 600 – 720 nm for PpIX or 500 – 720 nm for PpIX plus fluorescein) during violet-blue light exposure. Wavelength range selection was electronic and depended on the wavelength region of interest so as to cover the main spectral features of each imaging agent.

A 3D image cube (x, y, λ) was stored as a series of 2D spatial images (x, y) at a specific wavelength (λ). Each individual pixel’s spatial coordinate (x, y) corresponded to a location in the surgical field. We reconstructed spectrally-resolved (i.e., 1 nm resolution) full reflectance and fluorescence spectra for each pixel coordinate using a cubic spline interpolation, thereby generating a total of 723,840 spectra (696 x 520 × 2 exposures).

Tissue attenuation correction algorithm. The white light reflectance spectra corrected the detected fluorescence for the attenuation caused by tissue optical absorption and scattering^{22}. Spectrally-constrained dual band normalization was used to estimate the intrinsic fluorescence independently of the effects of tissue optical properties through the expression

\[
\Phi_{\text{Fluo, Corrected}}(\lambda) = \Phi_{\text{Fluo, Raw}}(\lambda) - \Phi_{\text{Fluo, Ref}}(\lambda)\times \left[ \frac{\Phi_{\text{Ref}}(\lambda)}{\Phi_{\text{Ref}}(\lambda)} \right]
\]

where \(\Phi_{\text{Fluo, Raw}}(\lambda)\) is the wavelength dependent, raw fluorescence intensity; \(\Phi_{\text{Fluo, Ref}}\) and \(\Phi_{\text{Fluo, Ref}}\) are the reflectance signals integrated over the ranges \(\lambda = 465 – 485 \text{ nm and } \lambda = 625 – 645 \text{ nm, respectively}\). \(\Phi_{\text{Ref}}\) is a calibration factor that depends on the light source power. The range for \(\Phi_{\text{Fluo, Raw}}\) was selected to be close to the excitation wavelength band (i.e., 405 nm) to approximate light attenuation from excitation, whereas the range for \(\Phi_{\text{Fluo, Raw}}\) was chosen for the main emission peak of PpIX around \(\lambda = 635 \text{ nm}\).

Our correction algorithm assumed that most of the light attenuation is due to absorption at the excitation wavelength, but scattering at the emission band can also be corrected with an empirical power function, \(\alpha, \Phi_{\text{Fluo, Raw}}(\alpha = 0.7)\). We acquired a pair of reflectance and fluorescence image cubes for each pixel coordinate (x, y), we integrated the reflectance spectrum to calculate \(\Phi_{\text{Fluo, Raw}}(\alpha)\) and \(\Phi_{\text{Fluo, Raw}}(\alpha)\), and estimated the corrected fluorescence, \(\Phi_{\text{Fluo, Corrected}}(\alpha)\), at each pixel by normalizing its raw fluorescence spectrum, \(\Phi_{\text{Fluo, Raw}}(\alpha)\) by these quantities. A least-squares estimate was applied to corrected fluorescence spectra to spectrally decompose the individual contributions of the main fluorophores: PpIX, fluorescein, and tissue autofluorescence through the expression

\[
C_{\text{Fluo}} = \left[ \begin{array}{ccc} B_{1} & \cdots & B_{N} \end{array} \right] \times C_{\text{Fluo}}^{\text{Corrected}}
\]

where \(B = [b_{1}, \ldots, b_{N}]\) is a matrix of basis spectra for \(N\) fluorophores components (e.g., PpIX, fluorescein, autofluorescence), and \(C_{\text{Fluo}}^{\text{Corrected}}\) is a relative concentration fluorophore vector. A system-specific calibration factor, \(S_{\text{Fluo}}\), derived from the least squares regression of phantom data of known PpIX concentrations converted the spectrally decomposed relative fluorophore concentrations into absolute concentrations for each pixel

\[
C_{\text{Fluo}}^{\text{Corrected}} = S_{\text{Fluo}}C_{\text{Fluo}}^{\text{Corrected}}
\]

Tissue phantoms. Liquid phantoms that simulate brain tissue (and tumor) optical properties at the excitation and emission wavelengths were fabricated from a dye (McCormick) as the main absorber and Intralipid as the scattering medium. A total of nine (9) phantoms of three (3) different optical absorption (\(\mu_{a,x} = 20, 40, \text{and } 60 \text{ cm}^{-1}\)) and scattering values (\(\mu_{s,x} = 8.7, 11.4, \text{and } 6.5 \text{ cm}^{-1}\)) were constructed (see Table 1). For each set of phantom optical properties, nine (9) PpIX concentrations covering the range found in brain tumors (0.019, 0.039, 0.078, 0.156, 0.313, 0.625, 1.250, 2.500, and 5.000 mg/ml) were added to produce a total of 81 phantoms of varying optical absorption, scattering, and PpIX concentrations.

We acquired a pair of reflectance and fluorescence image cubes for each phantom under the low-light conditions typically found in the operating room during fluorescence-guided surgery. Each pair was processed with the spectrally-constrained normalization to form a PpIX concentration image. A region of interest corresponding to (x, y) pixel coordinates in the raw fluorescence image cube and the calculated PpIX concentration image was identified for each phantom and used to (i) integrate the average area-under-the-curve intensity in the 610–720 nm range for the raw fluorescence spectra, \(F_{\text{RAW}} = \int_{610}^{720} \Phi_{\text{Fluo, Raw}}(\lambda) d\lambda\), and (ii) construct the average PpIX concentration, \(C_{\text{PPIX}}\), for the quantitative image map. The former represents the raw PpIX fluorescence, and is equivalent to using a bandpass filter to extract the signal in the range of interest. We also acquired images with the Zeiss Pento surgical microscope adapted for visual fluorescence imaging (vFI). Briefly, fluorescence light in the range 450–720 nm was collected on a RGB CCD camera, and qualitative assessments of the level of visible fluorescence were made.

\(F_{\text{QFI}}\) and qFI-derived \(C_{\text{PPIX}}\) were obtained as a function of the true PpIX concentrations in each phantom. Linear regression was used to define the linearity in the fluorescence response with fluorophore concentration and the detection limits of both approaches. Spectral decomposition of PpIX and fluorescence concentrations was evaluated with sixteen (16) additional phantoms at four different fluorescence concentrations (10, 5, 2.5, 1.25 µg/ml) and four PpIX concentrations (1.25, 0.625, 0.313, 0.156 µg/ml) with \(\mu_{a,x} = 40 \text{ cm}^{-1}\) and \(\mu_{s,x} = 11.4 \text{ cm}^{-1}\). For each phantom, reflectance and fluorescence image cubes were acquired and our correction algorithm applied.

Animal studies. These studies were approved by the Institutional Animal Care and Use Committee (IACUC) at Dartmouth. Eight (8) Lewis rats were anesthetized by inhalation of isoflurane (2–4%) in 100% O2. A 3 cm skin incision was made in the top of the cranium, a small burr hole drilled, and 5x10^5 CNS-1 tumor cells injected into the right striatum, and the wound closed using suture. The CNS-1 tumor cell line was chosen because it exhibits the histopathological hallmarks of human GBM, including invasion of parenchyma and periventricular spread with single cell infiltration^{36}. 
Tumors were grown for two weeks, and on day 14 post-implantation, rats were given an i.p. dose of 100 mg/kg of ALA (Sigma) two hours prior to imaging. Animals were anesthetized for surgery by inhalation of isoflurane (2–4%) in 100% O2 and a i.p. dose of 100 mg/kg of ALA (Sigma) two hours prior to imaging. Animals were tumor-free for two weeks, and on day 14 post-implantation, rats were given an i.v. dose of 100 mg/kg of 10% fluorescein sodium solution in deionized water was administered post-dura opening.

**Clinical studies.** The Committee for the Protection of Human Subjects (CPHS), which governs the participation of humans in research at Dartmouth, approved our studies, and all patients participated under informed consent. Approximately 3 hours prior to the induction of anesthesia, patients were given an oral dose (20 mg/kg) of ALA (DUSA Pharmaceuticals, Tarrytown, NY, USA) dissolved in 100 ml of water.

At the discretion of the surgeon (DWR), the OM light was switched from white to blue exposure, and digital images of visible fluorescence, and reflectance and fluorescence image cubes were acquired. Coregistered probe measurements and biopsy specimens were collected with the latter immediately processed for subsequent histopathological analysis.

**Histopathology.** Histopathological analysis was performed on human formalin fixed paraffin embedded tissue and processed for H&E staining.

**Data processing and analysis.** Data processing and analysis were performed with MATLAB® software (Version R2010a, The Mathworks, Inc., Natick, MA, USA) Linear regression yielded the coefficient of determination, $R^2$. Paired Student's t-test was used to compare differences. Pearson’s correlation coefficient was calculated to evaluate linearity between variables. Two-sided $P < 0.05$ was considered statistically significant.

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**Author contributions**

P.A.V., F.L., B.C.W., K.D.P., D.W.R. designed the experiments; P.A.V., F.L., B.C.W. performed clinical data acquisition; P.A.V., F.L., B.C.W. performed the clinical surgeries. P.A.V., F.L., B.C.W. designed the quantitative fluorescence imaging system. P.A.V. assembled the hardware and system software and performed the system performance and phantom studies. P.A.V., L.I.P., F.L., B.C.W., K.D.P., D.W.R. supervised the project and manuscript preparation. All authors edited the manuscript.

**Additional information**

Supplementary information accompanies this paper at http://www.nature.com/scientificreports

**Competing financial interests:** The authors declare a US provisional patent application serial No. 61/588,708 on some aspects of the technology used in this study.

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