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Fluorescence lifetime as a universal marker of solid tumors following injection of indocyanine green: First-in-human results

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Abstract

The ability to precisely label and visualize tumors in tissues can improve the accuracy of surgical resections compared to standard of care, which relies on visual inspection and palpation. While fluorescence intensity-based imaging is being evaluated for surgical guidance, variable tumor uptake and incomplete clearance of fluorescent probes reduces tumor vs. normal classification accuracy. Here we demonstrate that the fluorescence lifetime (FLT) of multiple types of solid tumors is longer than the FLT of healthy tissues in patients systemically injected with indocyanine green (ICG), an FDA approved near infrared dye. We show that this cancer-specific lifetime shift can distinguish tumor from normal tissue both at a cellular level using microscopy and in large specimens using wide-field imaging, with an accuracy of over 97% across multiple patients. Unlike intensity, which is a system-specific parameter that depends on tumor dye uptake and depth in tissue, FLT is a system-independent photophysical property that can be quantified in thick tissues. Our study suggests that FLT imaging with ICG can be immediately used to improve the accuracy of cancer surgeries.
The past two decades have seen a dramatic growth in the development of molecularly targeted fluorescent tracers and imaging systems for intraoperative tumor identification\textsuperscript{1-3}. Besides probes targeting tumor-specific molecular expression\textsuperscript{4-11}, fluorescent probes have been developed to target the aberrant tumor microenvironment, such as enzyme activity\textsuperscript{12-14}, hypoxia\textsuperscript{15}, increased lysosomal viscosity\textsuperscript{16-18}, and acidic pH\textsuperscript{19, 20}. The FDA approved near infrared (NIR) dye, indocyanine green (ICG), has also been extensively evaluated for cancer image guided surgery\textsuperscript{21-30}. Although not designed to be tumor-specific, ICG preferentially accumulates in most solid tumors due to enhanced permeability and retention followed by tumor cell internalization\textsuperscript{31}.

Despite improved performance over the standard of care visual inspection and palpation\textsuperscript{32}, identifying a single biomarker that is common to multiple cancer types has been challenging due to the heterogeneity of cancer biomarker expression\textsuperscript{33, 34}. As a result, tumor uptake of fluorescent probes can often be variable, with considerable off-target accumulation in normal tissues and organs, resulting in poor sensitivity and high false positive rates\textsuperscript{23, 34, 35}. Cellular specificity to multiple cancer types in humans has so far not been demonstrated using a single imaging agent. Even as newly developed imaging probes continue to improve in performance, it will be several years before their benefits are realized in the clinic due to the long and expensive regulatory pathway\textsuperscript{36}. An important factor that has limited the performance of existing probes is their use in conjunction with fluorescence intensity-based imaging, which cannot readily distinguish tumor-specific and non-specific fluorescence. Fluorescence intensity is measured in arbitrary units and depends on the size and depth of the tumor, dye uptake, detector efficiency, illumination power and other system-dependent parameters. As a result, it is difficult to quantify tumor-specific-contrast on an absolute scale across imaging systems and patients, potentially impeding broader clinical adoption and standardization of fluorescence imaging for surgical guidance\textsuperscript{34}.

On the other hand, fluorescence lifetime (FLT) is a photophysical property that is measured in absolute units of time\textsuperscript{37}, and is largely independent of system parameters, tumor probe uptake and depth in tissue\textsuperscript{38}. We have previously shown using animal studies that FLT imaging improves tumor vs. normal tissue classification accuracy by several-fold using systemically injected contrast agents\textsuperscript{39, 40}. Here we report the first clinical studies demonstrating that ICG undergoes a significant FLT shift in several types of solid tumors following systemic ICG injection. The FLT change is consistent across multiple subjects, especially for a given tumor type and can be detected both at a cellular level using microscopy and in macroscopic resection specimens using wide-field time domain imaging, providing more than 97% accuracy for tumor vs. normal tissue classification. We also show that malignant lymph nodes of ICG injected patients exhibit longer FLT\textsuperscript{38} than benign lymph nodes. \textit{In-vitro} studies with cancer cell lines suggest that the tumor-FLT enhancement of ICG can be attributed to a combination of ICG binding to serum proteins and the intracellular environment, likely the cancer cell lysosomes, where ICG is localized following internalization.
Results

FLT enhancement and cellular level specificity of FLT in human cancers

Liver cancer

We studied fresh specimens from patients undergoing surgery for primary and metastatic liver tumors. In poorly differentiated metastatic colorectal cancer (mCRC) (Fig. 1a), the fluorescence intensity was high along the rim of the grossly identified tumor boundary (Fig. 1a: center), indicating strong non-specific ICG accumulation and no apparent ICG uptake within the tumor. This phenomenon, termed as “rim-fluorescence”22, 23, has been attributed to ICG accumulation in hepatocytes and fibrotic tissue surrounding the tumor. On the other hand, our data indicate that ICG is indeed present within the tumor, which shows a significantly longer FLT (Fig. 1a: right, 1c) (0.62 ± 0.002 ns) compared to the FLTs within the bright normal liver parenchyma (0.54 ± 0.01 ns). In moderately differentiated hepatocellular carcinoma (HCC) (Fig. 1d), the fluorescence intensity within the tumor was heterogeneous with bright and dark regions (Fig. 1d: center), described previously as “partial fluorescence type”35,23, and significantly overlapped with the normal tissue fluorescence (Fig. 1e). On the other hand, the FLTs within the tumor (Fig. 1d: right) (0.65 ± 0.01 ns) were significantly longer than the FLTs in the surrounding normal liver parenchyma (0.52 ns ± .01 ns), (Fig. 1f).

To verify whether the tumor FLT enhancement occurs at a cellular level, we performed confocal fluorescence lifetime imaging microscopy (FLIM) and histopathology on thin tissue sections from the entire face of bread loafed liver specimens, when available. Confocal intensity images of a moderately differentiated HCC specimen showed low ICG fluorescence intensity (Fig. 1g: center) in the tumor with a high overlap with the intensity of the surrounding normal liver parenchyma (Fig. 1h). However, the FLTs in the tumor obtained with confocal FLIM were significantly longer than the surrounding normal liver (Fig. 1g: right; 1i). Notably, tumor infiltrating lymphocytes (TILs) located in the inflamed stroma displayed low ICG uptake and short FLTs (Fig. 1h and 1i) compared to the tumor and normal hepatocytes. FLIM images at high magnification (Fig. 1j) revealed FLT enhancement in individual liver cancer cells, while the normal hepatocytes did not show an enhanced FLT, even when the corresponding intensities were comparable. These data support the cellular origins of FLT enhancement in liver tumors and clearly indicate the improved performance accuracy for tumor vs. normal classification using FLT over fluorescence intensity.

An ROC analysis for tumor/normal classification using histology defined tumor and normal liver parenchyma (fluorescence intensity and FLT histograms corresponding to the specimen data in Fig. 1g are shown in Fig. 1k, l and ROC curves in Fig. 1m) resulted in an accuracy (AUC) of 98.5%, sensitivity of 94.3% (95% CI – 92%-96%) and specificity of 95% (95% CI – 92.7%-96.6%) corresponding to the highest J index (J = 89.6%) for FLT and an accuracy of 56.1%, sensitivity of 57.3% (95% CI – 53.1%-61.4%) and specificity of 49.8% (95% CI – 45.3%-54.2%) for fluorescence intensity (J = 13.9%). In cases when a full face of a bread loafed section was
not available (either due to the complex topology of the tumor or unavailability of tissue for banking), the patient-wise ROC analysis was performed using clinically defined gross tumor boundaries derived from color photographs of the specimens. The average accuracies (AUC) for tumor/normal classification based on the wide-field TD imaging (Supplementary Table 1) across multiple liver cancer patients (n = 7) was 98.7 ± 1.3% using FLT and 57.8 ± 39.5% using intensity. The average sensitivity and specificity were 95.8 ± 3.3% and 96 ± 3.3%, respectively (J = 91.8 ± 5.9%) for FLT, and 57.4 ± 38.1% and 60.3 ± 32.4%, respectively for intensity (J = 59.3 ± 32.8%).

**Head and neck cancer**

All specimens from head and neck (HN) cancer patients injected with ICG between 18 h to 50 h (n = 7) before surgery showed a significantly longer FLT in tumors compared to normal tissue (Fig. 1), except in one patient with spindle cell carcinoma consisting of unusual histomorphic features. In widefield fluorescence images, the long FLTs were contained within the grossly defined tumor boundary (Fig. 2a, b), while both the fluorescence intensities and FLTs of histologically tumor-free tissue (Fig. 2c) were comparatively lower. To determine whether the enhanced tumor FLT originates at a cellular level, we performed FLIM of tissue sections (~10 μm) cut from fresh specimens containing tumor and normal regions and compared against histology. A nearly one-to-one spatial correspondence was observed (Fig. 2d-f and Supplementary Fig. 1) between regions with longer FLTs (~0.85 ns, Fig. 2m) in the FLIM images and microscopic nests of infiltrative tumor cells on the histology images as identified by a pathologist blinded to the FLIM data. Large tumor clusters (Fig. 2d, dashed lines) also contained necrotic cores and terminally differentiated hyperparakeratotic tumor cells (Fig. 2d, arrowheads) with poor ICG perfusion and shorter FLT compared to the viable tumor cells. Non-tumor tissue within the tumor microenvironment (TME) (including desmoplastic stroma and muscle) showed a significantly shorter FLT than tumor cells despite the presence of non-specific ICG uptake in these regions (Fig. 2e, k, l). In some cases, the TILs in the TME showed long FLTs comparable to the adjacent tumor cells (Supplementary Fig. 2e, f, dotted arrows), while TILs in HN specimens generally showed short FLTs (Supplementary Fig. 2a, b, dotted arrows). The FLTs of dysplastic epithelial cells (Fig. 2f) were also longer than the benign/normal epithelium (Supplementary Fig. 2g, h). However, the longer FLTs in dysplastic epithelial cells and lymphocytes were confined to the TME and did not affect the overall accuracy of tumor vs. normal classification, which was based on the histograms of intensity and FLT distributions in tumor regions which included the TME (Fig. 2g, h). These data indicate that the ICG FLT enhancement in OSCC tumors across multiple HN cancer patients originates at a cellular level.

A patient-wise receiver operating characteristic (ROC) analysis (sensitivity vs. 1-specificity) of specimens from individual patients (Supplementary Table 2) resulted in a mean accuracy (AUC: area under the ROC curve) for FLT-based tumor vs. normal classification of 98.6 ± 1%, and a mean sensitivity and specificity of 97.4 ± 2.2% and 96.5 ± 2.6%, respectively, corresponding to the highest Youden’s J Index (J = 94 ± 2.7%). In comparison,
the mean accuracy, sensitivity, and specificity for intensity-based tumor vs. normal classification were 65.7 ± 25.8%, 64.5 ± 32.2%, and 66.9 ± 19.3%, respectively (J = 46.7 ± 24.8%). The mean tumor fluorescence intensity calculated for each patient showed a large variation across patients and was not significantly different from the normal tissue intensities (Fig. 2i), while the mean tumor FLT was consistently longer than the normal tissue FLT across all the patients included in the analysis (Fig. 2j).

We also performed a global, inter-patient analysis of confocal microscopy data from multiple HN cancer patients (n = 6). ICG fluorescence intensities (Fig. 2k) in muscle, blood vessels, and normal and desmoplastic stroma were comparable to the tumor, dysplasia, and TILs, indicating significant ICG retention in normal tissue. On the other hand, FLT's of tumor, dysplastic epithelium and TILs were significantly longer than several normal tissue types across all the patients (Fig. 2l). An ROC analysis across all patient specimens studied yielded a 98% accuracy (AUC) for tumor vs. normal classification using FLT contrast compared to 67.9% using intensity (Fig. 2n). The inter-patient ROC analysis allowed us to define a common threshold FLT of 0.7 ns (Fig. 2h, vertical dashed line; 2l, horizontal dashed line) for tumor-normal classification across multiple HN cancer patients, corresponding to the highest J index (J = 89.6%), with a corresponding sensitivity and specificity of 93.1% (95% CI – 85.8%-96.8%) and 95% (95% CI – 88.9%-97.9%), respectively. In comparison, a common intensity threshold of 100.1 AU (J = 26.3%) (Fig. 2g, vertical dashed line, 2k, horizontal dashed line) at the highest J index (J = 26.3%) resulted in a sensitivity and specificity of 67.5% (95% CI – 56.8%-76.6%) and 62% (95% CI – 52.2%-70.9%), respectively. It should be noted that for HN cancer as well as other cancers in this study, variations in microscopic tumor and normal tissue FLT's as seen in confocal FLIM are spatially averaged in the lower-resolution wide-field fluorescence images, resulting in a lower tumor and normal FLT's in wide-field images compared to the microscopy images.

**Bone and soft tissue cancers**

We studied cancers in the bone and soft tissue, including primary sarcomas and metastatic colon, breast, and renal carcinomas. FLIM images showed microscopic specificity of the enhanced FLT in primary sarcoma tumors such as high-grade leiomyosarcoma (Fig. 3a-e) and osteosarcoma (Fig. 3f-j, Supplementary Fig. 2), as well as bone metastasis of renal (Fig. 3k-o) and breast cancers (Supplementary Fig. 2), despite the presence of high non-specific ICG uptake within hemorrhagic areas, fibrous tissue, and muscle as seen in fluorescence intensity images (Fig. 3b, g, l). Interestingly, significant tumor vs. normal FLT contrast was observed in one patient with leiomyosarcoma even when ICG was injected at the induction of anesthesia before surgery (Fig. 3c). A patient-wise analysis (when paired tumor and normal tissues were available, n = 8) showed a wide distribution of average fluorescence intensity (Fig. 3p) with three patients showing higher ICG accumulation in the normal tissue compared to the tumors. However, all the patient-wise mean tumor FLT's were longer than the normal tissue FLT's. A patient-wise ROC analysis (Supplemental Table 3) resulted in a mean accuracy (AUC), sensitivity and specificity for FLT-based tumor vs. normal classification of 97.1± 1.3%, 94.2 ± 3.0% and
93.3 ± 2.2% (J = 86.1 ± 4.6%). In comparison, the mean accuracy (AUC), sensitivity and specificity for intensity-based tumor vs. normal classification were 54.4 ± 24.7%, 62.7 ± 31.6% and 51.7 ± 12.1% and (J = 32.4 ± 13.1%).

We next performed a global analysis of pooled FLIM data collected with specimens from 10 patients and represented the data as violin plots of intensity and FLT across various histologically identified tissue types (Fig. 3r, s). The fluorescence intensity of tumor was comparable to the non-cancerous tissue within (e.g., hemorrhage) or outside the tumor boundary (muscle, adipose tissue, connective tissue etc.), while the median FLT of the tumor (0.81 ns ± 0.04 ns) was consistently longer than all other normal tissues (hemorrhage: 0.51 ns ± 0.09 ns, connective tissue: 0.71 ns ± 0.07 ns, muscle: 0.6 ns ± 0.04 ns, adipose tissue: 0.61 ns ± 0.08 ns) (Fig. 3r, s, Supplementary Fig. 3). An inter-patient ROC analysis (Fig. 3t) yielded an accuracy (AUC) of 97%, and sensitivity and specificity of 93.9% (95% CI – 92.8%-94.9%) and 93.2% (95% CI – 92.1%-94.2%), respectively, for a common threshold of 0.76 ns corresponding to the highest J index (J = 85.3%). However, for a common intensity threshold of 94.05 AU (J = 19.5%) the accuracy was 61%, with a sensitivity and specificity of 72.9% (95% CI – 67.4%-77.8%) and 45.4% (95% CI – 41.5%-49.3%), respectively.

Sarcoma patients who received neoadjuvant radiation or chemotherapy showed significant treatment-related necrosis, cellular atypia, and inflammation, which gave rise to a heterogeneous ICG uptake within and outside the tumor boundary. Data from these patients were not included in the analysis above, since optimizing the ICG dosage and timings for patients with neoadjuvant therapy will require separate clinical studies. Nevertheless, we noted that the FLT within the clinically apparent viable tumor region were uniformly longer than the normal muscle in all specimens from treated patients (n = 7) (e.g., Supplementary Fig. 4: pleomorphic liposarcoma 3 hours post ICG injection (1 mg/Kg)), despite a heterogeneous ICG uptake within the visually identified tumor region. In these specimens, non-specific accumulation outside the tumor boundary resulted in low tumor vs normal intensity contrast.

Brain tumors

Glioblastomas (GBM) are highly infiltrative and distributed tumors\textsuperscript{25, 41}. The identification of tumor density at the infiltrative margin remains a critical challenge in the surgical management of these tumors. We imaged formalin fixed specimens from patients (n = 6) who underwent surgery for GBM 24 hours after ICG infusion (5 mg/kg)\textsuperscript{25}. A comparison of co-registered histology (Fig. 4a, left), confocal fluorescence intensity (Fig. 4a, center) and FLT images (Fig. 4a, right) indicated high ICG fluorescence intensity in tumor cells and hemorrhagic areas (Fig. 4a, dashed outlines), while a distinctly long ICG FLT was observed only in the tumor areas. Contrary to the sarcomas or the HN cancers, the necrotic tumor cores (Fig. 4a, arrows) in GBM showed high ICG retention (as observed previously\textsuperscript{41}) and long FLT, possibly due to a lower clearance rate of extravascular ICG in the brain compared to other well-perfused organs. In another GBM specimen devoid of necrosis, histologically identified
low and high tumor density areas (Fig. 4b, arrows) showed equivalent fluorescence intensity, but a sharp increase in FLT across the border between low to high tumor density areas (Fig. 4b, dashed line).

Since excised GBM specimens primarily consisted of tumor with varying densities of malignant glial cells, we aimed to study the ICG FLTs in multiple ROIs with various tumor densities rather than performing a tumor vs. normal binary classification as for the other tumor types. 150 ROIs obtained from 9 specimens collected from 6 GBM patients were graded by an experienced pathologist into four histologic groups as absent, low, moderate, and high (Fig. 4c-f, top row), based on a score reflecting the tumor cell density. ICG fluorescence intensities were comparable across the groups (Fig. 4c-f, middle row) with a weak negative correlation (Fig. 4g, \( R^2 = -0.18 \)) between fluorescence intensity and tumor density, indicating equivalent ICG uptake and retention in GBM regardless of the malignant cell density. However, the FLTs (Fig. 4c-f, bottom row) gradually increased from low to high tumor density ROIs, which resulted in a strong positive correlation (Fig. 4h, \( R^2 = 0.91 \)) between the mean FLT and tumor density. The ROIs classified as low-density groups contained only a few malignant cells (less than 20%) and therefore the mean FLT was not significantly higher from that of the tumor absent group. However, the high positive correlation between FLT and tumor density indicated that mean FLT may be employed to delineate moderate/high density tumor areas from the low-density areas at the tumor edge.

We sought to quantify the accuracies of fluorescence intensity and FLT in detecting tumor negative and positive areas. An inter-patient (n = 6) ROC analysis was performed for binary classification between the combined tumor-absent and low-density ROIs as a histologically negative group, and the combined moderate and high-density ROIs as a histologically positive group. This analysis resulted in an accuracy (AUC) of 97.5%, sensitivity of 90.7% (95% CI – 83.3%-95%), and specificity of 96.2% (95% CI – 87.2%-99%) (J = 84.3%) for FLT-based classification (Fig. 4i, red line) and an accuracy of 33%, sensitivity of 53% (95% CI – 40.6%-65.1%), and specificity of 20% (95% CI – 10.6%-31.2%) (J = 26.9%) for fluorescence intensity (Fig. 4i, green line). Since at the tumor margins, the likelihood of the presence of low-density ROIs is higher than that of the tumor negative ROIs, we performed an ROC curve analysis with only low-density ROIs as the histologically negative group. The resulting ROC curves (Supplementary Fig. 5) for both FLT and fluorescence intensity were comparable to the ROC curves that included the tumor-absent regions in the negative group (Fig. 4i), resulting in an accuracy of 97.7%, sensitivity of 90.7% (95% CI – 83.3%-95%), and specificity of 97.5% (95% CI – 87.1%-99.6%) (J = 86.3%) for FLT based classification (Supplementary Fig. 5, red), and an accuracy of 29%, sensitivity of 47.1% (95% CI – 34%-60.6%) and specificity of 20% (95% CI – 11.2%-30.5%) (J = 32.8%) for intensity (Supplementary Fig. 5, green). In summary, FLT measurements in ICG injected GMB specimens allowed us to differentiate moderate and high tumor density areas from low density (or tumor negative) areas with high accuracy and may be used to define the infiltrative tumor boundaries during GBM surgery.
Detecting metastatic lymph nodes

In addition to an accurate margin detection of primary tumors, stratification of tumor positive lymph nodes (LNs) from the negative ones during surgery can be highly beneficial\textsuperscript{42, 43}. We sought to determine whether wide-field FLT imaging can distinguish benign from malignant LNs, and to evaluate the microscopic specificity of FLT in metastatic tumor nodules. Widefield FLT imaging of two adjacent cervical LNs (Fig. 5\textit{a, b}), removed during a radical neck dissection revealed significantly longer FLT of ICG (0.64 ± 0.01 ns) in one of the LNs (Fig. 5\textit{a, b}, black dashed line), which was histologically malignant, while the LN with a short FLT (0.48 ± 0.02 ns) (Fig. 5\textit{a, b}, white dashed line) was histologically benign. The widefield imaging results were further supported by confocal FLIM (Fig. 5\textit{c-j}) of tissue sections, which confirmed that the LN with long FLT in the widefield image (Fig. 5\textit{a, b}: black dashed line) indeed contained metastatic tumor nodules, with the regions of long FLT (Fig. 5\textit{e}: dashed line) coinciding with histologically confirmed infiltrating tumor nests (Fig. 5\textit{f}: dashed line). The LN with a relatively short FLT (Fig. 5\textit{a, b}: white dashed line) showed no sign of microscopic tumor infiltration (Fig. 5\textit{g-j}).

Regional LNs excised during a partial hepatectomy of an mCRC patient showed a similar FLT enhancement (Fig. 5\textit{l}), which was associated with a histologically verified metastatic tumor nodule (Fig. 5\textit{m}: black dotted outline). Confocal FLIM from two regions labeled as “1” and “2” (Fig. 5\textit{l, m}) revealed the presence of infiltrative tumor clusters in “1” (Fig. 5\textit{n}: arrow) and normal lymphoid tissue in “2” (Fig. 5\textit{p}), in excellent spatial agreement with the histology of the same sections (Fig. 5\textit{o}: arrow, 5\textit{q}). A second lymph node specimen from the same patient also showed presence of long FLTs in small clusters of 5-10 tumor cells within the lymph node (Fig. 5\textit{r}) in agreement with histology (Fig. 5\textit{s}).

Overall, we examined 13 LNs (9 tumor-positive and 4 tumor-negative) from six patients (4 HN, 1 mCRC, 1 IHC). Excluding one tumor-positive LN which did not show any ICG uptake, all LNs (12 out of 13) were accurately identified using FLIM, with 8 LNs showing long FLTs (0.85 ns ± 0.06 ns) within infiltrating tumor nests. All tumor-negative normal LNs (n = 4) showed high ICG fluorescence intensity from the blood vessels (Fig. 5\textit{g, h}, arrows), however, the FLTs in the blood vessels and normal lymphoid tissue were shorter (0.66 ns ± 0.1 ns) compared to the tumor-positive LNs.

\textit{In-vitro} FLT enhancement in cancer cells and lysosomal co-localization

We sought to identify conditions \textit{in-vitro} that could drive the FLT enhancement of ICG in cancer cells. We first confirmed (Fig. 6\textit{a-c}, Supplementary Fig. 6) that ICG FLTs in multiple human cancer cell lines, namely HepG2, U2OS, HT29 and MDA-MB-231, were longer than the FLT in a normal cell line (MCF 10A), despite a near identical ICG uptake and fluorescence intensity (Fig. 6\textit{a, and Supplementary Fig. 6 center}). Furthermore, ICG was colocalized (r = 0.87) within the lysosomes\textsuperscript{31} of cancer cells (Fig. 6\textit{d, e}), suggesting that the intracellular environment of tumor cells contributes, at least in part, to the enhanced tumor FLTs of ICG observed in human tissue. To study the possible influence of lysosomal environment on ICG FLT, we tested the effects of
physiological parameters, such as pH and viscosity in modulating the FLT of ICG. The FLT of free ICG in solution was unaffected (Supplementary Fig. 7) within a wide range of pH (pH 3 - 11.9) \textit{in vitro}, presumably due to the unavailability of electron pairs for protonation in the physiologic pH range, suggesting that the acidic tumor environment is not likely to cause the observed FLT enhancement in tumors. On other hand, ICG FLT increased with solvent viscosity (Fig. 6f, g) within a physiologically relevant range\textsuperscript{44}, indicating that the higher intracellular viscosity of cancer cells\textsuperscript{17, 44} could play a role in regulating the intracellular FLT of ICG.

In addition to the environmental parameters, ICG fluorescence is affected by binding to serum proteins, such as albumin and increases its fluorescence quantum yield in presence of FBS\textsuperscript{45}. We verified that both the fluorescence intensity and FLT of ICG increase with FBS concentration (Fig. 6h-k). We further confirmed the effects of serum protein binding on intracellular ICG FLT by imaging tumor cells (HepG2) in presence of varying FBS concentration. We observed that the FLT of ICG in tumor cells increased with FBS concentration (Fig. 6l), over and above the FLT enhancement observed in the absence of FBS (Fig. 6a). Together, these data suggest that FLT enhancement in tumor cells could originate from a combination of ICG binding to serum proteins and a higher intracellular viscosity of tumor cells compared to normal cells.

**Discussion**

Our work suggests an important new clinical application of fluorescence lifetime as a universal imaging marker for solid cancers using ICG, an FDA approved NIR dye. Clinical data from patients systemically injected with ICG indicates that the FLTs of ICG in a wide range of solid tumors originating from the skin, oral cavity, liver, colorectum, bone, soft tissue, and brain, are longer than the FLT of non-specific dye uptake in normal tissues including blood vessels (Figs. 3c, 3m, 4c). Using rigorous comparisons of FLIM with standard histopathology, we show that the tumor FLT enhancement in human specimens is highly specific to cancer cells and is not merely reliant on enhanced permeability and retention within the tumor environment, the commonly attributed mechanism for ICG uptake in tumors\textsuperscript{21, 46}. The high specificity of tumor FLT enhancement enables the delineation of individual tumor cells with high resolution in thin tissue sections using confocal FLIM. FLT also allows the delineation of benign from malignant lymph nodes, and the classification of tumor from normal tissue in larger resection specimens with more than 97% accuracy using wide-field TD imaging in all the tumor types that we studied. A distinguishing aspect of this study, given the known challenges in target validation of molecular probes\textsuperscript{34}, is the direct one-to-one comparison of FLT imaging with histology, and quantitation of key performance metrics including sensitivity and specificity at a microscopic level.

In some cases, certain non-tumor tissues such as tumor infiltrating lymphocytes and the fibrous tumor capsule also showed enhanced FLT contrast that was comparable to the tumor FLT (e.g., Fig. 2l, 3s). Regions of necrosis within the tumor core also did not show ICG uptake or FLT enhancement (Fig. 2d) except for brain tumors (Fig. 4a). However, the poor FLT contrast within the non-viable tumor interior should not affect the utility
of FLT imaging since the boundaries of tumors are of most relevance for clinical tumor assessments and surgical resections\textsuperscript{41}. Our data also suggest that the FLT of desmoplastic stroma, which could be a cause of concern for tumor uptake of contrast agents\textsuperscript{3, 47}, exhibits significantly shorter FLTs than the tumors (Fig. 2d and e), thereby allowing clear delineation of the two tissue types.

\textit{In-vitro} data (Fig. 6) suggest that ICG is internalized in cancer cells and colocalized within the lysosomes. We also observed that ICG FLT strongly depends on viscosity (Fig. 6f, g) suggesting that the enhanced intracellular FLT of ICG could arise from the higher lysosomal viscosity of cancer cells. We also observed that ICG FLT is not affected by pH, suggesting that the tumor acidic environment is unlikely to cause the observed FLT shift. The FLT of ICG was longer in cancer cells compared to normal cells in the absence of FBS in the culture media (Fig. 6a). On the other hand, tumor FLT contrast in the presence of FBS was longer than the FLT without FBS (Fig. 6b), suggesting that both free and albumin bound ICG, if present in the cancer cells, could contribute to the observed FLT enhancement. Further detailed mechanistic studies are needed accurately pinpoint the origins of FLT enhancement in cancer cells.

Our study was not designed to evaluate the optimal ICG injection timing and dosage for achieving the highest tumor vs. normal FLT contrast. Excluding the brain studies, where a higher dosage of 5 mg/Kg was used, all the other studies used a standard 0.5 - 1 mg/Kg dosage. The injection timings were mostly determined by the availability of patients for an injection visit prior to surgery, varying between time of anesthesia induction (~15 min before surgical removal) and up to 1 week before surgery. It is plausible to expect that the tumor vs. normal FLT difference would be optimal for a suitable dosage and timing depending on the anatomical location of the tumor and the dye clearance rate from normal tissue. In our study, tumor and normal FLTs showed a clear separation even for injection at the time of surgery for some soft tissue sarcomas (Fig 3c), while for oral cancers, adequate FLT contrast was not observed until at least 12 hours post injection. A larger scale controlled clinical trial will help define a suitable range of ICG injection timing and dose to achieve the highest possible imaging performance. Nevertheless, the present study shows that a substantially improved tumor vs. normal classification accuracy is achievable using FLT compared to fluorescence intensity for a wide range of injection timings. This study was also not controlled to evaluate the effects of neo-adjuvant therapies including radiation and chemotherapy. In the few cases that we studied where patients had received prior treatments, we observed (Supplementary Fig. 4) heterogenous ICG uptake and FLT enhancement in necrotic and fibrous regions within the tumor core. However, we expect this variability to not adversely affect the performance of FLT imaging since these regions are usually associated with the tumor tissue and will therefore be resected. Future studies can provide further insights into the effects of prior treatments on the tumor FLT.

Pooled FLIM data from multiple subjects suggest that tumor and normal tissue can be separated at a microscopic level with \textit{>97\%} accuracy using a single FLT threshold, at least for a given tumor type.
Establishing a robust value of the FLT threshold (along with the associated optimal injection dosage and time point) would require larger scale clinical trials. While the inter-patient FLT thresholds in this work were based on microscopy (FLIM), it is conceivable that a separate threshold FLT could be defined for wide-field FLT imaging in future larger scale clinical studies. Once established, the threshold FLT could enable accurate and rapid determination of surgical resection margins without the need for referencing techniques, potentially resolving challenges in standardization currently faced by intensity-based systems and accelerating the adoption of this imaging technology as part of routine clinical care. In addition to real-time intraoperative imaging, a FLT threshold-based tumor vs. normal classification can enable rapid estimation of tumor burden in histological tissue sections, potentially automating comprehensive pathologic evaluation of large specimens immediately after surgery. FLT-based tumor quantification can complement structural contrast from standard histopathology and high resolution microscopy techniques.

Fluorescence lifetime imaging has been extensively applied in preclinical in-vitro, ex-vivo and in-vivo studies for tracking multiple biochemical processes and quantifying fluorescently labeled biomarkers such as cell surface receptors or enzymes in tissue. While visible FLIM has been evaluated for image guided surgery, endogenous tumor contrast in the visible spectrum is relatively lower than that using exogenous agents, and may not have an impact on surgical outcomes. NIR exogenous agents can detect sub-surface tumors due to the greater tissue penetration of NIR light compared to visible light. In addition, molecular imaging agents, including activatable probes and receptor targeted probes can detect and quantify receptor expression in tissue. The ability to quantify well known tumor-specific receptors such as EGFR or PD-L1, or other personalized molecular targets in tissue can complement ICG-FLT as a universal tumor label. Multiplexed FLT imaging in conjunction with targeted agents can allow simultaneous visualization of tumors and other important molecular markers or anatomic structures, thereby increasing diagnostic information content.

In addition to surgical applications, the deeper tissue penetration of NIR light can extend the application of tumor FLT contrast to non-invasive imaging of cancer in intact superficial lymph nodes and HN cancers, as well as to diagnostic breast imaging using algorithms for fluorescence lifetime tomography. To further enhance detection sensitivity of tumors in thick tissue, FLT imaging can be used in conjunction with ICG or other probes that emit in the second wavelength (NIR-II) window. The consistency of FLT enhancement of ICG across a variety of solid tumors and the availability of established techniques for FLT imaging from microscopic to macroscopic and tomographic whole-body imaging in thick tissue suggests a wide range of applications for NIR FLT imaging in cancer care, from diagnostics to intraoperative image guidance and post-operative histopathology.
Materials and Methods

**Chemicals:** Indocyanine Green (ICG) was purchased from the MGH Pharmacy. PBS, Trypsin-EDTA, high Glucose DMEM, fetal bovine serum (FBS), and Penicillin-Streptomycin were purchased from Fisher Scientific. The MEGM media kit for MCF 10A cells was purchased from Lonza. LysoTracker Deep Red was purchased from Invitrogen. Ethyl alcohol and Dexamethasone were purchased from Sigma-Aldrich. VECTASHIELD HardSet antifade mounting media containing DAPI (4,6-diamidino-2-phenylindole) was purchased from VECTOR laboratories. Water was obtained from a Millipore Q3 system. For a complete list of chemicals and reagents, refer to Supplementary Table 4.

**In-vitro studies:** To test the effects of pH on the FLT of ICG, a stock solution of ICG (30 mM in DMSO) was diluted to 1.5 mM ICG in 100 µl of 1:1 mixtures of PBS (1x) of varying pH and ethanol. The FLTs of 100 µl ICG at varying pH (3, 6.25, 7.25, 8.65, 11.9) were measured by imaging the solutions using a benchtop TD imaging system. Data are represented as the FLT mean and standard deviation obtained from the fluorescence lifetime maps of each ICG-filled tube.

To test the effects of viscosity on the FLT of ICG, a stock solution of ICG (30mM in DMSO) was diluted to 1.5 mM ICG in 100 µl mixtures of glycerol (0-95%), and PBS (95-0%). The composition of the ICG solutions along with their viscosities can be found in Supplementary Table 5. The FLTs of ICG (1.5 mM) in glycerol solutions of increasing viscosities were measured using the benchtop TD imaging system as described before and the data are represented as the mean and standard deviation obtained from the FLT maps of each ICG-filled tube.

To test the effects of serum protein binding to ICG, a stock ICG solution (10 mM in DMSO) was diluted to 50 µM ICG in 500 µl final volume in nine mixtures of DMEM and increasing concentration (0-100%) of fetal bovine serum (FBS). Subsequently, seven solutions with increasing concentrations of ICG (10 µM – 1 mM) were prepared in DMEM in the absence or presence of 10% FBS. The volumes of DMEM, FBS, and ICG used for each condition can be found in Supplementary Tables 6 and 7. TD fluorescence imaging of the ICG solutions at various FBS and ICG concentrations was performed, and the data are represented as the FLT mean and standard deviation obtained from the fluorescence lifetime maps (described in the Image Analysis section) of each solution.

**Cell lines and culture:** The cancer cell lines U2OS (osteosarcoma), HT29 (colorectal cancer), HepG2 (HCC), MDA-MB-231 (triple negative breast cancer), and the normal cell line, MCF 10A, (nonmalignant breast epithelial cells) were purchased from the American Type Culture Collection (ATCC). Unless otherwise mentioned, the cancer cell lines were cultured in high glucose DMEM supplemented with 10% FBS and 1% penicillin-streptomycin. MCF 10A was cultured in MEBM. All cell culture experiments were performed at 80%
confluency. All cell lines were maintained at 37°C in a 5% CO₂ incubator. For a complete list of cell lines and cell culture reagents, refer to Supplementary Table 4.

**ICG cellular uptake studies:** Cancer cells (U2OS, HT29, HepG2, and MDA-MB-231) and normal cells (MCF 10A) were cultured as described earlier. Cells were seeded into individual wells of Lab-Tek eight-well chamber slides at a density of 2.5 x 10⁴ cells per well for fast-growing cell lines (U2OS, HT29, and MDA-MB-231) and 4 x 10⁴ cells per well for slow-growing cell lines (HepG2 and MCF 10A). These initial seeding densities were chosen to ensure a comparable number of cells at the time of experiments. Cells were allowed to grow for 48 hours before experiments.

For the colocalization studies, 400 µl LysoTracker Deep Red (50 nM) diluted in prewarmed DMEM (MEBM for MCF 10A) was added to each well and incubated at 37°C for 1 hour. After 1 hour, the media containing LysoTracker Deep Red was removed and washed with fresh media (x1). Subsequently, 400 µl prewarmed media (DMEM with 10% FBS for all cell lines) containing ICG (10 µM) was added to each well and incubated at 37°C for 1 hour. After the incubation, ICG-containing media was removed, and the cells were washed with fresh media (x2). Cells were immediately fixed with 4% paraformaldehyde (PFA) for 10 minutes, followed by a wash with prewarmed PBS (x1). Finally, the chambers were carefully removed from the slides, and 20 µl mounting media with DAPI was added for each well. The slides were then mounted with a coverslip and secured with clear nail polish. All slides were imaged using a Leica Stellaris confocal FLIM system within 4 hours of mounting.

To test the effects of FBS on intra-cellular ICG lifetimes, HepG2 cells were grown for 48 hours on chambered slides as described before. Cells were then incubated (at 37°C for 1 hour) with 400 µl prewarmed ICG containing (10 µM) media with an increasing concentration of FBS (0%, 1%, or 5% FBS in DMEM). After the incubation, the cells were washed, and the slides were prepared for confocal FLIM imaging as described before.

For the comparison of ICG FLTs between normal (MCF 10A) and tumor (HepG2) cell lines, cells were grown for 48 hours on chambered slides as described before. Subsequently, 400 µl prewarmed media (FBS-free DMEM) containing ICG (10 µM) was added to each well and incubated at 37°C for 1 hour. FBS-free DMEM was used to exclude the effects of serum protein binding on the FLT of ICG. After the ICG incubation, the cells were washed, and the slides were prepared for confocal FLIM imaging as previously described. For MDA-MB-231, E0771 and HT29 cell lines (Supplementary Fig. 6), DMEM with 10% FBS was used.

**Clinical specimens and Patient characteristics:** We performed an Institutional Review Board (IRB)-approved clinical study with patients (n = 52) undergoing surgery for various cancers following intravenous injection of ICG. We studied both fresh resection specimens from surgeries performed at Massachusetts General Hospital (MGH) and Massachusetts Eye and Ear (MEE), and formalin-fixed specimens received from
prior surgeries conducted at The University of Newcastle and the University of Pennsylvania. At MGH and MEE, 31 patients undergoing surgery for hepatocellular carcinoma (HCC), metastatic colorectal cancer (mCRC) in the liver, head and neck (HN) cancer, soft tissue sarcoma, or glioblastoma (GBM) were enrolled in an IRB approved pilot clinical study (IRB Protocol #2019P000732, MGH; #NCT02280954, UPenn; Research ethics committee Reference Number: 17/NE/0361, Newcastle). The study was designed and performed in accordance with the Helsinki Declaration of 1975 and its amendments, and the laws and regulations of the United States. Patients were eligible if they were 18 years or older. Exclusion criteria were pregnancy, contraindications for fluorescent contrast agents, iodine hypersensitivity, patients with renal failure or uremia, and patients on dialysis. In addition to fresh specimens from MGH, formalin-fixed paraffin-embedded (FFPE) specimens from 15 soft tissue sarcoma patients and 6 glioma patients were obtained from the University of Newcastle and the University of Pennsylvania, respectively, using a material transfer agreement (MTA) associated with the MGH-IRB approved protocol for fluorescence lifetime imaging of resection specimens. Written informed consent was obtained from all the patients and ICG was systemically injected 15 minutes to 7 days before surgery. Lyophilized ICG powder was dissolved in clinical grade sterile water (provided with ICG) immediately before the injection and administered as an intravenous (IV) injection for 1 min (0.5-1 mg/kg, MGH and the University of Newcastle) or a 1-hour-long infusion (5 mg/kg, University of Pennsylvania). Further details on the dosage and timing of ICG injection along with the patient characteristics for each tumor type are summarized in Table 1. A power calculation for sample size estimation was performed to ensure a sufficient number of patients for each tumor type was included in the quantification. The sample size was calculated based on the mean FLT of ICG in tumor and normal tissue of 0.75 ns and 0.56 ns, respectively. With a standard deviation of 0.05 ns, we can achieve 85% power at α<0.05 with 4 patients per tumor type.

Widefield TD imaging: A custom-built time domain (TD) fluorescence imaging system was used for the imaging of resected clinical specimens. The TD imaging system consisted of a portable cart with a pulsed laser source (FFSMART-780, TOPTICA Photonics, 120 mW average power) providing 785 nm excitation, which was further filtered through a 769/40 nm bandpass filter. The excitation light was delivered to the sample using a multimode fiber (Thorlabs, Newton, New Jersey, United States), and fluorescence emission was collected in the reflectance mode through an 835/70-nm band-pass filter (AVR Optics). Time-resolved fluorescence images were captured using a gated intensifier (Picostar, LaVision) connected to a CMOS camera (Basler acA5472-17um, 0.1 to 1 second integration time). Imaging was performed with a 500 V gain, 500 ps gate width, and 200 ps steps for a total duration of approximately 6 ns per laser duty cycle of 12.5 ns. The average total power across the illumination area (approximately 4 cm diameter) was 10 to 20 mW. On the day of surgery, the research team was on standby in the frozen section pathology suite and TD imaging was performed on fresh specimens approximately within 30 minutes of surgical removal. Additional color photographs of the intact specimen were acquired and grossly identified (by a pathologist) tumor and normal tissue boundaries were
recorded on the photographs. Specimens were then inked to mark the surgical edge, bread loafed (only for HCC and mCRC), and imaged with the TD imaging system. For the HCC and mCRC specimens, bread loafed slices were fanned out to expose the tumors (Supplementary Fig. 8). After the imaging, grossly identified tumors and normal tissue were collected (1-5 cm in length) and immediately stored at -80°C in optimal cutting temperature (OCT) compound. Specimens were stored in OCT for up to 6 months.

**Tissue preparation for validation studies:** OCT-embedded tissues were sectioned using a cryo-microtome (CM1860 UV, Leica, Germany) into 8-10 µm thin sections and stored at -80°C. The specimens from the University of Newcastle and the University of Pennsylvania were obtained as 10 µm sections from FFPE tissue blocks. All sections (OCT and FFPE tissue blocks) were stored in the dark until confocal fluorescence lifetime imaging microscopy (FLIM) and histopathology were performed.

**Confocal FLIM:** A STELLARIS 8 FALCON (Leica, Germany) FLIM system was used for the NIR FLIM of the clinical specimens. NIR-FLIM was performed using 730 nm excitation with a 750 nm notch filter and detected with a HyD R detector operating within a 770-850 nm range. A 10x, 0.4 NA objective was used for image collection and digital images with 512x512 pixels (2.275 µm/pixel), 3 line repetitions, and 3 line averages were obtained. TD data was collected using time-correlated single photon counting. An automated image stitching algorithm was implemented in the FALCON/FLIM software to obtain large area (~ 3x2 cm) mosaics of the clinical specimens.

For the cell culture studies, confocal FLIM imaging was performed on the fixed and mounted cells using a 63x, 1.2 NA oil immersion objective. Three channels were set for simultaneous imaging of ICG (Ex/Em 730/770-850 nm), LysoTracker Deep Red (Ex/Em 542–582/604–644 nm), and DAPI (Ex/Em 330–385/420 nm). For the ICG channel, both fluorescence intensity and FLT images were acquired, while only fluorescence intensities were acquired for the LysoTracker Deep Red and DAPI channels. Images were of 16-bit depth and 512x512 pixels with 0.34 µm/pixel. 3-5 representative regions were imaged for each experimental condition.

**H&E staining and imaging:** After the confocal FLIM, the OCT and FFPE tissue sections were stained with hematoxylin and eosin (H&E) and mounted with coverslips. Images of H&E-stained tissue sections were obtained using an inverted Keyence BZ-X810 microscope (Keyence, Itasca, Illinois, United States). A Plan Apo 10x, 0.45 NA air objective (Nikon, Tokyo, Japan), and a monochrome CCD (colorized with LC filter) were used to capture images. Histology images were graded by experienced pathologists from MGH, MEE, and the University of Pennsylvania. The pathologists were blinded to the FLT imaging results at the time of histology evaluation.
Image analysis

Data exclusion criteria:

For the primary and metastatic liver tumors, 10 patients were enrolled for ICG administration and data from two patients (one benign lesion, one mCRC) were excluded from quantification. Only metastatic lymph nodes were available for imaging from one patient with intrahepatic cholangiocarcinoma. FLT quantification results from these lymph nodes were grouped with other metastatic lymph node data. Among the two patients excluded from quantification, one patient contained a benign lesion (hepatic biliary cystadenoma). The other patient specimen contained a small (1 - 2 mm) mCRC tumor, and was excluded from the analysis due to poor data quality of the wide-field TD images. FLT images from 7 (out of 10 enrolled) patients were included in the ROC analysis.

For the HN cancers, data from patients with ICG injection less than 18 h (n = 3) or greater than 50 h (n = 1) prior to surgery were excluded due to high ICG retention at short time points or low ICG retention at long time points, respectively. Within the patient population that received ICG between 18 h – 50 h prior to surgery (n = 7), only one patient showed poor ICG retention in the tumor that presented with spindle cell carcinoma with unusual histomorphic features. Data from 6 patients (out of 11 HN patients enrolled for ICG injection) were included in the ROC analysis.

For bone and soft tissue sarcoma, data from 6 patients (out of 22 enrolled) were excluded from the ROC analysis due to a prior history of radiation or chemotherapy. The patients who received neoadjuvant radiation or chemotherapy showed significant treatment-related necrosis, cellular atypia, and inflammation, which gave rise to a heterogeneous ICG uptake. Optimizing the ICG dosage and timings for patients with neoadjuvant therapy was not a focus of the current study and will be assessed in future large-scale clinical trials. Among the remaining 16 patients, data from 6 more patients were excluded due to poor fluorescence signal in archival FFPE tissue blocks. Data from 10 patients without neoadjuvant therapy were included in the ROC analysis.

For GBM, patients with a 1 h infusion of 5 mg/kg ICG (n = 6) were included in the ROC analysis, and the patients injected with a bolus of 0.5 mg/kg ICG (n = 3) showed poor ICG uptake in the tumors and were excluded from subsequent fluorescence image quantification.

Widefield TD image analysis: TD fluorescence images were analyzed in MATLAB (MathWorks, Natick, Massachusetts, United States) using a custom software. Fluorescence intensity images were represented as the sum of all time gates for each TD data. Pixels with fluorescence intensity below 10-20% of the maximum intensity of the field were excluded from further analysis, depending on the signal quality and dynamic range. TD data from individual pixels were plotted as time gate (nanosecond units) versus log(counts) (Supplementary Fig. 4d) and the FLT was obtained by fitting the decay portion (double-headed arrow;
Supplementary Fig. 4) of TD fluorescence profiles to a single exponential function, $e^{-t/\tau(r)}$, where $r$ denotes pixel location and $\tau(r)$ denotes the FLT of ICG at the location $r$. FLT values at each pixel location are plotted as FLT maps and the jet colormap in MATLAB was used for visualization. Color photographs of the specimens were co-registered with fluorescence intensity and FLT maps using an affine geometric transformation with manually identified fiducials.

The co-registered fluorescence intensity and FLT maps were first segmented into tumor and normal regions based on the grossly identified margin. When the histopathology of a full cross-section of the specimen was available, tumor boundaries were based on the histologically identified boundaries. The distribution of fluorescence intensity and FLT within the tumor and normal tissue regions were represented as histograms and violin plots.

**FLIM image analysis:**
The confocal FLIM data were first fitted to a single exponential decay as described before. The resulting FLIM and fluorescence intensity images were co-registered with the corresponding histology image using an affine geometric transformation. A direct correlation between the histology and fluorescence images can be drawn since the same tissue section was used for FLIM imaging and histological staining. The histology images were graded by pathologists who were blinded to the fluorescence images, and tumor-normal tissue boundaries were identified for each specimen. ROIs (~50x50 pixels/ROI) for different tissue types (e.g., tumor, dysplasia, tumor-infiltrating lymphocytes (TILs), normal epithelium/hepatocytes/glial cells, normal stroma, inflamed stroma, salivary glands, blood vessels, connective tissue, muscle, keratin, and adipose tissue) were outlined on the histology images using ImageJ (NIH, Version 1.48u) and approved by the pathologists. 30-80 ROIs per specimen were obtained, which provided over 400 ROIs across all patients per tumor type. The ROIs were then mapped onto the FLIM and fluorescence intensity images and saved as separate images. ROIs with less than 10% pixels represented by tissue were excluded from further analysis. ‘Zero’ pixel values in the remaining ROIs were replaced by ‘NaN’ and the mean fluorescence intensity and FLT values for each ROI were calculated. Fluorescence intensities from all ROIs from each patient were normalized to an 8-bit scale. The distribution of fluorescence intensity and FLT within the tumor and normal tissue regions were represented as histograms and violin plots.

For the cell culture studies, at least three representative images for each experimental condition and cell line were analyzed. The confocal FLIM images were first masked to segment the cytoplasm. FLT’s of the pixels represented as cytoplasm within the full image field were tabulated and plotted as histograms.

For the lysosomal colocalization of ICG, the LysoTracker Deep Red and ICG channels were first median filtered (radius = 2) followed by background subtraction. The resulting images were analyzed for pixel-by-pixel colocalization using the Coloc 2 plugin in ImageJ and a correlation coefficient (R) was calculated.
Histology: H&E stained slides were examined by a team of board-certified experienced pathologists (Liver: LZ, HN cancer: AP, WF, PS, Sarcoma: YH, Brain: MN, MA), and multiple regions of tumor, normal tissue (e.g., muscle, connective tissue, blood vessels, inflammation, adipose tissue, etc.) were identified. All pathologists were blinded to the FLIM images, and the identified tumor and normal tissue areas were outlined and saved in ImageJ.

Glioblastoma (GBM) scoring: GBMs typically present a variety of non-tumor cell types such as macrophages or neurons within any given tumor region owing to its distributed nature. Additionally, since surgeries are typically performed via routine internal debulking of tumor masses in a piecemeal fashion to minimize the excision of normal brain parenchyma\textsuperscript{41}, the excised tissues primarily consist of tumors with varying densities of malignant cells. Therefore, a binary classification of tumor and normal tissue based on histology was unattainable for GBM unlike other solid tumors (e.g., cancers of the liver, HN tumors, sarcomas etc.). We instead classified the tissue into multiple regions with varying tumor densities. 150 ROIs were scored by a pathologist (MN) on a scale of 0-10 for tumor content. A score of 0 was given when essentially no tumor was identified by a neuropathologist assessing tissue sections. A tumor score of 10 was given for dense and solid tumors, inclusive of malignant features such as pseudopalisading necrosis and microvascular proliferation. Intermediate scores were given based on tumor density and infiltrative features. After the initial scoring, ROIs were classified into four tumor density groups as follows: Absent (no tumor) – score 0; low (few atypical cells present but no definite tumor) – scores 1-4; moderate (tumor cells present, definite tumor mass) – scores 5-7; and high (abundant tumor cells) – scores 8-10. Two ROC curve analyses were performed by: 1) combining tumor-absent and low-density ROIs as histologically negative, and the moderate and high-density ROIs into a histologically positive group, and 2) considering only low-density ROIs as the histologically negative group and the moderate and high-density ROIs into a histologically positive group.

Statistics: Statistical analysis was carried out using the Mann-Whitney U test (two-tailed) to estimate p values for box plots. P values less than 0.05 were considered significant: *, P < 0.05, and **, P < 0.01. The experiments were not randomized, and the investigators were not blinded to allocation during experiments and outcome assessment. Results are presented as mean ± standard deviation.

ROC curves: ROC curve analyses were performed in two ways: 1) by calculating a separate ROC curve for each patient (patient-wise) when paired tumor and normal tissue were available, or 2) by combining data from all patients (inter-patient) for a global analysis. The fluorescence intensity and FLT from the histologically confirmed tumor and normal tissue ROIs were used to calculate probability distributions and receiver operating characteristic (ROC) curves. ROC curves were obtained by varying the threshold for intensity and FLT and computing sensitivity and specificity for each threshold. Sensitivity = (no. of pixels within the tumor with intensity or FLT above the threshold) ÷ (total no. of pixels within the tumor). Specificity (= 1 -
false positive rate) = (no. of pixels outside the tumor below the threshold intensity or FLT) ÷ (total number of pixels outside the tumor).

**Data Availability Statement:** The main data supporting the results in this study are available within the paper and its Supplementary Information. The raw data generated in this study are available upon reasonable request from the corresponding author.

**Code Availability Statement:** The custom Matlab codes applied to analyze the time domain fluorescence data are available from the corresponding author upon reasonable request.

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**Author Contributions:** R.P. performed designed and performed experiments and data analyses, in addition to co-writing the manuscript. T.M.L. assisted in clinical data acquisition, patient recruitment and project design. C.D.C. designed and performed experiments and analyzed the data. M.K., H.R.C, S.S., performed experiments and data analysis, A.L.K., provided nursing services for the clinical study. S.A.C. assisted with the IRB application, imaging system design and analysis. M.P.N., A.P., M.S.M., Y.P.H., M.M.A., L.Z., P.M.S., and W.C.F., provided pathology specimens and assisted in pathology data interpretation. B.V.N., A.L.F., K.S.E., J.Y.K.L., K.S.R., S.L., M.A.V., and K.K.T. provided surgical specimens, and assisted in data interpretation. A.T.N.K conceived the idea for the project, designed and performed the experiments, supervised the clinical studies, and wrote the manuscript.

**Competing Interests:** A.T.N.K., M.K. and R.P. are co-inventors of a pending US Patent application issued to The General Hospital Corporation. The patent might be the subject of a licensing agreement in the future. Y.P.H. received royalties from Elsevier publishing company unrelated to this study. The other authors declare no competing interests.
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Figure 1: FLT enhancement and microscopic cancer specificity in primary and metastatic liver cancers: 

a. Photograph, fluorescence intensity, and FLT map of a freshly resected metastatic colorectal cancer (mCRC) specimen obtained from a patient injected with ICG 72 h prior to surgery. Dashed lines indicate the clinically identified tumor boundary. Violin plots show distribution of intensity, b, and FLT, c, across pixels within the tumor and normal liver parenchyma for the specimen shown in (a). d, Photograph, fluorescence intensity, and FLT map of a freshly resected hepatocellular carcinoma (HCC) specimen obtained from a patient injected with ICG 72 h prior to surgery. Dashed lines indicate the visually identified tumor boundary. The liver tissue surrounding the primary tumor mass consisted of significant cirrhosis. Violin plots show distribution of intensity, e, and FLT, f, across pixels within the tumor and normal liver parenchyma. g, Histology, fluorescence intensity image, and FLT image of a 15 µm OCT embedded tissue section from an HCC specimen obtained from a patient injected with ICG 28 h prior to surgery. Histologically confirmed tumor boundary is shown in the histology (yellow line) and intensity (red line) images. Violin plots show the distribution of intensity (h), and FLT (i) across pixels within the tumor, inflamed stroma, and normal hepatocyte regions for the specimen shown in (g). Dark regions in the FLT map in (g) within the tumor area are due to artifacts from tissue sectioning or due to the presence of tumor-infiltrating lymphocytes that did not take up ICG. j, high resolution (20x magnification) histology (left column), fluorescence intensity (center column), and FLT (right column) images of normal and HCC tissue regions within (g). k, histogram of fluorescence intensity, and l, FLT within (red) and outside (green) the histologically defined tumor boundary in (g). m, ROC curves for tumor vs. normal tissue classification of histologically determined tumor boundary yielded an accuracy (AUC) of 98% using fluorescence lifetime (red) and 55% for intensity (green) based classification. Dashed lines represent the threshold intensity (h and k) or FLT (i and l) calculated from the ROC curves in (m) and correspond to the highest J index. n, Representative TD fluorescence decay profiles from the tumor cells (black) and normal hepatocytes (gray).

Figure 2: FLT enhancement and microscopic cancer specificity in head and neck (HN) cancers. a-c Color photographs (left), wide-field fluorescence intensity (center), and FLT maps (right) of whole resected specimens from patients with a, cutaneous SCC in the left ear, b, oral SCC in the tongue, and c, a histologically tumor-free tongue specimen, injected with ICG 18 - 50 h before surgery. Dashed lines indicate visually identified tumor boundaries based on color photographs. Histology, microscopic fluorescence intensity, and FLIM of: d, an OSCC specimen from the left jaw and e, a tongue OSCC specimen. Confocal FLIM was performed on frozen (OCT embedded) sections (15 µm) of the specimen using a 10x, 0.4 NA objective. Dashed lines in (d) indicate histologically identified boundaries of infiltrative tumor nests that also enclose necrotic cores and keratinized regions (arrowheads) with shorter FLT than tumor cells. The FLTs within individual microscopic cancer nests (open arrows on FLIM image and solid arrows on histology) are longer than the FLTs of non-tumor cells in the tumor microenvironment, including the stroma and muscle (outside the tumor boundaries). e, Invasive tumor cell clusters displayed longer FLTs compared to the surrounding desmoplastic stroma or muscle despite a comparable ICG fluorescence intensity. f, Long FLT values comparable to tumor cells are also observed in dysplastic epithelium adjacent to invasive tumors in an OSCC specimen from the tongue. Solid arrows: dysplasia at the basal epithelium (long FLT); Dashed arrow: invasive tumor nest (long FLT); Arrowhead: hyper-keratinized necrotic core (no ICG uptake); "* Mature and metabolically less active superficial epithelium (short FLT). Histograms of fluorescence intensity, g, and FLT, h, within tumor (red) and normal tissue (green) from a tongue SCC specimen show that tumor FLTs were significantly longer
than normal tissue FLTs while the intensities of tumor and normal show a high overlap. The patient-wise (n = 6) mean fluorescence intensity, $i$, and FLT, $j$, of tumor (red) and normal tissue (green) are shown as the mean of multiple ROIs (> 40) of histologically identified tumor and normal tissue in each patient. Mann-Whitney U test (two-tailed): *** $p < 0.001$. Violin plots showing the distribution of fluorescence intensity ($k$), and FLT ($l$) in HN tumors and normal tissue types across multiple patients (n = 6). NE: normal epithelium; NS: normal stroma; DS: desmoplastic stroma; SG: salivary glands. m, Representative TD decay profiles from OSCC tumor and normal oral muscle tissue. The long FLT of tumors was clearly visible in the slow fluorescence decay profile (red) in tumor compared to the decay profiles observed in skeletal muscle (green). n, Receiver operating characteristic (ROC) plot of sensitivity vs. false positive rate (1 - specificity) across all patients studied (n = 6) resulted in an area under the curve (AUC) of 0.98 and 0.68 for FLT-based and intensity-based tumor vs normal classification, respectively. It is noted that the tumor FLTs measured using confocal FLIM (d-l) were longer than the FLTs measured using widefield FLT imaging (a-c). The difference in FLTs can be attributed to the fact that while the microscopic images measure FLTs from smaller regions consisting of a few individual cancer cell clusters, the widefield images of tumor tissue represent an average over larger regions (because of loss in spatial resolution) which consist of both cancer cells and cancer associated tissue (e.g., desmoplastic stroma, TILs, normal stroma, muscle, blood vessels etc.), thereby reducing the overall FLT.

Figure 3: Fluorescence lifetime enhancement in primary and metastatic bone and soft tissue sarcomas.

a-e, FLT enhancement in leiomyosarcoma specimen from an ICG injected patient. a, H&E stained histology images of an 8 µm tissue section from a patient with leiomyosarcoma (injected with 75 mg ICG at induction) shown in lower magnification (10x, left) and higher magnification (20x, right). The dashed red line image shows the tumor (T) and normal (N) tissue boundary, and the arrow indicates extravascular blood accumulation due to a hemorrhage within the tumor. Right: hemorrhagic area shows a large pool of red blood cells (H). b, Confocal fluorescence intensity and c, FLIM in low (left) and high (right) magnification. The tumor/normal boundary is indistinguishable via ICG fluorescence intensity (b, left), while the normal muscle (a, N) shows significantly shorter FLTs compared to the FLTs in the tumor (c, left), clearly delineating the boundary. The pool of hemorrhagic red blood cells shows high ICG fluorescence intensity (b, right) but short FLTs (c, right) that are comparable to the normal tissue FLTs outside the tumor (0.5 ns). Histograms of intensity (d) and FLT (e) distribution in tumor (red) and normal (green) tissue in the same leiomyosarcoma specimen. f-j, FLT enhancement in osteosarcoma. f, Histology of an 8 µm tissue section from an osteosarcoma specimen resected 24 h after ICG injection (0.75 mg/kg). The red dashed line shows the T/N boundary. g, Fluorescence intensity image of the section in (f) showing high nonspecific ICG uptake, and h, FLT image showing a clear contrast between tumor and normal tissue. Histograms of intensity (i) and FLT (j) distribution in tumor (red) and normal (green) tissue. k-o, FLT enhancement in renal metastasis to the bone. k, Histology of an 8 µm tissue section from a patient with renal metastasis, indicating the T/N tissue boundary (dashed white). l, Fluorescence intensity of the same section as (k) shows high non-specific ICG uptake in normal tissue while FLT (m) clearly separates the tumor from the normal tissue. Short ICG FLT in a blood vessel within the tumor microenvironment is indicated by arrows in (k-m). Histograms of intensity (n) and FLT (o) distribution in tumor (red) and normal (green). The patient-wise (n = 8) mean fluorescence intensity, $p$, and FLT, $q$, of tumor (red) and normal tissue (green), calculated as the mean of multiple ROIs (> 20) of histologically identified tumor and normal tissue. Mann-Whitney U test (two-tailed): *** $p < 0.001$. Violin plots showing the distribution of fluorescence intensity, $r$, and FLT, $s$, in sarcoma cancer cells and several normal tissue types across multiple patients (n = 10). BV: blood vessels; CT: connective tissue; AT: adipose tissue. t, ROC plot of sensitivity vs 1-specificity for tumor vs normal classification in data from specimens across 10 patients. The AUC was 0.97 for
FLT vs 0.61 for intensity. Dashed lines represent the threshold intensity in d, n, and r and the threshold FLT in e, o, and s, and correspond to the highest J index as calculated from the ROC curves in (t).

**Figure 4: Fluorescence lifetime enhancement in high grade GBM.** a-b, Histology (left), fluorescence intensity (center) and FLIM (right) of 10 µm FFPE tissue sections of high grade GBM from two patients. Dashed outline in (a) indicates a pool of red blood cells originating from a hemorrhagic area with high ICG accumulation and strong fluorescence intensity (a, center) but a short FLT (a, right). The rest of the specimen consisted of high-density tumor and areas of necrosis (a, arrow), that showed high ICG uptake and longer FLT compared to the hemorrhagic area. The dashed lines in (b) indicates a transition from low to high tumor density. While fluorescence intensity (b, center) remained consistently high throughout the specimen, FLT (b, right) showed a clear transition at the boundary. c-f, Representative histology (top row), fluorescence intensity (middle row), and FLT (bottom row) images from four histologic categories, namely, tumor absent (c), low density (d), moderate density (e), and high density (f). g, scatter plots of fluorescence intensity, and h, FLT are shown for the four tumor density categories. Fluorescence intensity and FLT values are presented as mean and standard deviation across 150 ROIs from multiple patients (n = 6). Fluorescence intensity showed no correlation to tumor density (r²=-0.18), while FLT was strongly correlated (r²=0.91). Note that in both the FLT images (c-f, bottom row) and the FLT correlation plot (h) the mean FLT for low density category was close to the tumor absent category. The mean FLT for moderate and high density categories, however, were significantly (p < 0.01) higher than the tumor negative and low density categories. i, ROC curves of sensitivity vs 1-specificity using FLT (red) and intensity (green), with negative and low tumor density regions as the histologically negative group, and the moderate and high tumor density regions the positive group are shown. Gray-shaded areas represent 95% CI.

**Figure 5: Fluorescence lifetime imaging of tumor infiltrating lymph nodes.** a, Photograph of a lymph node (LN) specimen resected from a patient with oral SCC during radical neck dissection. Two LNs were identified in the specimen (dashed outlines), only one of which (the right LN) was later confirmed to be histologically positive for tumor. b, Widefield FLT image of the whole specimen showing a uniformly longer FLT (0.64 ± 0.01 ns) within the positive LN (right, black dashed outline) compared to the FLT (0.48 ± 0.02 ns) of the negative LN (left, white dashed outline). FLIM images of tissue sections from the tumor positive (c, e) and negative (g, i) LN along with the corresponding histology images of the same sections ((d, f) and (h, j)) are shown. FLT enhancement can be observed in individual tumor cell clusters within the positive LN (dashed outlines in e and f). Arrows in (g) and (h) indicate short FLT in blood vessels. k, Photograph of a LN specimen with mCRC infiltrates, resected from a patient undergoing liver surgery. I, FLT map of the mCRC LN specimen overlaid on a gray scale photo of the LN. Dotted black outline shows tumor boundary identified from co-registered histology shown in m. n, Confocal FLIM and o, histology of rectangular region “1” from (l) and (m) indicated that areas with long FLT (n, arrow) coincided with areas of infiltrating tumor cells (o, arrow). p, Confocal FLIM of region “2” in (l) and (m), just outside the histological tumor boundary showed shorter FLT compared to the region “1”. q, Histology confirmed the absence of tumor cells in region “2”. r, Confocal FLIM of a section from another LN (widefield FLT image not shown) obtained from the same patient displayed metastatic cancer cell nests (arrows) with long FLT surrounded by normal lymphoid tissue with short FLT. q, Histology of the region shown in r confirms the location of metastatic cancer cell nests (arrows).

**Figure 6: Mechanistic studies of fluorescence lifetime enhancement in tumor cell lines.** a, Representative fluorescence intensity and FLT images of HepG2 and MCF 10A cells grown in serum (FBS) free media. Fluorescence intensity images show equivalent ICG uptake in both cell lines, while FLT’s were significantly longer in the HepG2 cell line compared to the MCF 10A cell line. b, Representative fluorescence
decay curves from the cytoplasm of cancer cells, HT29 (red) and normal cells, MCF 10A (green) in serum free media showing an ICG FLT enhancement in cancer cells. 

**c, Histograms of FLT distributions in the cytoplasm of various cancer cell types compared to the FLT distribution in MCF 10A.**

**d, Fluorescence microscopy of U2OS sarcoma cells showing ICG localized in the lysosomes after 1 h incubation of cells with ICG.** Blue: nuclear staining with DAPI; Green: lysosomal staining with LysoTracker Deep red; Red: ICG; Yellow: colocalized LysoTracker and ICG fluorescence.

**e, Colocalization analysis of ICG with lysosomes as measured by the correlation (R = 0.87) of LysoTracker and ICG fluorescence intensity at multiple pixel locations.** Each data point in the scatter plot represents the LysoTracker and ICG fluorescence intensities at an individual pixel location.

**f, Dependence of ICG FLT on solvent viscosity.** FLT imaging was performed on a vial containing 1.5 mM ICG in 100µl mixtures of ethanol and PBS (1:1) of varying glycerol concentration (0-95%) encompassing a viscosity range of 1.01 - 523 Cp. FLT maps of the vials indicated increasing ICG FLT with increasing solvent viscosity (left - right).

**g, Scatter plot of ICG FLT versus viscosity.** ICG FLT increased from 0.3 ns to 0.72 ns between the viscosity range studied.

**h-l, Dependence of ICG FLT on serum protein binding.** Fluorescence intensity (h) and FLT (i) of ICG (50 µM) was measured in vitro for increasing FBS concentrations (% FBS). Scatter plots of ICG fluorescence intensity or FLT versus FBS concentration are presented in (j) and (k), respectively.

**l, Intracellular ICG FLT in HepG2 cells increased with increasing FBS concentration.** Note that the FLT image for the 0% FBS in l is the same image as the FLT image for Hep G2 in a. The nuclear stain, DAPI, is shown in blue. Intensity and FLTs are represented as mean ± standard deviation (SD).
Table 1
Summary of patient and tumor characteristics

<table>
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<tr>
<th>Patient Characteristics</th>
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<tbody>
<tr>
<td><strong>Gender</strong></td>
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</tr>
<tr>
<td>Female, n (%)</td>
<td>24 (48)</td>
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<tr>
<td>Male, n (%)</td>
<td>26 (52)</td>
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<tr>
<td><strong>Age, years</strong></td>
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<tr>
<td>Median (range)</td>
<td>64 (27-96)</td>
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<tr>
<td><strong>Tumor Location, n (%)</strong></td>
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<tr>
<td>Liver</td>
<td>10 (18)</td>
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<tr>
<td>HCC (2)</td>
<td></td>
</tr>
<tr>
<td>IHC (1 Lymph node)</td>
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<tr>
<td>Metastatic CRC (5 + 1 Lymph node)</td>
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<td>Benign (1)</td>
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<tr>
<td>Head and neck</td>
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<tr>
<td>OSCC - Tongue (5), Mandible (2), Buccal Mucosa (1)</td>
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<tr>
<td>Cutaneous SCC - Ear (1)</td>
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<tr>
<td>BCC – Skin (1)</td>
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<td>Melanoma – Scalp (1)</td>
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</tr>
<tr>
<td>Brain</td>
<td>9 (16)</td>
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<td>GBM – Left Parietal Lobe (4), Left Temporal Lobe (2), Right Temporal Lobe (1), Left Frontal Lobe (1), Occipital Lobe (1)</td>
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<tr>
<td>Bone and soft tissue</td>
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<tr>
<td>Liposarcoma – Thigh (1)</td>
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<tr>
<td>Myxofibrosarcoma – Thigh (3), Left Axilla (1), Forearm (1)</td>
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<td>Leiomyosarcoma – Thigh (3), Upper Arm (1)</td>
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<td>Osteosarcoma – Femur (1), Tibia (1)</td>
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<td>Chondrosarcoma – Pelvis (2), Humerus (2), Proximal Femur (1)</td>
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<td>Dermal sarcoma – Skin (1)</td>
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<td>Metastatic Breast – Hip (1)</td>
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<td>Metastatic Renal – Humerus (2)</td>
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<tr>
<td><strong>Time between infusion to surgery</strong></td>
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<tr>
<td>Range (median)</td>
<td>15 m-6 d (24-72 h)</td>
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</table>
Figure 2

(a) Photograph, Intensity, Lifetime
(b) OSCC - Skin
(c) OSCC - Tongue
(d) Negative - Tongue
(e) Histology, Intensity, Lifetime
(f) Intensity, Lifetime
(g) Intensity probability
(h) Lifetime probability
(i) Mean Intensity (AU)
(j) Mean Lifetime (ns)
(k) Intensity (AU)
(l) Lifetime (ns)
(m) Log(Counts) (a.u.)
(n) Sensitivity vs. 1-Specificity

OSCC: Oral Squamous Cell Carcinoma
AU: Arbitrary Units
ns: Nanoseconds
AUC: Area Under the Curve
n.s.: Not significant
***: Significant at p < 0.001
Figure 3

Leiomyosarcoma

Osteosarcoma

Renal Metastasis

k

n

p

q

r

s

t

Histology

Intensity

Mean Intensity [AU]

Mean lifetime [ns]

250

1.2

1

0

1

0

0.4

0.6

0.8

1

Tumor

Muscle

Inflammation

BV

CT

AT

Intensity [AU]

Lifetime [ns]

Sensitivity

Lifetime (AUC = 0.97)

Intensity (AUC = 0.61)

1-Specificity
Figure 4

(a) Histology
(b) Low
(c) Absent
(d) Low
(e) Moderate
(f) High

(g) Intensity (AU)
(h) FLT (ms)

(i) Sensitivity vs. 1-Specificity

Legend:
- Lifetime (AUC = 0.98)
- Intensity (AUC = 0.33)
Figure 5

Lymph nodes from neck dissection

Photograph

Tumor Positive

Lifetime

Tumor Negative

FLIM

Histology

FLIM

Histology

Lymph nodes from liver surgery of mCRC

Photograph

FLT overlay

Histology

FLIM

Histology
Figure 6
Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryData.pdf