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Implementation of FLIM and SIFT for improved intraoperative delineation of glioblastoma margin

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The aim of this study is to develop a novel technique for improving the intraoperative margin assessment of glioblastoma by examining the total extrinsic extracellular matrix (ECM) with eosin staining using fluorescence lifetime imaging microscopy (FLIM) and scale-invariant feature transform (SIFT) descriptor analysis. Pseudo-color FLIM images obviously exhibit ECM distributions, changes in sequential sections, and different regions of interest. Meanwhile, SIFT descriptors are first utilized for the discrimination of glioblastoma margins by matching similar ECM regions and extracting keypoint orientations from FLIM images obtained from a series of continuous slices. The findings indicate that FLIM imaging with SIFT analysis of the total ECM is a promising method for improving intraoperative diagnosis of frozen and surgically excised brain specimen sections.

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Surgery is usually the best option for removing a brain tumor\textsuperscript{2}. However, even for the most experienced surgeon, visual inspections are insufficient for them to determine whether the complete resection has been performed\textsuperscript{2}. By contrast, intraoperatively acquired images can aid the surgeon to conduct rapid and image-guided surgery in a more precise way. A variety of mapping techniques have been developed to assist surgeons to quantify when and where the complete resection has been completed. These techniques, including the ultrasound imaging\textsuperscript{2}, frame-based and frameless navigation systems\textsuperscript{2}, magnetic resonance imaging\textsuperscript{2}, and fluorescence imaging with 5-aminolevulinic acid\textsuperscript{2}, are essential in determining the tumor border delineation. However, the degree to which intraoperative data accurately reflect surgical reality for brain tumor resection is yet unclear. To date, the primary technical challenge for brain tumor imaging lies in identifying the brain tumor margin with enhancing specificity and sensitivity since existing imaging techniques do not offer sufficient information needed to properly delineate tumor boundaries.

Precisely surgical removal of brain tumors with minimal damage to surrounding normal tissues requires a series of excisions, and each excision should be inspected for the presence of any remaining brain tumor in order to reduce tumor recurrence. In particular, an intraoperative frozen section of suspected brain tumors remains an important diagnostic tool to distinguish effectively among different tumor categories and to optimize the surgical strategy. Previous studies have revealed that the diagnostic accuracy of the central nervous system intraoperative consultation based on the frozen section is generally higher than 85\textsuperscript{2}. Despite the advantages of the intraoperative frozen section, residual tumor due to the utilization of an incomplete margin near the resection cavity is the most common cause of tumor recurrence\textsuperscript{2}. For centuries, histologists have adopted the bright field microscopy to elucidate functional attributes of tissues by examining tissue architectures. In addition, label free pathology with second/third harmonic generation (SHG/THG), multiphoton microscopy (MPM)\textsuperscript{2}, and stimulated Raman scattering (SRS)\textsuperscript{10} have been used as complimentary tools to aid in the pathological diagnosis. For example, SRS images of massive collagen deposits in a fresh glioblastoma specimen were clearly visualized based on a very strong protein signal. More specifically, SHG/THG is the optimal method to image collagen with high specificity. However, MPM faces the weakness of weak endogenous autofluorescence signals, higher excitation laser power, and longer pixel dwelling time, while SHG/THG can only capture the collagen I/III. Likewise, SRS is only able to map the signals from lipid/protein deposits. As such, these nonlinear optical microscopies have limited application in neurosurgical setups for intraoperative examination. Further, although collagen levels in the normal adult brain are low, and glioblastoma does not express collagen I\textsuperscript{2}, previous studies have demonstrated that glioma cell lines deposit large amounts of collagens I, III, IV, and VI, whereas collagen XVI expression is upregulated in glioblastomas, and can further promote tumor cell adhesion\textsuperscript{2}. The optical microscopies mentioned above cannot offer a relatively simple approach for simultaneously imaging all these.
extracellular matrix (ECM) components in glioblastoma specimens\textsuperscript{11,14}. Consequently, it is essential to develop advanced optical imaging techniques for the precise brain tumor margin delineation and for tracking brain tumor migration.

Interestingly, eosin is an ideal colorant for acidophilic tissular components, which stains most of the proteins in the ECM (collagen and cell cytoplasm) pink and, thus, is widely used in conjunction with hematoxylin for hematoxylin and eosin (H&E) staining. Eosin is usually not considered as a fluorochrome, although high fluorescence emission has been detected\textsuperscript{15,16}. The absorption and emission maxima of eosin in an alcoholic solution are 527 and 550 nm, respectively. Fluorescence microscopic imaging of H&E-stained sections has been sporadically performed in medical fields for the morphometric evaluation of myocardial cells, liver cells, dermal elastic fibers, and spleen and collagen in the periodontal ligament\textsuperscript{6,17}. However, fluorescent signals involve multiple parameters besides intensity and spectrum. For instance, fluorescence lifetime, defined as the time for the intensity to decay to 1/e of its initial value, is sensitive to the ambient environmental variation of the excited dye, which, thus, is recognized as an excellent probe for local micro-environmental sensing. Moreover, compared to fluorescence intensity imaging, fluorescence lifetime imaging microscopy (FLIM) is independent of the excitation power, fluorophore concentration, and photo-bleaching\textsuperscript{18}, and therefore is able to quantify the pH value, ion concentration, viscosity, refractive index, and other biochemical or biophysical parameters in biological samples, such as cultured cells\textsuperscript{19}. In addition, FLIM can potentially be correlated to cell and tissue states during physiological and/or pathological processes\textsuperscript{20}.

To the best of our knowledge, the use of FLIM to simultaneously measure ECM from H&E-stained glioblastoma sections has not been explored yet. In this study, we first examined the accuracy of eosin fluorescence lifetime in the total ECM for assessing margins in glioblastoma resection, and then demonstrated an image analysis method to readily determine the fluorescence lifetime variance in sequential tissue sections, which are strongly correlated with histological changes. The analysis results indicate that the combination of FLIM imaging of H&E-stained frozen sections and the scale-invariant feature transform (SIFT) descriptor analysis method for total ECM eosin FLIM images pave a new avenue for intraoperative diagnosis of glioblastoma margins.

Fresh tissue was biopsied from a single patient undergoing brain tumor resection at the Sixth People’s Hospital of Shenzhen. Glioblastoma tissue was fixed in formalin, serially sectioned (8 μm in thickness), and stained with H&E using standard protocols. A histopathological examination (No. 15158924) was performed by a senior pathologist from the Sixth People’s Hospital of Shenzhen, and the corresponding tissue was identified as glioblastomas. This study was performed under a protocol approved by the Sixth People’s Hospital ethics committee. The patient gave informed consent to the use of the tissue specimen for medical research.

FLIM imaging was performed based on single photon excitation and time-correlated single photon counting (TCSPC). The FLIM system was setup on an inverted microscope (ECLIPSE TE2000-E, Nikon, Japan) equipped with a Plan APO 10x/NA0.45 air objective, a Plan APO 20x/NA0.75 air objective, and a Plan APO 100x/NA1.4 oil immersion objective. A picosecond super continuum (400–650 nm) laser with an acousto-optic tunable filter (AOTF) (SC400-4, Fianium, UK) was used for convenient selection of excitation light. A DCS-120 scanner (Becker & Hickl GmbH, Germany) was used to implement confocal scanning. Fluorescence lifetime measurement was determined by measuring the fluorescence collected by an objective, passed through a 495 nm long-pass filter and a 520–580 nm band-pass filter and detected by a photomultiplier tube (PMT, R10467U-40, Hamamatsu Photonics, Japan) that connected to a TCSPC module (Becker & Hickl GmbH). In addition, bright field images were obtained on the same microscope with a CRI VariSpec liquid crystal tunable filter (Cambridge Research and Instrumentation, USA) and a CCD camera connected to a different port.

FLIM data processing was carried out using the SPCImage software (Becker & Hickl GmbH). The mean lifetime $\tau_m$ for each pixel in each image ($256 \times 256$ pixels) was calculated with bi-exponential component fittings expressed as

$$I(t)/I(0) = a_1 \exp(-t/\tau_1) + a_2 \exp(-t/\tau_2),$$

where $a_1$ and $a_2$ denote the proportions, and $\tau_1$ and $\tau_2$ denote the lifetimes of the two components, respectively. Therefore, the mean lifetime was calculated using the equation,

$$\tau_m = (a_1 \tau_1 + a_2 \tau_2)/(a_1 + a_2).$$

A pseudo-color lifetime image was generated by assigning a specific color to a mean lifetime value at each pixel. The measurements yielded time resolved fluorescence profiles with 256 temporal channels ($\Delta t \approx 50$ ps), yielding $256 \times 256 \times 256$ data cubes.

The feature extraction and classification by SIFT descriptors was employed using the open source MATLAB package “VLFeat”, developed by Vedaldi and Fulkerson\textsuperscript{21}. The SIFT descriptor is a sparse feature representation that consists of both feature extraction and detection. To detect scale-invariant characteristic points, the SIFT approach uses cascaded filters, where the difference of Gaussians (DoG) is calculated on rescaled images, progressively. The points of interest are then described by gradient orientation histograms to assign every keypoint a 128-dimensional feature vector based on the gradient orientations of pixels in 16 local neighborhoods. Given an image, SIFT finds all the keypoints in the image with respect to the gradient feature of each pixel. Each
keypoint contains the information of its location, local scale, and orientation, and by collecting these data, the SIFT approach computes and generates a local image descriptor based on each keypoint. All the local descriptors are then combined, and the complete features can be obtained from the image.

Images of an H&E-stained glioblastoma biopsy sample were initially captured with bright field illumination [Fig. 1(A)] and then with single photon excitation FLIM: In Fig. 1(A), the area outside the white dashed line demarcates a more severe necrotic region in the glioblastoma tissue. Fluorescence intensity images generated from accumulating photons for each pixel showed distinct patterns of H&E fluorescence in the ECM of the glioblastoma [Figs. 1(B1), 1(C1), 1(D1), and 1(E1)]. However, due to the low fluorescence quantum yield of hematoxylin, many morphological alterations of the nuclei were not as easily distinguishable as in bright field micrographs of the H&E-stained sections. Therefore, it was concluded that the fluorescence originated primarily from eosin. Pseudo-color mean fluorescence lifetime images [Figs. 1(B2), 1(C2), 1(D2), and 1(E2)] of each region were achieved with SPCImage software, as previously described. According to the color map, shades of red correspond to the longest $\tau_m$ and blue the shortest. In these FLIM images, the cytoarchitectural hallmarks typical for glioblastoma multiform, such as hypercellularity and pleomorphism, necrosis and micro-vascular proliferation, were identifiable by the absence of intact nuclei and short fluorescent lifetimes. FLIM makes use of an alternative signal from eosin fluorescence lifetime, which is strongly associated with the local micro-environment, allowing easy identification of necrotic tissue from the micro-vascular region. The pseudo-color $\tau_m$ images revealed considerable differences in the components of ECM among micro-vascular regions, medium necrotic regions, and highly necrotic regions, as shown in Figs. 1(C2), 1(D2), and 1(E2), respectively.

Extraction of points of interest on the object from similarly matched regions of the ECM stained with eosin provides a “feature description.” SIFT matching consists of three major steps: keypoint detection, keypoint description, and keypoint matching. Firstly, the use of SIFT descriptors to find similar regions in two partially overlapping fluorescence lifetime images was verified. FLIM images rather than fluorescence intensity images were used due to not only the sensitivity of lifetime to the micro-environment, but also the ability to quantitatively reflect the environment, as mentioned above. The verification tests utilized the four $\tau_m$ images [Figs. 1(B2), 1(C2), 1(D2), and 1(E2)] as test images. In Fig. 1, different areas of the single H&E-stained glioblastoma section were selected. Figure 2 shows the matching results with SIFT descriptors between different pairs of these images. As Fig. 1(C2) is a region of interest in Fig. 1(B1), Figs. 1(B2), and 1(C2) have the most matching keypoints [Fig. 2(A)], as expected. Figures 1(C2) and 1(D2) are images of two partially overlapping regions as shown in Fig. 1(B1), and the SIFT result between them [Fig. 2(B)] illustrates the matching keypoints appearing in the overlapping margins. Figures 1(C2) and 1(E2) are images of two regions in parallel without overlap, and their matching keypoints appeared only at the neighboring margins [Fig. 2(C)]. The comparison of Figs. 1(D2) with 1(E2) indicated that there was no relationship between these images, and, as expected, their matching keypoints were rare [in Fig. 2(D)]. These results verified the accuracy of utilizing SIFT analysis on FLIM images, suggesting that matching points among multiple images of a series of continuous slices are a promising method for differential diagnosis of tumor margins during intraoperative surgical excision.

Fig. 1. (Color online) Single photon excitation fluorescence intensity and lifetime imaging of H&E-stained glioblastoma sections. (A) Bright field images. (B1) Eosin fluorescence intensity image corresponding to the marked region in Fig. 1(A) (blue box). (C1), (D1), and (E1), three regions of interest in (B1), respectively. (B2), (C2), (D2), and (E2), Pseudo-color $\tau_m$ images corresponding to (B1), (C1), (D1), and (E1), respectively. The corresponding continuous color coding scheme ranged from 60 ps (blue) to 180 ps (red).

Fig. 2. SIFT descriptors are used to find similar regions from $\tau_m$ images of different regions. (A), (B), (C), and (D) The matching results between Figs. 1(B2) and 1(C2), 1(C2) and 1(D2), 1(C2) and 1(E2), 1(D2) and 1(E2), respectively.
To demonstrate, margin assessment by eosin-stained ECM and SIFT were then performed. Four sequential 8-μm-thick sections were obtained and stained with H&E, simulating continuous slices for intraoperative assessment, as multiple slices lead to the change or disappearance of morphology in irregular regions of interest. As shown in the bright field images [Figs. 3(A1), 3(B1), 3(C1), and 3(D1)], glioblastoma cells in the same regions of interest decreased as the number of the slices increased, while blood vessel patterns eventually appeared, as shown in Fig. 3(D1). Figures 2(A2), 2(B2), 2(C2), and 2(D2) show the corresponding fluorescence intensity images of eosin in the ECM. The numbers of ECM and inflammatory cells increased as glioblastoma cells decreased. The pseudo-color $\tau_m$ images [Fig. 3(A3), 3(B3), 3(C3), and 3(D3)] again revealed considerable differences in the components of the ECM in the same region of interest in the four sequential slices. As expected, the ECM in blood had a longer fluorescence lifetime.

To date, there have been few reported cases where fluorescence lifetime has been used to examine H&E-stained sections for evaluation of the tissue microenvironment[23], although studies have highlighted that healthy human tissue exhibited parallel, rather than distorted, populations of collagen (the main constituent in ECM) running disorderly to themselves[24]. Figures 3(A4), 3(B4), 2(C4), and 3(D4) show keypoint orientations of the same region of interest in Figs. 3(A3), 3(B3), 3(C3), and 3(D3), respectively. It should be noted that the keypoint was created by first computing the gradient magnitude and orientation at each image sample point in a region around the keypoint location. A Gaussian weighting function with $\sigma$ equal to one half of the width of a 16 × 16 sample array was used to assign a weight to the magnitude of each sample point. These samples were then accumulated into orientation histograms, summarizing the contents over 4 × 4 subregions, with the length of each arrow corresponding to the sum of the gradient magnitudes near that direction within the region. Polarization-sensitive optical coherence tomography and SHG microscopy enabled measurements of the main orientation and the angular dispersion of collagen. Herein, the EMC orientations from fluorescence lifetime images of H&E-stained sections were directly extracted using SIFT. Thus, it could be determined whether the sequential cuts were out of the tumor border by the similarity of total ECM directions, which aided in the evaluation of the intraoperative resection. In addition, the $\tau_m$ histograms varied with the corresponding slices, which could also be correlated with histological changes in the sequential tissue sections. As shown in Fig. 3(E1), where the slices were cut further apart from the glioblastoma nidus, the corresponding eosin fluorescence lifetime was longer. In Fig. 3(E2), full width at half-maximum (FWHM) ($\tau_{\text{FWHM}}$) and probability distribution ($\tau_{\mu}$) of the $\tau_m$ histograms increased with the cutting number of the glioblastoma tissue.

Figure 4 shows the matching results from the SIFT descriptors’ analysis of the fluorescence lifetime images.
of the four sequential slices. The decreasing number of matching points in adjacent slices [Figs. 4(A), 4(B), and 4(C)] indicated that the glioblastoma was being excised gradually, showing the viability of this method for tumor margin assessment. However, the relatively high matching between the last two slices [Fig. 4(C)] suggested that the tumor boundary had not been reached, probably because the glioblastoma tissue was too thick, and only four sequential 8-μm-thick sections were insufficient. Furthermore, Fig. 4(D) shows that the matching between the first and the last slices is also high, although the two slices are morphologically distinct [Figs. 3(A2) and 3(D2)] with different fluorescence lifetime distributions [Fig. 3(E)]. The result showed that some points in the blood vessel in Fig. 3(D3) were similar to the points in the glioblastoma ECM in Fig. 3(A3), which may suggest that the blood contained glioblastoma ECM or metastasis from the glioblastoma to the blood already occurring, and, therefore, further sequential resections were needed. A small number of matching points between different pairs of slices will help indicate a complete resection of the tumor. However, the exact number or level of matching points verifying sufficient excision needs to be further studied.

In conclusion, this work is an attempt to develop a technique for intraoperative delineation of glioblastoma margins based on H&E-stained sections that are imaged with FLIM, followed by SIFT analysis of the overall ECM. Eosin is widely used in pathology as a colorant for H&E staining, while its fluorescent properties are, for the most part, neglected. Fluorescence lifetime imaging of eosin in H&E-stained tissue sections exhibits advantages, including simple operations without complex experimental conditions, and, thus, possesses important application values in clinical pathological diagnosis. The proposed method is also based on the knowledge that collagens play a key role in tumor invasion and the biophysical properties of collagen influence malignant outcome. Matching points between two or more images of a series of continuous scenes is a vital component in many pattern recognition tasks, and the results demonstrated that the SIFT descriptors serve as a useful tool for discrimination of glioblastoma margins by matching similar ECM regions and extracting keypoint orientations. Moreover, the method we proposed in this Letter relies solely on eosin staining of tissue sections, which suggests that the method is also suitable for intraoperative frozen section diagnosis of surgically excised brain specimens. In summary, fluorescence lifetime imaging with SIFT analysis has the potential to be applied in intraoperative assessment of brain surgical margins.

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