Multimodal non-linear optical imaging for label-free differentiation of lung cancerous lesions from normal and desmoplastic tissues

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Abstract: Lung carcinoma is the leading cause of cancer-related death in the United States, and non-small cell carcinoma accounts for 85% of all lung cancer cases. One major characteristic of non-small cell carcinoma is the appearance of desmoplasia and deposition of dense extracellular collagen around the tumor. The desmoplastic response provides a radiologic target but may impair sampling during traditional image-guided needle biopsy and is difficult to differentiate from normal tissues using single label free imaging modality; for translational purposes, label-free techniques provide a more promising route to clinics. We thus investigated the potential of using multimodal, label-free optical microscopy that incorporates Coherent Anti-Stokes Raman Scattering (CARS), Two-Photon Excited AutoFluorescence (TPEAF), and Second Harmonic Generation (SHG) techniques for differentiating lung cancer from normal and desmoplastic tissues. Lung tissue samples from patients were imaged using CARS, TPEAF, and SHG for comparison and showed that the combination of the three non-linear optics techniques is essential for attaining reliable differentiation. These images also illustrated good pathological correlation with hematoxylin and eosin (H&E) stained sections from the same tissue samples. Automated image analysis algorithms were developed for quantitative segmentation and feature extraction to enable lung tissue differentiation. Our results indicate that coupled with automated morphology analysis, the proposed tri-modal nonlinear optical imaging technique potentially offers a powerful translational strategy to differentiate cancer lesions reliably from surrounding non-tumor and desmoplastic tissues.

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1. Introduction

Lung cancer is the leading cause of cancer-related death in the United States. In 2012, there were 226,160 projected new cases and 160,340 estimated lung cancer related deaths, accounting for 29% of cancer deaths in men and 26% of cancer deaths in women [1]. The observed 5-year survival rate is less than 16%, and this number has not improved for decades despite the advances in therapeutics [2–4]. Although early detection and accurate diagnosis are considered the key elements for improving long-term cancer survival rates, current routine diagnostic techniques such as computed tomography (CT) and chest radiograph are not able to distinguish malignant lung carcinoma from benign lesions such as organizing pneumonia and tuberculosis [5]. Therefore, invasive tissue biopsies are still required for pathological analysis and definitive cancer diagnosis. CT-guided fine needle biopsies are usually performed to aspirate tissues on patients with soft tissue opacities. However, because of the insufficient resolution of CT images, the respiratory motion of patients, and tumor heterogeneity, it is sometimes difficult to obtain samples precisely at the neoplastic site for pathological analysis [6]. Therefore, imaging the biopsy site prior to tissue aspiration can help reduce sampling errors and even eliminate the need for invasive tissue removal.

In recent years, although needle biopsy and pathology analysis still remain the gold standard for definitive lung cancer diagnosis, nonlinear optical imaging techniques have attracted many research interests for on-site diagnosis and staging of lung cancer and many other diseases. Because pathological processes are often associated with altered cellular and extracellular properties in the diseased tissue, the slightly changed biochemical components and altered morphological features can be captured by fluorescence emission spectra or imaged histological features. Intrinsic fluorescence emission originates from a variety of intracellular molecules including NADH, flavins, porphyrins, and lipid-pigments, as well as certain extracellular components such as elastin and collagen [7]. Two-photon excited autofluorescence (TPEAF) allows on-site imaging of suspicious tissues by exciting endogenous fluorophores with two photons of low energy near infrared light, with minimized photodamage and increased penetration depth compared to one photon fluorescence imaging [8–12]. Second harmonic generation (SHG) occurs in systems without symmetric center but with overall hyperpolarizability. For ex vivo and in vivo tissue imaging, SHG signals arise from highly ordered and hyperpolarizable biological molecules such as collagen, microtubules, and myosin [13, 14]. Stromal changes are involved in neoplastic process to provide a tumor-promoting environment for cancer progression and metastasis [15]. As a major component of extracellular matrix, the architectural properties of fibrillar collagen can be obtained by SHG imaging to reflect stromal changes in diseased tissues. Autofluorescence and SHG analysis are performed in real time without the need of tissue fixation or staining.
For cancer imaging, SHG by itself or in combination with TPEAF provides a powerful tool for cancer diagnosis and differentiation based on intrinsic tissue properties [11, 12, 16].

Coherent anti-Stokes Raman scattering imaging (CARS) is a label-free imaging technique that captures intrinsic molecular vibrations and provides information for the biological and chemical properties of samples. CARS was invented in 1965 at the FORD Motor Company [17]. Because of its capability of real-time noninvasive tissue imaging, its applications in the biomedical area have attracted vast research interests in recent years. In the CARS process, a pump field (Ωp) and a Stokes field (Ωs) interact with the imaged sample via a wave-mixing process. When the beat frequency (Ωp - Ωs) is tuned to the vibrational frequency of Raman active molecules in the system, the resonant molecules are coherently driven to an excited state and produce a blue-shifted CARS signal at the frequency of 2Ωp - Ωs when coming back to the ground state [18]. The major advantage of CARS is that the signal is much stronger than conventional spontaneous Raman scattering, allowing image acquisition and analysis to be performed in real time. In the case of cancer imaging, when the beat frequency matches the symmetric vibrational frequency of CH2 bond at 2845 cm⁻¹, a subcellular level of contrast arises from the intrinsic distribution of CH2 bonds inside cells and tissues, providing video-speed imaging of pathological features such as the size and shape of cell nuclei, cell-cell distance, and formation of fibrous structures, without use of any exogenous contrast agents. We have shown that CARS imaging, when combined with a knowledge-based classification platform, effectively differentiates the neoplastic tissues of human lung, prostate, and breast from their normal counterparts in ex vivo experiments, suggesting the potential application of CARS imaging as a minimally invasive imaging modality for label-free cancer differentiation [19–21].

Elastin and collagen fibers are the major components of the normal lung connective tissue network. Elastin constitutes up to 50% of the lung connective tissue mass and acts as the elastic protein allowing the lung tissue to resume its original shape after stretching [22]. Elastin is a common extracellular fluorescence source, this property makes autofluorescence imaging a convenient tool for illustrating the microstructures of the elastin network [23]. Collagen fibers are well-aligned proteins that provide support to maintain the tensile strength of lungs during the respiration process. The presence of highly ordered ProHypGly repeats in collagen gives it an overall hyperpolarizability, leading to the generation of strong intrinsic SHG signals when excited [13]. TPEAF from elastin and SHG from collagen have been shown to be two major sources of intrinsic contrast for non-diseased lung tissues with excitation wavelengths at 860 nm and 780 nm [24, 25]. In normal lung tissue, elastin and collagen form a well organized network providing the lung with its elasticity and contraction strength, and the collagen and elastin network is essential for the maintenance and integrity of lung tissues [22]. However, in lung carcinoma, cancer progression is often associated with the destruction of normal extracellular collagen and elastin network. Malignant cancer cells secrete extracellular enzymes to degrade the original tissue network at the invasion front and promote tumor progression into surrounding tissues [26]. Unlike non-diseased lung with its rich fibrous structure, the extracellular matrix network is modified in the neoplastic process [27]. Among all lung cancer cases, adenocarcinoma is the most common type and accounts for 35% to 50% of total lung cancer incidences [28]. Squamous cell carcinoma is the second most common histological type of lung cancer and accounts for 30% of lung cancer diagnosed in the United States [28]. Cancers are composed of malignant cells and of a stromal environment that plays an important role in tumor growth. Tumorigenesis is not only determined by neoplastic cells, but also highly dependent on the tumor microenvironment. According to the “seed and soil” hypothesis, crosstalk between the tumor cells (seeds) and the stromal environment (soil) have major impacts on tumor growth and invasion [29]. For lung adenocarcinoma and squamous cell carcinoma, stromal changes during malignancy include activation of fibroblasts, increased deposition of extracellular collagen, and appearance of desmoplasia [30]. The growth of fibrous tissue around a neoplasm is usually characterized
with low cellularity and its presence can make it difficult to aspirate neoplastic tissues for pathology analysis and definitive diagnosis and cannot be detected by using CARS imaging alone.

The objective of this study thus is to assess the potential of differentiating human lung cancer tissues from normal and desmoplastic edge tissues with label-free multimodal optical imaging. In this study, we performed multi-modal ex vivo imaging and obtained label-free CARS, TPEAF, and SHG images for tumor, desmoplastic, and normal lung tissues. In this multimodal technique, microstructures of elastin and collagen were acquired with TPEAF and SHG, and texture features were analyzed. Imaging results show that normal lung is mainly composed of well-organized extracellular collagen and elastin fibers, neoplastic lung tissues have disrupted extracellular matrix network, and desmoplasia tissues are characterized by dense collagen growth. Our experimental results verified that label-free differentiation of neoplastic lung tissue can be achieved by the three combined nonlinear optics technologies. Furthermore, coupled with an appropriated miniature fiber optics probe, multimodal optical imaging will have the potential to detect and differentiate lung tissue on the spot during image-guided intervention of lung cancer patients.

2. Materials and methods

2.1 Tissue sample preparation

Lung tissues were obtained from lung cancer patients undergoing lung surgery at Houston Methodist Hospital, Houston, TX, following an institutional review board approval. The excised tissue samples were cut into 5 mm chunks and then immediately snap-frozen in liquid nitrogen for storage. Frozen tissue samples were passively thawed for 30 minutes at room temperature and then imaged with a CARS microscope.

2.2 Optical imaging system

The optical source of our microscopic system is a mode-locked Nd:YVO4 laser (High-Q laser, Hohenems, Astria) that provides a 7-ps, 76-MHz pulse train at 1064 nm, and a frequency-doubled 7-ps, 76-MHz pulse train at 532 nm. The 532 nm pulse train is used to pump an optical parametric oscillator (OPO, Levante Emerald, APE-Berlin, Germany), generating a 5-ps, 76-MHz pulse train, which is tuned to 817 nm. This 817 nm laser is used as the pump beam for the CARS process and as the excitation wave for TPEAF and SHG. For CARS, the 1064 nm pulse train is used as the Stokes beam. The Stokes beam and the pump beam are overlapped both temporally and spatially to generate CARS signals. This is achieved by adjusting a time-delay line (DL) in the temporal domain and using a long-pass dichroic mirror (q10201pxr, Chroma, VT) in the spatial domain. A dichroic mirror is used to separate emission signals from excitation laser beams. The upright scanning microscope is modified from an Olympus FV300 confocal microscope adopting a 2D galvanometer. A 1.2-NA water immersion objective (60X, Olympus) is used for sample imaging. A red-light-sensitive photomultiplier tube (PMT, R3896, Hamamatsu, Japan) is used as the detector. Specific Bandpass filters are placed before the PMT detector to obtain corresponding CARS, TPEAF, and SHG images.

2.3 Image acquisition

The tissue samples were placed on a 170-µm cover slide (VWR) and then inverted on an imaging chamber to avoid possible compression. For CARS imaging, the pump beam was tuned to 817 nm and the Stokes beam was fixed at 1064 nm to probe the symmetric stretching frequency of CH2 bond at 2845 cm⁻¹. CARS signals were generated at 663 nm. A bandpass filter of hq660/40m-2P (2mm diameter, Chroma Inc.) was used to block unwanted background signals and collect CARS emissions specifically. For TPEAF and SHG processes, the 817 nm laser beam was used as the excitation wavelength, bandpass filters hq550/100m-
2P (2mm diameter, Chroma Inc.) and hq410/10m-2P (2mm diameter, Chroma Inc.) were used to capture autofluorescence signals from elastin in the range of 500-600 nm and SHG signals from collagen at 408.5 nm, respectively. The acquisition time was about 4 seconds per imaging frame of 512 × 512 pixels. For display purposes, we selected green pixels to represent 500-600 nm autofluorescence signals from elastin and blue pixels to represent SHG signals from collagen. We used grey scale to illustrate the CARS signals from symmetric CH2 stretching. The average power on samples was about 75 mW for TPEAF and SHG. For CARS, the average power was about 75 mW for pump beam and 35 mW for Stokes beam. Image was displayed with the Olympus FluoView v5.0 software. After imaging, all samples were marked to indicate the sampling locations, fixed with 4% formaldehyde, paraffin-embedded, sectioned through imaged locations, and stained with hematoxylin and eosin (H&E). Bright-field images of the H&E slides were examined with an Olympus BX51 microscope as a standard control.

2.4 Image segmentation and analysis

Both TPEAF and SHG images were segmented to extract features of elastin and collagen fibers. Preprocessing was performed before the segmentation. First, the inhomogeneous background caused by different imaging conditions was removed. The “rolling ball” algorithm proposed by Sternberg Stanley [31] was applied here. Basically, the background value is adaptively estimated for every pixel by averaging over a large ball around the pixel. The background subtraction was followed by denoising, where a median filter with the window size of 3 was used. The autofluorescence images were then preprocessed to find the “tube-like” structures. The eigenvalues of the Hessian matrix were used to calculate the measurement of “tubeness” [32]. For 2D images, if the large eigenvalue is negative, its absolute value is returned as “tubeness”, otherwise return 0. Robust Automatic Threshold Selection (RATS) was then performed for further segmentation [33]. Basically, the input image needs to be subdivided into a quadtree. A regional threshold is then calculated within each of the smallest subregions, which is the gradient weighted sum of the pixels. For extracting and quantitating collagen fibers in SHG images, a maximum entropy algorithm [34] was used using maximal inter-class entropy.

3. Results

3.1 Label-free multimodal imaging of human lung tissues

Figure 1 shows the representative TPEAF, SHG, and CARS images for normal lung tissues. For the processes of TPEAF and SHG, the tissue samples were excited with the pump beam at 817 nm. Signals of two-photon excited autofluorescence emissions from elastin are detected in the green light (500-600 nm) region, showing the fine details of the looped pattern for normal lung elastin network (Fig. 1(A), green). The collagen network is illustrated by intrinsic SHG signals at 405-415 nm (Fig. 1(B), blue). The collagen fibers show a wavy conformation and their overall spatial arrangement overlaps with the elastin framework (overlapped image in Fig. 1(D)). In the CARS process, the 817 nm pump beam and 1064 nm Stokes beam interact with tissue samples via a four wave mixing process, driving the symmetric stretching mode of CH2 bond to an excited state. CARS signal is obtained at the wavelength of 647 nm, providing intrinsic distribution of CH2 bonds inside cells and tissues and allowing video-speed imaging of pathological features without any exogenous labeling. Figure 1(C) shows the CARS image of non-diseased lung tissue. Because cell nuclei have less CH2 bonds compared to cell membrane and cytoplasm, they appear as dark spots in CARS images. One of the cell nuclei in the CARS image is indicated by a yellow arrow. Elastin and collagen fibers are shown as the bright fibrous structures (blue arrow). The imaging results show that normal lung is mainly composed of well-organized elastin and collagen fibers as the supporting matrix, similar to the H&E staining result for the normal lung tissue (Fig. 1(F)).
Fig. 1. TPEAF, SHG, CARS, and H&E images of normal lung tissue. (A) TPEAF image. (B) SHG image. (C) CARS image. (D) Superimposed TPEAF and SHG image. (E) Superimposed TPEAF, SHG, and CARS images. (F) H&E image of the same lung tissue but at a different location. Green indicates the TPEAF of elastin fiber in the range of 500-600 nm. Blue indicates the SHG signal from collagen fiber at 408.5 nm. CARS image (grey scale) shows signals from cells and fibrous structures of elastin and collagen. Yellow arrow and blue arrow point to a cell with a central dark nucleus and fibrous protein, respectively. Scale bar: 50 µm.

3.2 Differentiate lung cancer tissue

In this study, we use TPEAF, SHG, and CARS imaging to distinguish the neoplastic tissues of lung from normal non-diseased tissues. The imaging result for a representative lung adenocarcinoma case is shown in Figs. 2(A)-2(F). Cancer cells are illustrated in the CARS image (yellow arrow in Fig. 2(C)). The adenocarcinoma tissue contains mostly cancer cells forming glandular structures. Only a few broken elastin and collagen fibers are present in the tissue when imaged with TPEAF and SHG channels (Fig. 2(A) and Fig. 2(B)). The broken fibrous structure is also shown in the CARS image (Fig. 2(C), indicated by the blue arrow). The imaging result for a lung squamous cell carcinoma case is shown in Figs. 3(A)-3(F). Significantly fewer elastin and collagen fibers are present in the squamous cell carcinoma tissue compared to non-diseased (Figs. 3(A) and 3(B)). A cluster of tumor cells is shown with CARS imaging (Fig. 3(C)). The cell size of squamous cell carcinoma appears larger because tumor cells are usually bigger than normal and the size can appear bigger or smaller at various imaging/sectioning planes. In our previous study of lung cancer diagnosis with CARS, we quantified the distribution of cell sizes and developed a classification platform for definite lung cancer diagnosis [19]. The imaging results for both adenocarcinoma and squamous cell lung cancer correlate well with H&E stained histological features of the same specimen (Fig. 2(F) and Fig. 3(F)).
Fig. 2. TPEAF, SHG, CARS, and H&E images of adenocarcinoma tissue. (A) TPEAF image of adenocarcinoma tissue. (B) SHG image of adenocarcinoma tissue. (C) CARS image of adenocarcinoma tissue. (D) Superimposed TPEAF and SHG image of adenocarcinoma tissue. (E) Superimposed TPEAF, SHG, and CARS images of adenocarcinoma tissue. (F) H&E image of the same adenocarcinoma tissue. Green indicates the TPEAF of elastin fiber in the range of 500-600 nm. Blue indicates the SHG signal from collagen fiber at 408.5 nm. CARS image (grey scale) shows signals from cells and fibrous structures of elastin and collagen. Yellow arrow and blue arrow point to a cell with a central dark nucleus and fibrous protein, respectively. Scale bar: 50 µm.
3.3 Stromal changes in cancerous lung tissues

For non-small cell lung carcinoma, the desmoplastic response makes it difficult to precisely aspirate tissues with useful diagnostic information when performing prevalent CT-guided needle biopsy. To investigate the application of our multimodal imaging technique for label-free detection of desmoplasic in non-small cell lung carcinoma, we conducted TPEAF, SHG, and CARS imaging of desmoplastic tissues obtained from adenocarcinoma and squamous cell lung cancer patients undergoing surgery. Figure 4 shows the imaging results for a representative desmoplastic site. Dense collagen deposition is visualized with SHG imaging (Fig. 4(B)), and only a few broken elastin fibers are detected (Fig. 4(A)). Cell nuclei are indicated by a blue arrow in the CARS image (Fig. 3(C)). The multi-modal imaging result of dense collagen deposition at the desmoplastic site correlates well with the H&E result (Fig. 4(F)).
3.4 Quantitative analysis of connective tissue network

Elastin and collagen fibers in TPEAF and SHG images of human lung tissue were quantitatively analyzed. Altogether, there were six images of normal tissues, 13 images of desmoplastic edge tissues, and 33 images of tumors. The TPEAF and SHG images were segmented automatically to extract the histological features of elastin and collagen fibers. Figure 5 shows the segmentation results of elastin and collagen fibers in the normal human lung tissue sample of Fig. 1 using RATS and ME methods mentioned in Section 2.4.
In addition, Fig. 6 shows the statistical measurement of collagen and elastin fibers in SHG and TPEAF images respectively. Total area is the summation area of all segmented collagen fibers, which represents the total amount of collagen fibers in each SGH image. The average area of the top 10% biggest collagen fiber blobs is also calculated. The bigger the average area, the less broken the collagen fibers are. All values are normalized by the maximal value for the purpose of illustration. In TPEAF images, the total length and average length of the elastin fibers are calculated instead of the area. It is clearly shown in Fig. 6(A) that in comparison with the normal tissue, less collagen fibers exist in the tumor while significant larger amount of collagen fibers exist in desmoplastic area with respect to both total amount and fiber blob size (p<0.05). On the other hand, Fig. 6(B) shows that elastin fibers are significantly reduced in both desmoplastic and tumor tissues compared to those in normal tissue (p<0.05) while there is no significant difference between desmoplastic and tumor tissues.

4. Discussion

In this study, we examined the potential of label-free multimodal optical imaging for the differentiation of lung cancer tissues from non-diseased and desmoplastic tissues. We combined three non-invasive label-free imaging techniques, namely, TPEAF, SHG, and CARS, for direct tissue differentiation without the need of tissue fixation or staining.
Molecule-specific signals are generated at video speed based on the intrinsic chemical and biological properties of the specimen, and subcellular level of contrast is achieved for fast and accurate tissue differentiation. We were able to directly image pathological patterns of tumor cell nuclei using CARS, elastin using TPEAF, and collagen using SHG for human lung tissues. Current results show that label-free tri-modal optical imaging is capable of characterizing lung cancer, non-diseased, and desmoplastic tissues and has the potential to serve as a powerful tool for video-speed tissue differentiation.

In our *ex vivo* experiments, endogenous TPEAF signals from elastin and SHG signals from collagen were captured with tissues from lung cancer patients. Since both lung tumor and a margin of surrounding non-diseased tissues are removed during surgery, we performed label-free multimodal imaging with the excised non-diseased tissues first. TPEAF and SHG images of non-diseased lung clearly illustrated the looped pattern of elastin and collagen for the lung connective tissue network. CARS was tuned to detect the symmetric stretching signals from CH$_2$ bonds in this study. Cell nuclei appear as dark spots, whereas elastin and collagen appear as bright fibrous structures in CARS images. The imaging results demonstrate that fibrous elastin and collagen are the major components of non-diseased lung tissue, and they work together to form a well-organized supporting matrix to ensure the tensile and contraction performance of normal lung. However, the original lung connective tissue network is likely to be degraded by the extracellular enzymes secreted by tumor cells at the neoplastic site, thereby promoting the growth and invasion of tumor. In this study, we demonstrate that cancer tissues contain mostly tumor cells and significantly less fibrous structure is present compared to non-diseased lung tissues. The other major difference between cancer and non-diseased tissues is that the latter has a well-organized looped fibrous structure while elastin and collagen are mostly degraded and exist as a broken pattern in cancerous tissues.

Cancers are composed of not only malignant cells, but also a stromal environment that is altered to allow cancer progression and invasion. For non-small cell lung carcinoma, stromal changes during malignancy lead to strong desmoplastic response, which causes dense collagen deposition around the tumor that may make it difficult to accurately sample neoplastic cells for pathological analysis [30, 35, 36]. CT-guided needle biopsy is often directed toward desmoplasia because the increase of lung density. However, highly desmoplastic areas of tumor may have low cellularity, resulting in difficulty getting adequate samples for pathological analysis. This results in unnecessary repeated biopsy for patients until correct sampling and diagnosis can be made. Therefore, imaging the biopsy site prior to invasive tissue aspiration is expected to significantly reduce sampling errors.

CARS imaging alone cannot reveal desmoplasia. We thus examined the potential of the proposed tri-modal imaging technique for label-free detection of desmoplasia in non-small cell lung carcinoma by conducting TPEAF, SHG, and CARS imaging of desmoplastic tissues obtained from adenocarcinoma and squamous cell lung cancer patients. Our experimental data indicated that dense collagen deposition can be visualized and differentiated with SHG imaging and that desmoplastic tissues are also characterized by broken elastin fibers and poor cellularity.

Our long-term goal is *in vivo* microscopy imaging for real-time structural and functional cellular imaging at a suspicious site prior to sampling tissues for pathological analysis. The laser power of 75mW for pump beam and 35mW for Stokes beam is same as the laser power we used in previous work and does not cause photodamage [21]. In human body, the tolerance to laser power is much higher than that in thawed tissues, therefore less photodamage is expected and the current laser power is expected to be suitable for clinical applications. In CARS images, the cell nuclei appear as dark spots and the common pathological features can be extracted for cancer diagnosis. Previously our group has demonstrated that CARS, when combined with a knowledge-based classification platform, distinguishes cancerous tissues from normal and non-neoplastic tissues with high sensitivity, and small cell carcinoma from
non-small cell carcinomas with high sensitivity in \textit{ex vivo} experiments \cite{19}. However, that technique fails to extend to detect desmoplasia. We also noticed our CARS images were rather uneven, most likely due to the chromatic aberrations. However, as long as the unevenness is consistent in every image, this will not affect the results of computerized image analysis.

To address this issue, this study demonstrated that the tri-modal, label-free, non-linear optical imaging technique can distinguish normal lung, desmoplastic edge, and neoplastic tissue for non-small cell lung carcinoma. With image-guided needle biopsy, we expect to directly differentiate normal lung tissues, desmoplastic edges, and neoplastic tissues during the process of needle biopsy, thus increasing sampling accuracy, especially in settings where immediate or on-the-spot diagnosis by a cytopathologist is not available. Because cell structures are clearly visualized by CARS imaging, the differentiation of cancer subtypes can be based on the common pathological characteristics utilized for cancer diagnosis, such as nuclei shape and size, cell volume, and cell-cell distance. In the current study, we used a tri-modal imaging technique to distinguish cancerous tissues from normal and desmoplastic areas for non-small cell lung cancer which can be associated with strong desmoplastic response. An automated image analysis algorithm has also been developed to quantitatively extract the histological features of elastin and collagen fibers and utilize them for differentiation of normal, desmoplastic, and tumor tissues.

Our ongoing work includes combination of previous and current tissue classification methods for precise location of neoplastic tissues and on-site differential diagnosis of lung cancer subtypes. We expect that the label-free tri-modal optical imaging technique can potentially be applied to fresh excised or biopsied tissue specimens on the spot during intraoperative or interventional procedures and save precious time in sending tissue specimens for sectioning, staining, and diagnosis in a pathology laboratory. We are also designing and fabricating micro-optics probes to extend the \textit{ex vivo} multimodal optical imaging system for \textit{in vivo} image-guided needle biopsy or optical biopsy. As a result, this label-free multimodal imaging approach can potentially improve lung cancer diagnosis and serve as a powerful tool for video-rate real-time tissue differentiation.

5. Conclusion

CARS imaging can provide high quality microscopic images at the cellular level without using contrast agents or fluorescence reporters and thus well position for \textit{in vivo} clinical applications, which is quite infeasible for other conventional microscopic imaging modalities such as bright field microscopy or fluorescent microscopy.

For non-small cell lung carcinoma, stromal changes during malignancy lead to strong desmoplastic response and cause dense collagen deposition. This phenomenon can make it hard to sample neoplastic cells accurately for pathological analysis. We demonstrated the feasibility of label-free tri-modal optical imaging for quantitative differentiation of cancerous lung tissues from non-cancerous and desmoplastic tissues. By combining three non-invasive label-free imaging techniques, TPEAF, SHG, and CARS, we directly imaged pathological patterns of tumor cell nuclei, elastin, and collagen in \textit{ex vivo} experiments with human lung tissues. An automated image analysis algorithm was developed to extract and quantitate the histological features of elastin and collagen fibers and utilize them for differentiation of normal, desmoplastic, and tumor tissues.

The work reported herein indicates that the combination of three label-free non-linear optics imaging techniques is effective in differentiating normal lung, desmoplastic edge, and neoplastic tissues. Our ongoing work is to translate the proven \textit{ex vivo} imaging technology into an \textit{in vivo} imaging setting by integrating with micro-optics probes and combining quantitative tissue classification methods for \textit{in vivo} precise location of neoplastic tissues and on-site differentiation of lung cancer subtypes. The new label-free microendoscope device, if successfully implemented, will have the potential to offer fast, efficient, and reliable cancer
diagnosis without the need of invasive and sometimes repetitive and potentially harmful tissue removal.

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