Differential Diagnosis of Lung Carcinoma With Coherent Anti-Stokes Raman Scattering Imaging

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Aimed at bridging imaging technology development with cancer diagnosis, this paper first presents the prevailing challenges of lung cancer detection and diagnosis, with an emphasis on imaging techniques. It then elaborates on the working principle of coherent anti-Stokes Raman scattering microscopy, along with a description of pathologic applications to show the effectiveness and potential of this novel technology for lung cancer diagnosis. As a nonlinear optical technique probing intrinsic molecular vibrations, coherent anti-Stokes Raman scattering microscopy offers an unparalleled, label-free strategy for clinical cancer diagnosis and allows differential diagnosis of fresh specimens based on cell morphology information and patterns, without any histology staining. This powerful feature promises a higher biopsy yield for early cancer detection by incorporating a real-time imaging feed with a biopsy needle. In addition, molecularly targeted therapies would also benefit from early access to surgical specimen with high accuracy but minimum tissue consumption, therefore potentially saving specimens for follow-up diagnostic tests. Finally, we also introduce the potential of a coherent anti-Stokes Raman scattering-based endoscopy system to support intraoperative applications at the cellular level.


Lung carcinoma is the most prevalent type of cancer in the world and is responsible for more deaths than other types of cancer.1 When symptoms occur, the disease often has already progressed to an advanced stage, which is the major cause of its low survival rate.2,3 Five-year survival rates after diagnosis are between 8% and 12% in Europe4,5 and less than 15% in North America.6 Early detection of lung cancer has attracted major research interest because it dramatically increases the survival rate.5,6 Conventional detection strategies like sputum cytology7–9 and x-ray10–12 confront significant limitations because of their low detection rates.10–12 As a result, new diagnostic technologies have been actively developed in the past few decades; some of them have been used in clinical applications.

Low-dose spiral computed tomography has been shown to be capable of detecting lung nodules at an earlier stage than conventional chest x-ray.15–16 Nodules as small as 2 to 3 mm7 can be detected with low-dose spiral computed tomography, such that many lung cancers can be detected at stage 1. However, the low specificity of this method leads to a large number of false-positive readings and unnecessary follow-up evaluations in a significant number of tested patients.17 To further assess the pulmonary nodules (>7–8 mm) detected by computed tomography, positron emission tomography has been deployed,18,19 particularly in characterizing the staging and possible metastasis of cancer.20,21 Nevertheless, this technique suffers from low spatial resolution22 and poor susceptibility to respiratory motion.23 As a result, a tissue biopsy (normally fine-needle aspiration) is always included as a follow-up test after the detection of a nodule. The problem encountered by fine-needle aspiration, however, is the difficulty of performing biopsy on nodules smaller than 10 mm,17 making the diagnosis of small lung lesions a continuing difficulty.

Clinical problems in early lung cancer detection have driven the development of bronchoscopies. Whereas conventional white-light bronchoscopy is based on the detection of alterations in tissue surface structure, autofluorescence bronchoscopy aims at exploiting the spectral difference between normal and precancerous/early cancerous tissues.24 The only approved clinical device (Xillix Technologies, Richmond, British Columbia, Canada) takes advantage of the fact that cancerous lesions possess a higher level of backscattered red light than normal tissue when excited with a violet laser. Size and specificity are the major limiting factors of this technique because smaller–fiber-optic instruments (<1 mm) are needed for diagnosis of peripheral lesions.25

As an emerging label-free technique, optical coherent tomography formulates optical contrasts using the interference of 2 temporally incoherent lights, with a resolution of 2...
to 10 μm and an imaging depth of 2 to 3 mm. Micron-level resolution allows in vivo investigation and screening for possible lung lesions using light reflected from within tissue to generate cross-sectional images. In addition, optical coherent tomography–based probes can be used with a conventional bronchoscope, thereby increasing biopsy yield and providing preliminary diagnostic results. Although micrometer resolution combined with label-free features has enabled optical coherent tomography to achieve broad applications in the biomedical sciences, limited spatial resolution and specificity still hinder its accuracy, especially for small lung nodules or early-stage tumors. Therefore, there is a great demand to develop a fast, reliable, and cost-effective differential diagnostic tool for the early detection of lung cancer in order to increase accuracy, avoid unnecessary biopsy, and reduce patients’ suffering.

COHERENT ANTI-STOKES RAMAN SCATTERING IMAGING

Coherent anti-Stokes Raman scattering (CARS) microscopy was first reported in the 1980s by a group at the Navy Research Laboratory. It captures intrinsic molecular vibrations to create optical contrast. In the CARS process, a pump field (ωp), a Stokes field (ωs), and a probe field (ωp) interact with the samples through a four-wave mixing process. When the frequency difference, ωp − ωs (beating frequency), is in resonance with a molecular vibration, an enhanced signal at the anti-Stokes frequency, ωs = ωp − ωs + ωp, is generated in a direction determined by the phase-matching conditions. The major advantage of CARS is that the signal yield is much higher, typically several orders of magnitude, than the signal yield obtained through the spontaneous Raman scattering process. The intensity of CARS depends nonlinearly on the two incident intensities: I_CARS ~ I_p^2I_s. As such, high peak powers are necessary and CARS signals are tightly restricted to the focal plane, allowing a 3-dimensional (3-D) segmentation capability, similar to 2-photon microscopy.

In CARS excitation, the pump and Stokes fields coherently drive all resonant oscillators in the excitation volume at ωp − ωs generating a third-order polarization at the anti-Stokes frequency.

The capability of generating optical contrast from endogenous chemical structures has led to a rapid expansion of CARS microscopy for high-resolution imaging in biomedical research. It has been used to visualize various tissue structures, such as skin, DNA, and retina. In addition, CARS has been used in the imaging of brain sections. Using the CH2 stretching frequency, brain anatomy was revealed quite well at micron-level resolution. Collectively, CARS microscopy offers many advantages. First, it generates optical contrast using molecular vibrations, which are intrinsic features of the specimen. Because no natural or artificial fluorescent probes are required, CARS effectively avoids the toxicity, photobleaching, and artifacts associated with the staining process. By tuning the beating frequency, CARS provides chemically selective excitation of characteristic vibrational resonances, allowing imaging of particular chemical structures at will. Second, because of its coherent nature, CARS microscopy is several orders of magnitude more sensitive than spontaneous Raman microscopy. As a result, CARS requires a much lower average power for excitation (~0.1 mW), minimizing the phototoxicity caused by the imaging process and enabling its long-term in vivo imaging applications. Third, as nonlinear microscopy, CARS offers 3-D sectioning capability because CARS excitation is restricted to the focal volume. Combined with a fast scanning platform, this feature allows imaging of tissue and cell structures on a 3-D scale in real time. Finally, the optimum light sources for CARS are picosecond-pulse lasers. Compared with the use of femtosecond lasers, this feature reduces the chance of 2-photon–induced photodamage and increases the light penetration depth in thick tissue.

CARS IMAGING FOR LUNG CANCER DIAGNOSIS

Currently, in order to accurately delineate the type of lesions for definitive treatment, pathologists routinely stain lung biopsy tissue to examine changes in cellular and histologic features such as cell size, cell–cell distance, and formation of fibrous structures. However, although this method is subject to interobserver variations, the CARS technique already provides high-resolution images that can clearly detect these features, without tissue staining with exogenous agents. Therefore, a pattern recognition method was developed in conjunction with CARS whereby such images were used as a basis for the quantitative classification of these cellular features in a way that would lead to a differential analysis of lung cancer. A total of 1014 CARS images were acquired from 92 fresh-frozen lung tissue samples. The established pathologic workup and cellular diagnostics were used as prior knowledge for establishment of a knowledge-based CARS system using a machine learning approach. This system functioned to separate normal, nonneoplastic, and subtype lesions of lung cancers based on extracted quantitative features describing fibrils and cell morphology. Figure 1 shows representative CARS images and corresponding hematoxylin and eosin stain results of normal tissue, cancer, and noncancerous lesions.

The normal lung is predominantly composed of well-organized fibrous structures, consisting of the bronchi and supporting matrix for alveoli (Figure 1, A and B). Cancer regions showed much denser cellularity compared with normal regions, and the size and configuration of the cells corresponded with these parameters, as shown by hematoxylin and eosin stain (Figure 1, C through H). Meanwhile, organizing pneumonia and interstitial fibrosis (Figure 1, I through L), 2 types of noncancerous lesions serving as controls, showed dense fibrous structures with distortion of normal lung architecture, similar to those shown by the corresponding hematoxylin and eosin stains.

Fibril and cell structures were used to separate carcinous from benign and normal samples because changes in these structures are closely related to different kinds of lung lesions, including cancer, pneumonia, and interstitial fibrosis. In contrast, cancerous samples show a high density of cancer cells whose nuclei can be identified by CARS because of their low CH2 level (Figure 1). Therefore, a computerized segmentation algorithm was used to precisely delineate the boundaries of cell nuclei. Measurements of morphologic characteristics, including nuclear size, cell volume, and cell-cell distance, were performed after segmentation for classification analysis of cancer subtypes. Figure 2 illustrates the 3D spatial distribution of normal, benign, and cancer samples (Figure 2A), as well as cancer subtypes (Figure 2B) using partial least square regression analysis, in which most of the disease groups are well separated. A support vector machine with recursive feature
elimination–based classification analysis further showed the system’s ability to distinguish lung cancer from normal and nonneoplastic lung tissue with 91% sensitivity and 92% specificity. Small cell carcinomas were distinguished from non–small cell carcinomas with 100% sensitivity and specificity. As an adjunct to submitting tissue samples to routine pathology, this novel system recognizes the patterns of fibril and cell morphology, enabling medical practitioners to perform differential diagnosis of lung lesions in mere minutes.

In spite of this progress, the developed platform showed only limited accuracy (around 70%–75%) for differentiating between 2 major cell types of non–small cell lung carcinoma, adenocarcinoma and squamous cell carcinoma. This difficulty is not surprising, and it corresponds with the clinical difficulty in differentiating these 2 cell types using morphology alone. However, the advent of molecularly targeted therapies makes identification of the various histologic cell types and subtypes of lung cancer more important. Definitive diagnosis of these cell types is increasingly required for effective molecular treatment. As a result, an efficient diagnostic strategy using small biopsies or cytology specimens, with minimum tissue consumption, will greatly benefit additional molecular tests that are frequently involved in reaching a definitive diagnosis for targeted therapies. Accordingly, the potential of CARS has been further exploited by extending the previously developed 2-dimensional (2-D) image analysis framework into a 3-D nuclear segmentation, feature extraction, and classification platform. Using appropriate reconstruction and interpolation methods, a z-stack (a series of images acquired from different imaging depths) was modeled into a 3-D volume. Similar pathology-related features were measured in 3-D instead of 2-D to reach improved accuracy by eliminating certain 2-D artifacts. Thus, a 3-D measurement would provide access to the nucleus as a whole and allow measuring real nuclear size represented by volume.

In the work by Gao et al, different mouse lung cancer models were developed to build a 3-D platform for nuclear segmentation and cancer cell type classification. Superpixel-based local clustering was used to perform segmentation and produce clusters that capture actual image boundaries with great accuracy in 2-D images and was then extended to 3-D images, in which the clusters are called supervoxels. A modification of the simple linear iterative clustering

Figure 1. Ex vivo images of human lung lesions. Coherent anti-Stokes Raman scattering (CARS) and hematoxylin and eosin images of (A and B) normal lung, (C and D) squamous cell carcinoma, (E and F) adenocarcinoma, (G and H) small cell carcinoma, (I and J) organizing pneumonia, and (K and L) organizing pneumonia (original magnifications ×90 [A, C, E, G, I, and K] and ×125 [B, D, F, H, J, and L]. Reprinted from Cao et al, 2011, with permission from SPIE.
The algorithm was designed to compute 3-D supervoxels. In this clustering process, the 3-D volume is partitioned into small regions based on local affinity information and voxels are grouped into one cluster according to the similarity of their features. The clustered data set was used to extract pathologically relevant features about the cell nuclei, which were then used to perform classification of lung cancer cell types. Figure 3 illustrates a representative case of the fitting results on all nuclei in an adenocarcinoma volume, where all ellipsoids are plotted with their color representing different depths within the volume.

Figure 2. Spatial distributions of (A) cancer, normal, and benign cells and (B) cancer subtypes using partial least square regression analysis. Reprinted from Gao et al., 2011, with permission from SPIE.

Figure 3. Three-dimensional nuclear distribution of an adenocarcinoma volume after supervoxel-based nuclear segmentation. All ellipsoids are plotted with their color representing different depths within the volume.
Figure 4. Visualization of 2-dimensional (2-D) and 3-dimensional (3-D) classification results. A, Adenocarcinoma and squamous cell carcinoma using 2-D data analysis. The threshold for classification is the straight line $y = 0.5$. Points with $y \geq 0.5$ were classified as adenocarcinoma and points with $y < 0.5$ were classified as squamous cell carcinoma. B, Similarly, classification results from the 3-D data analysis are plotted.

Figure 5. Coherent anti-Stokes Raman scattering (CARS) images of mouse (A) kidney and (B) ear tissues using a CARS microscope with a multimode fiber–delivered laser (original magnifications ×90). Reprinted from Wang et al., 2010, with permission from The Optical Society.
Classification results from the 3-D data analysis are plotted in Figure 4, B, where clear separation between cell types allows for data visualization on the same graph. Quantified classification accuracies were a 99.56% and 97.78% true-positive rate for adenocarcinoma and squamous cell carcinoma, respectively. False-positive classifications were 0.45% and 2.22%. The results showed that features extracted from 3-D data analysis provided information that significantly enhanced the classification accuracy and thus demonstrated proof of concept that information extracted from 3-D image analysis and segmentation can be used for the automatic diagnosis of lung cancer subtypes.

DEVELOPMENT TOWARD CARS MICROENDOSCOPY

Like conventional optical imaging technologies, CARS faces the barrier of limited imaging depth caused by strong scattering inside the specimen; thus, the imaging depth is typically limited to less than 1 mm. Therefore, the development of CARS endoscopy is required for deep tissue imaging. A flexible endoscopy system consists of a light source unit, a detection unit, a control and display unit, and a fiber probe.\(^{50,51}\) The latter is commercially unavailable for constructing a CARS endoscope. Compared with CARS, significant progress has already been made in developing 2-photon excitation fluorescence fiber microendoscopy.\(^ {52-57}\) The lag existing between the two is because 2 excitation lasers are required for CARS excitation whereas only 1 is needed for 2-photon excitation fluorescence. The additional laser beam creates many challenges, including efficient delivery of 2 ultrafast lasers using optical fibers, efficient collection of CARS signals, minimization of nonlinear optical effects, efficient design of chromatic aberration–corrected miniature optics, and miniaturization of laser scanning mechanisms.\(^ {58-61}\)

Several groups have reported progress toward developing optical-fiber–based CARS endoscopy systems. The first benchtop CARS fiber probe prototype was demonstrated by researchers at Harvard University in 2006.\(^ {58}\) They illustrated a proof-of-principle demonstration of CARS endoscopy by using a single-mode fiber (SMF) with a focusing lens attached to the fiber tip. They used a 2-dimensional translational stage to raster scan the sample and achieved imaging of polystyrene beads. To address the strong nonlinear effect inside fibers, that is, the four-wave-mixing (FWM) effect, researchers\(^ {62}\) at the University of California, Irvine, proposed a fiber probe design that incorporates 2 separate fibers for excitation light delivery and for signal detection in 2010. They investigated the probe design challenges, including capturing the backscattered CARS signals in the sample and the nonlinear effects of fibers for delivering the laser pulses. They illustrated that different biological tissues could be imaged ex vivo by using their fiber-delivered probe for CARS imaging. In addition, a new CARS probe design and its optical modeling results were reported by researchers at the University of Washington, INO, and University Health Network in Canada in 2010.\(^ {41}\) They illustrated a miniature microscope objective design with a new spiral scanning fiber imaging technology and evaluated its anticipated performance through optical modeling. Their technique has the potential to make a CARS probe with a small footprint, an adjustable field of view, and high spatial resolution. The closest design to a real CARS endoscope to date is a laboratory prototype built by researchers\(^ {50}\) at Harvard University (Cambridge, Massachu-
These reported strategies were based on extraction and calibration of a series of disease-related morphology features for diagnosis using a machine learning approach. With continuing progress to development of CARS-based microendoscopy, another benefit of the reported image quantitation system lies in its potential for in vivo differentiation of lung carcinoma cell types and possibly subtypes during biopsy. With the advent of molecularly targeted therapies, avoiding sampling errors to allow complete identification of the various histologic cell types and subtypes within a given lung cancer becomes even more important. Coherent anti-Stokes Raman scattering allows selecting biopsy sites within a mass or nodule to minimize sampling error. Blind aspiration biopsy or fluoroscopic guided biopsy may sample surrounding tissues rather than the cancer and may not sample different cell types or subtypes within a cancer; thus, they may not be representative of the cancer as a whole. In contrast, CARS will not only ensure sampling of all cell types and subtypes within a heterogeneous lung cancer for molecular analysis.

Figure 6. Coherent anti-Stokes Raman scattering (CARS) images of mouse (A) skin and (B) liver tissues using the polarization and dual-wavelength wave plate strategy in the fiber-delivered CARS to suppress four-wave mixing (original magnifications ×150 [A] and ×90 [B]. Reprinted from Wang et al. 2011, with permission from The Optical Society.

Figure 7. A, Coherent anti-Stokes Raman scattering (CARS) spectrum of polystyrene beads (PEB) using our CARS endoscopy prototype. B, An acquired CARS image of fat cells in the mouse skin (original magnification ×150). Reprinted from Wang et al. 2011, with permission from The Optical Society.
at the time of biopsy, but also facilitate the whole diagnostic process while providing more abundant biopsy samples of the correct tissues for biomarker studies.

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