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Baowei Fei a,b,c,d, Guolan Lu b, Xu Wang e, Hongzheng Zhang e, James V. Little f, Kelly R. Magliocca f, Amy Y. Chen e

a Department of Radiology and Imaging Sciences, Emory University, Atlanta, GA
b Department of Biomedical Engineering, Georgia Institute of Technology and Emory University
c Department of Mathematics & Computer Science, Emory University, Atlanta, GA
d Winship Cancer Institute of Emory University, Atlanta, GA
e Department of Otolaryngology, Emory University, Atlanta, GA
f Department of Pathology and Laboratory Medicine, Emory University, Atlanta, GA

ABSTRACT

We are developing label-free hyperspectral imaging (HSI) for tumor margin assessment. HSI data, hypercube $(x,y,\lambda)$, consists of a series of high-resolution images of the same field of view that are acquired at different wavelengths. Every pixel on the HSI image has an optical spectrum. We developed preprocessing and classification methods for HSI data. We used spectral features from HSI data for the classification of cancer and benign tissue. We collected surgical tissue specimens from 16 human patients who underwent head and neck (H&N) cancer surgery. We acquired both HSI, autofluorescence images, and fluorescence images with 2-NBDG and proflavine from the specimens. Digitized histologic slides were examined by an H&N pathologist. The hyperspectral imaging and classification method was able to distinguish between cancer and normal tissue from oral cavity with an average accuracy of 90±8%, sensitivity of 89±9%, and specificity of 91±6%. For tissue specimens from the thyroid, the method achieved an average accuracy of 94±6%, sensitivity of 94±6%, and specificity of 95±6%. Hyperspectral imaging outperformed autofluorescence imaging or fluorescence imaging with vital dye (2-NBDG or proflavine). This study suggests that label-free hyperspectral imaging has great potential for tumor margin assessment in surgical tissue specimens of H&N cancer patients. Further development of the hyperspectral imaging technology is warranted for its application in image-guided surgery.

Keywords: Hyperspectral imaging, image-guided surgery, tumor margin assessment, cancer detection, image classification, image quantification, head and neck cancer, label free, fluorescence imaging

1. INTRODUCTION

Of the 15.2 million new cases of cancer in 2015, over 80% of cases will need surgery, some several times [1]. Surgery cures approximately 45% of all patients with cancer [2, 3]. To cure a cancer patient by surgery, the surgeon must remove the entire tumor at the time of surgery. Unfortunately, up to 39% of patients who undergo surgery leave the operating room without a complete resection due to positive or close margins [2, 4, 5]. It has been reported that a complete resection is the single most important predictor of patient survival for almost all solid cancers [6]. In breast, head & neck, lung, colon, and pancreatic cancers, complete resection is associated with a 3-5 fold improvement in survival compared to a partial or incomplete resection [7-10].

Various methods have been developed for cancer margin assessment. Visual appearance and palpation are often used by a surgeon to differentiate between malignant and benign tissue [11]. However, this visual assessment is subjective. Intra-operative frozen margin evaluation is commonly used to optimize surgical margin delineation at initial surgery [12]. Small samples from the surgical bed are selected to evaluate presence or absence of residual cancer [13]. However, intraoperative frozen section diagnosis may suffer from errors that occur during sampling and histological interpretation. In addition, histological processing can take time [14], which is labor intensive and prolongs surgery time. Fluorescence-guided imaging to navigate cancer resection has been shown to improve the number of complete resections and progression free survival [15-21]. In most cases, fluorescence-based approaches require the injection of a fluorescence contrast agent. There are clinical needs to develop label-free imaging technology and quantification methods to aid decision making during image-guided surgery.
Hyperspectral imaging, also called imaging spectrometer [22], was originated from remote sensing and has been explored for various applications by NASA [23]. With the advantages of acquiring two dimensional images across a wide range of electromagnetic spectra, HSI has been applied to numerous areas including archaeology and art conservation [24] [25], vegetation and water resource control [26] [27], food quality and safety control [28] [29], forensic medicine [30] [31], crime scene detection [32] [33], and biomedical area [34] [35].

Hyperspectral imaging has been emerged as a relatively new imaging modality for medical applications. This label-free imaging technology does not require a contrast agent and offers great potential for objective assessment of cancer margins. Light delivered to the biological tissue undergoes multiple scattering from inhomogeneity of biological structures and absorption primarily in hemoglobin, melanin and water as it propagates through tissue [36] [37]. The absorption, fluorescence and scattering characteristics of tissue change with the progression of diseases [38]. Therefore, the reflected, fluorescent and transmitted lights from tissue, where are captured by HSI, carry quantitative diagnostic information about tissue pathology [39] [40] [38, 41]. Spatially resolved spectra obtained by HSI provide diagnostic information about the tissue physiology, morphology, and composition. Recent advancements of hyperspectral cameras, image analysis methods and computational power make it possible for many exciting applications of hyperspectral imaging, such as cancer detection and image-guided surgery [42].

In this study, we developed a pipeline of preprocessing and quantification tools for hyperspectral image data. We developed image classification methods to differentiate tumor from benign tissue. We evaluated the hyperspectral imaging and classification method in surgical tissue specimens from human patients undergoing cancer surgery.

2. METHODS AND MATERIALS

2.1 Hyperspectral imaging system

A Maestro (PerkinElmer Inc., Waltham, MA) imaging system was used to acquire hyperspectral dataset. This is a wavelength-scanning system consisting of a Xenon light source, a solid-state liquid crystal filter and a 12-bit high-resolution charge-coupled device (CCD). Details about this system has been described in previous papers [43] [44]. This system is capable of obtaining reflectance images over the range of 450 nm – 950 nm with 2 nm increment, as well as fluorescence images under different excitation light sources [45].

2.2 Surgical specimen collection

Head and neck cancer patients who underwent surgery at Emory University Hospitals Midtown were recruited into the study. All tissues were collected under the clinical protocol approved by the Institutional Review Board of Emory University. During surgery, fresh surgical specimens were collected and sent to pathology for cancer assessment. Three tissue samples, i.e., i) clinically visible tumor, ii) surrounding normal tissue, and iii) tumor with adjacent normal tissue, were collected from the main specimen of each consented subject.

2.3 Hyperspectral image acquisition

Fresh surgical specimen were scanned with hyperspectral imaging in the following steps: 1) Acquire white and dark reference hypercube before tissue imaging. White reference image cubes are acquired by placing a standard white reference board in the field of view. The dark reference cubes are acquired by keeping the camera shutter closed. 2) Acquire reflectance hyperspectral images of the specimen from 450-900 nm with 5 nm intervals. 3) Acquire fluorescence imaging with 2-NBDG (Cayman Chemical, Ann Arbor, Michigan) by incubating tissue as described in [46]. 4) Acquire florescence imaging with proflavine (Sigma Aldrich, St. Louis, MO) similarly as described in Step 3).

2.4 Histological processing and analysis

After imaging, the resected specimen were fixed in formalin overnight and then sent to pathology for the standard histologic processing with hematoxylin and eosin (H&E) staining. The pathologic slides were digitally scanned, and an experienced pathologist outlined the tumor border on the digitized images for validation.

2.5 Hyperspectral data normalization

The purpose of data normalization was to remove the spectral non-uniformity of the illumination device and the influence of dark current. The raw data is normalized using the following equation:

$$
\text{Normalized Data} = \frac{\text{Raw Data} - \text{Dark Reference}}{\text{White Reference} - \text{Dark Reference}}
$$
\( I_{\text{reflect}}(\lambda) = \frac{I_{\text{raw}}(\lambda) - I_{\text{dark}}(\lambda)}{I_{\text{white}}(\lambda) - I_{\text{dark}}(\lambda)} \)

Where \( I_{\text{reflect}}(\lambda) \) is the calculated normalized reflectance value at the wavelength \( \lambda \). \( I_{\text{raw}}(\lambda) \) is the intensity value of the sample pixel. \( I_{\text{white}}(\lambda) \) and \( I_{\text{dark}}(\lambda) \) are the corresponding pixel intensities from the white and dark reference images at the wavelength \( \lambda \).

2.6 Glare detection and removal

Glare regions are formed due to specular reflection from the moist tissue surface and do not contain useful diagnostic information of the tissue. As we reported in [47], the glare detection method includes the following steps: 1) Estimate the first-order derivatives of spectral curves with a forward difference method; 2) Calculate the standard deviation (SD) of each derivative curve and generate an SD image for each hypercube. Glare pixels show higher SD than normal pixels. 3) Compute the intensity histogram of each SD image, fit the histogram with a loglogistic distribution, and then experimentally identify a threshold that separates glare and non-glare pixels.

2.7 Hyperspectral image classification

We analyze the spectral data to classify each pixel into normal and cancer tissue. To reduce computational time without reducing accuracy, spectral curves were averaged in non-overlapping blocks of 5×5 to yield a spectral signature per block. All of the spectral information available in the hyperspectral data was utilized. Blocks containing glare pixels were excluded from classification process. Each block was assigned a label as cancerous or normal. We implemented ensemble linear discriminant analysis (LDA) as the classifier using MATLAB (MathWorks, Natick, MA) [48].

2.8 Pathological validation

We used the pathologic images of the same surgical specimen to validate the cancer detection with hyperspectral image classification. On the digitized H&E-stained pathological slides, the tumor margin was outlined by an H&N pathologist. To reasonably assess the performance of the classification, we chose the regions of interest (ROIs), where the tumor or normal tissue were histopathologically confirmed by the pathologist, for quantitative analysis and validation.

2.9 Performance metric

The sensitivity and specificity of the classifiers for each patient were calculated based on the number of correctly classified tumor and normal pixels/blocks of all the specimens belonging to this patient. Furthermore, we calculated how many normal pixels/blocks were correctly classified for a normal specimen, how many tumor pixels/blocks were correctly classified for a tumor specimen, as well as the sensitivity and specificity on a tumor-normal interface specimen for each patient. We evaluated the performance of the hyperspectral image classification using the areas under the curve (AUC), accuracy, sensitivity, and specificity, as defined in the following equations (TN: true negative, TP: true positive, FP: false positive, FN: false negative):

\[
\text{Accuracy} = \frac{TP + TN}{TP + FP + FN + TN};
\]

\[
\text{Sensitivity} = \frac{TP}{TP + FN}; \quad \text{Specificity} = \frac{TN}{TN + FP};
\]

3. RESULTS

Fresh surgical tissue specimens were collected from 16 head and neck cancer patients. These patients included 7 oral cancer, 1 maxillary sinus cancer, 5 thyroid cancer, 1 parotid cancer, and 2 larynx cancer. As described above, we collected three types of tissue specimens from each human subject, which include: i) Clinically visible tumor tissue without necrosis, ii) Normal tissue, and iii) Tumor with adjacent normal tissue at the tumor-normal interface. Figure 1 shows the three tissue specimens and their corresponding histological sides.
For each patient, we used the images of the tumor and normal tissue to train the classification algorithms and then used the tumor with adjacent normal tissue to test the performance of the classification methods. In another word, the classification method built the training model with the spectral features extracted from the tumor and normal tissue and was then evaluated on the tumor-normal interface tissue of the same patient. Using the reflectance spectra from hyperspectral imaging, the hyperspectral imaging method was able to distinguish between cancer and normal tissue of the oral cavity with an average accuracy of 90±8%, sensitivity of 89±9%, and specificity of 91±6%. For tissue specimens from the thyroid, the method achieved an average accuracy of 94±6%, sensitivity of 94±6%, and specificity of 95±6%. As shown in Table 1, hyperspectral imaging outperformed autofluorescence imaging, 2-NBDG and proflavine fluorescence imaging for both cancer sites.

Table 1. Classification performance of HSI, autofluorescence imaging, and fluorescence imaging with 2-NBDG and proflavine.

<table>
<thead>
<tr>
<th>Cancer Site</th>
<th>Imaging Method</th>
<th>AUC</th>
<th>Accuracy</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oral Cavity</td>
<td>HSI</td>
<td>0.94±0.06</td>
<td>90±8%</td>
<td>89±9%</td>
<td>91±6%</td>
</tr>
<tr>
<td></td>
<td>Autofluorescence</td>
<td>0.83±0.19</td>
<td>80±18%</td>
<td>78±21%</td>
<td>86±14%</td>
</tr>
<tr>
<td></td>
<td>2-NBDG</td>
<td>0.86±0.16</td>
<td>83±15%</td>
<td>81±19%</td>
<td>85±11%</td>
</tr>
<tr>
<td></td>
<td>Proflavine</td>
<td>0.72±0.25</td>
<td>70±21%</td>
<td>71±20%</td>
<td>70±22%</td>
</tr>
<tr>
<td>Thyroid</td>
<td>HSI</td>
<td>0.98±0.03</td>
<td>94±6%</td>
<td>94±6%</td>
<td>95±6%</td>
</tr>
<tr>
<td></td>
<td>Autofluorescence</td>
<td>0.74±0.33</td>
<td>70±34%</td>
<td>76±23%</td>
<td>77±35%</td>
</tr>
<tr>
<td></td>
<td>2-NBDG</td>
<td>0.80±0.20</td>
<td>76±20%</td>
<td>75±22%</td>
<td>79±19%</td>
</tr>
<tr>
<td></td>
<td>Proflavine</td>
<td>0.86±0.16</td>
<td>82±16%</td>
<td>79±18%</td>
<td>85±13%</td>
</tr>
</tbody>
</table>
Figure 2 shows the photographs of the tumor and normal tissue as well as the tumor with adjacent normal tissue for a typical case. The three types of tissue demonstrate different spectral curves. The tumor margin as assessed by the classification method was close to that of the histological image outlined by the pathologist.

Figure 2. Tumor margin detection of surgical specimens from an H&N cancer patient. After hyperspectral image acquisitions, the tissue were processed histologically and tumor margins were outlined on the pathologic image (bottom right) by a pathologist (JVL), which was used to validate the results of the classification (top-right). The average spectral curves are shown at the bottom left for each type of tissue (tumor, normal, and tumor with adjacent normal tissue).

4. DISCUSSION

In this study, we reported automated tissue classification methods that use the spectra from 450 to 900 nm to extract diagnostic information. Each hyperspectral image contains over 2 million reflectance spectral signatures. The reflectance spectra capture the alteration of absorption and scattering properties of the tissue associated with malignant transformation. Molecular fingerprinting based on inverse modeling of reflectance spectra may shed new light on our understanding of cancer biology.

Although frozen section diagnosis is commonly used to guide surgical resection during surgery, it only samples a small portion of tissue in the resection area, which may lead to underestimation and does not guarantee margin-negative resection. Moreover, this procedure is time-consuming and labor-intensive. HSI is a wide-field imaging modality that can cover a large field of view, thus can provide rapid assessment of complete resection margins.

In this surgical specimen study, the label-free hyperspectral imaging is superior to autofluorescence imaging or fluorescence imaging with vital dye (2-NBDG or proflavine) for the detection of head and head cancer. A recent study has shown that wide-field fluorescence imaging with 2-NBDG can accurately distinguish the pathologically normal and abnormal biopsy tissue of head and neck cancer patients [49]. Proflavine has also been applied for distinguishing between benign and neoplastic mucosa in the head and neck [50]. We previously demonstrated the utility of hyperspectral imaging for head and neck cancer detection in a subcutaneous cancer animal model [47, 51] and a chemically-induced oral cancer model [52]. One prominent advantage of hyperspectral imaging is that it does not require
the use of an exogenous contrast agent. Therefore, this noninvasive imaging technology can be rapidly translated from *ex vivo* tissue specimens to *in vivo* human studies, such as clinical trials of hyperspectral image-guided surgery.

5. CONCLUSION

Hyperspectral imaging is an emerging imaging modality for medical applications. This label-free imaging technology does not require a contrast agent and offers great potential for cancer detection and image-guided surgery. Hyperspectral big data contain both spatial and spectral information. Our hyperspectral image quantification tools are able to distinguish cancer from normal tissue in fresh surgical specimens of head and neck cancer patients. Further development of the hyperspectral imaging technology is warranted for its application in image-guided surgery.

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