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A comparison of mid-infrared spectral regions on accuracy of tissue classification

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Abstract

Infrared (IR) spectroscopic imaging, utilizing both the molecular and structural disease signatures, enables extensive profiling of tumors and their microenvironments. Here, we examine the relative merits of using either the fingerprint or the high frequency regions of the IR spectrum for tissue histopathology. We selected a complex model as a test case, evaluating both stromal and epithelial segmentation for various breast pathologies. IR spectral classification in each of these spectral windows is quantitatively assessed by estimating area under the curve (AUC) of the receiver operating characteristic curve (ROC) for pixel level accuracy and images for diagnostic ability. We found only small differences, though some that may be sufficiently important in diagnostic tasks to be clinically significant, between the two regions with the fingerprint region-based classifiers consistently emerging as more accurate. The work provides added evidence and comparison with fingerprint region, complex models, and previously untested tissue type (breast) – that the use of restricted spectral regions can provide high accuracy. Our study indicates that the fingerprint region is ideal for epithelial and stromal models to obtain high pixel level accuracies. Glass slides provide a limited spectral feature set but provides accurate information at the patient level.

Introduction

The current gold standard for diagnosing cancer and further determining the course of disease is a biopsy, followed by a pathologist examining prepared samples from the tissue. Morphometric features such as the cell shapes, nuclear features and tissue architecture are commonly used to determine the presence of tumor and its grade(1). This process requires expert opinions, which are variable, can result in mistakes and does not leverage the power of modern computing. Hence, there is a need to complement the current pipeline with digital tools enabling comprehensive tumor analysis to increase accuracy, automation for better

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processes and enhanced recognition of disease. The use of combination diagnostics offering rapid and accurate tissue analysis will help in better decision making and therefore better patient outcomes. Fourier Transform Infrared (FT-IR) spectroscopic imaging coupled to digital pattern recognition tool has previously been reported for aiding in diagnostic decision making by providing objective and reliable tissue visualization and profiling(2–8). Recently, FT-IR spectroscopy has also been used to determine patient response to treatment especially chemotherapy(9,10) Further, it has been utilized to estimate the extent of malignancy(11). IR imaging captures the spatial detail of tissue as well as the biochemical signatures of the contained cellular constituents(12). The major spectral regions for tissue segmentation have been the 800 - 1800 cm^{-1} (fingerprint) region that typically consists of the bending vibrational modes of molecules and spans typical imaging detector cut-offs up to the carbonyl stretching mode ($\sim 1730 \text{ cm}^{-1}$), a cell and tissue “silent” region 1800-2700 cm^{-1} and a higher wavenumber region i.e. 2700 - 3800 cm^{-1} where there is significant information from N-H, S-H, O-H and C-H stretching vibrations(13). Since the same molecules lead to complementary stretching and bending modes, the fingerprint and stretching regions are often considered to contain redundant information for small molecules, though they might be independently informative for complex mixtures of molecules that comprise cells and tissues.

In most tissue segmentation studies, the entire spectrum is utilized for simultaneous analysis of chemical changes and alterations in tissue morphology. The idea of using discrete features was popularized(14) when data sets got very large and precluded full spectral analyses in reasonable times but increased significantly in importance with the advent of discrete frequency IR imaging(15,16). One direction has been focused on variable selection(17–19) to speed up biomedical imaging by reduced data acquisition(20,21). Emerging quantum cascade laser based instruments are largely focused on the fingerprint regions and may record spectra over the tunable laser’s full range(22,23). Using only the fingerprint region for histopathologic classification, several studies show accuracy in analyses(15,16,24–27). A comprehensive set of studies on use of limited spectral regions is not available for this emerging technology. For FT-IR imaging, in which the entire spectral range is available, the effect of different data parameters like signal to noise ratio (SNR) and spectral resolution has been studied(28) but there is a need to elucidate the potential of the two IR spectral regions independently for tissue classification. Another motivation for such a study is a very practical approach that has focused on analyzing only the stretching region by imaging samples on glass slides(29). Though a feasible path for routine pathology, it precludes the recording of the fingerprint region due to absorption by glass of wavelengths longer than $\sim 5 \mu\text{m}$. Although, the use of higher wavenumber region for classifying IR images acquired from tissue on glass substrates has been previously reported(29,30), the use of spectral features in this range for complex order tissue classification still remains to be investigated.

From the standpoint of obtaining a practical yet fully useful assay, it is important to first understand and quantify the predictive power of each of these two spectral regions as well as compare their potential to the full spectral range. That result can allow an informed choice in model development and data acquisition. Second, there is a need to understand the limits of performance if the spectral region is limited. This can allow an intelligent choice of trade-off between practicality (e.g. glass slides) or using rapid laser-based imaging (e.g. for discrete

frequency) or of future approaches with limited bandwidth. In this study, we alter previously developed classification models (26) to incorporate spectral metrics from the fingerprint and the high wavenumber region separately. The two models are compared with each other for overall performance, quantified by the area under the curve (AUC) values for receiver operating characteristic (ROC) curves.

Methods

Bioethical Approval

This study used de-identified human subject specimens. All protocols were performed according to the approved project by the University of Illinois at Urbana-Champaign Institutional Review Board i.e. IRB 06684. All experiments were performed in accordance with the University of Illinois at Urbana-Champaign's Institutional Review Board (IRB) as approved for study # 06684

Sample preparation and data collection

A breast tissue microarray (BR1003) was obtained from US Biomax Inc. Multiple sections were acquired for IR imaging and staining for obtaining histopathological ground truth. This array contains a distribution of patients of different disease states i.e. normal, usual ductal hyperplasia, atypical hyperplasia and invasive carcinoma. A detailed description of the patients is provided in table 1 given below. This offers an opportunity to build diagnostic models relevant for risk stratification and hence better treatment strategies. These are formalin fixed and paraffin embedded tissue specimens. The section to be imaged is first deparaffinized using hexane bath. Previous studies on breast and bone tissues have reported that dewaxing does not alter the IR profile in a statistically significant manner thereby making it appropriate for downstream analysis(31,32). The adjacent section is stained with Hematoxylin and Eosin (H&E) which is then annotated by a pathologist for important cellular markers. IR images are acquired using an Agilent Stingray Imaging System. A total of 100 patients' data is acquired at a spectral resolution of 4 cm^{-1} and a nominal pixel size of 1.1 microns, as reported previously(26). For each patient, a background is collected at an empty space on the slide to ratio the single beam intensity and the effect of atmospheric changes.

Data Preprocessing

Acquired IR images consist of 128×128 pixels; the sample area for each patient is ~120 fields of view (tiles). These tiles are stitched together in ENVI + IDL 4.8 and minimum noise fraction (MNF) is implemented using in house programs(33) to improve the SNR of the acquired data. This is especially critical for high definition data that is typically noisier than standard data for accurate classification downstream. After applying MNF, no smoothing was implemented. Finally, all patient cases are mosaicked together to generate a single image of the tissue microarray (TMA) containing all 100 cases. An initial set of specific spectral metrics based on the known biochemical signatures is identified and data is converted to the metrics thereby reducing the dimensionality of the data. Since only height and area ratios were used for class differentiation, no additional baseline correction or normalized was needed.

Data Analysis

Classification Model—Two 6 class models characterizing the tumor (6E) and its microenvironment (6S), which were previously reported(26), are used here as the reference model for comparisons. In the 6-class stromal model (6S), the epithelial compartments are only divided into benign and malignant while the stroma is separated into loose, dense and desmoplastic. All the other cell types like mucin, secretions, red blood cells (RBCs), necrosis and lymphocytes are clubbed together into the “others” class. The 6-class epithelial model (6E) on the other hand focuses on the subtle alterations of the benign ductal proliferations, for instance, the benign epithelium is further divided into usual and atypical hyperplasia. The choice of these classes is governed by the pathological significance of epithelial and stromal subtypes indicative of patient risk and prognosis.

Feature Selection—Next, features from the different spectral regions were isolated based on the 134 spectral metrics (peak height and peak area ratios) identified and described in our previous study(26). In this study, we utilize the same ground truth data and classification model while utilizing only a subset of features in different spectral regions to quantitatively estimate their predictive power. Out of the 134 metrics, 16 biomarkers spanned the high wavenumber region and 47 definitions belonged to the fingerprint region. These two feature sets were then used to build two classification models for both the 6S and 6E class separating, generating in total 4 classification schemes.

Performance Assessment—For estimating the area under the receiver operating characteristic (ROC) curves (AUC) values, annotations on the stained images are mapped onto the IR slice (regions of interest) for ground truth data to train and validate the supervised algorithm. Each class is represented by 24000 randomly chosen pixels from annotated tissue microarray. These pixels are then randomly divided into half for subsequent training and validation. The validation pixels are then used to estimate the ROC curves. The AUC values are compared across the high wavenumber and the fingerprint feature set for both the 6S and 6E models. Since, the ROC curves are evaluated on a subset of data pixels, model projections onto the entire tissue microarray of 100 cases containing 56 million pixels are evaluated for the different models. These two measures together give a precise estimation of the model performance for all the 4 classification schemes in different spectral regions.

Results

Classification comparisons using a six class model (6S) focusing on the stroma

While many comparisons and classification tests focus on relatively simple determinations that rely on large spectral differences (e.g. a two-class model to separate epithelial cells from stroma), we focus here on a relatively sophisticated example in which subtle chemical changes are expected between multiple classes that make classification challenging under all circumstances. We chose to compare the efficacy of IR spectral metrics drawn from different spectral regions for breast tissue characterization. First, we investigated a stromal model reported previously (26), which consists of a six-class model identifying the tumor and the accompanying desmoplastic reaction. This model, termed 6S for its six classes and focus on the stroma, consists of two classes associated with a simple disease determination [benign

epithelium, malignant epithelium] as well as four classes of stroma that span the association with normal or diseased abundant stroma and other constituents [dense stroma, loose stroma, desmoplasia, “other”]. We marked regions of TMA samples used for training the algorithm with pixels of each of these classes and examined their average IR spectra. A total of ~780,000 pixels were averaged for the 6 class stromal model with per class distribution as benign epithelium (~36000), malignant epithelium (~130000), dense stroma (340000), loose stroma (59000), desmoplasia (26000) and others (190000). As shown in Fig. 1A, both the spectral regions demonstrate differences between the six classes. We then used only spectral features from the regions indicated in the solid boxes to develop de novo classifications protocols. As seen in Fig. 1B and 1C, good class separation and disease identification is obtained for both regions. This attests to the extensive ability inherent in IR spectra to classify tissue and provide confidence that more complicated models, than previously reported, can be successfully developed using glass slides and limited spectral regions. However, the fingerprint region (Fig. 1C) provides higher accuracies for all classes with a major increase in the AUC value for desmoplasia (tumor-associated stroma along with immune reaction) and loose stroma. Thus, at least in this comparison, it is likely that the fingerprint region is more informative and needed if very high pixel level accuracies are desired. This higher accuracy likely arises from the more informative fingerprint region. Given the tissue structure and scattering-induced spectral changes(34–37) commonly observed in tissue, the higher accuracy may also be aided by lower scattering in the fingerprint region(38,39). Since these are FT-IR measurements, we do not anticipate effects of noise in the spectrum to be significantly different in the two regions. Similarly, any potentially differential effects of water vapor are carefully mitigated by purging the instrument during data acquisition and a small computational water vapor correction.

While it is informative to examine spectra and classification accuracy at the single pixel level, images are used in practice to observe features and make determinations of disease. Somewhat different pixel level accuracies can still provide images whose quality is similar and diagnostic ability is maintained. Hence, as a second measure of comparison, we examined the classified images resulting from the use of the two spectral regions. Fig. 2 shows the spatial performance of the high wavenumber (Fig. 2A) and the fingerprint (Fig. 2B) regions. The projections for the normal and invasive ductal carcinoma patient cases are similar but there are significant differences in the discussed atypical and lobular carcinoma cases. For the atypical core, the high frequency region identifies some epithelial pixels as the malignant class while the fingerprint classification labels it as predominantly benign. Even though atypical ductal hyperplasia has been known to be associated with high risk of progression to malignancy(40), the quantitative association of atypical epithelial cells with that of the tumor cells is unknown. For the invasive lobular carcinoma (ILC) case shown below, the fingerprint region (Fig. 2B, ILC) precisely identifies the two epithelial ducts (evident on H&E stained image in Fig. 2C) with one being benign and the other malignant. On the other hand, the glass region classification (Fig. 2A, ILC) identifies one of them as benign epithelium accompanied by the “others” class (contains lymphocytes) and the other as a cocktail of benign cells, tumor cells and others. This can be attributed to the extensive lymphocytic response surrounding these two ducts as evident from the H&E stained image (Fig. 2C, ILC). Thus, while the results are generally comparable, there are significant

differences in performance that vary with the pathologic state of the tissue. Again, the comparison points to the superiority of the fingerprint region in the classification of tissue and provides evidence for the need to carefully examine varying pathologies in the development and comparison of protocols for histopathology using IR imaging.

Classification Comparisons of six-class epithelial (6E) model

While stromal changes accompanying breast cancer are important and their subtle transformations(41,42) are a challenging test to compare IR imaging capabilities, conventional disease diagnoses are based only on epithelial cells. Hence, we evaluated a six class disease model(26) that largely focuses on epithelial transformations [hyperplasia, atypical, malignant, normal, stroma, others] for the two spectral domains. This model, termed 6E for its six classes and focus on epithelium, was evaluated on the same TMA discussed for the 6S case. We followed the same protocols as for the six-class stromal model and developed both classified images and ROC analyses for this epithelial-focused model. Average spectra from each of these classes is previously reported in our study(26). Fig. 3A and Fig. 3C (Glass Region, ROC) illustrate the classification performance of the high wavenumber spectral domain. Fig. 3B and Fig. 3C (Fingerprint Region, ROC) shows the predictive power of the IR metrics in the fingerprint spectral domain. Again, while the images show the effects on various pathologies, the pixel level accuracies are indicated in the ROC curves. As compared to the 6S model, the projections for both the regions are similar spatially, with the fingerprint region performing only slightly better than the glass region for the different epithelial states, stromal and other extracellular compartments. The invasive lobular carcinoma patient case is still better predicted by utilizing the fingerprint domain, as evident in the figure below. Again, the results are similar and show the striking ability of even the limited spectral region to show relatively high accuracies. Again, for consistently high accuracy and accurate spatial detail, the fingerprint region is desirable.

Tissue classification using glass slides, and using the high frequency region as a consequence, has been shown to be very effective and a potentially practical route to clinical translation of IR imaging (29) for prostate tissue. However, the demonstrations have been on relatively simple models. Here, the results demonstrate that the concept largely holds for sophisticated models as well. The caveat is that higher accuracies and more faithful spatial detail is obtained by using the fingerprint region. Further, the demonstration on breast tissue provides increased confidence that the use of glass slides and limited spectral regions may prove to be a generally useful strategy. The reasons for the relatively similar performance between the spectral regions is not unexpected as both regions contain contributions, though varying in an unspecified manner, from the same molecules and vibrational modes in both regions reflect the same species. Similarly, the superiority of the fingerprint region is not unexpected, given the higher level of detail in this region. One common aspect of the use of limited frequencies is that significantly accurate results can be obtained from limited spectral regions, providing an impetus for discrete frequency imaging that can further speed up analyses by only measuring smaller spectral bandwidths. We have not examined the effects of noise, use of substrates, scattering or costs here. These could be important considerations in the choice of a specific protocol.

Conclusions

A debate around the feasibility and virtues of using limited spectral regions, dictated by different experimental constraints, was examined using a standardized data set. We found that there is high potential of the high frequency region of the IR spectrum for extensive tumor and microenvironment analyses; however, the fingerprint region shows higher accuracy in two scenarios of complex models. The epithelial and stromal projections presented above demonstrate the ability of IR conserved biomarkers in different frequency domains for patient stratification based on the disease states. This study provides further support for protocols that enable the use of different sample substrates, especially glass, for rapid integration into the current clinical workflows.

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Figure 1:

Evaluation of different spectral features for breast tissue classification for tumor and its microenvironment identification using the 6S model [benign epithelium, malignant epithelium, dense stroma, loose stroma, desmoplasia, other]. A. Average spectra of the different classes as reported previously(26) in the model with the spectral region of interest highlighted. B. The corresponding ROC curve for the 6-class model using the fingerprint region. C. The ROC curve with the AUC values using the high wavenumber region.

Figure 2:

Classification images of the two spectral regions for four different pathologies [Normal, atypical, intraductal carcinoma (IDC), intralobular carcinoma (ILC)]. A. Classified images of the four cases of different disease states using features from the high wavenumber region. B. Classified images of the same cases using features from the fingerprint region. C. The corresponding H&E stained images for gold standard comparison.

Figure 3:
Classification projections for the two spectral regions for disease stratification. A. Classified images of four patient cases of different disease states using features from the high wavenumber region. B. Classified images of the same cases using features from the fingerprint region. C. The corresponding ROC curves for the two models with the AUC values representing model performance.

Table 1:

Patient cases with their corresponding disease states used to build the classification models.

Disease State	Number of Patients	Description	Additional Comments
Hyperplasia	20	Benign but with 1.5 fold risk of developing invasive cancer as compared to the reference population	None
Atypical Hyperplasia	20	Benign but 4-5 fold higher risk	Epithelial cells from these patients are not used as ground truth for the benign or normal class. However the final classifier is projected onto these patients to confirm the disease label
Malignant	40	20 belonging to ductal and 20 to lobular carcinoma	Ground truth pixels for desmoplasia were only taken from these patients
Normal	20	Benign: Reference Population	None