Differential diagnosis of breast cancer using quantitative, label-free and molecular vibrational imaging

Yaliang Yang,1,5 Fuhai Li,1,2,5 Liang Gao,1,2,3,5 Zhiyong Wang,1 Michael J. Thrall,4 Steven S. Shen,4 Kelvin K. Wong,1 and Stephen T. C. Wong,1,2,3,4,*

1Department of Systems Medicine and Bioengineering, The Methodist Hospital Research Institute, Weill Cornell Medical College, Cornell University, Houston, Texas 77030, USA
2NCI-ICBP Center for Modeling Cancer Development, The Methodist Hospital Research Institute, Weill Cornell Medical College, Cornell University, Houston, Texas 77030, USA
3Department of Bioengineering, Rice University, Houston, Texas 77005, USA
4Department of Pathology and Laboratory Medicine, The Methodist Hospital, Weill Cornell Medical College, Cornell University, Houston, Texas 77030, USA
5Authors contributed equally to this work.
*stwong@tmhs.org

Abstract: We present a label-free, chemically-selective, quantitative imaging strategy to identify breast cancer and differentiate its subtypes using coherent anti-Stokes Raman scattering (CARS) microscopy. Human normal breast tissue, benign proliferative, as well as in situ and invasive carcinomas, were imaged ex vivo. Simply by visualizing cellular and tissue features appearing on CARS images, cancerous lesions can be readily separated from normal tissue and benign proliferative lesion. To further distinguish cancer subtypes, quantitative disease-related features, describing the geometry and distribution of cancer cell nuclei, were extracted and applied to a computerized classification system. The results show that in situ carcinoma was successfully distinguished from invasive carcinoma, while invasive ductal carcinoma (IDC) and invasive lobular carcinoma were also distinguished from each other. Furthermore, 80% of intermediate-grade IDC and 85% of high-grade IDC were correctly distinguished from each other. The proposed quantitative CARS imaging method has the potential to enable rapid diagnosis of breast cancer.

OCIS codes: (170.3880) Medical and biological imaging; (170.4580) Optical diagnostics for medicine; (180.4315) Nonlinear microscopy; (300.6230) Spectroscopy, coherent anti-Stokes Raman scattering microscopy

References and links
1. M. Heron, “Deaths: leading causes for 2004,” Natl. Vital Stat. Rep. 56(5), 1–95 (2007).
2. American Cancer Society, Cancer Facts & Figures 2011 (American Cancer Society, Atlanta, GA, 2011).
3. M. J. Beresford, A. R. Padhani, N. J. Taylor, M. L. Ah-See, J. J. Stirling, A. Makris, J. A. d’Arcy, and D. J. Collins, “Inter- and intraobserver variability in the evaluation of dynamic breast cancer MRI,” J. Magn. Reson. Imaging 24(6), 1316–1325 (2006).
4. P. Robbins, S. Pinder, N. de Klerk, H. Dawkins, J. Harvey, G. Sterrett, I. Ellis, and C. Elston, “Histological grading of breast carcinomas: a study of interobserver agreement,” Hum. Pathol. 26(8), 873–879 (1995).
5. P. K. Gupta, S. K. Majumder, and A. Uppal, “Breast cancer diagnosis using N2 laser excited autofluorescence spectroscopy,” Lasers Surg. Med. 21(5), 417–422 (1997).
6. S. K. Majumder, N. Ghosh, and P. K. Gupta, “N2 laser excited autofluorescence spectroscopy of formalin-fixed human breast tissue,” J. Photochem. Photobiol. B 81(1), 33–42 (2005).
7. Y. Yang, A. Katz, E. J. Celmer, M. Zarawaska-Szczepaniak, and R. R. Alfano, “Fundamental differences of excitation spectrum between malignant and benign breast tissues,” Photochem. Photobiol. 66(4), 518–522 (1997).
8. A. S. Haka, K. E. Shafer-Peltier, M. Fitzmaurice, J. Crowe, R. R. Dasari, and M. S. Feld, “Diagnosing breast cancer by using Raman spectroscopy,” Proc. Natl. Acad. Sci. U.S.A. 102(35), 12371–12376 (2005).
1. Introduction

Breast cancer is the second leading cause of cancer-related deaths in women (1 in 8 women; about 13%) and accounts for approximately one-third of all cancers diagnosed among women in the United States [1]. The American Cancer Society estimated 230,480 new cases of invasive breast cancer and 57,650 new cases of in situ breast cancer, as well as approximately 39,520 breast cancer-related deaths, in women in 2011 [2]. Therapeutic decisions are based on imaging studies and pathologic diagnosis, neither of which has perfect sensitivity or specificity [3,4]. As the gold standard for clinical diagnosis, surgical pathology examines multiple histological features of tissues or cells (cell size, shape, and density or the formation of specific patterns) removed by surgeons or radiologists to characterize cancer lesions and their subtypes. The diagnostic process usually begins with a breast biopsy of either abnormal calcification or mass lesion, which is often performed by open surgery that removes the entire lesion, or by minimally-invasive core-needle biopsy that removes 5-12 cores of tissues to ensure adequate sampling. The excised tissues are then fixed, sliced, stained, and finally examined under a microscope by pathologists to make a diagnosis, resulting in a turnaround time ranging from hours to days. Frozen sections are more rapid, but are usually not performed on breast specimens because fatty tissue does not perform well in this technique. As a result of the long turnaround time for conventional histology, another procedure is often necessary because biopsies need to be repeated or margins need to be re-excised. Resulting delays or misdiagnosis in this process could directly lead to a missed opportunity to treat lesions early or unnecessarily aggressive therapies with harmful side-effects. Since diagnosis of cancer lesions plays a critical role in breast cancer prevention and treatments, a more rapid diagnostic technique could potentially reduce the number of repeated procedures while facilitating the whole process by allowing on-the-spot recognition of inadequate biopsies or positive margins.
In light of this, a variety of optical imaging techniques, such as fluorescence and Raman spectroscopies, have been explored to improve breast cancer diagnosis. Fluorescence spectroscopy has been demonstrated as a useful tool in breast disease correlations through ex vivo imaging experiments [5–7]. Although fluorescence imaging provides relatively high signal-to-background ratio, the small number of endogenous fluorophores in breast tissue and their overlapping spectra limit its applications [8]. Raman spectroscopy is another modality that has been investigated for disease diagnosis. It functions to identify disease lesions by capturing intrinsic chemical changes within tissues [8]. Previous study has successfully demonstrated its usefulness in identifying carcinomas by having a sensitivity of 94%, a specificity of 96% and an overall accuracy of 86% [9]. However, this technique is limited by its long acquisition time (> 1 s/pixel) with high excitation power, preventing its applications from fast scanning of large surface areas with high spatial resolution [10]. Collectively, then, there is considerable interest in developing a fast, less invasive, and more objective method for the screening and diagnosis of breast cancer [11].

As a molecular imaging technique, coherent anti-Stokes Raman scattering (CARS) microscopy has been demonstrated as a powerful tool for label-free imaging with sub-wavelength spatial resolution [12–15]. CARS imaging formulates contrast by probing resonances from specific chemical bonds in unstained samples, enabling its chemical selectivity. Its coherent nature further renders CARS signal several orders of magnitude stronger than the conventional Raman signal, thus offering video-rate imaging speed [16,17]. Therefore, this imaging modality has been successfully applied to a variety of biomedical applications, including the imaging of viruses, cells, tissues and live animals, as well as drug delivery [12,18–25]. In the field of cancer detection, a recent study showed the use of multiplex CARS for interferometric imaging of breast cancer for identification of cancer margins [26]. In this study, breast tissues were evaluated using their spectrum profile for construction of a digitized image for identification of tumor boundaries. The strategy was based on the chemically-selective modality of the CARS technique, but did not use its high spatial resolution in capturing cellular structures.

Current pathology examination of stained breast biopsy samples focuses on changes in such cellular and histological features as cell size, cell-cell distance, and formation of fibrous structures [27]. Accurate identification of these features will lead to delineating the type of lesions for definitive treatment. However, conventional pathology examination is still subject to interobserver variations [4]. The CARS technique provides high-resolution images which can clearly detect individual cells without using any exogenous agent to stain tissue. Therefore, we hypothesized that a cell/tissue pattern recognition method could be developed using established pathological workup and diagnostic features as a basis for the quantitative classification of different types of breast lesions, leading, in turn, to a fast examination strategy for the analysis of breast cancer samples. Accordingly, in this study, such disease-related features as cell size, cell-cell distance and presence of fatty and fibrous structures were used for classification analysis. Cancerous lesions were initially separated from normal tissue and benign proliferative lesion using visual features such as the presence of fatty and fibrous structures. To further separate cancer subtypes, cellular features related to the morphology and distribution of cancer cell nucleus were extracted from ex vivo CARS images of human breast tissues, including ductal carcinoma in situ (DCIS), invasive ductal carcinoma (IDC), and invasive lobular carcinoma (ILC), and used to quantitatively characterize different cancer subtypes through a classification strategy based on machine learning techniques. To the best of our knowledge, this pilot study demonstrates the first diagnostic platform with label-free and fast imaging properties with the potential to distinguish breast cancer from normal and benign tissues on the basis of quantitative cellular and tissue features applied to a computerized classification system.
2. Materials and methods

2.1. CARS microscopy

As shown in Fig. 1, the schematic of our CARS microscopy consists of a laser source and a beam-scanning microscope. A mode-locked Nd:YVO₄ laser (High-Q Laser, Hohenems, Austria) provides a 7-ps pulse train at 1064 nm and a frequency-doubled, 5-ps pulse train at 532 nm with repetition rate of 76 MHz. The 1064 nm pulse train is used as the Stokes wave for CARS process, while the 532 nm pulse train is used to pump an optical parametric oscillator (OPO) (Levante Emerald, APE-Berlin, Germany). The OPO generates a 5-ps pulse train output, which has a tunable wavelength range of 680-980 nm and is used as the pump wave for CARS process. The pump and Stokes beams are overlapped by adjusting a time-delay line in temporal domain and by the long-pass dichroic mirror DM1 (q1020lpxr, Chroma, VT) in spatial domain to satisfy the precondition for producing a CARS signal. The scanning microscope is modified from an Olympus FV300 confocal microscope adopting a 2D galvanometer. A red-light-sensitive photomultiplier tube (R3896, Hamamatsu, Japan) is used as the detector, which can sensitively detect the major spectral range of our CARS emission. A 60X, 1.2-NA water-immersion microscope objective (IR UPlanApo, Olympus) was used for this study. The lateral and axial resolution is estimated to be approximately 0.4 µm and 0.9 µm, respectively [28].

![Fig. 1. Schematic of CARS microscopy. M: mirror, OPO: optical parametric oscillator, DL: delay line, DM: dichroic mirror, L: lens, MO: microscope objective, BT: breast tissue, BPF: band-pass filter, PMT: photomultiplier tube.](image)

2.2. Sample preparation

Breast tissues were obtained from female patients undergoing surgical biopsy and surgery at The Methodist Hospital (TMH), Houston, TX, following Office of Human Subjects Research approval from The Methodist Hospital Research Institute (TMHRI). The excised tissues were immediately snap-frozen in liquid nitrogen for storage. A total of nineteen patients were enrolled in this study, including 4 cases of fibroadenoma, 2 cases of DCIS, 8 cases of IDC (2 cases of intermediate-grade (IG-) IDC and 6 cases of high-grade (HG-) IDC), and 5 cases of...
ILC. Twelve normal tissue samples were also collected from the same patients with IDC and ILC. Frozen tissue samples were thawed at room temperature and then imaged *ex vivo* using the CARS microscope. Two to five sampling points were imaged for each specimen and a total of 48 sampling points were examined (9 from DCIS, 11 from IG-IDC, 17 from HG-IDC and 11 from ILC patients). At each sampling point, three images were acquired from different imaging depths, resulting in a total of 144 images were used in this study. After CARS imaging, imaged places were marked with India blue, and samples were fixed in buffered formalin, sliced 5-μm thick, and finally stained with H&E, as a standard control of the disease type.

2.3. Image acquisition

Tissue samples were placed on a 170-μm cover slip and inverted on a rubber ring to form a sample chamber to avoid possible compression and associated morphologic changes of tissues during imaging [29]. The pump wavelength was tuned to 816.8 nm and the Stokes wavelength was fixed at 1,064 nm to reach a beating frequency of 2,845 cm\(^{-1}\), probing the symmetric CH\(_2\) stretching band. The CARS signal at 663 nm was collected by the same objective, i.e., using a backward (Epi-) detection scheme. Then it was separated from the excitation waves by the long-pass dichroic mirror DM2 (770dcxxr, Chroma, VT). Unwanted residual signals were blocked using a band-pass filter (BPF) (hq660/40m-2p, Chroma, VT). Image processing was performed using the Olympus Fluoview V5.0 software. Z-stack images with 1-μm step size were acquired at two digital zooms. Low power views at zoom 1.5X (~0.30 μm/pixel) with overall architectural information would allow us to clearly observe morphological features, while high power views at zoom 3.0X (~0.15 μm/pixel) with detailed cellular information would be used for precise segmentation of cells. Average power on sample was ~70 mW and ~35 mW for the pump and Stokes beams, respectively. This power combination is higher than that typically used for CARS imaging. It is due to the fact that solid tumor tissues normally possess a lower lipid level than normal tissues. As a result, a higher excitation power is required to provide enough image contrast for observation of cellular structures in tumor tissues. The acquisition time was about 4 seconds per frame with 512 x 512 pixels. Bright-field images of their corresponding H&E slides were captured by using an Olympus BX51 microscope and examined by a pathologist to determine the type of lesions as a standard control.

2.4. Quantitative image analysis

Cell nucleus segmentation: A semi-automated segmentation algorithm was developed to accurately delineate boundaries of cell nuclei [30]. The process consists of one manual step and four automated steps which take approximately 5 minutes for images from each patient. 1) Manually select a point within a cell nucleus. 2) Crop an image patch (a square window containing the interested object) centered at the selected point containing the nucleus. 3) Apply a seeded watershed algorithm [31–33] to segment the image patch into background and foreground, and thus obtain a rough region of the cell nucleus. 4) Use intensity threshold \([m-1.75\times\delta, m + 1.75\times\delta]\) for identification of another rough nucleus region, where \(m\) and \(\delta\) are the average intensity and standard deviation in a neighborhood of the center point. 5) Delineate the nuclear regions by overlapping the rough regions obtained from watershed and threshold methods (steps 3 and 4), and fit the result with an ellipse using the least square criterion [34] for a final boundary. An average of 27 cells for DCIS, 18 cells for IG-IDC, 16 cells for HG-IDC, and 12 cells for ILC per image were used for parametrical calculation. Figure 2(B) provides an illustration of cell nuclear segmentation of an IDC sample shown in Fig. 2(A).
Validation of cell nuclei segmentation: One hundred cells were randomly selected from 10 CARS images for each subtype of four breast cancer lesions: DCIS, IG-IDC, HG-IDC, and ILC, to validate the semi-automated segmentation algorithm. The semi-automated segmentation results were compared with the manual segmentation results by calculating three scores: precision, recall and f-score. They are given as follows:

\[
p = \frac{S \cap S'}{S'}, \quad r = \frac{S \cap S'}{S}, \quad f = \frac{2pr}{p + r},
\]

where \(S\) is the ground truth (manual segmentation result) of the \(i\)-th cell manually measured, and \(S'\) is the semi-automated segmentation result measured by the software used in this study. Figure 3 shows the validation results of cell segmentation in terms of precision, recall and f-score. It can be seen that all three indexes are close to 90% for the 400 individual nuclei from four cancer subtypes, indicating the high accuracy of our cell nuclear segmentation algorithm.

2.5. Extraction of disease-related features

We designated seven pathological features to characterize the difference among breast cancer subtypes: nuclear size, lengths of major (long) and minor (short) axes of cell nucleus, Voronoi tessellation (Fig. 2(C)) size (approximation of cell size) \([35,36]\), as well as average, major (maximum) and minor (minimum) neighbor distances of cells in the Delaunay triangulation graph (Fig. 2(D)) \([37,38]\). Moreover, five parameters, including mean value, standard deviation, skewness, kurtosis, and entropy, were employed to evaluate the distribution of each feature. The mathematical definitions of skewness \(\gamma_1(x)\), kurtosis \(\gamma_2(x)\), and entropy \(H(x)\) are as follows:

\[
\gamma_1(x) = \frac{1}{b-a} \int_a^b [(x - \mu)/\sigma]^3 p(x) dx,
\]

\[
\gamma_2(x) = \frac{1}{b-a} \int_a^b (x - \mu)^4 p(x) dx \left[\frac{\int_a^b (x - \mu)^2 p(x) dx}{\left(\int_a^b p(x) dx\right)^2}\right]^2 - 3
\]

and

\[
H(x) = -\frac{1}{b-a} \int_a^b \log_2(p(x)) p(x) dx,
\]

where \(x\) is a random variable whose observations are within \([a, b]\), \(p(x)\) is the probability density function of \(x\), and \(\mu\) and \(\sigma\) denote the mean and
standard deviation of $x$, respectively. Consequently, a total of thirty-five features were extracted to describe each CARS image.

2.6. Differential diagnosis analysis

We performed two analyses to investigate the separation of cancer subtypes: partial least square regression (PLSR) and semi-supervised learning (SSL) classification. PLSR is used for 3-D data visualization, and SSL is used to classify different subtypes of breast lesions. The basic idea of PLSR is to build a regression prediction model between the observation variables $X$ (independent variables) and the dependent variables $Y$. Since there might be many independent variables (when the dimension of $X$ is high), it will make the prediction model complicated and sensitive to the noise. To overcome this problem, the PLSR approach functions to reduce a high dimension independent variable space into a lower dimension space (dimension reduction), which can be represented by only a few coordinates (latent components). Then a linear regression prediction model is built between the latent components (by projecting the data points from the high dimension space onto the latent components to obtain new coordinates of the data points in the new space) and the dependent variables. So, technically, the PLSR analysis is to predict dependent variables $Y$ from independent variables $X$ by extending the idea of principal component analysis (PCA). The detailed implementation of PLSR can be found in [40]. In brief, this algorithm employs weight vectors $c$ and $w$ to maximize the correlation $\text{cov}(u, t) = [\text{cov}(Y, X) w]^T$, where $u = Yc$ and $t = Xw$ are called score vectors for $Y$ and $X$, respectively. After obtaining the $i$-th score vectors $u_i$ and $t_i$ (the projection coordinates of $X$ and $Y$ on the $i$-th latent components), the process is then applied to the residual matrices $Y_i$ and $X_i$ to get the next set of score vectors $u_{i+1}$ and $t_{i+1}$, where $Y_i = Y_{i+1} - u_i q_i^T$, $X_i = X_{i+1} - t_i p_i^T$, and $p_i = X_{i+1}^T t_i / t_i^T t_i$, $q_i = Y_{i+1}^T u_i / u_i^T u_i$.

After data visualization using PLSR, SSL classification analysis was performed to separate cancer subtypes. The idea of SSL is to make use of both the training data and the data structure information embedded in the unlabeled data. SSL is straightforwardly used to smooth classification results. In other words, SSL prefers that the nearby samples should belong to the same class, and the labeled samples transfer their label information outward to their nearby unlabeled neighbors gradually layer by layer. An intuitive example is the two moons shape data provided in [43,44]. Mathematically, the processes of SSL are as follows. Given $m$ are data points $X = \{x_1, x_2, \ldots, x_m\}$ which belong to $c$ class ($C = \{1, 2, \ldots, c\}$). The first
$m_1$ data points are labeled as $y_i \in C$, and the other data points are unlabeled. SSL analysis finds a non-negative matrix $F_{mc}$, which will be used to generate the labels of unlabeled data points, such as $y_i = \arg \max_{j \in C} F_{ij}$. The cost function of $F_{mc}$ is defined as follows [43,44]:

$$
\Phi(F) = \frac{1}{2} \left( \sum_{i,j=1}^{m} W_{ij} \left\| F_i \sqrt{D} - F_j \sqrt{D} \right\|_2^2 + \mu \sum_{i=1}^{m} \left\| F_i - Y_i \right\|_2^2 \right)
$$

(1)

where $W_{ij} = \begin{cases} \exp\left(-\frac{\|x_i - x_j\|_2^2}{2\sigma^2}\right), & i \neq j; \\ 0, & i = j. \end{cases}$ is an affinity matrix, $D$ is a diagonal matrix with $D_{ii} = \sum_{j=1}^{m} W_{ij}$, $F_i$ and $F_j$ are the $i$-th and $j$-th row vectors of $F$, $Y_i$ is the $i$-th row vector of $Y$, and $Y_{ij} = 1$, $y_i = j$; and $Y_{ij} = 0$ for the unlabeled data points. On the right-hand side of the above equation, the first term is the smoothness function which requires the neighboring data points to belong to the same class, while the second term is the fitting function which limits the labeled data points in order to be consistent with their original labels. Then the optimal $F^*$ satisfies $F^* = \arg \max_{F} \Phi(F)$. Differentiating $\Phi(F)$ at $F^*$, the equation $\left( \partial \Phi / \partial F \right)_{F^*} = F^* - SF^* + \mu (F^* - Y) = 0$ can be obtained, where $S = D^{-1/2}WD^{-1/2}$. Therefore, $F^* - SF^*/(1 + \mu) - \mu Y/(1 + \mu) = 0$. Letting $\alpha = 1/(1 + \mu)$ and $\beta = \mu/(1 + \mu)$, the relation $F^* = \beta (I - \alpha S)^{-1} Y$ can thus be obtained [43,44].

To validate the classification algorithm, a leave-one (patient)-out cross-validation analysis was conducted. In this process, the data from one of the patients were used for testing, while the remaining patients’ data were used to train the classifier. Since three z-stack CARS images were captured for each sample, we used the voting method to manage the conflicting results among individual stacks. The sample’s subtype was determined according to the classification results of the majority of the z-stacks. For example, if two of three z-stacks were classified into the same class, this sample would be recognized into that class regardless of the result from the third stack.

3. Results

3.1. CARS Images of breast tissues

Figure 4 shows a comparison of CARS images of normal breast tissue, benign proliferative lesion and carcinomas with their H&E stained photomicrographic images. On H&E stained images, normal breast tissues predominantly consist of adipose and fibrous structures (Figs. 4(B) and 4(D)). These structures possess strong CARS signals and can be clearly recognized in CARS images, as shown in Figs. 4(A) and 4(C), respectively. No obvious cells were identified in the normal tissues with CARS, possibly because of the overwhelming CARS signals from the fat and fibrous tissue components. Fibroadenoma is a common benign biphasic fibroepithelial tumor. One of its unique features is the intracanalicular pattern, in which the compressed duct shows linear branching pattern with slit-like lumen, as indicated by the arrow in the H&E stained image shown in Fig. 4(F). The same pattern is also clearly identified in the CARS image, as indicated by the arrow in Fig. 4(E).

Figure 4(H) shows the H&E stained image of a solid subtype of DCIS. The tumor cells are confined within the basement membrane and nearly fill the entire duct space. There are prominent cytoplasmic borders with sharp outline. These features are also presented in the
CARS image shown as Fig. 4(G). Similarly, IG-IDC consists of tumor cells growing in cords, nests, tubules, and anastomosing cell clusters, invading into the surrounding stroma, as shown in Fig. 4(J). All these features are distinctly observed in the CARS image shown in Fig. 4(I). The CARS and H&E stained images of HG-IDC are shown as Figs. 4(K) and 4(L), respectively, in which tumor cells are arranged singly or in small clusters, but without noticeable tubule or gland formation. Figure 4(N) shows an H&E stained image of a classic ILC, with characteristic infiltrative pattern with single or rows of cells (Indian filing) invading into the stroma. This pattern is clearly presented in the CARS image, as shown in Fig. 4(M). It is worth noting that in some foci, the single filing infiltrative pattern of ILC is inconspicuous, and the tumor cells may just be dispersed in the stroma in an irregular fashion.

![Fig. 4. CARS images of human breast tissues taken at Raman shift of 2845 cm⁻¹ and their H&E stained images from similar regions. Images of (A) adipose and (C) fibrous structures in normal tissues and their H&E stained images (B) and (D). Image (E) of a kind of fibroadenoma (a benign lesion) and its H&E stained image (F), in which a compressed duct can be clearly seen as a linear branching pattern with slit-like lumen, as indicated by the arrow. Image (G) of DCIS and its H&E stained image (H). Images of (I) IG-IDC and (K) HG-IDC and their H&E stained images (J) and (L). Image (M) of ILC and its H&E stained image (N). Scale bars: 10 μm.](image-url)
Fig. 5. Distributions of the seven features of four subtypes of breast cancer: (A) nuclear size, (B) major and (C) minor radii, (D) Voronoi tessellation size, as well as (E) average, (F) major and (G) minor neighbor distances.
3.2. Differential diagnosis of breast cancer

In most cases, histological evaluation alone is sufficient to separate \textit{in situ} carcinoma from invasive carcinomas and to separate ductal and lobular carcinoma subtypes. However, in some instances, the differential diagnosis of histological subtype of breast cancer may be difficult and cannot be reliably made with conventional H&E staining of histological sections, even by an experienced pathologist. Therefore, using breast tissue samples with histologically well-characterized lesions by H&E staining, we explored whether an algorithm could reproduce identical or near-identical morphological characterizations using CARS images. Figure 5 provides the distributions of the seven features for four subtypes of breast cancer. From Fig. 5(A), 5(B) and 5(C), it can be seen that IG-IDC has larger nuclear size and longer major and minor radii with wider distribution ranges than other subtypes. HG-IDC has narrower distribution range while IG-IDC has wider distribution range than other subtypes in Voronoi tessellation size, as shown in Fig. 5(D). IG-IDC and ILC have longer average and minor neighbor distances with narrower distribution ranges than other subtypes, as shown in Fig. 5(E) and 5(G). IG-IDC has longer major neighbor distance with a wider distribution range than other subtypes, as shown in Fig. 5(F). Figure 6 shows the global spatial distributions of four subtypes of breast cancer using PLSR analysis. Here, it can be visually seen that DCIS is mostly separated from IG-IDC and ILC but partially overlaps with HG-IDC, and ILC is well separated from other subtypes while IG-IDC and HG-IDC have partial overlapping.

The quantitative analytical results of differential diagnosis of breast cancer subtypes are listed in Table 1, while the classification overview is illustrated in Fig. 7. The accuracies of separating \textit{in situ} carcinoma from invasive carcinoma are shown in Table 1(A). While 100% of the \textit{in situ} carcinoma is correctly identified and 18% of the invasive carcinomas are erroneously classified as \textit{in situ} carcinoma, having an overall accuracy of 92%. This result could be visualized in the 3-D distribution of these cases in Fig. 6. Based on this result, a
A classification algorithm was developed to separate DCIS from IG-IDC and ILC, and the results are shown in Table 1(B). By this algorithm, 96% of DCIS and 95% of IG-IDC and ILC samples are correctly classified with an overall accuracy of 96%. The accuracies of separating IDC from ILC are shown in Table 1(C). They are 100% separated from each other, which is also illustrated in the 3-D visualization results in Fig. 6. As shown in Table 1(D), 80% of IG-IDC and 85% of HG-IDC were correctly separated with an overall accuracy of 83%.

Table 1. Classification accuracy of separating cancer subtypes from each other (Accuracy = (true positive + true negative) / total testing samples)

| A. Separating in situ carcinoma from invasive carcinoma | B. Separating DCIS from IG-IDC and ILC |
|--------------------------------------------------------|---------------------------------------|
| In situ       | Invasive | DCIS       | IG-IDC & ILC |
| 100%          | 0        | 96%        | 4%           |
| 18%           | 82%      | 5%         | 95%          |
| Accuracy      | 92%      | Accuracy   | 96%          |

| C. Separating ILC from IDC | D. Separating IG-IDC from HG-IDC |
|----------------------------|---------------------------------|
| ILC           | IDC    | IG-IDC    | HG-IDC |
| 100%          | 0      | 80%       | 15%    |
| 0             | 100%   | 20%       | 85%    |
| Accuracy      | 100%   | Accuracy  | 83%    |

4. Discussion

In this exploratory study, we have demonstrated the feasibility of using CARS microscopy to distinguish breast cancer from normal tissue and benign proliferative lesion, as well as different cancer subtypes. High quality ex vivo images were obtained for normal, benign (fibroadenoma), DCIS, IDC and ILC breast tissues by using a custom-built CARS microscope. Our results show that CARS microscopy is capable of characterizing breast tissue structures and cell types in a manner similar to H&E staining of conventional histological sections. On CARS images, normal breast tissues present predominantly adipose and fibrous structures, while fibroadenomas possess unique morphological features in accordance with pathological criteria. On the other hand, cancer tissues exhibit distinct cellular features with high cellularity. These disease-related features can be used to distinguish cancer lesions from normal and benign tissues. In addition, the cells of different cancer subtypes also present unique features, e.g., the cords and tubules for IG-IDC, the solid pattern for HG-IDC, and the single filing pattern for ILC. CARS microscopy was also shown to discriminate these cellular features to further separate cancer subtypes. A computerized platform was developed to
perform nuclear segmentation and classification of different types and subtypes of breast lesions. Our results showed a good distinction of cancer from normal tissues and benign lesions, as well as cancer subtypes. Compared to H&E analysis, however, our approach presents a much faster strategy and eliminates the need for sample processing and the use of exogenous contrast agents, thus significantly reducing diagnostic time. The separation of some breast lesions, such as atypical ductal hyperplasia and DCIS, is more subtle and will likely continue to require conventional histological analysis. Nonetheless, we have demonstrated that CARS imaging is reliable, sensitive and specific in discriminating between different subtypes of breast cancer, e.g., non-invasive *in situ* vs. invasive, different histological subtypes, e.g., DCIS vs. IG-IDC & ILC, and different histological grades of carcinoma, e.g., intermediate vs. high grade. Because they have direct impact on prognosis, choice of treatment modalities and monitoring response to therapy, such distinctions are critical.

The detailed reasons for further separating cancer subtypes from each other are as follows. 1) Separating *in situ* carcinoma from invasive carcinoma: *in situ* carcinomas have an excellent prognosis and are generally treated with lumpectomy and sometimes radiation, whereas invasive carcinomas have poorer prognosis and are generally treated with surgery (lumpectomy or mastectomy with or without lymph node removal), chemotherapy, and sometimes radiation. 2) Separating ILC from IDC: Rates of mastectomy compared to breast-conserving surgery in ILC are slightly higher than for IDC [45], and ILC is also not a good candidate for neoadjuvant chemotherapy because pathologic complete response-rates are much lower for ILC (3%) than for IDC (15%) [46]. Two large series with long follow-up observation [47,48] have revealed trends showing that the prognosis of ILC in the early years is somewhat better than the prognosis for IDC, while this trend is reversed in later years. That is, after about 6 years, relapse of ILC catches up with IDC. 3) Separating IG-IDC from HG-IDC: High-grade means that tumor cells are poorly differentiated in the Bloom-Richardson grading system, and poorly differentiated cancers have a worse prognosis. Patients with poor prognosis are usually offered more aggressive treatment, such as extensive mastectomy and one or more chemotherapy drugs, while patients with a good prognosis are usually offered less invasive treatments, such as lumpectomy and radiation or hormone therapy.

Sometimes identifying cancer subtypes is difficult using the CARS technique because the histological features may be difficult to observe based on the limited field of view in the CARS image. As an adjunctive diagnostic approach, the quantitative analysis of cancer cells facilitates more accurate identification of cancer subtypes. To enable the implementation of this quantitative approach, cell nucleus segmentation was performed, followed by extraction of seven pathology-related features with 5 evaluation indexes, a total of 35 features, to describe each image. The distributions of seven features for four subtypes of breast cancer are shown in Fig. 5, which indicates differences among subtypes. Moreover, the global spatial distributions of four subtypes using the 35-feature set under PLSR analysis are shown in Fig. 6, and the results show the robustness of the algorithms in separating cancer subtypes. Finally, a quantitative analysis of the differential diagnosis of cancer subtypes was conducted, and the results show high accuracies for the separation of cancer subtypes.

As a future study direction, 3D imaging and differential diagnosis of breast cancer using CARS microscopy is attractive. It can provide more information than 2D images, and allow tracking of features from different levels to identify 3D architecture and low contrast structures that are difficult to appreciate from single images [49]. The 3D imaging capability of the CARS technique makes this aim achievable based on its nonlinear nature. Nonetheless, prospective studies with a larger sample size are necessary for subtypes of cancer (DCIS in particular) to further evaluate the efficacy of our method. Current study is still limited by the number of samples and might experience a larger bias.
5. Conclusion

We demonstrated that, for the first time, the feasibility of integrating label-free CARS microscopy and quantitative data analysis to classify breast cancer from normal tissue and benign proliferative lesion, as well as further separate cancer subtypes. This study suggests that quantitative CARS microscopy has the potential to be used as a routine examination tool to rapidly identify breast cancer ex vivo. For future studies, the label-free and fast imaging properties of CARS could propel this technique to become a non-invasive approach for in vivo and real-time diagnosis of breast cancer without the need for histological staining or administration of exogenous contrast agents. Although conventional histological analysis would remain the gold standard and would remain necessary for difficult cases requiring the analysis of subtle pathologic features or immunohistochemistry markers, the fact that CARS seems to be able to delineate major diagnostic entities shows its promise to greatly increase the amount of information timely available to patients and physicians during biopsy or excision procedures.

Acknowledgments

The funding of this research was initiated and supported by the Department of Systems Medicine and Bioengineering of The Methodist Hospital Research Institute, Weill Cornell Medical College and John S Dunn Research Foundation to STCW. The authors would like to acknowledge David Bernard, MD, and Pam McShane from the tissue bank of The Methodist Hospital for their assistance.