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Abstract. Fourier transform infrared imaging (FTIRI) combined with chemometrics algorithm has strong potential to obtain complex chemical information from biology tissues. FTIRI and partial least squares-discriminant analysis (PLS-DA) were used to differentiate healthy and osteoarthritic (OA) cartilages for the first time. A PLS model was built on the calibration matrix of spectra that was randomly selected from the FTIRI spectral datasets of healthy and lesioned cartilage. Leave-one-out cross-validation was performed in the PLS model, and the fitting coefficient between actual and predicted categorical values of the calibration matrix reached 0.95. In the calibration and prediction matrices, the successful identifying percentages of healthy and lesioned cartilage spectra were 100% and 90.24%, respectively. These results demonstrated that FTIRI combined with PLS-DA could provide a promising approach for the categorical identification of healthy and OA cartilage specimens. [DOI: 10.1117/1.JBO.20.6.060501]

Keywords: Fourier transform infrared imaging; articular cartilage; osteoarthritis; partial least squares-discriminant analysis.

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1 Introduction

Collagen and proteoglycan (PG), two main macromolecules in articular cartilage, play important roles in maintaining cartilage's biophysical and mechanical properties. A disruption of the collagen network or a reduction in PG content would lead to cartilage diseases, mainly marked by the functional incapacitation of articular cartilage accompanied by joint pain and loss of mobility for the patients.[1] Articular cartilage can be conceptually subdivided into three structural zones from the surface to subchondral bone superficial zone (SZ), transitional zone (TZ), and radial zone (RZ).[2,3] Both collagen and PG are inhomogeneously distributed in these zones.[4] In addition, the PG content, especially in SZ,[5,6] shows clear differences between healthy and osteoarthritic (OA) cartilages.

Fourier transform infrared imaging (FTIRI), which is a newly developed spectroscopic imaging technique with high resolution and sensitivity, can quantify the collagen and PG contents in articular cartilage. FTIRI provides the possibilities to study the concentration variations of collagen and PG with microscopic resolution. Most FTIRI studies in the literature are limited to the qualitative analysis,[7] which is due to the significant overlapping among the IR characteristic bands of the principal macromolecules. Recently, the absorption bands of the collagen and PG in cartilage were analyzed by principal component regression (PCR), which has produced the distribution profiles of both principal macromolecules.[4]

Partial least squares (PLS) regression method combines the features of principal component analysis and multiple regression algorithm,[8] which uses the entire range of the spectrum rather than the individual peak absorption bands. PLS-discriminant analysis (PLS-DA), a classification method based on PLS, possesses a robust and objective ability in classification since it extracts information from both the predictor matrix (spectral matrix) and response matrix (categorical matrix).[9] By replacing the concentration matrix in PLS with a categorical matrix in PLS-DA, the PLS-DA approach has been successfully used for discriminating analysis in biomedical fields.[10,11]

Following our recent work that successfully quantified the percentage concentrations of collagen and PG in both healthy and OA cartilages by FTIRI combined with PCR[4] or PLS,[12] the main goal in this research was to classify the healthy and OA cartilages. This would be the first report that the hyphenated methods of FTIRI with PLS-DA were employed to differentiate healthy and OA cartilage samples.

2 Materials and Methods

2.1 *Sample Preparation*

The articular cartilages were obtained from 10 mature dogs (five healthy and five OA) that had similar individual features both in weight and age. The five dogs for OA preparation were scarified 2 year after the anterior cruciate ligament (ACL) transection in one knee, which was approved by the Institutional Review Committees.

Each cartilage was cut into several consecutive blocks with a size of 2 mm × 2 mm × 2 mm. After being washed in the saline and quickly frozen by liquid nitrogen, these blocks from the same locations of the animals were sectioned by a Cryostat (Leica CM 1950, Germany) into 10-μm sections in thickness at −20°C, and then picked up on MirrIR slides (Kevley Technologies, Chesterland, Ohio). These sectioned specimens were dried in the air for 2 h before FTIRI experiments to minimize the effects of water absorption on FTIRI experiments.

2.2 *FTIRI Experiment*

FTIRI experiments were carried out by using a PerkinElmer Spotlight-300 infrared imaging system, which includes an FTIR spectrometer and an infrared microscopy. The cartilage sections placed on the MirrIR slides were mounted on the scanning stage

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of the FTIRI system. Infrared imaging data of the cartilage sections were collected by an mercury cadmium telluride array detector at 6.25-μm pixel size and an 8-cm^{-1} wavelength spacing over a range of 4000 to 744 cm^{-1}. The visible images were obtained by visible imaging mode and showed a region of interest for FTIRI.[4] Background spectra were also collected for the correction of the imaging data in the same spectral range.

2.3 *Partial Least Squares-Discriminant Analysis*

PLS-DA was carried out in Unscrambler X software (CAMO SOFTWARE INC., New Jersey), which is a multivariate data analysis software. In PLS-DA, the PLS regression model is built to relate the infrared spectral matrix to the categorical matrix. Since this categorical matrix is actually a category vector depending on the spectral matrix, the numbers of 0, 1, and a cut-off value of 0.5 were used for designating the classes of the cartilages.[11,13] If the prediction value is higher than 0.5, the sample is assigned to class 1 (healthy cartilage). In contrast, it is assigned to class 0 (OA cartilage) if the prediction value is lower than 0.5. Classification of an unknown sample would depend on the prediction category values of the PLS-DA model.

IR spectra were extracted from FTIR images of all cartilage sections (five healthy and five OA). Each extracted spectrum was the averaged IR spectrum of the corresponding selected areas in the FTIR images, which is consistent with our previous results.[4,7] The data were obtained from a 0- to 100-μm depth under the surface with 10-μm intervals. A total of 100 spectra were obtained from all specimens and numbered from 1 to 100. Eighteen spectra (10 from healthy and eight from OA) were randomly selected from the 100 spectra to constitute the calibration matrix of PLS model that includes at least one spectrum for each sample. The residual spectra (40 from healthy and 42 from OA) were prepared for the prediction as the prediction matrix.

Spectral preprocessing, including baseline offset, normalization, and multiplicative scatter correction, was performed for the spectral matrix to improve the reliability and stability of the discriminant model. Leave-one-out cross-validation was performed in the PLS model. The predictive ability of this model can be tested through the fitting relation between the predicted and actual values.[8] The fitting coefficient (Pearson's r) of 0.95 indicates an excellent linear fitting relationship of spectral and categorical matrices and a good representative of this PLS model. One-way analysis of variance (ANOVA) test was performed for the PLS-DA calculated category values by using the software of Origin 8.0 (OriginLab Corp., Massachusetts)

3 Results and Discussion

Figure 1 shows the visible and total absorption IR images of healthy and an OA cartilage sections, respectively. Some chondrocytes are interspersed in cartilage sections and shown as irregular circles or shapes in the visible images; the quantity of the cells becomes less in SZ, TZ, even the upper part of RZ for OA cartilage [Fig. 1(b)]. In addition, the cells in the subchondral areas of OA cartilage were bigger than these in healthy cartilage [Fig. 1(a)].[14] When comparing the total absorption images of healthy and OA cartilages, the absorption intensity was significantly reduced by 10% in SZ and TZ of OA cartilage (100-μm depth beneath the cartilage surface). The main differences between OA and healthy cartilages, therefore, appear to be in SZ and TZ.[5] In the processing of the PLS modeling, IR spectra would be extracted from SZ and TZ. The PLS-DA calculated results are shown in Fig. 2.

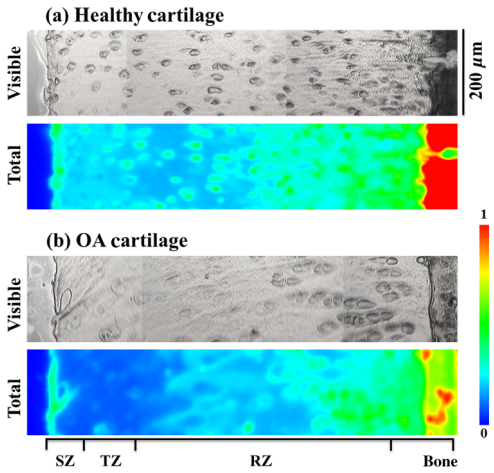

Fig. 1 Visible and total absorption infrared images of (a) a healthy and (b) an osteoarthritic (OA) whole-thickness cartilage from the surface to subchondral bone (size = 900 μm × 160 μm). The absorption limits of healthy and OA cartilages were 0 to 1. The color bar shows the relatively absorbance intensities of Fourier transform infrared images.

In Fig. 2, spectra #1 to 50 come from the healthy cartilage, which are shown as the rectangular points in this plot; spectra #51 to 100 originate from the OA cartilage, shown as the round points. There are 100 calculated values in the plot, including the calibration and prediction matrices. Prediction accuracies of the calibration and prediction matrices by the PLS-DA model are 100% and 90.24%, respectively.

The discriminant accuracy of healthy cartilages was 100%, which means that no healthy cartilages spectra were misjudged by this model. By comparison, there were eight misjudged spectra in the prediction matrix from the OA cartilage. The predicted category values of these eight misjudged OA spectra were over the cut-off value of 0.5, which were shown as the solid circles above the dashed line in Fig. 2. According to the spectral number in the prediction matrix, these eight misjudged spectra appear in the surface part of OA cartilage (0 to 20 and 80 to 100 μm positions).

In addition, one-way ANOVA tests were carried out for the cartilage sample. The statistical analyses based on the calculated

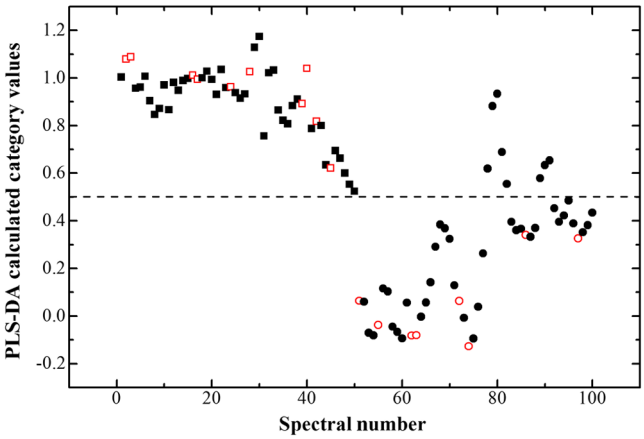

Fig. 2 Partial least squares-discriminant analysis (PLS-DA) calculated category values of the spectral matrix, including 100 spectra sequenced from 1 to 100. Healthy (open square) and OA (open circle) cartilages spectra of calibration matrix were predicted in cross-validation; healthy (filled square) and OA (filled circle) cartilages spectra of prediction matrix were predicted by PLS-DA.

values from PLS-DA showed that differences among the healthy cartilage were unremarkable but became clear for the OA cartilage. There were also significant differences between the healthy and OA cartilage groups, which confirms the significant decrease in the PG content in OA cartilage when compared with the healthy cartilage.[5]

The procedure of ACL transection is known to induce a mechanically instability in knee and change in the joint reactive force, which causes cartilage damage.[5] Although all OA cartilages were obtained from the 2-year ACL transection knee joints, differences among the individual specimens might contribute to the misjudgment. For example, the OA degree of some misjudged OA samples might be weaker than those of other OA samples, where the OA degeneration did not developed deeper to TZ for these sections. PG loss could consequently become unremarkable in this area.[5] In addition, the SZ of some individual OA samples may be seriously damaged, which causes difficulties in defining the actual surface of the OA cartilage section in imaging. Since the PG loss in RZ occurs later in the degradation process, an inaccurate identification of the surface location may cause the last 2 to 3 spectra to be extracted from deeper locations than 80 to 100 μm,[4,5] which results in misjudging in the spectra from the 80- to 100-μm location.

For the surface misjudgment (the 0- to 20-μm region), the peripheral regions of cartilage sections may curl up slightly during the air-drying process. In addition, the changes of chondrocyte in the OA cartilages[15] might cause stronger diffusion reflections and scattering effects in SZ than those in the intermediate layer. These practical factors might contribute to the misjudged cases in the surface position for OA cartilage.

4 Conclusions

FTIRI combined with PLS-DA was used successfully to classify healthy and OA cartilages, with accuracies of 100% and 90.24% for calibration and prediction matrices, respectively, which closely approached the Fisher's discrimination results obtained in our lab (unpublished). To the best of our knowledge, this hyphenated technique of FTIRI and chemometrics discriminance had not yet been applied in research of articular cartilage and OA. This approach could become a promising classification-discriminant technique for discriminating between healthy and OA specimens, though it is currently limited in section research in lab.

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