Native fluorescence spectroscopy reveals spectral differences among prostate cancer cell lines with different risk levels

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Abstract. The spectral changes of native fluorophores among normal fibroblasts and cancer cell lines of different metastatic ability are investigated by fluorescence spectroscopy. The normal (fibroblast), moderately metastatic (DU-145), and advanced metastatic (PC-3) cell lines were each selectively excited at 300 nm, and their fluorescence emission spectra are analyzed using principal component analysis to explore the differences of the relative contents of tryptophan and reduced nicotinamide adenine dinucleotide in these cell lines. The results show that the tryptophan emission featured predominantly in the fluorescence spectra of the advanced metastatic cancer cells in comparison with the moderately metastatic cancer and normal cells. © 2013 Society of Photo-Optical Instrumentation Engineers (SPIE) [DOI: 10.1117/1.JBO.18.8.087002]

Keywords: normal, moderately metastatic and advanced metastatic cell lines; native fluorescence spectra; optical biopsy; metastasis-relevant key fluorophores; tryptophan; NADH.

Paper 130178RR received Mar. 27, 2013; revised manuscript received Jun. 27, 2013; accepted for publication Jul. 2, 2013; published online Aug. 2, 2013.

1 Introduction

Native fluorescence (NFL) spectroscopy for cancer detection is a promising technique proposed as “Optical Biopsy” by Alfano in the mid-1980s. Researchers can detect the fingerprints of intrinsic fluorophores or extrinsic target contrast agents, which may be valuable for cancer detection in vivo. The key intrinsic fluorophores include tryptophan, collagen, elastin, reduced nicotinamide adenine dinucleotide (NADH), porphyrins, and flavin adenine dinucleotide (FAD). The major intrinsic diagnostic molecules in cells absorb and emit in the ultraviolet to visible spectral range. For example, NADH and FAD are involved in the oxidation of metabolic molecules; therefore, direct monitoring of their fluorescence dynamically can be used to report the metabolic activity of cells. Combined with blind source separation methods, this technique has been used to investigate the intrinsic tissue fluorescence spectral alterations due to the induction of morphological and molecular changes during disease development or chemotherapy. These key fluorophores within cells and tissues may serve as biomarkers to study the intrinsic differences between normal and cancerous tissues.

In this paper, NFL spectroscopy was shown to be an effective approach to distinguish two prostate cancer cell lines with different metastatic ability, based on the changes in their fluorophore compositions. The measured NFL spectra of a normal fibroblast cell line, a moderately metastatic (DU-145), and an advanced metastatic (PC-3) prostate cancer cell line were analyzed using principal component analysis (PCA). The experimental and the analyzed results indicate that NFL spectroscopy can be used to monitor the changes of the key fluorophores of different cell lines with different risk levels. The method introduced here may form the basis of a new method to augment the information content in cancer staging.

2 Material and Methods

The cell lines used in this study are normal human fibroblast cells (Coriell Cell Technologies, Camden, New Jersey, USA), moderate metastatic prostate cells (DU-145), and advanced metastatic prostate cells (PC-3). The two metastatic prostate cell lines to be investigated were purchased from the American Type Culture Collection (ATCC) in Manassas, Virginia, and stored in 95% air/5% CO2 at 37°C. The cell lines were further sub-cultured to increase the cell population. The normal cells, fibroblast, were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM: Life Technologies, Carlsbad, California, USA) with 10% fetal bovine serum (FBS; Gibco BRL, Cleveland, Ohio), 5% Penicillin Streptomycin, and 2 mM L-glutamine. The moderate metastatic prostate DU-145 cells were cultured in Minimum Essential Medium Eagle (MEM; Sigma Chemical Co., St. Louis, Missouri, USA), with 10% FBS, 2 mM L-glutamine, 1.5 g/L Na-bicarbonate, 4.5 g/L glucose, 0.1 mM NEAA, and 1.0 mM Na-pyruvate. The advanced metastatic PC-3 cell line was cultured in Ham’s F-12K medium (Life Technologies, Carlsbad, California, USA),...
supplemented with 20% FBS, 1.5 mg/ml NaHCO3, 1.5 g/L sodium and 2 mM L-glutamine. All cell lines were incubated in a humidified atmosphere of 4% CO2 at 37°C. At above 95% cell confluence (~5 days for PC-3 cells to 20 days for fibroblasts depending on their population doubling times), the cells were harvested by the treatment with Trypsin-EDTA (5% Trypsin and 5.3 × 10−6 M EDTA: Gibco BRL, Cleveland, Ohio) for 5 to 10 min, diluted in the medium, then isolated by centrifugation. The cells were re-suspended and washed once in phosphate buffered saline (PBS, Sigma-Aldrich, St. Louis, Missouri). After centrifuging and re-suspending the cells at ~9 × 10⁶ cells/ml PBS, the cells were transferred to a 1 × 1 × 4 cm³ (the inside dimensions) quartz cuvette (NSG Precision Cells, Inc. Farmingdale, New York) for the fluorescence experiments. The cell suspensions were vortexed evenly before each measurement was taken.

The NFL spectra of the cells were measured using the Fluorolog®-3 spectrofluorometer system. The excitation light with 2-nm spectral width was focused on samples with a spatial size of ~3 × 1 mm² and 0.5 μW power deposition. Each scan was ~1 min at a scan speed of 300 nm/min. The fluorescence was collected with a spectral resolution of ~1 nm. To assess possible background fluorescence due to cell sample preparation, the emission spectra of the quartz cuvette, Trypsin-EDTA and PBS were measured. The 300 nm excitation wavelength was selected for a number of reasons: (1) it excites the key native biomarkers in cells. Based on our previous knowledge of the spectra of the key fluorophores, the spectral changes during tumor evolution, and spectral changes from chemotherapy of malignant breast cells can be detected with 300 nm excitation. (2) Spectral measurements of the quartz cuvette, Trypsin-EDTA and PBS did not show measurable background fluorescence under 300 nm excitation. (3) Among the key building block molecules, tryptophan has a higher quantum yield (QY) of 0.2 to 0.35 when excited at ~280 nm depending on the solvents rather than the other fluorophores, such as NADH (QY of 0.019 to 0.05) and flavin (QY of 0.03). As cells and tissues contain several other fluorophores of interest (e.g., NADH and flavins) whose emission fingerprints may be buried by the strong signal from tryptophan at 280 nm excitation, the cell suspensions were excited at 300 nm to enhance the detection of other fluorophores over tryptophan. These measurements were particularly useful in exploiting differences among cancer cell lines with different metastatic ability due to the changes of their fluorophores compositions using the PCA method.

It is important to minimize photo-toxicity and prevent mutagenicity in patients by the excitation wavelength. Therefore, the exposure time and dose of the 300 nm excitation light should be carefully controlled. There are two most commonly used safety standards for photo-toxicity are the threshold limit value (TLV) and minimum erythermal dosage (MED). The TLV for exposure at 300 nm is 10 mJ/cm² (Ref. 16), and for MED, a commonly used index for skin damage due to light exposure between 295 and 300 nm, is 20 mJ/cm² (Ref. 17). Given the spot size (A = 3 mm × 1 mm), duration time (t = 1 min) and power of light (P = 0.5 μW) used in our experiment, the energy density of the incident light was calculated as

\[
F = \frac{P \cdot t}{A} = \frac{0.5 \times 60}{0.3 \times 0.1} = 100 \ (μJ/cm²) = 0.1 \ (μJ/cm²),
\]

which is two orders of magnitude less than the two commonly used safety standards. This indicates that the NFL spectra with the selected excitation wavelength of 300 nm may be safe for human use and particularly for in vivo prostate cancer staging.

3 Results and Discussions

The NFL spectra of 16 different fibroblast cell samples as well as 17 different DU-145 and PC-3 cell samples were each measured with 300 nm excitation. The average fluorescence spectral profiles of PC-3, DU-145 and fibroblast cells are shown in Fig. 1(a) as solid, dashed, and dotted lines, respectively.

The main emission peaks of all three-cell lines were found at ~340 nm, which is attributed to the emission peak of tryptophan. Another shoulder peak at ~465 nm was observed for all of the three cell lines, known as the characteristic NADH fluorescence peak. The major difference of the profiles between the advanced metastatic cancer cell line, PC-3, and the nonadvanced metastatic cell lines, DU-145, and normal fibroblasts is that the fluorescence intensity of PC-3 at 340 nm was significantly higher than for the other two cell types [Fig. 1(a)]. In order to illustrate the salient difference between the spectral profiles of DU-145 cells and the fibroblasts, their emission spectral profiles are shown in Fig. 1(b). It is noticeable as the reversal of the peak intensities of ~I₃₄₀ and ~I₄₆₅, i.e., ID > IF at ~340 nm but IF < ID at ~465 nm, where ID and IF is the emission intensity of DU-145 and fibroblast cell line, respectively. The emission spectral differences may reflect the change of key fluorophores in cells with different metastatic ability.

![Image](https://example.com/image.png)  
Fig. 1 NFL spectra of (a) all three cell lines to be investigated, e.g., PC-3 (solid), DU-145 (dash) and fibroblast (dot); and (b) the comparison between DU-145 (dash) and fibroblast (dot). The emission spectra of all three cell lines are obtained using the excitation of 300 nm with standard deviation error bars at key wavelengths.
risk levels. In order to understand these changes, PCA and linear discriminant analysis (LDA) were employed to analyze the NFL spectra of cell lines to unravel the key bio-molecular components.

The extracted variances of the first two principal components (PCs) obtained using the PCA and the NFL spectra of the three cell lines are shown as solid lines in Fig. 2(a). The emission spectra of tryptophan and NADH were measured individually with the excitation of 300 nm, and the results are shown as dashed lines in Fig. 2(a). A comparison of the extracted variances of the PCs in the cell samples with the actual fluorescence spectra of tryptophan and NADH shows good agreement of the first PC (1st PC) to the tryptophan profiles and the second PC (2nd PC) to the NADH profile, indicative of these two biomolecules being the major contributors to the two significant PCs. To quantify the relation between the fluorophores and the corresponding PCs, the correlation coefficients of NFL spectral features of the fluorescent fluorophores and the variances of the corresponding PCs were calculated and given in Table 1.

It can be seen from Table 1 that the correlation coefficient of tryptophan and the 1st PC is 0.918, indicating a strong linear relationship between the spectral features of tryptophan and the variance of the 1st PC. The correlation coefficient of NADH and the 2nd PC is 0.479, indicating a moderate linear relationship between spectral features of NADH and the variance of the 2nd PC, and indicating overlapping contributions from other fluorophores\(^8\) albeit not resulting in significant separate PCs. Figure 2(a) and Table 1 demonstrate that NFL spectral features of PCs (e.g., peaks, troughs, and spectral shapes) are similar to those of fluorophores that contributed to the NFL spectra of cells. The two PCs derived from PCA analysis and assigned to tryptophan and NADH by comparison account for the major spectroscopic feature changes caused by the variance of these key fluorophores. Assuming that a cell consists of a number of fluorophores/PCs of interest involved in cancer development, the spectrum of a cell can be considered as a linear combination of the spectra of all fluorophores.\(^9,18\) This is based on two assumptions: (1) The spectrum of the \(k\)th fluorophore can be represented by an \(N\) dimensional vector \(c_k\), where the dimension \(N\) is indexed by wavelength; and (2) the vector \(c_k\) is unrelated or nearly unrelated with each other for all \(K\) fluorophores without loss of generality, where \(k = 1, 2, \ldots, K\).

Therefore, the NFL spectrum of cells for one measurement can be written as

\[
s = \sum_{k=1}^{K} a_k c_k + n = C a + n, \tag{1}
\]

where \(C = (c_1, c_2, \ldots, c_k)\) is the matrix of native spectra of the key fluorophores and \(a = (a_1, a_2, \ldots, a_k)^T\). Here, \(a_k\) is a constant proportional to the quantity of the \(k\)th fluorophore in the cell and \(n\) is the noise.

The NFL spectra collected from the three cell models using 300 nm excitation and analyzed by PCA showed that there were two distinct significant PCs, the 1st PC—tryptophan versus the 2nd PC—NADH, contributing to their emission spectra. Thus, Eq. (1) can be reduced to \(s = a_1 c_1 + a_2 c_2 + n\). The relative contribution from the 1st PC (tryptophan) and the 2nd PC (NADH) as reflected by their constant coefficients, \(a_1\) and \(a_2\), can be calculated using PCA. The calculated results of constant coefficients of the 1st PC—tryptophan and the 2nd PC—NADH are shown in Fig. 2(b) as scatter plots for each cell sample (50 independent experiments in total; 16, 17, and 17, for fibroblasts, DU-145, and PC-3 cells, respectively). It can be seen that the scatters corresponding to the normal (circle, fibroblasts), moderate metastatic cancer (hexagon, DU-145) and the advanced metastatic cancer (square, PC-3) cells are distributed in different regions. The data for the PC-3 cells are located to the right of the data for the normal fibroblasts as well as the DU-145 cells. This indicates that the relative fluorescence contribution of tryptophan is higher in PC-3 cells than in the other cell types. In addition, DU-145 cells and normal fibroblasts are more strongly separated along the 2nd PC, indicating a significant variation in NADH fluorescence. These profiles may be used as a potential criterion to distinguish cancerous cells from the normal, and separate advanced metastatic cancer cells from less aggressive cancer cells using LDA method. The significant PCs using the

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**Table 1** Correlation coefficients of NFL spectra of fluorophores and variances of the corresponding PCs.

<table>
<thead>
<tr>
<th>Fluorophores</th>
<th>1st PC</th>
<th>2nd PC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptophan</td>
<td>0.918</td>
<td>–</td>
</tr>
<tr>
<td>NADH</td>
<td>–</td>
<td>0.479</td>
</tr>
</tbody>
</table>

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![Fig. 2](https://www.spiedigitallibrary.org/journals/Journal-of-Biomedical-Optics)
1st PC and the 2nd PC for classification of NFL spectra between normal and cancerous cell lines are illustrated in Fig. 2(b). The dashed line in Fig. 2(b) was generated by LDA of the two groups formed by the normal cells (circle) and cancer cells (square and hexagon, respectively). This separating line was used to create a criterion for categorizing the true or false positive and negative results in our NFL spectral study. Subsequently, LDA was applied to obtain maximum separation between advanced metastatic cancer cell (square) and the other two cell models (circle and hexagon), resulting in the solid line displayed in Fig. 2(b).

The accuracy for the classification of cell lines using significant PCs of the 1st PC and the 2nd PC can be evaluated by the criteria generated by the LDA separating lines. The calculated sensitivity and specificity of this analysis procedure are summarized in Table 2.

The performance of a two-group classification is typically evaluated by a receiver operator characteristic (ROC) curve, which is a graphical plot of true positive rate versus false positive rate. Accuracy of the performance is measured by the area under the ROC curve (AUC). The ROC curves shown in Fig. 3(a) and 3(b) for cancer versus healthy cell lines, and advanced metastatic versus moderate metastatic cell lines, respectively, were generated from the scatter plot of data sets of normal cells (circle for fibroblasts) and cancerous cells (squares for PC-3 and hexagons for DU-145) shown in Fig. 2(b). The AUC values calculated from the ROC curves shown in Fig. 3(a) and 3(b) are 0.95 and 0.97, respectively, demonstrating the excellent efficacy of using NFL spectra and the PCA combined with LDA as a promising classification tool for distinguishing different cancer cell lines with different risk levels as well as healthy normal cells.

It is recognized that the variances of PCs recovered by the PC analyses comprise the naïve fluorescence spectra of the cells and account for the most spectral power/strength.\textsuperscript{15} Table 1 shows that the recovered variances of PCs are related to the spectra of two key fluorophores, tryptophan, and NADH. Subsequent LDA demonstrated excellent separation between normal fibroblasts, the moderately aggressive cancer cells (DU-145) and the metastatic prostate cancer cell (PC-3).

The analyzed results help us capture the interaction of light with biological cells, and therefore understand the relationships between measured NFL signals and underlying the changes of biology or morphology of cells with different metastatic ability. The variances of the 1st PC and the 2nd PC exhibited by the solid lines in Fig. 2(a) actually reflect the NFL spectra of the key fluorophores, e.g., tryptophan and NADH, respectively. The scatter plot of Fig. 2(b) is $a_{1}$ versus $a_{2}$ that are the constants proportional to the contribution of fluorescence of tryptophan and NADH to the cell fluorescence, respectively, obtained from all 50 ($16 + 17 + 17$) independent experimental runs for the three cell lines. It can be seen from Fig. 2(b) that the most cancerous data sets of the 1st PC are located in the right side of the normal data sets of the 1st PC, indicating that the relative fluorescence contribution of tryptophan to cancer cells is higher than that of the normal cells. Furthermore, the advanced metastatic cancer data of the 1st PC are located in the right side of the nonaggressive cell data for the 1st PC, demonstrating that higher fluorescence contribution of tryptophan was observed in the advanced metastatic cancer cell line (PC-3) than in the fibroblast and DU-145 cell lines.

Identifying the metastatic potential of cancer is critical in cancer detection and staging. In the fiscal year 2012, distinguishing aggressive from indolent prostate cancer was one of two overarching challenges for Prostate Cancer Research Program (PCRP) of Congressionally Directed Medical Research Programs (CDMRP) from Department of Defense (DoD).\textsuperscript{19} This study is highly relevant and serves as a key step to predict the metastatic potential of prostate cancer using NFL. This research will advance the understanding of using NFL to identify metastatic prostate cancer even using intact tissue specimens. Further analysis could uncover metastasis-related key fluorophores that may be used as native biomarkers to predict metastatic prostate cancer in their early stages. Based on this study, a new optical technique may be developed for classifying and determining tumor stages.

**Table 2** Sensitivity and specificity of NFL spectral difference among cell lines evaluated by PCA and LDA analysis.

<table>
<thead>
<tr>
<th>Evaluated components</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cancer versus normal</td>
<td>88</td>
<td>100</td>
</tr>
<tr>
<td>Advanced versus nonadvanced</td>
<td>82</td>
<td>97</td>
</tr>
</tbody>
</table>

Fig. 3 Accuracy evaluated by ROC curve using the 1st PC versus the 2nd PC, for distinguishing significant PCs to classify cell lines into two groups for (a) normal cells (fibroblast) versus cancerous cells (PC-3 and DU-145); and (b) nonadvanced cells (fibroblast and DU-145) and advanced metastatic cancer cells (PC-3).
4 Conclusion

To the best of our knowledge, this is the first native spectral study to distinguish different cell lines with different risk levels using the NFL spectra of cells analyzed by PCA. By comparing the PCA-recovered variances of PCs with the spectra of key fluorophores known to be present in cells/tissues, the emission spectral features among different cell lines underscore the potential role of intrinsic proteins and fluorophores in cancer development. The PCA method can provide useful information for classification of cells with different risk levels while significantly reducing sample dimensionality. In addition, the LDA was employed to create a criterion for separating the normal cells, moderate metastatic cancer cells, and the advanced metastatic cancer cells. This research indicates that NFL spectroscopy may be used as a “fingerprint” or criterion for monitoring the different grades of risk level of prostate cancer.

Acknowledgments

This research is supported in part by U.S. Army Medical Research and Material Command grants numbered W81XWH-11-1-0335 and W81XWH-08-1-0717, and the National Institutes of Health (NIBIB R01 EB008111 and NCI R01 CA171651). Yang Pu thanks deeply Ms. Brenda Phelps at the administration of the Optical Radiology Lab at Washington University School of Medicine for her help and support during his working on this project at Washington University. Yang Pu also acknowledges Ms. Natalia Kruchevsky at Memorial Sloan Kettering Cancer Center (MSKCC) for her help in cell culture techniques.

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