Detecting cervical cancer progression through extracted intrinsic fluorescence and principal component analysis

Seema Devi
Prasanta K. Panigrahi
Asima Pradhan
Detecting cervical cancer progression through extracted intrinsic fluorescence and principal component analysis

Seema Devi,a Prasanta K. Panigrahi,b and Asima Pradhan,c,a,*

In Indian Institute of Technology, Department of Physics, Kanpur 208016, India
bIndian Institute of Science Education and Research (IISER), Kolkata 741246, India
cIndian Institute of Technology, Center for Lasers and Photonics, Kanpur 208016, India

Abstract. Intrinsic fluorescence spectra of the human normal, cervical intraepithelial neoplasia 1 (CIN1), CIN2, and cervical cancer tissue have been extracted by effectively combining the measured polarized fluorescence and polarized elastic scattering spectra. The efficacy of principal component analysis (PCA) to disentangle the collective behavior from smaller correlated clusters in a dimensionally reduced space in conjunction with the intrinsic fluorescence is examined. This combination unambiguously reveals the biochemical changes occurring with the progression of the disease. The differing activities of the dominant fluorophores, collagen, nicotinamide adenine dinucleotide (NADH), flavin adenine dinucleotide (FAD), and its derivatives and collagen.2,3 Both NADH and FAD play a major role in the metabolic activities of the cell, whereas structural changes in the tissue are produced by changes of the collagen.4,5 The ratio of NAD+ and NADH is known to change significantly in diseased tissue. NADH is bound to proteins in normal tissues as compared to the unbound one in tumors, exhibiting different behaviors as a result.6,7 The collagen network, which forms the supporting backbone in the stroma, systematically breaks down with the progress of the disease.8,9 These changes result in differing emission properties of the tissue in normal and diseased conditions and have been captured through fluorescence spectroscopy and imaging.10,11 However, absorption by blood and scattering from cells and nuclei in tissue result in masking the weak fluorophore activities, making it difficult to observe these changes. Hence, isolating these environmental effects will undoubtedly help in identifying the differing optical activities, particularly in the early stage of cancer where these changes are expected to be small.

Various techniques have been used to correct for the attenuation due to scatterers and absorbers from the measured fluorescence of the tissue samples. Schemes developed with judicious combinations of measurements like an intensity ratio of the fluorescence to scattered light,12 ratios of diffuse reflectance,13,14 and ratios of autofluorescence15,16 at different wavelengths are used to extract intrinsic fluorescence. However, their accuracy in correcting for the attenuation is limited since the wavelength dependence of optical parameters is not taken into account.1 In this direction, several groups have developed transfer functions in terms of optical transport parameters of tissue17 using Kubelka-Munk theory,18 diffusion theory19,20 photon migration theory,21,22 and Monte Carlo simulations.23 Most of these techniques apply only to a limited wavelength range (500 to 800 nm), where the absorption coefficient ($\mu_a$) is less dominant than the reduced scattering coefficient ($\mu'_s$). The photon migration theory covers the entire bio-optimally relevant wavelength region, which includes UV ($\mu_a \geq \mu'_s$), visible, and near-infrared ($\mu_a \ll \mu'_s$), however, prior knowledge of the optical parameters of the targeted samples are necessary.23,24 Liu et al.27 reported the development of a compact point-detection fluorescence spectroscopy system and an empirical ratiometric method to quantify the intrinsic fluorescence redox ratio and hemoglobin concentrations in in vitro orthotopic brain tumor rat model. We make use of a novel approach, developed earlier by our group, in effectively combining the measured polarized fluorescence with the polarized elastic scattering to remove the effects of absorption and diffuse scattering.28,29 The main advantage of this correction technique is the recovery of both line shape and intensity information in a turbid medium with reasonable accuracy for the range of optical transport parameters,28 where the ratio of $\mu_a / \mu'_s$ lies between 0 and 1.8.

1 Introduction

Early detection of cancer can significantly improve chances of patient survival. Conventional techniques are often not sensitive enough to detect subtle changes that occur in the early stages of disease. Optical methods, however, are known to be sensitive to biochemical and morphological changes that occur during the progress of the disease. Biochemical changes occurring at a cellular level originate from biomolecules, e.g., nicotinamide adenine dinucleotide (NADH), flavin adenine dinucleotide (FAD), and its derivatives and collagen.2,3 Both NADH and FAD play a major role in the metabolic activities of the cell, whereas structural changes in the tissue are produced by changes of the collagen.4,5 The ratio of NAD+ and NADH is known to change significantly in diseased tissue. NADH is bound to proteins in normal tissues as compared to the unbound one in tumors, exhibiting different behaviors as a result.6,7 The collagen network, which forms the supporting backbone in the stroma, systematically breaks down with the progress of the disease.8,9 These changes result in differing emission properties of the tissue in normal and diseased conditions and have been captured through fluorescence spectroscopy and imaging.10,11 However, absorption by blood and scattering from cells and nuclei in tissue result in masking the weak fluorophore activities, making it difficult to observe these changes. Hence, isolating these environmental effects will undoubtedly help in identifying the differing optical activities, particularly in the early stage of cancer where these changes are expected to be small.

Various techniques have been used to correct for the attenuation due to scatterers and absorbers from the measured fluorescence of the tissue samples. Schemes developed with judicious combinations of measurements like an intensity ratio of the fluorescence to scattered light,12 ratios of diffuse reflectance,13,14 and ratios of autofluorescence15,16 at different wavelengths are used to extract intrinsic fluorescence. However, their accuracy in correcting for the attenuation is limited since the wavelength dependence of optical parameters is not taken into account.1 In this direction, several groups have developed transfer functions in terms of optical transport parameters of tissue17 using Kubelka-Munk theory,18 diffusion theory19,20 photon migration theory,21,22 and Monte Carlo simulations.23 Most of these techniques apply only to a limited wavelength range (500 to 800 nm), where the absorption coefficient ($\mu_a$) is less dominant than the reduced scattering coefficient ($\mu'_s$). The photon migration theory covers the entire bio-optimally relevant wavelength region, which includes UV ($\mu_a \geq \mu'_s$), visible, and near-infrared ($\mu_a \ll \mu'_s$), however, prior knowledge of the optical parameters of the targeted samples are necessary.23,24 Liu et al.27 reported the development of a compact point-detection fluorescence spectroscopy system and an empirical ratiometric method to quantify the intrinsic fluorescence redox ratio and hemoglobin concentrations in in vitro orthotopic brain tumor rat model. We make use of a novel approach, developed earlier by our group, in effectively combining the measured polarized fluorescence with the polarized elastic scattering to remove the effects of absorption and diffuse scattering.28,29 The main advantage of this correction technique is the recovery of both line shape and intensity information in a turbid medium with reasonable accuracy for the range of optical transport parameters,28 where the ratio of $\mu_a / \mu'_s$ lies between 0 and 1.8.

*Address all correspondence to: Asima Pradhan, E-mail: asima@iitk.ac.in

Journal of Biomedical Optics 127003-1
December 2014 • Vol. 19(12)
Decoupling the effects of scatterers and absorbers improves diagnostic efficacy since subtle biochemical changes that take place during the progress of tumors are visible in the intrinsic fluorescence. To further highlight these subtle changes, a principal component analysis (PCA) is applied which disentangles the collective behavior from smaller correlated clusters in a dimensionally reduced space. The use of PCA with suitable classification functions has been found useful in differentiating various classes of human tissues. It is worth pointing out that Ramanujam et al. have developed a multivariate statistical algorithm for the screening and diagnosis of cervical precancers. To illustrate the general applicability of our approach, we focus here on cervical precancers. Studies on the detection of cervical tissue precancers have been pursued by several groups using different optical techniques and statistical methods, both in vivo and in vitro. In vivo measurements by Nordstrom et al. have shown that UV fluorescence provides a better discrimination of precancers from normal squamous cervical tissue than diffuse reflectance. A sensitivity of 86% and 87% specificity has been achieved by them in normal versus CIN1 and 91% sensitivity and 93% specificity for normal versus CIN2/3. Huh et al. have discriminated the high grades of CIN 2/3 with 90% sensitivity using in vivo data from both fluorescence and reflectance. A binary classification of different sets of cervical tissue is reported by Kortum et al. They have reported a better discrimination with combined reflectance and fluorescence measurements (83% sensitivity and 80% specificity) compared to exclusively using reflectance spectroscopy in in vivo studies of squamous normal and high grades squamous intraepithelial lesions (HGSIL) of cervical tissue. Isolating chronic inflammation from precancers is necessary for accurate detection. Roder et al. have discriminated chronic inflammation and low grades of squamous intraepithelial lesions from HGSIL with 89% sensitivity and 100% specificity using fluorescence spectroscopy. Other optical techniques like optical coherence tomography and Raman spectroscopy have also been utilized for cervical precancer detection. The absorption coefficient and reduced scattering coefficient obtained through in vivo diffuse reflectance spectroscopy measurements and Monte-Carlo simulations by Ramanujam et al. have shown differences among normal, CIN1, and CIN2 cervical tissue. Fluorescence from body fluids have also displayed a potential for early detection. Most of the groups mentioned above have also reported in vivo measurements on different sets of cervical tissue using PCA.

In this study, the merit of coupling experimentally extracted intrinsic fluorescence with PCA is clearly demonstrated in cervical precancers. Elimination of both diffuse scattering and absorption effects unambiguously reveals signatures of fluorophores in intrinsic fluorescence spectra as has been validated in our earlier work on tissue mimicking phantoms. The combined approach highlights subtle biochemical changes in the activities of NADH, flavins, collagen and porphyrin. Careful examination of the sectorial behavior of the dominant eigenvectors reveals the relative contributions of NADH, flavins, collagen, and porphyrin. To further classify, the Mahalanobis distance has been calculated using the scores of selected principal components.

2 Methods and Materials

2.1 Data Acquisition

A commercial spectrofluorometer (SPEX, Fluorolog 3, Model FL3-22), which uses a 450 W xenon lamp as the source, is used for acquisition of data from cervical tissues. The polarized fluorescence spectra of fresh cervical tissue specimens are recorded with excitation at 370 nm. All measurements are made at an angle of 22.5° with respect to the incident polarized light. The epithelial layer of the tissue is exposed to the incident light during the recording of the spectra. The scanning range of polarized fluorescence is 400 to 600 nm with an integration time of 0.1 s and a spectral resolution of 1 nm. In addition, corresponding polarized elastic scattering spectra are also recorded. Keeping the excitation polarizer vertical, copolarized (VV) and cross-polarized (VH) fluorescence and corresponding polarized elastic scattering are recorded with the emission polarizer in the vertical and horizontal positions, respectively.

2.2 Protocol Used to Extract Intrinsic Fluorescence

The intrinsic fluorescence is extracted by using the measured polarized fluorescence and polarized elastic scattering data, which remove the effects of diffuse scattering and absorption. The intrinsic fluorescence intensity (I_{int,fl}) is given by

\[ I_{int,fl} = \frac{\left[ I_{VV}(\lambda) - G(\lambda) \times I_{VH}(\lambda) \right]}{\left[ R \times \left( I_{VV}(\lambda) - G(\lambda) \times I_{VH}(\lambda) \right) \right]^{1-x}}. \]  

Here, subscripts fl and scat refer to the fluorescence and elastic scattering, respectively, with \( I_{VV} \) and \( I_{VH} \) representing the intensities of co- and cross-polarized channels. \( G(=I_{VV}/I_{VH}) \) corrects for the difference in instrumental response to vertically and horizontally polarized light. Elastic scattering \( I_{VH} = G \times I_{VH,scat} \) at the excitation wavelength is denoted by \( R \). On an average, fluorescence and elastic scattering contribute equally to the entire path traversed by the excitation photon inside the tissue. The factor \( x = 0.5 \) takes into account this contribution. The validation of this technique has been previously reported on tissue mimicking phantoms.

2.3 Sample Collection and Handling

This in vitro study involved fresh human cervical tissue provided by G.S.V.M. Medical College, Kanpur, within 2 h of excision. All samples are taken from hysterectomy cases and a written consent form is obtained from each patient by the doctors involved in this study. The protocol number for this work from the Institute Ethical Committee (IEC) of IIT Kanpur is IITK/IEC/2012-13/1/3. These samples are labeled as normal and abnormal on the basis of visual inspection. Sample dimensions are \( \sim 2 \text{ mm} \times 5 \text{ mm} \). The samples are rinsed with saline water to remove superficial blood before performing the experiment. Data acquisition time for each measurement is 3 min. After completing the signal acquisition, these tissue samples are sent back to the medical college for histopathology analysis.
and to confirm classification as normal, CIN1, CIN2, and cancerous tissue.

2.4 Data Analysis

Polarized fluorescence spectral data, collected from 99 patients of varying ages and economic backgrounds, are categorized into different groups as normal (21 samples), CIN-1 (47 samples), CIN-2 (22 samples), and cancerous (9 samples) on the basis of histopathological reports. Intrinsic fluorescence is extracted for emission wavelengths from 400 to 609 nm using the earlier mentioned protocol. Subsequent to the extraction, a principal component analysis (PCA) is applied to the intrinsic fluorescence spectra for the dimensional reduction of the data without loss of its features. In addition to dimensional reduction, PCA identifies the spectrally prominent regions through the eigenvector components of the dominant eigenvalues. It also characterizes the noise using results from random matrix theory, making the spectral features unambiguous. To identify the principal components (eigenvectors) of the spectra containing variance of the complete data, an empirical correlation matrix $C$ is constructed.

$$C_{ij} = \frac{1}{N} \sum_{k=1}^{N} \delta I_i^T(k) \delta I_j(k) = \frac{A^T A}{N}. \quad (2)$$

Here, $\delta I_j^T(k) = A_{jk}$ is the mean subtracted intensity divided by the standard deviation, computed over the samples at each wavelength. Index $i$ varies from 1 to 210, representing the wavelength range from 400 to 609 nm and $k$ gives the number of samples. Subsequently, the principal components PC1, PC2, and PC3 corresponding to the three highest eigenvalues of the correlation matrix are calculated, which capture ~99% variance of the original data. These eigenvalues lie well above the noise threshold. Further, probability density functions are used to study the scores of the principal components for careful analysis of the distribution. In order to estimate the probability density function $f$ of the scores $x$, a kernel-smoother density estimation is used:

$$\hat{f}_h(x) = \frac{1}{nh} \sum_{i=1}^{n} K \left( \frac{x - x_i}{h} \right). \quad (3)$$

where the entire data are divided into $n = 100$ equally spaced points $x_i$. Here $h$ is the smoothing parameter, known as bandwidth, and $K$ is the normal distribution given as

$$K \left( \frac{x - x_i}{h} \right) = \frac{1}{\sqrt{2\pi}} e^{-\left(\frac{x - x_i}{h}\right)^2/2 h^2}. \quad (4)$$

Scores of the selected principal components of each tissue class are then divided into training and validation data sets. Mahalanobis distances of validation set data points ($v$) from the mean of training set data points ($t_m$) are calculated as given below:

$$r = \sqrt{\left(v - t_m\right)^T c_i^{-1} \left(v - t_m\right)}, \quad (5)$$

where $c_i$ is the covariance matrix of training set data points. The different tissue types are classified on the basis of $r$. Ten samples each from normal and CIN2, 24 samples from CIN1, and 4 cancer samples are randomly selected for inclusion in the training data sets. The remaining 11 normal, 23 CIN1, 12 CIN2, and 5 cancer samples are used as the validation data sets. Mahalanobis distances are computed using training data sets for each grade, and these distances are then used to classify the validation sets of the corresponding grades. This process is repeated twice taking randomly selected samples in training and validation data sets, and this made no significant difference in classification. Finally, the above-computed Mahalanobis distance is used to classify the complete data set (which included both training and validation data sets).

3 Results and Discussion

Figures 1(a) and 1(b) display co- and cross-polarized components of the fluorescence and of the elastic scattering spectra of cervical tissue, respectively. Area normalized intensities of copolarized, cross-polarized, and intrinsic fluorescence spectra are shown in Fig. 1(c). The cross-polarized component travels a larger path inside the tissue and bears more absorption and diffuse-scattering effects as compared to the copolarized component. As a consequence, the absorption dips at 410 nm are enhanced in the cross-polarized components, as seen in Fig. 1. The removal of the dip in the intrinsic fluorescence spectrum is clearly visible in Fig. 1(c).

The technique, thus, successfully extracts intrinsic fluorescence by eliminating the effects of absorption and diffuse scattering. This can now be used to study the biochemical behavior of normal and different grades of cervical tissue samples with further statistical analysis. Figure 2(a) shows the area normalized mean intrinsic fluorescence spectra of normal, CIN-1, CIN-2, and cancerous cervical tissue samples. The regions in the wavelength ranges from 400 to 420 nm and 452 to 470 nm are attributed to the contribution from collagen and NADH, respectively. These ranges are magnified in

![Fig. 1 Typical plots of co- and cross-polarized components of (a) fluorescence spectra, (b) elastic scattering spectra, and (c) typical area normalized spectra of copolarized, cross-polarized, and intrinsic fluorescence emission for human cervical tissue.](image-url)
Figs. 2(b) and 2(c) to highlight the reduction of collagen and increase in NADH contributions with development of the disease, respectively. The reduced collagen emission corresponds to the breaking of collagen matrix structures and the increased NADH emission is due to the enhanced metabolic activity of cells with the growth of abnormal cells. \(^5\)–\(^9\) It is pertinent to note at this point that even on extraction of the intrinsic fluorescence, the spectra do not display significant changes as the disease progresses.

Figures 3(a), 3(e), and 3(f) depict the correlation maps for intrinsic, co- and cross-polarized fluorescence components of the normal tissues. With 370-nm excitation, the spectra are scanned from 400 to 609 nm. The wavelength shift in the correlation map is calculated from 400 nm. Significantly high fluorescence, the spectra do not display significant changes as the disease progresses.

Fig. 3 Correlation maps of intrinsic fluorescence for (a) normal, (b) CIN-1, (c) CIN-2, and (d) cancerous cervical tissue. (e) and (f) show co- and cross-polarized fluorescence correlation matrices for normal cervical tissue. One observes well-demarcated domains in normal and different grades of precancer in the correlation maps.
correlation domains are seen in the correlation map of intrinsic fluorescence compared to the domains of co- and cross-polarized components. The high correlation occurs because the fluorescence is now free from the effects of absorption and diffuse scattering. The importance of extracting intrinsic fluorescence is clearly visible in these correlation maps where the domains of scattering. The contribution of intrinsic fluorescence of CIN-1, CIN-2, and cancerous cervical tissues are displayed in Figs. 3(b), 3(c), and 3(d). In the correlation map of the normal tissue, three distinct domains are visible. These correspond to the fluorescence emission of collagen (400 to 420 nm), NADH (452 to 470 nm), and FAD (500 to 550 nm), as is evident from the wavelength regions they occupy.

The size of the first domain, which corresponds to the activity of collagen, decreases monotonically when going from normal to CIN-1, CIN-2, and cancer samples. The second domain corresponding to NADH activity is red-shifted in the case of CIN-1 and CIN-2 relative to the normal one and is most dominant in the correlation map of the cancer case. The contribution of the third domain, which represents the activity of FAD and its derivatives, reduces sequentially in the abnormal tissues relative to the normal ones. A small domain appears at approximately 580 nm in CIN-2 and cancer cases; this may be due to porphyrin contributions. There is evidence of porphyrin fluorescence in breast tumors from recent studies. Thus, in spite of a large standard deviation as seen in Fig. 2, distinct differences are still observed in the corresponding correlation maps of intrinsic fluorescence. This demonstrates the strength of PCA in highlighting the molecular information of already corrected spectra.

We now proceed to select the significant eigenvalues and corresponding eigenvectors of the correlation matrices, which contain the maximum information of the original data. The first three highest eigenvalues chosen are listed in Table 1. Other eigenvalues have been found to be quite small and, hence, have not been considered. The eigenvectors (principal components) corresponding to these three highest eigenvalues are used to represent the complete data. This is evident in the spectra, reconstructed using eigenvectors corresponding to the first two and the first three highest eigenvalues and shown in Figs. 4(a) and 4(b), respectively. The reconstructed spectrum captures the original spectrum more accurately since the eigenvector corresponding to the third eigenvalue is included.

Table 1 First, second, and third highest eigenvalues for normal and different grades of cervical tissues showing distinct differences between tissue types.

<table>
<thead>
<tr>
<th>Eigenvalues</th>
<th>Normal</th>
<th>CIN-1</th>
<th>CIN-2</th>
<th>Can</th>
</tr>
</thead>
<tbody>
<tr>
<td>First</td>
<td>206.0</td>
<td>199.2</td>
<td>198.1</td>
<td>203.7</td>
</tr>
<tr>
<td>Second</td>
<td>2.5</td>
<td>7.8</td>
<td>4.6</td>
<td>3.1</td>
</tr>
<tr>
<td>Third</td>
<td>0.9</td>
<td>1.6</td>
<td>3.5</td>
<td>2.0</td>
</tr>
</tbody>
</table>

Probability densities of the PC1, PC2, and PC3 scores have been systematically analyzed, as shown in Figs. 5(a), 5(b), and 5(c), respectively. A kernel-smoother has been used to get the normal distributions of the scores of PC1, PC2, and PC3. It is observed that probability distribution curves of the scores of PC1, PC2, and PC3 are able to separate different tissue grades. The distribution curves of these groups have some overlap. However, the peak separation of the distribution curves of PC3 scores is more distinct as compared to the other two, bringing out the subtle differences more distinctly.

The principal components can be visualized as new coordinate axes along which the given data are optimally represented. Hence, any parametric relation between these can have empirical implications. Considerably different curvatures are observed in the three-dimensional scatter plots of PC1, PC2, and PC3 for different tissue types, as is clear in Fig. 6(a). Scatter plots for CIN-2 and cancerous tissue types are more dispersed compared to normal and CIN-1. The probability density function and scatter plots both show remarkable differences among different tissue types.

It is evident that the eigenvector entries and their distributions provide valuable insight into the relative contribution of different fluorophores to the emission spectra. The eigenvector entries of PC1, PC2, and PC3 corresponding to the first three largest eigenvalues are shown in Fig. 7 for normal and different grades of cancerous tissue. As is well known, the dominant eigenvalue corresponds to the collective motion, akin to the motion of the center of mass. It can be observed that PC1 bears the reflection
of the structures in the correlation matrices as it retains most of the information of the original data. These modes in a financial time series are known as market modes, representing the correlated behavior of the entire market. It is clear from Fig. 7(a), which depicts the eigenvector entries of the dominant eigenvalue, that these are broadly distributed and show characteristic collective differences in the structure of the correlation matrices. The other smaller eigenvalues provide information about the subdominant correlated sectors. In the context of a financial time series, these refer to correlated sectors arising from the cluster of companies of similar kind. In the present context, these refer to different fluorophore activities and their interrelations. To systematically identify the correlated substructures, the eigenvalue entries are plotted with prior demarcation of different sectors corresponding to the activity of different fluorophores, namely collagen (400 to 420 nm), NADH (452 to 470 nm), FAD (∼530 nm), and porphyrin (∼580 nm).

The eigenvector entries corresponding to the second and third largest eigenvalues shown in Figs. 7(b) and 7(c) clearly segregate the domains of activity of different fluorophores.
The eigenvector entries (PC3) corresponding to the third largest eigenvalue show that collagen activity in the wavelength range from 400 to 420 nm is significantly reduced from normal to cancer. The NADH activity corresponding to the range from 452 to 470 nm shows a rapid increase in CIN-1 and then a gradual decrease as the disease progresses. The traces of its increased activity can then be tracked in the eigenvector entries of the second largest eigenvalue. In CIN-2, the levels of porphyrin activity corresponding to the range from 580 to 609 nm are increased considerably and remain high in cancer cases. Hence, the biochemical information is faithfully captured by the principal components and clearly demonstrates the changes as normal tissue progressively deteriorates to the higher grades.

Further, for a quantitative estimation of the spectral properties of tissue, the Mahalanobis distance has been utilized for discrimination. The sensitivity and specificity of the intrinsic fluorescence are found to be 100% for classifying the normal from CIN1, CIN2, and cancerous tissue samples. The Mahalanobis distances of the copolarized fluorescence show very high scatter, while unpolarized data points are clumped together around the origin, although with 100% sensitivity and specificity.

The sensitivity and specificity for copolarized elastic scattering are (100%, 100%), (90%, 95%), and (100%, 100%), in differentiating normal from CIN1, CIN2, and cancerous samples [Figs. 8(g), 8(h), and 8(i)], respectively. The cross-polarized components of both fluorescence and elastic scattering also show similar trends as copolarized data (not shown here).
intrinsic fluorescence scores display a clear separation with almost no dispersion. The fact that intrinsic fluorescence shows maximum separation indicates its usefulness in reliable discrimination.

4 Conclusion
In conclusion, the polarization-based technique presented here successfully extracts intrinsic fluorescence from the cervical tissue spectra after eliminating the absorption and diffuse scattering effects. In combination with PCA, the experimentally extracted intrinsic fluorescence clearly unmasks the contributions of collagen, NADH, FAD, and porphyrin during the progression of the disease. It is worth emphasizing the effective role of PCA, which separates the collective behavior through its eigenvectors corresponding to first three highest eigenvalues, thereby accurately demonstrating the relative change of the fluorophore activities in the different grades of precancer. The spectral features of these fluorophores manifest in different domains of the correlation matrices. The correlation maps of the intrinsic fluorescence reveal distinct differences among the normal and the different grades as compared to the subtle differences observed in their spectral profiles. Furthermore, the importance of extracting intrinsic fluorescence can be observed from the correlation map of intrinsic fluorescence, which gives three distinct domains that are not clearly visible in co- and cross-polarized components. The eigenvectors corresponding to the first three highest eigenvalues of the correlation matrices containing maximum information of the original data highlight the broad spectral structures and their differences in normal and different grades of different tissues. It can be observed that eigenvectors corresponding to the first three highest eigenvalues capture the relevant spectral features of the correlation matrices and clearly discriminate different tissue types. The sectorial behavior of the eigenvector entries of these dominant eigenvalues unambiguously highlights the differing activities of collagen, NADH, FAD, and porphyrin in different grades of precancer. In particular, the eigenvectors corresponding to the second and third eigenvalues clearly reveal the relative contribution of different fluorophores to the correlation matrix. The contribution of the third eigenvalue may be emphasized, as it proved vital in remarkably pointing out the activities of the fluorophores with disease progression. In future, this study may be extended to other forms of cancer and to in vivo studies. Further quantification with Mahalanobis distance analysis has shown the reliability of using intrinsic fluorescence as a discriminating tool. Though the sensitivity and specificity of intrinsic, co-, and cross-polarized data turn out to be 100%, the dispersion in data of co- and cross-polarized states is high, while the intrinsic fluorescence displays maximum separation between normal and different grades, respectively. In conclusion, it is pertinent to note that in vivo results need not always follow those of excised tissue. The major concerns are the oxidation of major fluorophores and the weaker absorption by blood in in vitro conditions. These concerns may be answered only through in vivo studies. Nevertheless, validating the proposed technique does provide an insight into the possibility of using it in clinical environment. To take this study further, a polarization-based probe has been fabricated and is in the preliminary stage of testing.

Acknowledgments
The authors acknowledge Dr. Kiran Pandey and Dr. Asha Agarwal of GSVM Medical College, Kanpur, for providing well-characterized tissues for preliminary studies and Dr. Anита H. Gharekhani of C. U. Shah Science College, Ahmedabad, India, for helping in statistical analysis. Authors also acknowledge Mr. Swapnil Barmase, an integrated MS student of the Department of Chemical Sciences, ISER Kolkata, Mohanpur Campus Nadia, India, for his help in the sectorial representation of eigenvector entries.

References

Seema Devi is pursuing her PhD in the Department of Physics at the Indian Institute of Technology (IIT), Kanpur, India. Her areas of interest include intrinsic fluorescence spectroscopy, synchronous fluorescence spectroscopy, wavelet transform, principal component analysis, confocal imaging, and fluorescence life-time imaging for early detection of cancer.

Prasanta K. Panigrahi is currently a professor at the Indian Institute of Science Education and Research (IISER) Kolkata, India. He received his doctoral degree from the University of Rochester, New York, in 1988. His research interests lie in Bose-Einstein condensates, cold fermions, nonlinear dynamics, quantum computation and quantum information, wavelet transform, and field theory.

Asima Pradhan is a professor in the Department of Physics at the Indian Institute of Technology (IIT), Kanpur, India, and is also associated with the Center for Lasers and Photonics. She received her PhD from City University of New York in 1991. Her areas of interest include laser spectroscopy and biophotonics. Her expertise is in fluorescence spectroscopy, light scattering spectroscopy, time-resolved fluorescence, polarization-based fluorescence, and Mueller matrix imaging of biosamples.