Stokes shift spectroscopy highlights differences of cancerous and normal human tissues

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The Stokes shift spectroscopy (S3) offers a simpler and better way to recognize spectral fingerprints of fluorophores in complex mixtures. The efficiency of S3 for cancer detection in human tissue was investigated systematically. The alterations of Stokes shift spectra (S^3) between cancerous and normal tissues are due to the changes of key fluorophores, e.g., tryptophan and collagen, and can be highlighted using optimized wavelength shift interval. To our knowledge, this is the first time to explicitly disclose how and why S3 is superior in comparison with other conventional spectroscopic techniques.   © 2012 Optical Society of America
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Fluorescence spectroscopy has been widely investigated for diagnosing cancer since the study by Alfano *et al.* in the 1980s [1]. The difference between the emission and absorption peaks is known as the Stokes shift interval, $\Delta\lambda_{ss}$. A spectroscopic method was proposed to acquire the fluorescence signal by a fixed wavelength shift interval ($\Delta\lambda_i$) between the excitation λ_{exc} and emission λ_{em}, which is termed as Stokes shift spectroscopy (S3) by Alfano and Yang [2]. Although this spectral approach was used in multiple fluorophore analysis in tissue, no other groups in the United States are applying it in cancer detection. This is because the previous studies did not investigate the reason why S3 is superior over absorption, fluorescence, and excitation-emission matrix (EEM) measurements. Therefore, few researchers are aware of the excellence of S3 technique in cancer diagnostic application in tissues.

This Letter will demonstrate the efficiency of S3 to recognize spectral fingerprints of fluorophores in complex mixtures and its application to highlight the difference between cancerous and normal tissues. We will also show how to select an optimal wavelength shift interval to obtain the best Stokes shift spectra (S^3) for the purpose of cancer detection, and discuss why S3 is superior over other conventional spectroscopic techniques.

As an example, the averaging S^3 of 15 pairs of cancerous (solid line) and normal (dash line) breast tissues were recorded by setting $\Delta\lambda_i = 40$ nm in the synchronized scan mode of a spectrometer (Perkin-Elmer LS 50), and shown in Fig. 1. The scan speed was 300 nm per minute. Each spectral profile was normalized to a unit value of 1 (i.e., the sum of squares of the intensity elements in each emission spectrum was set as 1). The salient difference of S^3 between cancerous and normal breast tissues can be observed as two reverses of the peak intensities at $\sim I_{294}$ and $\sim I_{340}$: (1) $I_c > I_n$ at ~ 294 nm, while $I_c < I_n$ at ~ 340 nm, where I_c and I_n are the intensity of cancerous and normal tissues, respectively; and (2) $I_{294} > I_{340}$ for cancer, while $I_{294} < I_{340}$ for normal, where I_{294} and I_{340} are the intensity at 294 and 340 nm, respectively. A tiny peak can be seen at 385 nm for the cancerous tissue by enlarging the S^3 profile in the range of 365 nm to 420 nm, as shown in the insert of Fig. 1.

These differences may reflect tissue fluorophores' change during the evolution of cancer. In order to understand which components mainly contribute to these changes, S^3 of the main fluorophores in breast tissue, e.g., tryptophan, collagen, NADH, and flavin, need to be measured.

The S^3 of a mixture solution of tryptophan, NADH, and flavin with $\Delta\lambda_i = 40$ nm is displayed as solid line in Fig. 2(a). The S^3 of collagen for same value of $\Delta\lambda_i$ was superposed as a dashed line. Since collagen is not soluble in water, it is hard to obtain the S^3 of the mixture including collagen. The aqueous collagen suspension was shaken evenly before the measurements. Compared with Fig. 1, Fig. 2(a) shows that the main peak at ~ 290 nm for the S^3 of the breast tissues is from tryptophan. The secondary main peak at ~ 340 nm corresponds to collagen, and the very tiny peak at ~ 380 nm stands for NADH. No obvious peak of flavin was observed. Therefore, the S^3 profiles of breast tissues acquired with $\Delta\lambda_i = 40$ nm are mainly contributed from tryptophan, collagen, and NADH.

To study the relative content changes of fluorophores, an analytical method, namely the nonnegative least square (NNLS) method, was applied to the S^3 of the
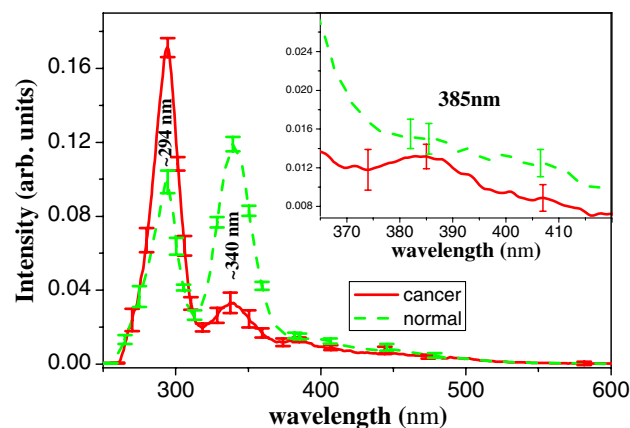

Fig. 1.   (Color online) Average Stokes Shift spectra of cancerous (solid) and normal (dash) breast tissues acquired by the selective $\Delta\lambda_c = 40$ nm.

cancerous and normal breast tissues, shown in Fig. 1 to extract relative contents of the fluorophores, e.g., tryptophan, collagen, and NADH, using the measured S^3 signal acquired with $\Delta\lambda_i = 40$ nm shown in Fig. 2(a). Figure 2(b) shows the scatter plot of the relative content for tryptophan versus collagen of cancerous (square) and normal (circle) breast tissues. A separating line on the scatter plots was loaded by the Linear discriminant analysis (LDA) model for the diagnostic significance of tryptophan versus collagen. The sensitivity and specificity were calculated as 80% and 86.7%, respectively. The most salient feature of Fig. 2(b) is that all data points for the normal tissue locate in the upper-left side in comparison with the data for the cancerous tissue, indicating that the relative contribution of collagen to the S^3 signal in the normal tissue is higher that of the cancerous tissues, while the relative contribution of tryptophan in normal tissue is lower than that in the cancerous tissue.

Reproducible results were observed for other kinds of human tissue studies, such as prostate and lung cancers. Similarly, two inverse spectral properties of (1) $I_c > I_n$ at ~294 nm, while $I_c < I_n$ at ~340 nm for 100% and (2) $I_{294} > I_{340}$ for cancer, while $I_{294} < I_{340}$ for normal for ~60%, were observed in S^3 measurements for cancerous and normal tissues.

In order to explicitly understand the diagnostic significance of these two inverse properties, the S^3 of mixed solution of tryptophan, NADH, and flavin with the concentration of ~0.4 mg/cm^3 was measured with different $\Delta\lambda_i$ from 20 to 140 nm with a step increase of 20 nm. The spectra of this mixture solution for $\Delta\lambda_i = 20$, 60, and 80 nm are displayed as solid, dashed, and dotted lines, respectively, in Fig. 3(a). For the visual reason, the S^3 obtained by $\Delta\lambda_i = 20$ nm was magnified by 10 times. Figure 3(b) exhibits the S^3 of the same mixture solution for $\Delta\lambda_i = 100$, 120, and 140 nm displayed as solid, dashed, and dotted lines, respectively. The spectrum of the mixture acquired by $\Delta\lambda_i = 40$ nm was shown in Fig. 2(a) previously. All curves are acquired under same experimental condition except using different $\Delta\lambda_i$.

From the $\Delta\lambda_i$-dependent S^3 of the mixture shown as Figs. 2(a), 3(a), and 3(b), it can be seen that the peak intensities of three fluorophores ascend with the increase of $\Delta\lambda_i$, at first, and then drop down at different $\Delta\lambda_i$. The full width at half maximum (FWHM) of S^3 profiles for all fluorophores expands monotonously with growth of $\Delta\lambda_i$.

Fig. 3. (Color online) S^3 of mixed solution of tryptophan, NADH, and flavin obtained by (a) $\Delta\lambda_c = 20$ nm (solid), 60 nm (dash), and 80 nm (dot); and (b) 100 nm (solid), 120 nm (dash), and 140 nm (dot).

When $\Delta\lambda_i$ reaches 120 nm, the NADH signal almost takes over the flavin signal.

To quantitatively study the changes of S^3 with $\Delta\lambda_i$, the FWHM and the peak intensities of S^3 profiles as a function of $\Delta\lambda_i$ for three fluorophores in the solution are shown as Figs. 4(a) and 4(b), respectively. Figure 4 is very useful for researchers to choose an optimal $\Delta\lambda_i$. For signal processing, two most important properties determine the quality of signals: resolution and magnitude. The values of FWHM stand for the inversing resolutions, the larger FWHM, and the worse resolution. Figure 3(a) actually reflects that the resolutions for tryptophan (square–solid), NADH (circle–dash), and flavin (hexagon–dot) decrease monotonously with the increase of $\Delta\lambda_i$, indicating that the smaller $\Delta\lambda_i$, the higher the resolution of the S^3 signal. Figure 3(b) exhibits (1) when $\Delta\lambda_i = 20$ nm is chosen, the three fluorophores have approximately the same peak intensities; (2) as $\Delta\lambda_i$ grows up, the peak intensities of three flurophores ascend at first, but descend at different critical values of $\Delta\lambda_i$. The curve for tryptophan (square–solid) falls at $\Delta\lambda_i = 80$ nm, for flavin (hexagon–dot) drops at $\Delta\lambda_i = 60$ nm, and for NADH (circle–dash) decreases at $\Delta\lambda_i = 120$ nm.

One should recognize that S3 actually acquires the signal of fluorescence. The S^3 profile of each biomolecule is determined by its corresponding peak positions of absorption and emission. When synchronized scan wavelength shift interval $\Delta\lambda_i$ approaches the Stokes shift interval, $\Delta\lambda_{ss}$, the S^3 signal magnitudes will access the maximum. While $\Delta\lambda_i$ crosses $\Delta\lambda_{ss}$, the intensity of S^3 will fall down. To understand Fig. 4(b), the S3-related parameters of different fluorophores of interest are listed in Table 1. As a comparison, the values of $\Delta\lambda_{drop}$ for the fluorophores observed in our experiment are also listed.
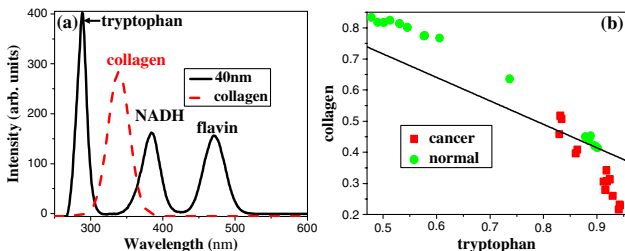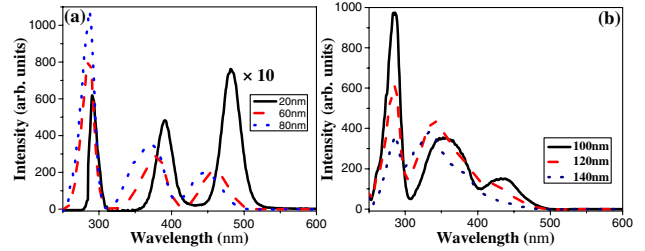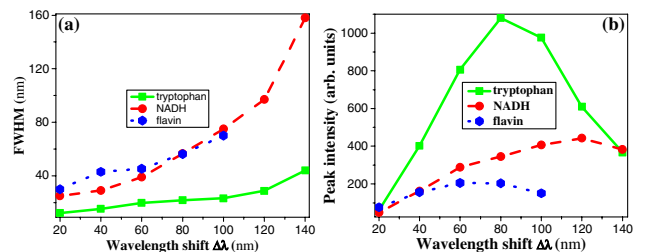

Fig. 2. (Color online) (a) S^3 of mixed solution of tryptophan, NADH, and flavin obtained by $\Delta\lambda_c = 40$ nm (dash) and aqueous suspension collagen; (b) The relative content of tryptophan versus collagen in cancerous and normal breast tissues by analyzing their S^3 obtained with the selective $\Delta\lambda_c = 40$ nm. The separating lines were calculated using the LDA method.

Fig. 4. (Color online) (a) FWHM and (b) S^3 peak intensities of tryptophan (square–solid), NADH (circle–dash) and flavin (hexagon–dot) in the mixed solution changed as a function of $\Delta\lambda_c$.

Table 1.   S3-Related Parameters of Key Fluorophores

molecule	tryptophan	collagen	NADH	flavin
λ_{abs} (nm)	280	340	340	450
λ_{em} (nm)	340–350	380–390	440–460	520
λ_{ss} (nm)	70–80	40–50	100–120	70
$\Delta\lambda_{drop}$ (nm)	80	—	120	60

Table 1 can be used to explain the changes of the S^3 peak intensities with $\Delta\lambda_i$ for different fluorophores. When S^3 was acquired by $\Delta\lambda_i = 20$ nm, the excitation of all three fluorophores is far from the Stokes shift interval, $\Delta\lambda_{ss}$, which results the smallest S^3 signal intensity. Since the Stokes shift interval, $\Delta\lambda_{ss}$ of flavin and tryptophan is $\sim$70 nm and $\sim$70–80 nm, respectively, the magnitude of S^3 signals from these two fluorophores is boosted with the increase of $\Delta\lambda_i$ within the range of $\Delta\lambda_{ss}$ at first, but the intensity drops at $\Delta\lambda_i = 60$ nm for flavin and 80 nm for tryptophan after it exceeds their $\Delta\lambda_{ss}$. The same reason causes the peak intensity of NADH to regress back at $\Delta\lambda_i = 120$ nm after it increases. The observed $\Delta\lambda_{drop}$ is in good agreement with $\Delta\lambda_{ss}$. The drop point for collagen is expected to be $\sim$40–50 nm.

Tryptophan, collagen, NADH, and flavin are key molecules in cancer diagnosis using spectroscopy [3]. For breast cancer, the most common grading system used in the U.S. is the Scarff–Bloom–Richardson (SBR) system [4], which is a breast cancer staging system to determine how aggressive and invasive the cancer is [4]. According to the features described by the SBR system, the higher cell density is the hallmark of breast cancer; therefore the increase of fluorescence from the main fluorophores inside cells—e.g., tryptophan, NADH, and flavin—should be expected. The primary fluorophore in the breast tissue extracellular matrix is collagen [5]. For invasion and subsequent metastasis, tumor cells degrade the surrounding extracellular matrix (ECM), which is composed mainly by collagen [4]. Understanding these changes during breast cancer evolution is critical to reveal the contributions of the fluorophores in tissues using spectroscopic techniques.

Based on Figs. 2 and 3, one should choose the optimal $\Delta\lambda_i$ as small as possible if resolution is the only consideration. However, in order to enhance the signal-to-noise ratio (SNR), the optimal $\Delta\lambda_i$ should be chosen as close as to $\Delta\lambda_{ss}$. In particular application in breast cancer detection, the alterations of fluorophores due to the cancer development direct an optimal $\Delta\lambda_i$. In the spectral analysis on cancer detection, it is very difficult to calculate the absolute concentration of the fluorophores. One needs to find an unchanged component to be a reference to observe the changes of the fluorophores of interest. It is better to use our approach because of evidence of an increase of tryptophan and a decrease of collagen in cancerous tissue [4,5]. Since $\Delta\lambda_{ss} = 40$–50 nm for collagen and $\Delta\lambda_{ss} = 70$–80 nm for tryptophan, $\Delta\lambda_i = 40$ nm should be chosen as an optimal scan wavelength interval, which gives balance between the resolution and the SNR. Furthermore, the S^3 with $\Delta\lambda_i = 40$ nm investigates the signal arisen from tryptophan and collagen in tissue, which change inversely in cancerous and normal tissues.

Therefore, the S^3 with selective $\Delta\lambda_i = 40$ nm highlights the difference between cancerous and normal tissues and causes the two inverse spectral properties exhibited by Fig. 1.

The conventional spectral methods applied in tissue optics studies are absorption (O.D.), emission, excitation, and EEM measurements. In the absorption measurements, only a few chromophores can match the detectable level in tissue. The fluorescence spectroscopy, either emission or excitation, can detect low level fluorophores, but because of the fixed pump (detecting) wavelength, the strongest emission (excitation) signal can be acquired only for one or two fluorophores. In addition, the emission signals of most fluorophores have very wide FWHM. So emission and excitation measurements provide poorer resolution and much less information than S3. Although EEM can be used to ensure the coverage of all endogenous fluorophores, the acquisition is extremely time consuming, and thus not suitable for clinical application. Furthermore, redundant information of EEM conceals alteration of the spectral fingerprints of cancerous and benign breast tissues. In contrast, S3 measurements can be used to acquire enough information of multiple key fluorophores in a much lower concentration and relatively higher resolution by employing a single scan (compared with EEM). This approach thus can dramatically reduce data acquisition time and keep the classification accuracy reasonably high.

In summary, the S3 method offers an efficient way to rapidly measure spectral fingerprints of complex mixtures such as tissue and highlights the differences between cancerous and normal tissues. To our knowledge, it is the first time to study why and how the wavelength interval $\Delta\lambda_i$ should be optimized to obtain better S^3 for cancer detection in human tissue. This study demonstrates that the S3 measurements can be used to acquire information for different key fluorophores in one scan and used to investigate the changes of the relative contents of the key fluorophores in breast tissues due to the development of cancer.

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