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Article *in* Optics Letters · January 2014

DOI: 10.1364/OL.39.000243 · Source: PubMed

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Quantitative Mueller matrix fluorescence spectroscopy for precancer detection

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Received September 23, 2013; revised November 27, 2013; accepted December 2, 2013;
posted December 4, 2013 (Doc. ID 198092); published January 6, 2014

Quantitative fluorescence spectroscopic Mueller matrix measurements from the connective tissue regions of human cervical tissue reveal intriguing fluorescence diattenuation and polarizance effects. Interestingly, the estimated fluorescence linear diattenuation and polarizance parameters were considerably reduced in the precancerous tissues as compared to the normal ones. These polarimetry effects of the autofluorescence were found to originate from anisotropically organized collagen molecular structures present in the connective tissues. Consequently, the reduction of the magnitude of these polarimetric parameters at higher grades of precancer was attributed to the loss of anisotropic organization of collagen, which was also confirmed by control experiments. These results indicate that fluorescence spectral diattenuation and polarizance parameters may serve as potentially useful diagnostic metrics.

OCIS codes: (120.5410) Polarimetry; (240.2130) Ellipsometry and polarimetry; (170.6935) Tissue characterization; (300.6280) Spectroscopy, fluorescence and luminescence.

http://dx.doi.org/10.1364/OL.39.000243

Fluorescence spectroscopy has emerged as a promising tool for noninvasive and early diagnosis of cancer [1–4]. The parameters of autofluorescence from tissue that has been exploited for diagnosis include differences in the static spectra, decay kinetics, and polarization [1–4]. While volumes of literature are available on exploiting the former two parameters of fluorescence, there are only a few reports on the polarization properties of autofluorescence from tissue [5–7]. These limited studies have shown that the measurement of the depolarization of autofluorescence or polarized autofluorescence may yield additional diagnostic information [5–7]. However, these studies are primarily based on the conventional measurement of fluorescence polarization anisotropy (the ratio of the polarized fluorescence component to the total intensity, a measure of depolarization [8]) with linear polarization excitation alone [5–7]. The fluorescence polarization anisotropy determined by using such a conventional approach reflects "lumped" effects arising from several simultaneously occurring polarizing/depolarizing interactions taking place during the fluorescence emission process [5,9]. Specifically, for fluorescence emission from a turbid medium like tissue, the emitted fluorescence is additionally (in addition to the intrinsic causes of depolarization such as the rotational diffusion of the fluorophore's random orientation of the fluorophore molecules, the radiationless energy transfer among fluorophores, etc.) depolarized due to multiple scattering effects [5]. Further, when the emission takes place from fluorophores having anisotropically oriented/organized molecular structures, the other polarimetry characteristics may also contribute significantly [9]. The prominent effects that may additionally exhibit in fluorescence from such anisotropic systems are diattenuation (the differential excitation of fluorescence with orthogonal linear and circular polarization states) and polarizance (the differential emission of fluorescence having orthogonal linear and circular polarization states) [9–11]. The simultaneous occurrences of all these polarization effects (as usually encountered in tissues [10]) contribute in a complex interrelated way to the conventionally measured polarization anisotropy [9] masking the potentially interesting effects and hindering their interpretation. The measurement of the full 4×4 fluorescence Mueller matrix may prove to be useful for this purpose. Once measured, the fluorescence Mueller matrix can be analyzed further to extract the individual fluorescence polarimetry characteristics containing information on the structure and organization of the contributing fluorophores [9,10]. The extracted fluorescence polarimetry parameters may thus reveal additional biochemical information, which may be exploited for enhancing the diagnostic ability of the fluorescence-based approach. Therefore, we have investigated this possibility by measuring 4×4 fluorescence spectral Mueller matrices from human cervical tissues (from the connective tissue region) having different grades of precancers. The connective tissue (stroma) region is specifically chosen because collagen, the major fluorophore in connective tissue [1,2,12], is known to possess oriented/organized molecular structures in the form of cross-links between the individual molecules [13]. Moreover, recent studies have demonstrated that alterations in stromal biology play an important role in stimulating neoplastic progression in preinvasive disease [1,13,14]. Monitoring the alterations in a collagen structure via its characteristic fluorescence polarimetry parameters may thus prove to be useful for the prognosis of the disease.

The specifics of the experimental system for the fluorescence spectroscopic Mueller matrix measurement can be found in our previous paper [15]. Briefly, the system is comprised of a polarization-state generator (PSG) unit and a polarization-state analyzer (PSA) unit. The PSG consists of a horizontally oriented fixed linear polarizer

and a rotating quarter-wave retarder. Analogously, the PSA unit consists of the same system arranged in the opposite order with the analyzer oriented vertically. The strategy for recording the fluorescence spectroscopic Mueller matrix measurement is based on recording 16 sets of spectra for four different optimized orientations of rotatable quarter-wave retarders of the PSG and PSA units [15]. The system has been eigenvalue calibrated to correct for the wavelength response of the PSG and PSA units, ensuring high accuracy of the Mueller matrix measurements in the entire wavelength range of 400–800 nm [15]. For recording the 4×4 fluorescence Mueller matrices, the 405 nm line of a diode laser was used as an excitation source. The spot size of the laser at the sample site was kept at ~1 mm. The tissue sections were histopathologically characterized [cervical intraepithelial neoplasia (CIN) or dysplasia grades I, II, and III] biopsy samples of human cervical tissues with normal counterparts obtained from G. S. V. M. Medical College and Hospital in Kanpur, India. The unstained tissue sections (thickness ~5 μm, lateral dimension ~4 mm × 6 mm) were prepared on glass slides. The standard method employed was tissue dehydration, embedding in wax, sectioning under a rotary microtome, and subsequent dewaxing. The fluorescence spectroscopic Mueller matrices were recorded both from the precancerous tissue sections and from their normal counterparts. The control experiments were performed on the extracted bovine collagen (collagen from bovine achilles tendon, type I, C4387 SIGMA) samples. The collagen was treated with 5% (weight by volume) acetic acid and the fluorescence Mueller matrices were recorded both before and after (30 min) treatment. The acetic acid treatment was employed to study the effect of the breaking of collagen cross-links [16] on the resulting fluorescence Mueller matrix elements and on the derived fluorescence polarimetry parameters.

Figure 1 shows the typical fluorescence spectral Mueller matrices recorded with 405 nm excitation from the connective tissue region of a grade-III precancerous tissue sample. Several interesting trends are apparent. The first element M_{11} (which represents the unpolarized fluorescence emission) shows a characteristic peak around 500 nm, which has been previously identified primarily due to the emission from collagen [1–4]. The considerably weaker intensities of the diagonal elements (M_{22}, M_{33}, and M_{44}) underscore the strong depolarizing nature of the fluorescence from tissue that arises

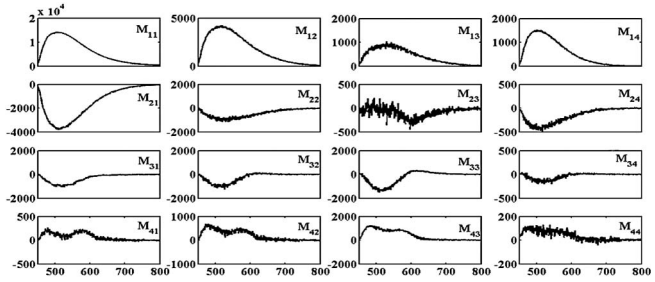

Fig. 1. 4×4 fluorescence spectroscopic (λem = 475–750nm) Mueller matrix recorded (in the exact backscattering configuration) with 405 nm excitation, from the connective tissue region (stroma) of a typical grade-III precancerous tissue section.

due to both the intrinsic and extrinsic (due to multiple scattering) causes of depolarization [5].

The elements M_{24} (M_{42}) and M_{34} (M_{43}) in a Mueller matrix typically characterize the phase retardance effect. Since the fluorescence process is inherently associated with strong dephasing effects, the intensity values of these elements are also consequently quite weak. Moreover, such dephasing also induces randomness to the corresponding intensities of these elements (manifested as larger fluctuations at around 500 nm, corresponding to the fluorescence peak).

In contrast, the elements of the first row and first column of the Mueller matrix show significant intensities with spectral variations similar to the collagen emission. While the M_{12} and M_{13} elements of the first row represent a horizontal/vertical and +45°/ – 45° linear diattenuation (fluorescence linear dichroism) effect, the M_{14} element characterizes (left/right) a circular diattenuation (fluorescence circular dichroism) effect. Analogously, M_{21} and M_{31} represent linear polarizance effects, and M_{41} represents a circular polarizance effect (linearly and circularly polarized emissions, respectively) [9,10]. In order to gain some quantitative information on the contributing polarization effects, the spectral Mueller matrices were decomposed into basis matrices of constituent polarimetry effects, namely, depolarization matrix M_{Δ}, retardance matrix M_R, and diattenuation matrix M_D, using the polar decomposition [10,17]. However, we would like to note that such a sequential decomposition might not be strictly valid for analyzing the Mueller matrix of a complex process like fluorescence from a turbid medium. Specifically, the contributing depolarization process may be far more complex than representing it via a single sequential depolarization matrix. In addition to the intrinsic depolarization mechanisms of fluorescence, this will have contributions of multiple scattering-induced depolarizations at the excitation and the emission wavelengths exhibited in a complex distributed fashion. The formulation of an appropriate *inverse* analysis method that can encompass all these complexities is a formidable task, and such a method does not currently exist. Thus, we have initially explored the widely used polar decomposition approach [17] for decoupling the polarimetry effects (fluorescence diattenuation and polarizance effects are of particular interest). As expected, the decomposition process yielded quite a high magnitude of depolarization (the magnitude of the depolarization coefficient Δ was ~0.9 over the entire spectral range) and rather weak magnitude of retardance (~0 rad, not shown here). Moreover, as also anticipated, the decomposition yielded significant values for both the linear and circular diattenuation (d^{fl}_{lin}, d^{fl}_{cir}) and polarizance (p^{fl}_{lin}, p^{fl}_{cir}) parameters of fluorescence. These are shown in Fig. 2, where the spectral dependence of the extracted fluorescence diattenuation [Fig. 2(a)] and polarizance [Fig. 2(b)] parameters for the precancerous and normal tissue samples are displayed. Here, the mean values for these parameters for 15 precancerous [grade I (four), grade II (six), and grade III (5)] and five normal tissue sections are plotted; the error bars represent the corresponding standard deviations.

Both the precancerous and normal tissues are observed to exhibit the fluorescence linear diattenuation and linear polarizance effects; the corresponding circular

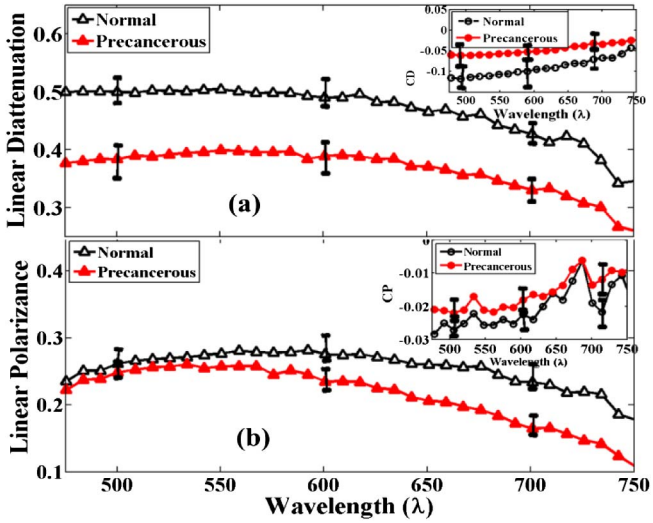

Fig. 2. Spectral variation of (a) fluorescence linear diattenuation $d_{\text{lin}}^{\text{fl}}$ and (b) fluorescence linear polarizance $p_{\text{lin}}^{\text{fl}}$ parameters (shown by triangles) for normal (open symbol) and precancerous (solid symbol) connective tissue sections. The insets of (a) and (b) show the fluorescence circular diattenuation and circular polarizance parameters, respectively (shown by circles). The values of the parameters reported here are the mean values over 15 precancerous and five normal tissue sections, respectively, and the error bars represent corresponding standard deviations.

effects (shown in the inset of the figures) are relatively weaker. Interestingly, the magnitudes of both $d_{\text{lin}}^{\text{fl}}$ and $p_{\text{lin}}^{\text{fl}}$ are observed to be significantly reduced in the precancerous tissues, with the difference being more pronounced for the linear diattenuation effect as compared to the linear polarizance effect. The linear diattenuation parameter is a manifestation of the differential excitation of fluorescence by the orthogonal linear polarization states (horizontal/vertical and $+45°/-45°$). Similarly, circular diattenuation arises due to the differential excitation of fluorescence by left/right circularly polarized light [9]. These effects may be interpreted as fluorescence-detected linear and circular dichroism, respectively, and may, accordingly, be related to the anisotropic (linear and circular) absorption properties of the ground molecular state of the emitting fluorescent species [9]. The considerably high magnitude of linear diattenuation may thus originate due to the anisotropic absorption properties arising from the linear anisotropic organization (cross-linked fibrous structures) of collagen in the connective tissue. The relatively weaker (not negligible though) magnitude of circular diattenuation possibly arises due to the presence of chiral moiety in collagen [18]. The polarizance effects, on the other hand, deal with anisotropic emission properties of the excited molecular state (preferential emission of fluorescence having orthogonal linear and circular polarization states) [9]. Like the linear diattenuation effect, the prominence of the linear polarizance effect (as compared to circular polarizance) also underscores the dominant role of the cross-linked molecular structure of collagen to the resulting linear anisotropic fluorescence emission. Nevertheless, the significant reduction in the magnitudes of both the fluorescence linear diattenuation $d_{\text{lin}}^{\text{fl}}$ and the linear polarizance $p_{\text{lin}}^{\text{fl}}$ in higher grades of precancer indicates the loss of anisotropic organization/orientation of collagen molecular structures in the connective tissue regions of the precancerous tissues. This may arise due to the destruction of collagen cross-links and the resulting randomization of the fibrous collagen structures with the progression of precancers [1,12,13].

In order to comprehend the above hypothesis, we conducted preliminary controlled experiments on extracted bovine collagen samples by treating them with 5% (weight by volume) acetic acid. An acetic acid treatment is known to degrade the organized collagen fibrous structures (microfibrils and fibers) by breaking the molecular cross-links [16]. The aim of these control experiments was not to mimic the exact nature of the morphological alterations in the collagen structures induced by neoplastic transformations of connective tissue [14]; rather, the idea was to gain qualitative understanding about the resulting effects of collagen molecular structure degradation on the fluorescence diattenuation and polarizance parameters.

The results of these control experiments are shown in Fig. 3. Both the fluorescence linear diattenuation $d_{\text{lin}}^{\text{fl}}$ and linear polarizance $p_{\text{lin}}^{\text{fl}}$ parameters of the type-I bovine collagen samples were observed to decrease significantly after the acetic acid treatment. The fluorescence Mueller matrices from these collagen samples also exhibited nonzero values of circular diattenuation and polarizance effects, with the magnitudes being considerably lower as compared to the corresponding linear effects (a trend that has been observed from the connective tissue samples as well). These results confirmed that the degradation of a collagen fibrous structure (as a result of breaking of the molecular cross-links) leads to an appreciable reduction in the fluorescence diattenuation and polarizance parameters, which may thus be exploited for probing structural changes in collagen. In addition to the reduction of the $d_{\text{lin}}^{\text{fl}}$ and $p_{\text{lin}}^{\text{fl}}$ parameters, an overall decrease of the total fluorescence intensity was also observed after the acetic acid treatment. This was expected, because the fluorescence emission is known to originate from the collagen molecular cross-links, the breakage and degradation of which leads to a decrease of the fluorescence quantum yield. Note that due to the presence of collagen in the connective tissue layer, one would expect a similar reduction of the absolute fluorescence intensity with a degradation of the cross-linked collagen fibrous structures [1,2,12,19]. However, the quantification of such absolute changes in the

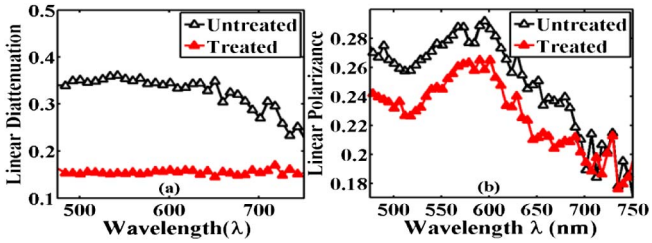

Fig. 3. Spectral variations of (a) fluorescence linear diattenuation $d_{\text{lin}}^{\text{fl}}$ and (b) fluorescence linear polarizance $p_{\text{lin}}^{\text{fl}}$ for the untreated (open triangle) and acetic-acid-treated (solid triangle) type-I bovine collagen samples.

fluorescence intensity as a signature of the structural alterations of the collagen in actual tissue would be rather complex. This follows because the recorded fluorescence intensity from actual tissue is contributed by other tissue fluorophores having overlapping fluorescence emission bands (e.g., with 405 nm excitation, tissue fluorescence is also contributed by nicotinamide adenine dinucleotide hydride (NADH) and flavins, etc. [1,2,4,12]), and it is also strongly modulated by the wavelength-dependent absorption and scattering properties of tissue [4,6,12]. The isolation and quantification of the changes in the absolute fluorescence intensity due to collagen structural alterations alone is thus a formidable task. In contrast, the fluorescence linear diattenuation and linear polarizance effects are not expected to be influenced by the emission from isotropic molecules such as NADH or flavins; thus, these parameters bear an exclusive signature of the anisotropic organization of the collagen fibrous structures in connective tissue.

To summarize, the results presented demonstrate that the fluorescence emission from anisotropically organized/oriented molecular structures (like collagen present in connective tissue) manifests itself as fluorescence linear diattenuation and linear polarizance effects. These intrinsic fluorescence polarimetry parameters can be interpreted by the fluorescence spectral Mueller matrix measurement and its subsequent analysis via the Mueller matrix decomposition. The initial studies performed on the connective tissue regions of human cervical tissue sections revealed significant differences in these fluorescence polarimetry parameters between precancerous and normal tissues. The magnitudes of these parameters were found to be significantly reduced at higher grades of precancers, which were attributed to the loss of the anisotropic organization of collagen present in connective tissues. Our current studies are directed toward the development of a more encompassing approach for the analysis/interpretation of the fluorescence Mueller matrix. Nevertheless, our initial exploration shows considerable promise of the fluorescence linear diattenuation and linear polarizance parameters as novel diagnostic metrics for precancer detection.

The authors thank Dr. Asha Agarwal and Dr. Chaynika Pantola, G. S. V. M. Medical College and Hospital, Kanpur, India for providing the histopathologically characterized tissue samples.

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