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ORIGINAL ARTICLE

Spatially-offset Fluorescence Spectroscopy (SOFS) Using Ring Illumination and Point Collection for Sub-surface Measurements in Layered Tissue

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Abstract
Purpose To report development of a depth-sensitive fluorescence spectroscopy system based on the configuration of point collection fixed at the centre of an illumination ring on the surface of a target sample.
Methods The system makes use of an axicon for converting the collimated laser beam into ring shaped illumination and achieves the ability of subsurface interrogation by varying the radii of the illumination rings thereby introducing spatial offsets between collection and illumination. The system was validated on a non-biological phantom comprising a thin tissue paper overlaying a thick dye card and also on a biological tissue sample that was a chicken leg tissue consisting of a thin epithelial membrane on the top of a much thicker muscle tissue.
Results It was found that while the fluorescence spectra corresponding to zero spatial offset are dominated by the fluorescence emission bands associated with the top layer of the layered samples, the fluorescence spectra measured with spatial offsets contain higher contribution of fluorescence signatures characteristics of the bottom layer as compared to the spectrum measured with the zero offset.
Conclusions The SOFS based system is able to interrogate subsurface depths beyond the reach of the conventional confocal fluorescence by simply varying the position of the axicon in the excitation arm. Due to the simplicity in its instrumentation, the approach has the potential to be developed into a portable system for in situ measurement in a tissue.

Keywords Spatially-offset fluorescence spectroscopy (SOFS), Axicon lens, Depth-sensitive measurement, Layered tissue sample

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INTRODUCTION

Fluorescence spectroscopy, in recent years, has been suggested and validated as a powerful tool for automated, non-invasive and real-time diagnosis of cancer of various organ systems [1, 2]. The majority of the research investigating the applicability of the approach has used it either in point spectroscopy mode [1] by measuring fluorescence sequentially from various sites on the surface of a target tissue following illumination of these sites with light of appropriate wavelength, or in imaging mode [2] by detecting fluorescence emitted from a comparatively larger area of a tissue surface following its illumination. The above techniques, though, deserve merit in their own right and have been shown to be useful in certain situations, their major limitation is that they seek to derive information only from the surface of a tissue, although the sub-surface tissue layers also are known to undergo various morphological and biochemical changes (as tissue transforms from normal to neoplastic) quite different from that of the surface [3, 4]. Further, the resulting changes in optical properties that get manifested in the fluorescence signatures of the surface and sub-surface tissue layers are also not the same [5]. Since the fluorescence emission at a given point or a region on the surface of a tissue is volume

integrated (over the sub-surface depths), it does not necessarily contain complete information of the tissue state due to the averaged out fluorescence contributions from the sub-surface tissue layers having different fluorescence characteristics. Obtaining the depth-wise fluorescence information is important because it may facilitate a more detailed analysis of the biochemical (and morphological) state of a given tissue thereby leading to an improved diagnostic feedback.

In recent years a large array of studies has been reported on depth-sensitive fluorescence measurements in tissue [5-11]. For example, Pfefer *et al*. [6] extensively studied the technique based on source-detector separation for sub-surface investigation. In this technique, the fluorescence spectra are measured from the points (lying on the surface of the sample) which are spatially away from the point of light incidence. The rationale is that the signals originating from the deeper layers are spread over a wider area due to multiple scattering in comparison to that from the shallower layers. They showed that the relative sensitivity to the sub-surface layers was significantly affected by how the fiber-optic probe was designed. They also demonstrated how oblique incidence (i.e. angular illumination) led to the same effect of source-detector separation and could facilitate selective interrogation of subsurface fluorescence up to depth of ~500 μm [7]. Zhu *et al*. [8], by using Monte Carlo simulations, theoretically compared the performance of a fiber optic probe developed using the principle of source-detector separation with that developed using the cone illumination-geometry (variable aperture method) in human cervical tissues and predicted a higher depth-selectivity of the probe developed based on the source-detector separation. Recently, Schwarz *et al*. [9] reported a fiber-optic probe (designed based on the principle of source-detector separation) capable of probing three different zones of depths, shallow, medium and deep, and showed that the incorporation of this kind of depth selective feature could improve the detection of precancerous lesions. Despite having the ability to probe sub-surface fluorescence, a general limitation of all these fiber-optic probes based on source-detector separation is an increasingly poorer signal-to-noise (SNR) ratio with increasing separation between the illumination and the collection fibers. The use of confocal fluorescence spectroscopy (CFS) [5], though can overcome the limitation of SNR, it cannot be used in its conventional form (cone-illumination and cone-collection) for probing larger (~few hundred microns) depths beneath the surface of a turbid medium like tissue. This is because being based on the principle of collecting the fluorescence photons generated within the focal zone of the illumination beam, it rapidly loses its depth selectivity beyond the depth in a tissue where excitation photons get completely diffused due to the effect of multiple scattering. Two novel configurations based on cone-shell illumination and cone collection through the use of axicons have recently been proposed by Ong *et al*. [10, 11] to probe depths beyond the reach of conventional confocal fluorescence. However its applicability is yet to be validated in a biological tissue.

We report here development of a depth-sensitive fluorescence spectroscopy system using the concept of varying illumination zones in the form of concentric rings of variable radii while keeping the point of collection fixed at the centre of the illumination rings on the surface of the target sample. The technique is a variant of source-detection separation and closely resembles the principle of inverse spatially-offset Raman spectroscopy (I-SORS) proposed by Matousek *et al*. [12]. By making use of an axicon, the collimated fluorescence excitation beam is incident onto the sample surface in the form of concentric illumination rings of varying radii and the emitted fluorescence exiting the sample surface at the centre of the concentric rings is collected for detection. The radii of the rings onto the sample surface are varied by giving displacement to the axicon lens along the axis of the illumination beam. The ability of the system to measure fluorescence spectra of the subsurface layer was demonstrated using layered non-biological phantom and biological tissue sample.

MATERIALS AND METHODS

Instrumentation

A schematic of experimental setup of spatially-offset fluorescence spectroscopy (SOFS) is shown in Fig. 1. The

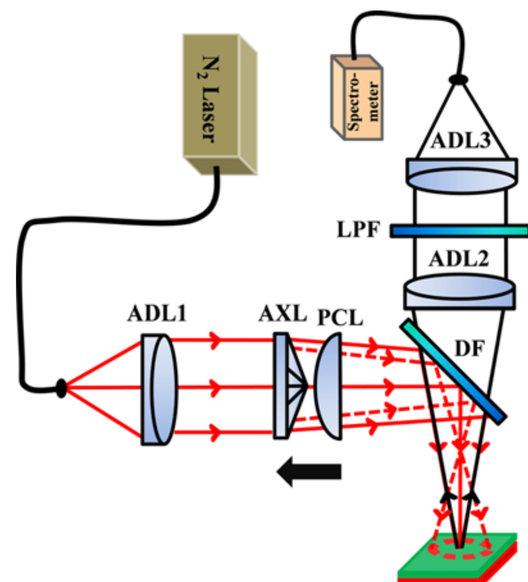

Fig. 1. Schematic of the experimental setup of depth-sensitive fluorescence spectroscopy based on SOFS. The abbreviations are as follows: ADL-Achrpmat doublet lens, AXL–Axicon lens, PCL–Plano-convex lens, DF–Dichroic filter and LPF–Long-pass filter.

system uses a home-built N_2 laser (λ_{ex} = 337 nm) as the fluorescence excitation source. The laser emits 10 ns pulses with a repetition rate of 10 Hz at pulse energy of 300 µJ. The output of the laser beam through a multi-mode fiber of core 400 µm is collimated by ADL1 (ACA254-100-UV, Thorlabs Inc.) of focal length 100 mm. AXL of apex angle 170° (1-APX-2-UV-E254, UAB Altechna) in combination with a plano-conex lens, PCL of focal length 50 mm, mounted on a translational stage, converts the collimated laser beam into a ring shaped beam. DF (Di01-R355-25x36, Semrock Inc.) kept at an angle of 45° with respect to the axis of the axicon steers the ring-shaped collimated beam on to the sample surface. The zero-offset refers to the position of the lens-axicon assembly (closer to the dichroic filter) which condenses the ring-shaped illumination beam into a point illumination on the sample surface. The non-zero offsets refer to the positions of the lens-axicon assembly which form concentric rings of illumination on the sample and can be obtained by displacing the assembly away from the dichroic filter (in the direction of arrow). The radii of the rings are the quantitative measure of the amount of non-zero spatial offsets. The fluorescence signal emitted from the sample is transmitted through the same dichroic filter, collimated by ADL2 (AC254-060-A-ML, Thorlabs Inc.) of focal length 60 mm and then passed through LPF (BLP01-355R-25, Semrock Inc.) before it is coupled to a multi-mode fiber of core 100 µm with the help of ADL3 (AC254-035-A-ML, Thorlabs Inc.) of focal length 35 mm. The light coming out of the distal end of the fiber is fed to a chip based spectrometer (USB 4000, Ocean Optics) for depth-sensitive fluorescence spectral measurements. The Stoke shifted fluorescence emission is always collected from the centre of the concentric rings of illumination.

Experimental measurements

The non-biological phantom was a green dye card of thickness ~2.0 mm with a layer of tissue paper of thickness 0.15 mm pasted on its top. The tissue sample was a chicken leg tissue consisting of a thin epithelial membrane (thickness ~0.25 mm) on the top of a much thicker muscle tissue (thickness ~10 mm). The depth-sensitive fluorescence spectra were measured with an integration time of 1 s and 5 s for the non-biological phantom and the biological tissue samples, respectively. The spot size was estimated by an edge response method [13, 14] and this came out to ~0.24 mm. All the measured fluorescence spectra were processed to remove instrumentation induced variations and to yield calibrated spectra. The resultant fluorescence spectra were further corrected for the non-uniform spectral response of the detection system. Following data processing, each spectrum was normalized with respect to its integrated intensity (ΣI) over the spectral range of 350-700 nm to remove the absolute intensity information from the spectrum that might be affected by many unavoidable experimental factors.

RESULTS AND DISCUSSIONS

Fig. 2a shows the fluorescence spectra measured from the non-biological phantom corresponding to two different spatial offsets, zero offset (thick line) and 2.0 mm offset (thin line). Each spectrum is the average of the fluorescence spectra corresponding to three different lateral positions and the error bars in grey background represent 1 standard deviation. It is apparent from the figure that though both the spectra are characterized by two distinct spectral bands around 435 nm and 515 nm, significant differences are seen in their spectral

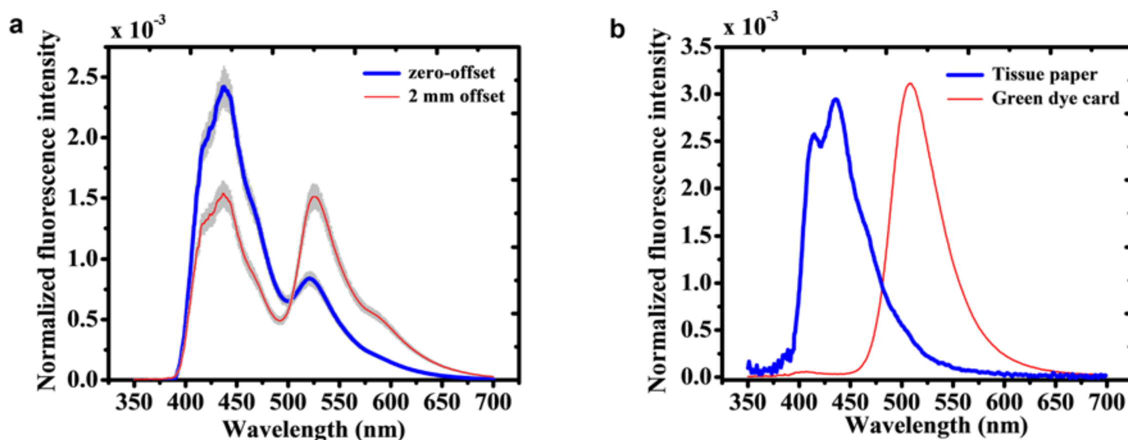

Fig. 2. (a) Fluorescence spectra of non-biological phantom (a dye card of thickness ~2.0 mm with a layer of tissue paper of thickness 0.15 mm pasted on its top) measured for zero offset (thick line) and 2 mm offset (thin line). Each spectrum is the average of the fluorescence spectra corresponding to three different lateral positions and the error bars in grey background represent ±1 standard deviation. (b) Reference fluorescence spectra of only tissue paper (thick line) and only dye card (thin line) measured from the same experimental setup with zero offset.

intensity distribution. For example, while the 435 nm band is significantly more intense as compared to the 515 nm band in the spectrum corresponding to the zero offset, the band intensities are almost similar in the spectrum corresponding to the spatial offset of 2 mm. Fig. 2b shows the reference fluorescence spectra measured with zero offset with the same experimental system separately from the tissue paper and the green dye card.

A comparison of Fig. 2a with Fig. 2b clearly reveals that the spectrum corresponding to the zero offset is dominated by the fluorescence emission band associated with the top layer of tissue paper and this is the why fluorescence band around 435 nm, found only in tissue paper (Fig. 2b, thick line) but not in green dye card, is found to be significantly higher in the measured spectrum corresponding to the zero offset (Fig. 2a, thick line). This is quite expected because here the emitted fluorescence signal is always collected from the same spot on the surface of the sample onto which the excitation laser beam is focused. Further, one can see that the fluorescence spectrum measured with 2 mm spatial offset contains considerably higher contribution of fluorescence signatures characteristics of the bottom layer as compared to the spectrum measured with the zero offset and this explains why the fluorescence band at ~515 nm, seen to be present only in the fluorescence emission spectrum of the green dye card (Fig. 2b, thin line), is observed to be considerably intense in the measured spectrum corresponding to 2 mm offset (Fig. 2a, thin line). This becomes further evident from the computed ratio of the intensity of the characteristic fluorescence emission peak of tissue paper (435 nm) to that of the characteristic emission peak of green dye card (515 nm). The ratio of intensities at 435 nm and 515 nm (I_{435}/I_{515}), a quantitative measure of the relative contribution of top vs. bottom layer, was found to be significantly higher (I_{435}/I_{515} ~3.2) for the fluorescence spectrum measured with zero offset as compared to the corresponding ratio value (I_{435}/I_{515} ~1.1) calculated for the spectrum corresponding to 2 mm offset.

After validating the SOFS system in the non-biological phantom, its performance was evaluated in a biological tissue (chicken leg) having an epithelial membrane covering a relatively thicker muscle tissue. Fig. 3a shows the fluorescence spectra measured from the tissue sample corresponding to two different offsets, zero offset and 4 mm offset. Each spectrum is the average of three spectra measured from three different lateral positions on the surface of the tissue sample with error bars representing ±1 standard deviation. It is apparent from the figure that like the non-biological phantom, here also, there are significant differences in the spectral intensity distribution between the emission bands in the spectra corresponding to the two spatial offsets. For example, in the spectrum corresponding to the 4 mm offset the intensity of the peak at ~395 nm, known to be characteristic of the structural protein collagen, is seen to be considerably higher than the intensity of the peak at ~450 nm believed to be due to coenzyme reduced nicotinamide adenine dinucleotide (NADH) [1]. In contrast, the intensity of the peak at ~450 nm is found to be substantially enhanced in the spectrum corresponding to the zero spatial offset. It becomes further evident if one computes the ratio of the characteristic emission peak of collagen (~395 nm) to that of the characteristic peak of NADH (~450 nm). The ratio of fluorescence intensities at 395 nm and 450 nm (I_{395}/I_{450}), a quantitative measure of the relative contribution of collagen vs. NADH, was found to be significantly higher for the fluorescence spectrum corresponding to 4 mm offset as compared to the spectrum with zero offset. Fig. 3b shows the plot of the I_{395}/I_{450} as a function of spatial

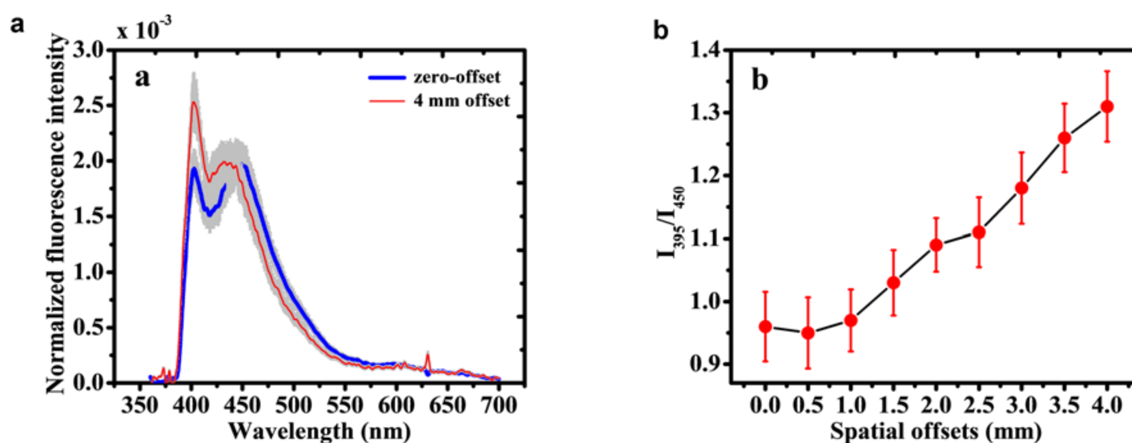

Fig. 3. (a) Fluorescence spectra of chicken leg tissue measured for zero offset (thick line) and 4 mm offset (thin line). The tissue had an epithelial membrane (thickness ~0.25 mm) on the top of a muscle of thickness ~10 mm. Each spectrum is the average of the fluorescence spectra corresponding to three different lateral positions and the error bars in grey background represent ±1 standard deviation. (b) Plot of the ratio of fluorescence intensities at 395 nm and 450 nm (I_{395}/I_{450}) against the spatial-offsets between the collection point and the illuminations rings. The ratio is a quantitative measure of the relative contribution of collagen vs. NADH present in the two layers of the tissue.

offsets between the illumination and the collection point on the surface of the sample. It is evident from the figure that when the spatial offset is zero the ratio value is the lowest (~0.96) signifying larger contribution of NADH as compared to collagen as is expected in the epithelium [4, 15]. As the offset increases, the I_{395}/I_{450} values are seen to increase up to a maximum value of ~1.32, indicating an increased contribution of collagen (as compared to NADH) as one can expect in a muscle tissue (bottom layer of the sample).

It is important to note that SOFS uses diffuse photons for fluorescence excitation and intrinsically seeks to collect only the multiply scattered fluorescence photons that are away from the illumination zone thereby allowing one to probe deeper (few hundred microns) beneath the sample surface. In contrast, the underlying mechanism of sub-surface probing with CFS is not based on the diffuse component of the excitation light. CFS predominantly collects fluorescence photons generated within the focal zone of the excitation beam thereby confining the probing depth to only few tens of microns beneath the sample surface [5, 16]. At progressively larger depths the multiply scattered fluorescence photons cause the pinhole aperture increasingly lose its ability to reject light scattered outside the focal zone, especially within the region (inside the collection cone) between the surface and the focal plane. In fact, as the excitation beam penetrates deeper in the sample, the exponential increase in the ratio between the multiply scattered and un-scattered light intensities [17] causes it to totally lose its focusing ability making the excitation beam overly blurred and causing CFS to completely lose its depth-selectivity. To confirm that CFS loses its depth-selectivity when the excitation light is totally diffused, an experimental arrangement for CFS measurement was set up [16] and the same non-biological mock-up sample used in the present study was used to measure the fluorescence signal from a 2 mm thick dye card through a layer of tissue paper of thickness of ~0.15 mm. No fluorescence signal characteristic of green dye card could be detected with CFS even with integration time of >100 s. However, SOFS, as has already been seen, was able to recover fluorescence signal of dye card in the same non-biological sample.

The results of the study described above demonstrate the potential of SOFS (with ring illumination and point collection) as a viable technique for carrying out depth-sensitive fluorescence spectroscopy in a layered sample. Though, in principle, it is a derivative of the standard method of source-detector separation (in an inverse configuration), widely used for depth-sensitive fluorescence measurements in various tissue types [6-9], SOFS offers several advantages in its comparison. For example, in the conventional source-detector separation setups, the depth-sensitive fluorescence measurements are done through fiber-optic probes in which the source-detector separation layout is implemented by arranging multiple fibers with a pre-fixed spatial separation between the fibers either in a linear array with the illumination fiber at either of the ends, or in concentric circular arrays with the central fiber providing illumination. Thus, one is left only with the option to monitor fluorescence of sub-surface depths in a discrete manner. In contrast, in the SOFS setup, the probing depths can be varied in a continuous manner by simply varying the position of the axicon (along the laser beam axis) in the excitation arm (see Fig. 1). Since this axicon is independent of the sample arm, it essentially provides a remote handle in continuously varying the probing depths inside a sample without requiring any manipulation inside the sample arm. This is a significant advantage for *in situ* measurement of fluorescence from a tissue. The other important advantage of SOFS is the SNR which is found to be fairly good at a reasonable integration time (1-5 s) even with larger offsets (i.e. larger depths). This is possible because of the ring illumination which leads to a much larger number of fluorescence photons that can diffuse through before being detected as compared to point illumination where the corresponding number of photons is expected to be much less. It is important to note here that the SNR rapidly degrades with separation between illumination and collection (i.e. at increasing depth) in the conventional source-detector separation based systems.

A limitation of the present scheme of implementation of SOFS is that it cannot obtain in one go the spectral information from all the depths and requires carrying out multiple measurements in a serial mode for acquiring fluorescence spectra from the different depths of a layered sample. As a consequence, it leads to an increase in the overall acquisition time as compared to conventional source-detector separation measurements involving simultaneous multiple detection. However, the multiple detection schemes have several inherent characteristics that limit the obtainable signal-to-noise levels and consequently the accessible depths. For example, a major limitation of the conventional fibre probe source-detector separation concept stems from its reliance on the collection of multiple fluorescence spectra on different Charge-coupled device channels. The spectra thus collected exhibit small distortions due to imaging imperfections present at various levels within a spectrograph thereby limiting the sensitivity of the approach and consequently its capacity to recover weak fluorescence signals from the deeper layers of a sample. In contrast, SOFS has the advantage of collecting fluorescence spectra free from artifacts that are experienced in conventional source-detector separation involving multiple-detection. It also has the potential for accessing the highest possible SNRs. Further, since the concept uses a wider illumination zone available on the sample surface, it allows for interrogation of samples at a lower power density thereby reducing any adverse effects due to sample heating [12].

This is particularly beneficial when probing biological tissues *in-vivo* where safe illumination intensity levels may need to be adhered to.

Although the capability of the SOFS system in measuring the depth-sensitive fluorescence spectra from a layered tissue sample has been successfully demonstrated, further efforts are required for improving the performance of the developed system before it can be eligible for the routine tissue analysis. A major limitation is the benchtop design of the system which precludes its transportation to a clinic required for the analysis of tissue in a clinical situation. In order to demonstrate the added utility of the system, the design of an endoscopic probe is critical. The objective of our future endeavor will be to develop a SOFS system having a handheld probe and use it *in-vivo* on human tissues to evaluate its efficacy for probing the depth resolved structure. Further, we plan to conduct a small scale pilot study on patients with potentially cancerous lesions of oral cavity to explore and evaluate the capability of the approach for *in-vivo* probing of epithelial and stromal layers in tissues of human oral cavity.

CONCLUSIONS

To conclude, a SOFS based depth-sensitive fluorescence spectroscopy system has been developed. The system achieves its ability for sub-surface measurement by using spatial offsets between illumination and collection through an axicon. The use of the system in interrogating sub-surface depths is shown in non-biological phantom as well as in biological tissue sample. Due to the simplicity in its instrumentation, the approach has the potential to be developed into a portable system with a hand-held probe for measuring depth-sensitive fluorescence in situ from a tissue for differential diagnosis.

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CONFLICT OF INTEREST STATEMENTS

Khan KM declares that he has no conflict of interest in relation to the work in this article. Kumar R declares that he has no conflict of interest in relation to the work in this article. Krishna H declares that he has no conflict of interest in relation to the work in this article. Ghosh N declares that he has no conflict of interest in relation to the work in this article. Majumder SK declares that he has no conflict of interest in relation to the work in this article.

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