LIF spectroscopy of stained malignant breast tissues

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Abstract: We employ laser induced fluorescence (LIF) spectroscopy to discriminate between normal and cancerous human breast (in-vitro) tissues. LIF signals are usually enhanced by the exogenous agents such as Rhodamine 6G (Rd6G) and Coumarin 7 (C7). Although we observe fluorescence emissions in both fluorophores, Rd6G–stained tissues give notable spectral red shift in practice. The latter is a function of dye concentration embedded in tissues. We find that such red shifts have a strong dependence on the dye concentration in bare, in stained healthy, and in malignant breast tissues, signifying variations in tubular abundances. In fact, the heterogeneity of cancerous tissues is more prominent mainly due to their notable tubular densities– which can provide numerous micro-cavities to house more dye molecules. We show that this can be used to discriminate between the healthy and unhealthy specimens in different biological scaffolds of ordered (healthy) and disordered (cancerous) tissues. It is demonstrated that the quenching process of fluorophore molecules slows down in the neoplastic tumors according to the micro-partitioning, too.

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1. Introduction

Invasive breast cancer is the most common carcinoma that affects more than ten percent of American female population [1]. Although the true origins of the occurrence of breast tumors are not fully understood, some of risk factors are known; such as family history, age, genetic defects, breast density, estrogen fluctuation, and radiation exposure [1].

In general, there are two main categories of breast malignancies: invasive and noninvasive cancers. The latter includes the ductal and lobular carcinoma in situ, and the former has three types; invasive ductal (ID) which is the most common type of invasive breast cancer representing 50% to 80% of all breast carcinomas, invasive lobular (IL), and inflammatory carcinomas [2]. In practice, some specific information is sought by the pathologist to

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determine the grade of neoplastic tumors. These include the degree of gland formation, the nuclear features, and the mitotic activity [3].

Mammography is the standard screening technique and a common tool for detecting the nontangible breast cancers [4]. However, this method utilizes X-ray (ionizing radiation) that is harmful for the live organs. Moreover, this method suffers from a number of false signals that may prompt an unnecessary biopsy. X-ray may be followed by ultrasound, CT scan, and MRI imaging techniques as complementary diagnostic procedures. However, the higher cost of MRI and the relatively low sensitivity of ultrasound seriously limit their applications [5]. The positron emission tomography (PET) may be advised in some cases for a very high cost. If a lesion is found through various clinical breast examinations, a biopsy is routinely administered. The biopsied tissue is fixed and stained, and the ensuing data are analyzed by a pathologist [6]. Inevitably, this proves to be time consuming and often requires a lengthy multi–stage process—thus a high likelihood of an erroneous diagnosis. On the other hand, the laser spectroscopic techniques are promising to become competent enough to render the unnecessary biopsy, while being a minimally invasive procedure and giving a more accurate data [7, 8]. Such techniques usually require the application of suitable coherent beams and high resolution spectrometers, or appropriate imaging cameras.

In LIF spectroscopy, the molecular systems are stimulated by an appropriate laser line. After the vibrational relaxation and successive internal conversion, the excited molecular transitions emit the fluorescence photons due to electrons cascading from the excited singlet states to the ground level at longer emissive wavelengths compared to the laser [9]. This is equally valid for exogenous as well as endogenous agents (auto–fluorescence). The auto–fluorescence of the biological tissues is detectable due to the existence of natural fluorophores in the live tissues and organs; such as nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FAD), etc [10]. Since the auto–fluorescence signals are not usually intense, however the exogenous agents lead to strong fluorescence emissions, whose spectrum provide valuable information about the biomaterials. This technique has been already applied to the various biological samples [11, 12].

It is worth noting that the adopted Lambert–Beer equation gives the variation of the fluorescence intensity in terms of the fluorophores’ concentrations, where \( I_f \), \( \beta \), \( C \), \( \alpha \), \( k \) and \( l \) identify the fluorescence intensity, proportionality factor, concentration, extinction coefficient, self-quenching parameter, and the cuvette length of the biomaterial, respectively. The Stocks shift \( \Delta \) (the difference between the absorption peak \( \nu_{ex} \) and that of fluorescence emission line \( \nu_{f} \)) is given by [13]:

\[
\Delta = \nu_{ex} - \nu_{f} \tag{1}
\]

This innate shift mainly depends on the molecular structure of the fluorophore and the solvent polarity. The fluorescence intensity is inversely related to the Stocks shift [14], such that \( I_f = I_o \eta_f \sigma_f / (\Delta + \nu_{f} ) \), where \( I_o \), \( \eta_f \) and \( \sigma_f \) are the absorbed intensity, quantum yield and the averaged frequency of fluorescence emission at the peak of excitation frequency \( \nu_{ex} \).

The elevated fluorescence signal arises from the larger population of the molecular transitions, whereas the decrease in amplitude is mainly due to static/dynamic quenching including self-quenching, collisional effects, as well as the aggregation of monomers to dimmers, particularly at dense solutions. This can be expressed by the Stern–Volmer equation: \( F_0 / F = 1 + KQ \), where \( F_0 / F \), \( K \) and \( Q \) ascertain the fluorescence intensity in the absence (presence) of quencher, quenching constant and the quencher density, respectively. In addition, the mean separation of the fluorophore molecules becomes relatively shorter at
higher concentrations allowing a larger Forster resonance energy transfer \cite{11, 14, 15}. This leads mainly to a further self-quenching. On the other hand, the small Stocks shift usually induces a notable spectral shift \cite{13, 16}, which is the focus of our work, where distinct characteristics of several fluorophores such as Rd6G are sought.

Palmer et al observed significant differences in the fluorescence properties of the normal and cancerous human breast cells \cite{17}. In general, the cancerous cells gain a number of receptors as a distinguishable characteristic unlike normal cells. The receptors show strong affinity to dye molecules. They utilized a xenon arc lamp to excite the transitions of endogenous fluorophores to record the auto-fluorescence spectra. Gupta et al have emphasized the observed changes in the auto–fluorescence of normal, benign, and cancerous breast tissues \cite{11}. Although, it gives out the differential diagnosis, the auto–fluorescence suffers from a low fluorophore abundances which lead to a faint output signal. In addition, this method for most part is unable to discriminate among the different types of carcinoma. The competition amongst numerous natural fluorophores reduces the resolution within the congested fluorescence spectra \cite{10}. Furthermore, the absorption and emission peaks of the endogenous agents usually lie in the visible spectral range. However, the spectral window in which a tissue is nearly transparent appears in NIR region which belongs to a larger penetration depth \cite{18}. In order to overcome these drawbacks, the exogenous agents are usually employed to enhance the signals \cite{18}. Some nano biomaterials exhibit positive innate properties for imaging or therapeutic applications; such as metal or polymeric nanoparticles which may function as the nanocarriers, delivering photosensitizers into the selective tissues. The effective results of application of these are usually confirmed by other methods, such as by magnetic resonance imaging (MRI), photothermal therapy (PTT), and photodynamic diagnosis (PDD) \cite{19–21}. Several biocompatible fluorophores such as Indocyanine green (ICG) \cite{5}, Cyanine7 (Cy7) \cite{22–24}, dialkylcarbocyanine fluorophores \cite{25}, and Methylene blue are also examined for the purpose of fluorescence imaging.

In fact, cancer is supposed to be a chaotic phenomenon and a disorder in tissue. Fractal geometry reveals how an object with irregularities can be explained by inspecting how the number of features of one size is related to the number of similarly shaped features of other sizes. By focusing on the irregularity of tumor growth rather than on a single measure of size—such as diameter or volume—fractal geometry is well suited to quantify morphological characteristics of tumors \cite{26–28}. It is known that a fractal is a mathematical object characterized by $D_F$ as a fractional (non-integer) dimension. The number of boxes $N$, which are needed to cover sample surface is identified by $N \propto L^{D_F}$, where $L$ is the box width. Then, a fractal dimension is given by $D_F = \log N / \log L$ \cite{28}. This analysis is widely applied in order to identify the geometry of malignancies based on their apparent irregular border and the random patterns of vascular growth \cite{26–28}. In fact, a tumoral tissue with higher $D_F$ usually gains higher degree of disorder as well.

To our best of knowledge, no report is available to study the LIF of in-vitro human breast tissues, particularly to demonstrate the spectral shift in the neoplastic tissues of interest. In this work, the LIF spectroscopy is explored to measure the spectral shifts of the stained breast tissues by virtue of Rd6G fluorophores to positively identify the malignancy \cite{29, 30}. The bare Rd6G solutions (without tissue) show a red spectral shift at low concentrations that face a changeover to blue shift at adequately dense solutions. Conversely, we have shown when Rd6G molecules are embedded in healthy and cancerous tumors, the spectral red shift survives even at dense concentrations. Furthermore, laser scanning confocal microscopy (LSCM) is utilized to attest that the fluorophore molecules are evenly penetrated into depth of tissues. We shall conclude that such spectral shifts mainly arise from the higher fractal dimensions and the structural disorder properties of the malignant breast tissues.
2. Methods and materials

2.1 Preparation of tissue samples and dye solutions

More than 30 normal and 30 invasive ductal carcinoma (grade II) breast tissue samples are examined in-vitro. According to modified Scarff-Bloom-Richardson (SBR) grading system established by Elston and Ellis, almost 10%–75% of glands/tubules are formed in grade II of IDC [31], exhibiting the abundance of tubules in the breast cancer respect to healthy tissues. Those are obtained from more than 30 patients undergoing surgical resections or biopsies while histologically malignancies were suspected. The ethics committee of Sina Hospital, Tehran approved the protocol for the use of tissue samples. Breast tissue sections for LIBS investigations were prepared as follows. Each sample is kept in 10% neutral buffered formalin immediately after surgery or biopsy to prevent any unwanted deformation. The slices of ~5mm × 5mm × 2mm dimensions are cut before the laser examination. The non–neoplastic tissues are sampled from the uninvolved areas far enough away from the tumor margin to assure that the tissues are healthy and free from carcinoma in situ. The healthy tissues are yellowish in appearance and contain plenty of fat, while tumors appear stiff and almost white. The paraffin-embedded tissues are prepared for LSCM images. The tissues are cut into 6µm thick slices with 2mm cross-sections, using a microtome.

Rd6G (C_{27}H_{29}ClN_{2}O_{3}) and C7 (C_{20}H_{19}N_{3}O_{2}) are used as the exogenous fluorophores of choice to dye the tissues. Ethanol is used as the solvent for both Rd6G and C7. The solution (fluorophore + solvent) is well homogenized in the ultrasonic bath (DT-102-H model made by Bandelin Co. having 120 W power, 35 kHz frequency) for ~15 minutes. Before carrying out LIF experiments, each sample is stained by immersing it in the dye solutions with different concentrations (0.5–4mM) for 30 minutes. The specimens are then dried in air before laser exposures. An image acquisition software package for tissue integrity investigation (image pro–insight software) is utilized to assess the degree of integrity of tissues. Image-Pro Insight is an advanced image acquisition software package controlling a variety of microscopy cameras for capturing the highest quality images. It also benefits of advanced particle detection and measurement steps acquiring with the image analysis solution provided by Media Cybernetics.

2.2 Experimental array

A second harmonic of 150 mW CW Nd:YAG laser at 532 nm (green) is employed to excite the Rd6G fluorophores. A 75mW CW diode laser at 405 nm (blue) induces the C7 transitions as well. The fluorescence emission is collected by the optical fiber into the spectrophotometer (Avantes Ava Spec 2048, NA = 0.22) over the spectral range of 200–1100 nm, 300 lines/mm with 0.1 nm spectral resolution as well as by the optical fiber reflection probe FCB-UV400-2-ME-SR (400 nm core diameter). Figure 1 illustrates the arrangement for LIF experiments. Laser scanning confocal microscopy (Nikon Ti, Japan) is exploited to take images of typical stained-tissues in order to study the homogeneity of dye penetration throughout the tissue. The laser at 543 nm is used to excite Rd6G fluorophores.
3. Results and discussion

The systematic experiments on the (fluorophore + normal) and (fluorophore + cancerous) tissues were carried out based on LIF spectroscopy using the well-known C7 and Rd6G fluorophores. The experiments are divided into two categories: (i) LIF spectra taken from the bare fluorophore solutions (without tissue), (ii) LIF emissions of the stained tissues (tissue + fluorophore). At first, the spectral shift of the bare fluorescence emissions is measured as a function of the concentration ranging from 0.05 to 3 mM. Then, these are compared with the LIF data of the stained tissues. Figure 2(a) illustrates the red shift and the corresponding FWHM of overlapping area of the absorption and fluorescence spectra in terms of C7 concentration. Inset of Fig. 2(a) displays the C7 fluorescence spectra versus concentrations. Note that in this case, the fiber is fixed at right angle position ($\phi = 90^\circ$). The emissions are spectrally shifted to longer wavelengths as fluorophore concentration changes from 0.05 to 3 mM. Subsequently, the corresponding FWHM of the overlapping area notably decreases as a function of the fluorophore concentration, accompanying a sensible red shift as long as the overlap exists. Figure 2(b) depicts the similar spectral shift and corresponding FWHM as a function of Rd6G concentration at $\phi = 90^\circ$. This attests that significant red shift takes place at low concentrations, following a subsequent changeover to blue shift for denser solutions. In fact, FWHM initially falls off with concentration, corresponding to a rise in the red shift. Then, at higher concentrations, FWHM increases as a result of transition of red shifts to blue shifts. Inset of Fig. 2(b) displays the corresponding Rd6G fluorescence spectra versus concentrations.

The detected spectral shift happens for the fluorophores with sufficiently small Stocks shift, as a consequence of the large overlapping between the fluorescence and the absorption spectra, according to Eq. (1) [13–15, 32]. In fact, the latter is taken into account as the dominant factor as to the reabsorption of emitted photons takes place by the adjacent molecules. The Stocks shift arises from the displacement of the potential energy surfaces (PESs) of the ground and excited states in equilibrium condition due to the anharmonic electron-phonon interactions in the course of an electronic transitions [13]. The strength of electron-phonon coupling is determined by Huang–Rhys $S$-parameter, which is proportional to the square of Stocks shift $\Delta$ according to $S = \Delta^2 / 2$ [33]. Therefore, a high $S$-parameter corresponds to a larger Stocks shift, leading to a notably smaller overlap, and vice versa [13]. The Rd6G faces a small $S$-parameter, corresponding to a large overlap that in turn results in strong red/blue shifts of emissions. Conversely, the encirclement of dye molecules due to dense concentrations usually reduces the reabsorption rate. The aggregation of the dye molecules at larger concentrations may lead to the formation of dimers [34]–which lessens the population of fluorophore molecules. Consequently, both the encirclement of dye
molecules and the formation of dimers result in smaller reabsorption rate that gives rise to the blue shift, eventually.

The spectral measurement is obviously sensitive to the angle of detection (ψ) during the successive experiments. Besides, in case of (fluorophore + tissue), the emissions are optimally detected by placing the fiber in front of the samples at ψ = 45°. Thus, the fluorescence spectra of both C7 and Rd6G bare solutions (in the absence of tissue) are initially recorded for concentrations of 0.25 to 4 mM at this new arrangement before doing experiments on (fluorophore + tissue). No sensible spectral shift is observed inspecting the data taken from various emissive C7 solutions. Furthermore, the FWHM remains nearly invariant in terms of the C7 concentration as shown in Fig. 2(c). Conversely, Fig. 2(d) depicts the notable spectral shift in terms of Rd6G solutions. The insets of Figs. 2(c) and 2(d) display the corresponding fluorescence spectra versus C7 and Rd6G concentrations, respectively. In fact, when the fiber is placed in right angle position (ψ = 90°), it collects the fluorescence emissions from the depth of sample. However, in the case of setting fiber position at ψ = 45°,
the emissions are collected from the surface layer, because the excitation–laser faces molecular scattering. This effect becomes significant at shorter wavelengths due to the Rayleigh scattering. Moreover, at denser concentrations, there is a shorter penetration depth of photons and the excited molecules are populated close to the surface in front of the sample. These facts explain why the fluorescence emissions are not reabsorbed by the adjacent molecules–thus no detectable spectral shift can occur in the case of C7. The nature of the short penetration depth at 405nm, as well as the relatively small overlapping region between the C7 absorption and the fluorescence emissions, intensify this result. On the other hand, the absorption coefficient of the Rd6G is lucidly large at 532 nm [13, 16], thus the photons undergo more reabsorption events, leading to a sensible red shift, in comparison.

Afterwards, the LIF experiments are carried out on the stained tissues. Figures 3(a) and 3(b) illustrate the histological images of typical normal and cancerous stained breast tissues, respectively. An obvious heterogeneity is shown in the case of malignant tissues due to the increased density of the tubular structures [31]. There are numerous micro-cavities which clearly attest to the micro-partitioning structures of the cancerous tissues versus the healthy ones. Figure 3(c) illustrates the degree of integrity determined by the image pro–insight software, which enables us to distinguish the structural heterogeneity of tissues of interest. Unlike the cancerous ones, the healthy tissues enjoy of a higher degree of integrity. The higher grade of disorder up to ~25% is measured for neoplastic tissues compared with the normal ones according to Fig. 3(c). As a consequence, the integrity assessment is in good agreement with the fractal theory [27, 35], where the abnormal features of cancerous tumors is correlated with the irregular pathological shapes. The malignant tissue gains a higher fractal dimension and a notable structural disorder [3, 35]. Several studies have been done on the fractal dimensions of normal and malignant breast tissues [35, 36], reporting a typical mean value of $D_f ~1.45$ for the fractal dimensions of the malignant tissues, which is significantly higher (by ~21%) than that of the normal ones ($D_f ~1.21$) [26, 35].

Specimens of normal and cancerous breast tissues are separately prepared by immersing them into four distinct concentrations of the Rd6G and C7 solutions (i.e., 0.5, 1.5, 2.5 and 4mM) for 30 minutes. After drying, the samples (Rd6G + tissues) are irradiated by CW Nd:YAG laser at 532 nm, 150 mW, and the other specimens (C7 + tissues) are exposed by the CW diode laser at 405nm, 75mW. It is worth noting that the auto-fluorescence of the tissues is negligible mainly due to the strong fluorescence emissions of exogenous dyes. Note that there is no detectable spectral shift for the case of (C7 + tissue) similar to that for bare C7 solutions, as discussed above. Figure 4(a) illustrates the fluorescence emissions of the fluorophore molecules embedded in the normal and cancerous tissues as a function of Rd6G
concentration from 0.5 mM up to 4 mM. The fiber is fixed in front of the samples at $\varphi = 45^\circ$.

Emissive wavelengths of (Rd6G + cancerous) tissues obviously show the sensible red shift respect to those of (Rd6G + healthy) samples. By increasing the dye molarity, the max red shift of ~8 nm appears. Furthermore, it reveals a mean spectral red shift of ~3 nm for the Rd6G-stained healthy tissues, and ~1 nm for Rd6G-malignant tissues in terms of the dye concentration. In comparison, the data taken from the bare Rd6G solutions is also shown in the figure, where no dye-stained tissues are used. It depicts that the red shift continues as a function of Rd6G concentration up to 1 mM, when a switch to the blue shift begins to occur. Similarly, Fig. 4(b) illustrates a typical red shift of ~8 nm at 4 mM when the specimens (Rd6G + malignant) and (Rd6G + healthy) tissues are compared. Figure 4(c) displays a rise in the spectral red shift versus Rd6G concentrations in the cancerous and normal tissues, whereas the corresponding FWHM of overlap area between the absorption/emission spectra slightly decreases indicating the higher sensitivity of fluorescence emissions to the spectral red shift. Furthermore, LIF spectroscopy is carried out for typical cancerous tissues of the other organs. However, those tissues did not show a sensitivity to the dye concentrations. This is mainly arises from the special nature of breast tumors which vary in tubules size and density according to Fig. 3(b).

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**Fig. 4.** a) Emissive wavelengths of the (Rd6G + normal) and (Rd6G + cancerous) in-vitro breast tissues at the certain Rd6G concentrations of 0.5, 1.5, 2.5 and 4 mM accompanying the bare fluorescence spectra for the 0.25–4 mM Rd6G solutions (in the absence of tissue), (b) A typical fluorescence spectra of the (Rd6G + normal) and (Rd6G + malignant) breast tissues at 4 mM indicating the lucid red shift of the fluorescence emission of the cancerous tissue, (c) Spectral shift and the corresponding FWHM of overlapping region in terms of Rd6G concentration, d) Emissive wavelength of Rd6G stained a typical plant tissue (Solanum toberosum–potato) emphasizes similar red shift in terms of Rd6G concentration, inset: SEM image of the stained plant tissue representing micro-cavity sites [37]. The honey–hive like architectures acts as a micro-partitioning structure during LIF. In all cases the fiber was set at $\varphi = 45^\circ$.  

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Moreover, the LSCM is employed to take the cross-sectional images of typical healthy and malignant tissues, stained by 0.5 and 2.5mM Rd6G solutions. Figure 5(a), 5(b) and 5(c) illustrate the LSCM images of cancerous tissues (without dye) in addition to the stained ones in 0.5 and 2.5mM Rd6G concentrations, respectively. Figure 5(d), 5(e) and 5(f) show the similar images are depicted for the normal tissues. Tissues are cut by the microtome into 6µm-thick slices after the preparation. The tumor depth is measured to be ~2mm. Fluorescence emissions are collected along the tissue thickness. This attests the evenly penetration of dye concentration along the tissue depth. In fact, the dye is homogenously distributed throughout tissue such that the laser can reach the depth of tumor. The inverse of the absorption coefficient for Rd6G ($\alpha$~2cm$^{-1}$ [14]), is referred to the laser penetration depth $L = 1/(\sigma_{dye} \alpha) = 1/\alpha$, to be ~5mm for the typical Rd6G-stained tissue. L is given to be longer than the tissues thickness of interest. Moreover, Fig. 5(b) and 5(c) lucidly exhibit higher fluorophore abundance in the malignant tissues with respect to the healthy ones as shown in Fig. 5(e) and 5(f).

| Rd6G concentrations (mM) | Breast       |     |     |
|--------------------------|--------------|-----|-----|
| 0 (without dye)          | a)           | b)  | c)  |
| 0.5                      | d)           | e)  | f)  |
| 2.5                      | g)           | h)  | i)  |

Fig. 5. LSCM images of a) Cancerous breast tissues without dye, b) Breast tumor stained in 0.5mM Rd6G solution, c) Breast tumor stained in 2.5mM Rd6G solution, d) Normal breast tissue without dye, e) Normal breast sample stained in 0.5mM Rd6G solution and f) Normal breast tissue stained in 2.5mM Rd6G solution.

Furthermore, the LIF spectroscopy on solanum toberosum (Potato) is also carried out, in comparison. The honey-hive like architecture of plant tissues of interest are shown in the inset of Fig. 4(d) [37]. The alteration of spectral shift is quite similar to that of normal and cancerous tissue as shown in Fig. 4(a) to be quite different from that of bare Rd6G solution. This elucidates that the dominant mechanism most likely arises from the micro-partitioning effect of honey-hive like scaffold to avoid the molecular collisional events leading to smaller self-quenching rates, keeping the red shift at intense Rd6G solution. We come to conclusion that numerous micro-cavities either in the form of tubules abundant in the IDC breast tissues or in the plant scaffold micro-structures give rise to the continual red shift even at dense solution unlike what takes place for the bare solution. The larger quenching rate and the subsequent blue shift resemble to be the key parameters to discriminate the healthy tissues from breast malignancy, too.

The spectral data are analyzed via the standard SPSS v19 statistical package using t–student distribution, revealing that the emissive fluorescence of the breast malignant tissues are larger than those of healthy ones with 99% confidence level.
In fact, the breast tissues are known as a highly scattering media [6]. The red shift of the fluorescence emissions in the (Rd6G + cancerous) tissues respect to (Rd6G + normal) ones arises from the elevated rates of the reabsorption as the emissive photons traverse a longer path as compared to those in (Rd6G + normal) tissues. The fluorescence emission/reabsorption rates are significantly increased inside a scattering media having a higher degree of irregularity. The addition of nano-scatterers lengthens the photon random walk, as they increase the likelihood of multiple scattering. The longer is the random walk, the higher would be the reabsorption rate—which subsequently leads to a larger red shift [13].

In general, three competing mechanisms take place in dye solutions when laser irradiation is applied at large concentrations. Self-quenching increases due to a rise in collision rates between the fluorophore molecules that results in the reduction of the fluorescence emission according to modified Lambert–Beer equation. In addition, the refractive index and random walk increases which give rise to the elevated reabsorption events and subsequent measurable red shift. Conversely, the Stern-Volmer equation states that the higher population of the fluorophore molecules leads to the aggregation and a compound formation which may reduce or stop the red shift process. The formation of the numerous micro-cavities is initiated at the onset of the abnormal cell proliferation due to the structural alteration of tubular architecture [38]. The latter resembles to induce the micro-partitioning effect [16, 32]. Here, the micro-partitioning architecture of the cancerous scaffold slows down the aggregation events such that the micro-cavities do trap further fluorophore molecules. This indicates the notable reduction of the self-quenching events. That is why the relatively larger mean separation of the fluorophores at higher dye concentration induces smaller Forster resonance energy transfer, eventually allowing a detectable spectral red shift. In the other words, the micro-partitioning effect among the cancerous tissues prohibits the fast quenching of the fluorescence emissions and slows down the collisional rates of the fluorophore molecules. This effect is leveled off within the normal tissues resulting in the higher quenching rate, smaller the reabsorption rate and the smaller red shift accordingly, as shown in Fig. 3(a).

Here, we have assessed the fluorescence spectral shifts as a key parameter to diagnose in-vitro cancerous breast tissues for the purpose of clinical applications. This technique may be extended for real-time examination of in-vivo breast tumors and minimally invasive LIF spectroscopy using a selective laser line and a biocompatible fluorophores.

4. Summary

Here, the utility of laser induced fluorescence LIF spectroscopy is investigated for a number of in-vitro breast samples. Systematic experiments are carried out based on the LIF spectroscopy in order to discriminate the normal and cancerous breast tissues due to their differences in tubular densities. LIF of exogenous agents is strongly dependent on the dye concentration and tissue structure as the scaffold for the fluorophore materials. We have shown that fluorescence emission properties are dictated by regular scaffold (healthy tissue), or disordered structure (malignant tissues). Therefore, the fluorophore molecules that are not embedded in any tissue clearly exhibit drastically different spectral properties. Rd6G bare solutions show red shifts in terms of density of dilute solutions (≤1mM), while it switches to blue shifts at denser concentrations (>1mM). Furthermore, a detectable red shift due to the fluorescence emission of the Rd6G–stained cancerous tissues appears respect to the Rd6G–stained healthy samples. In general, the spectral shift arises from high rate of the reabsorbing events within the neoplastic tissues. The intercellular micro-cavities allow the exogenous agents (Rd6G) to be trapped leading to a larger red shift when the molecular transitions are excited at 532 nm. The micro-partitioning nature of the cancerous tissues originating from proliferation of tubules contributes to the slow-down of the self-quenching rate of fluorophores–unlike the normal ones. These observations are not seen in other tumors than the breast tissues, because no tubules or micro-cavity structures are found to be present in others.
In summary, LIF in bare dense solutions deals with three competing mechanisms: (i) the collision rate increases at higher concentration, leading to self-quenching of the excited fluorophores, (ii) the simultaneous rise in the index of refraction leads to a longer optical path and a lengthier random walk, giving rise to a higher reabsorption rate, leading to a measurable red shift. Furthermore, (iii) higher concentrations of fluorophores may agglomerate the molecules to form new compounds, thus reducing the dye concentration and switching to blue shifts. However, this is drastically different in the case of stained tissues, where the imbedding of fluorophore molecules in the biological scaffolds acts to significantly subdue the collisional rates. The separating effect of the higher number of tubules in a tissue can prohibit the molecular agglomeration and thus hinder any density reduction which can cause spectral red shifts switch to blue shifts. As a consequence, this allows LIF of (tissue + fluorophore) at higher dye concentration enjoy a detectable red shift up to 8 nm, as seen.

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