Laser-induced fluorescence: Progress and prospective for *in vivo* cancer diagnosis

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Cancer diagnosis and treatment are of great interest due to the high death rate of cancer. To improve the cure rates of cancer, a diagnostic tool which can detect and treat cancer at initial stages is great needed. Laser-induced fluorescence (LIF) is an adequate analytical technique with advantages of high sensitivity, low sample consumption, short testing time, and suitable for *in situ* testing. Therefore, it has become one of the most widely used spectroscopic methods for cancer *in vivo* diagnosis in recent years. This review mainly focuses on the applications of *in vivo* LIF to distinguish premalignant, malignant from normal tissues in a variety of organ systems, such as lung breast, colon, cervix, esophagus, and bronchus. The potential influence factors for cancer diagnostics and the subsequent suitability of the method to different applications are well discussed. Meanwhile, the technical merits and weaknesses of the LIF technology for cancer diagnosis are also evaluated. Furthermore, different exogenous fluorophores, endogenous fluorophores, and fluorophores synthesized in the tissue are compared on their active principle and effect contrast. The technical potentials of LIF for further development and future applications are also presented as well in this review.

Cancer is a group of disease characterized by uncontrolled growth and spread of abnormal cells. It is caused by both external factors such as tobacco, infectious organisms, chemicals, radiation and internal factors such as inherited mutations, hormones, immune conditions, and mutations that occur from metabolism. Now, cancer is the second most common cause of death in the US, only after heart disease. According to the statistics data from the American Cancer Society for 2012, about 1638910 new cancer cases were diagnosed in 2012. This data does not include noninvasive cancer such as urinary bladder, basal, and squamous cell skin cancers. According to the report, in 2012, about 577190 Americans die of cancer, more than 1500 people a day. In the US cancer accounts for nearly 1 of every 4 deaths [1]. Our world has an estimated population of around 6 billion, about 0.1 billion people have cancer. By extrapolation, cancer is fatal to millions of people around the world. However, almost all cancers from all anatomical sites have different diagnostic problems. Because many cancers have vague symptoms and signs, some anatomical sites are difficult to visualize deeper inside the body. There is a high mortality rate of the different types of cancers throughout the world because they usually are diagnosed and treated at a late stage. Thus, the diagnose of cancer at early stage is of great need, which can diagnose premalignant or malignant tissues at an early stage to reduce the mortality rate [2].

In recent years, many fluorescence spectroscopy measurement techniques, such as laser-induced fluorescence (LIF), fluorescence correlation spectroscopy (FCS), photodynamic diagnosis (PDD), and photodynamic therapy (PDT) are applied in biological and biomedical investigations and disease detection. In which, LIF is one of the most widespread spectroscopic methods, especially in diagnosis of the cancer [3–6]. LIF is a promising new adjunctive technique

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for *in vivo* tissue diagnosis. Due to its many advantages such as safe, non-invasive, high sensitivity, short testing time, low sample consumption and *in situ* testing, which have been reported in an enormous of LIF application papers, LIF becomes an adequate analytical technique for tumorous diagnosis [7–11]. In fact, LIF has a long history. Initially, Lannuzzi [12] made a research on the polarization of laser-induced fluorescence in anthracene in 1965. Since then, LIF began to be applied in measuring the spectroscopy of diversiform elements, compounds, and metallic oxldes. In 1970, Johnson et al. [13] used a pulsed nitrongen laser to induce resonance fluorescence in BaO, MgO, and PbO. Hickman and Moore [14] used laser to induced the fluorescence in rhodamine B and algae. In 1977, Jackson et al. [15] using LIF to detection the stained rodent blood of plasmodium yoelli, trypanosoma gambiense, and trypanosoma equiperdum. Since that time, LIF began to be applied in biomedicine area. In 1993, Kohl et al. [16] used LIF to image superficially growing tumor, laying a foundation for the detection of cancer via LIF imaging method. Till now, LIF was widely used for distinguishing premalignant, malignant from normal tissues in a variety of organ systems, such as lung [17,18], breast [18,19], colon [20,21], cervix [20,22], esophagus [17,23], bronchus [17,24], head and neck [17,24].

As a kind of high efficiency analytical technique with great impact on the diagnosis of diversified diseases including the cardiovascular disease, a detection limit of LIF can reach to 10^{-13} mol/L and even single molecule level for the fluorescent probe with high fluorescence efficiency [25–27]. For the aim of a more effective detection result and more widespread biochemical *in vivo* application, LIF technique has been coupled to a number of other detection methods frequently. Microdialysis on-line with capillary electrophoresis with LIF detection technique can be applied to monitor naphthalenedicarboxyaldehyde(NDA)-derivatives or amino acids to diagnose many neuro-debilitating diseases, such as epilepsy and Alzheimer's disease [28]. LIF coupled with optical coherence tomography (OCT) has been investigated for a vast array of applications such as intracoronary stenting and imaging the anterior chamber of eyes [29]. Time-resolved laser-induced fluorescence spectroscopy (TR-LIFS) is an adjunctive tool for delineation of tumor from surrounding normal tissue and the intraoperative rapid evaluation of brain tumor specimens. This technique can be used as a tool for the identification of meningiomas [30]. Other techniques coupled with LIF were used to diseases diagnosis such as LIF and mass spectrometry (MS) with high performance liquid chromatography (HPLC) which could use in metabolism of doxorubicin [31]. In future, the multifarious technique coupled with LIF will be one of the great important development directions to LIF with the application in biochemical and clinical disease diagnosis areas.

The high sensitivity and specificity of LIF and the speed with which the assay can be performed have made LIF a powerful technique with numerous applications such as detection of the cancer, monitoring of pollutants, nuclear waste products, combustion products, food detection, and plasmas [32]. But the application in tumorous diagnosis has the great important meaning for a timely cure and higher survival rate to cancerous patients. Therefore, numbers of researchers focus on the researches about how to use LIF technique for an effective and accurate detection on various kinds of fatal cancer.

1 Feasibility of LIF applications in cancer diagnosis

1.1 Fluorescence spectroscopy

Fluorescence spectroscopy technique involves the illumination of tissue with light and the collection of the light returned from the tissue, then generates a spectrum with intensities over a specified wavelength range.

Figure 1 shows the basic principles of flourescence spectroscopy [2]. Fluorescence is typically the emission of light from aromatic molecular depending on excited states. Fluorescence is the luminescence in the molecular which absorb a photon to a higher energy state (the excited state). This state triggers the emission of another photon with a longer wavelength. The electron in excited orbital is paired to the second electron in the ground state orbital. When returning to the ground state it is spin allowed and occurring rapidly by emission of a photon [33]. A fluorophore will not fluoresce in its stable configuration (ground state or a relatively low-energy state). Since the fluorophore is unstable at high-energy configurations, it will reduce to the lowest-energy excited state by undergoing several non-radiative
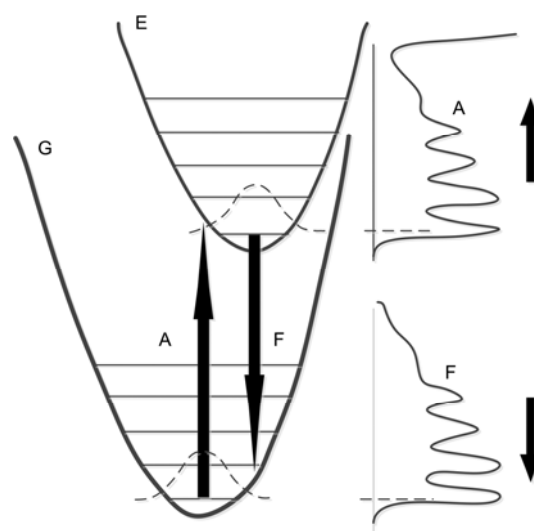

Figure 1 Basic principles of fluorescence spectroscopy (F). A, absorption spectroscopy; G, the electronic ground state; E, the lowest eletronic excited state of the molecule. Reprinted with permission from [2], copyright 2013, Taylor & Francis Inc.

deactivation processes (semi-stable) then decays back to the ground state and release or emitted the excess energy as light. The lifetime of fluorescent states is approximately 10^{-5} to 10^{-10} s. The fluorophore can absorb light energy again and repeat the entire process. The energy of the fluorescence photon is lower than the energy of the absorbed excitation photon generally due to the radiative loss in the excited state. Thus, the wavelength of the fluorescence is longer than that of the excitation. The magnitude of the Stokes shift is determined by the chemical environment of the fluorophore and the electronic structure. The Stokes shift is fundamental to the sensitivity of fluorescence techniques as it allows the emission light to be separated from the excitation light. The common factors which affect the fluorescence intensity of the emission spectrum are temperature, solvent effect, pH, dissolved oxygen, concentration, and the structural rigidity of the fluorophore [33,34].

Fluorescence spectroscopy has been successfully applied at research and clinical levels to detect the pathologic and physiological transformations of biological tissues such as neoplasia, dermal lesions, and atherosclerosis plaques with high sensitivity and specificity [35–39]. The main advances in the use of fluorescence as a diagnostic and analytical tool are the fact that high sensitivity fluorescence can be detected versus a dark background. This makes fluorescence one of the most sensitive tools available for the detection trails. As a sensitive, non-invasive method, fluorescence is often used in microscopy and the measurement of the emission intensity of the sample at very low concentration of fluorophores in biological systems [40]. Applying specific dyes to a sample which generate light of one wavelength range in response to the excitation light is capable of generating impressive contrasts and resultant images, whereas, the application of extrinsic dyes makes them less suitable for many *in vivo* applications. Auto-fluorescence makes it possible for a number of *in vitro* and *in vivo* biomedical diagnostic applications.

1.2   Fluorophores

Exogenous fluorophores, endogenous fluorophores and fluorophores synthesized in the tissue from a precursor molecule that is given externally are three main type of fluorophores used for cancer diagnostic in LIF. Cancer tissues have an increased rate of metabolism and gives rise to alternation in endogenous fluorophores. As a result, its spectral characteristics are different from normal tissue. Then, using LIF spectroscope for the diagnosis of tissue pathologies is possible. Firstly, the detection discrimination algorithms are based on varying of concentration of biological chromophores. On the other hand, the detection of the disease tissue based on the fact that the exogenous chemical probe has the ability to selectively accumulate in the diseased areas [41,42]. Tissue fluorescence originates from several endogenous fluorophores including structural pro-

teins (elastin, collagen), amino acids (tryptophan, tyrosine and phenylala-nine), porphyrins, enzyme cofactors (NADH, FAD), and flavins. The emission of these fluorophores has been investigated both *in vitro* and *in vivo* [32,35,43–45]. Chromophores including NADH, collagen, elastin, porphyrins, and carotenoids, which are produced in excess from pathologies in tissues or presented in a normal state, contribute to the emission spectrum using for interpretation of diagnostic results along with other parameters. The endogenous fluorophores such as collagen, elastin, amino acids, vitamins, lipids, porphyrin, (NA(P)DH), FAD, and riboflavin have a significant variation of concentration in different tissue types. These differences in the local environment within the tissue are essential for the discrimination between tumor and normal tissues by fluorescence spectroscopy. Compared with non-dysplastic tissues, the high-grade dysplastic lesions had a low collagen and high NAD(P)H fluorescence for *in vivo* detection of dysplasia in the cervix, esophagus, colon, bladder, breast, lung, and oral cavity. This trend and some other *in vitro* researches have demonstrated the possibility of LIF as a real-time and non-invasive diagnostic. Characterizing biological samples can be performed by steady-state fluorescence measurements such as cells or tissues in terms of overall intensity, spectral shape, and peak wavelength [40,46]. However, there may be possibilities of photodegradation of the analytes led by the high light intensity and the autofluorescence of the polymer chip can decrease the detection limit. Therefore, indirect fluorescence detection was demonstrated by means of incorporating the fluorophore into the background buffer, but this method is less sensitive than fluorescence.

Measuring a simple solution of several fluorescence could achieve a better effect than the situation that the increase of several complications make the fluorescence measurement in tissue more complicated [47]. While using LIF (usually with UV excitation) to detect intrinsic tissue fluorescence of auto-fluorescence, care must be taken to minimize the light exposure, especially to internal tissue [18]. The most appropriate wavelengths to excite protoporphyrin IX, coproporphyrin and uroporphyrin of protoporphyrin IX, coproporphyrin and uroporphyrin in clinically neutral and mild acidic conditions is 358–405 nm. Protoporphyrin IX (PpIX) is the most commonly used photosensitizer as an immediate precursor of heme. The rate of PpIX synthesis is calculated by the rate of synthesis of 5-aminolevulinic acid (ALA) in some tissues [48]. PpIX is selectively accumulated in cancer cells due to high metabolism rate. This property is being employed for 5-ALA-induced PpIX fluorescence detection of the cancer tissues. 5-ALA-induced PpIX belongs to the fluorophore synthesized in the tissue from a precursor molecule. Auto fluorescence measurements combined with 5-ALA-induced PpIX fluorescence measurements will greatly increase the sensitivity and specificity. Protoporphyrin IX, coproporphyrin, and uroporphyrin might contribute to the fluorescence of

carious tissue. Matošević et al. [49] used LIF to study the excitation of protoporphyrin IX (PP), coproporphyrin (CP), and uroporphyrin (UP) under different pH conditions, then compare LIF spectra of PP, CP, and UP with various PHs to the fluorescence of natural carious lesion. At last, they concluded that pH has an important influence on the light emission and absorption of porphyrins. While penetrating into the superficial mucosal cells and applying generally and topically, 5-ALA is considered as a good route for administration as it avoids the systemic side effects and only induces local photosensitization [50,51].

Most point fluorescence measurements make use of intrinsic fluorescence, but many exogenous fluorescence trails for *in vivo* diagnosis were reported as well. Nilsson et al. [52] used LIF to characterize the localization of a trimethoxylated carotenoporphyrin [CP(OME)$_3$] and trimethylated carotenoporphyrin [CP(ME)$_3$] in an intramuscularly transplanted malignant tumor and healthy muscle in female mice. Using a polychromator equipped with an image-intensified CCD camera to record fluorescence is in the region of 455–760 nm. [CP(OME)$_3$] and [CP(ME)$_3$] are shown to demarcate malignant from normal tissue, surrounding tissue at ratios of about 5:1 and 6:1, carotenoporphyrins are taken up by the kind of tumor and already show a consistent and good demarcation after 3 h administration and possible to clinically use. [CP(OME)$_3$] exhibited a more intense fluorescence than [CP(ME)$_3$] at the main peak, indicating the possibility of dose reduction and this could be of value in a clinical situation in the reticuloendothelial system which accumulates inherent substance characteristic. They suggest that CPs could be potential candidates for *in vivo* LIF clinical diagnosis [52].

Three years later, Nilsson et al. [53] investigated the biodistribution of [CP(OME)$_3$] and [CP(ME)$_3$] in mice by means of LIF via the of injection [CP(OME)$_3$] and [CP(ME)$_3$] into 38 tumor bearing mice and measured the carotenoporphyrin fluorescence of tumoral and peritumoral tissues as well as abdominal, thoracic, and cranial cavities tissues. A nitrogen laser pumped dye laser was used to induce the fluorescence at the emitting light of 425 nm and was analyzed by a polychromator which equipped with an image intensified CCD camera of 655 and 720 nm fluorescence.

Fluorophores in body could be considered as the medium to achieve the objective of the diagnosis, also the bridge connecting the macroscopic and microscopic in tumorous diagnosis. With the help of appropriate fluorophores, we can distinguish the cancered tissues from the normal ones. A series of effective, uninjurious, and novel fluorophores are expected to be developed in future.

1.3 LIF for cancer diagnosis

Recently, accurate diagnosis of disease as a method to evaluate the pathological changes in tissues has a great contribution to the clinical histopathological diagnosis. At pre-

sent, the universal method is biopsy [54]. But, one of deficiencies to biopsy is that the tissue sample of the body will be removed. Although biopsy can act as one of disease detection methods, it brings a lot of physical pain to patients. Only use this method may be subjective due to the discrepancy when tissue is *in vivo* or *in vitro*. Furthermore, this method may be time-consuming sometimes, even the final diagnoses may be delayed or inaccuracy in some complex cases. Additional potential motivation to eliminate the need for the surgical environment which required taking biopsy samples and histology is that the patients want reduced the health-care cost. Hence, the immediacy of diagnostic information is needed with the preponderance of minimizing the time of the patient for awaiting an answer and simultaneously reducing spiritual harm to patients [42]. For the past few years, people begin to focus on a new way for disease detection, which is "*in vivo*" disease diagnosis worked with optical method. The fluorescence technique can be automated and can offer real time detection and differentiation with precision, sensitivity, and selectivity. LIF have a major impact on the detection of cancers as a non-invasive diagnostic tool, which can identify diseased tissue sites in real time and in site [55]. Generally, the predictive accuracy of spectra is better than the prediction based on biopsy solely. Laser-induced fluorescence spectroscopy can minimize the need for repetitive biopsy [56–59].

As one of the optical technique to *in vivo* oncology diagnosis, LIF detection technique has disparate ways to differentiate premalignant and malignant lesion. One way that a group of researchers have taken is the method utilized exogenous or endogenous fluorophores and then detected the appropriate anatomical sites by fiber-optic probe in their trials, after the inducing by laser with specific wavelength, they will differentiate the normal and cancerous tissues according to the fluorescence intensity, the wavelength of the fluorescence or the fluorescence intensity ratio of primary and secondary peak for maximum accumulation in tissue [60–62]. This method belongs to single-point diagnosis. Another diagnostic method used by many other groups is that they utilized exogenous or endogenous fluorophores and an endoscope-based system bonding with a LIF detection system and an optical imaging system to get the image of the whole tissue. Therefore, such a method can be called imaging diagnosis. For instance, OCT is usually taken advantage as the imaging system to visualize mucosal thickening or abnormal mass development over the imaging time points [63–65]. In addition, time-resolved autofluorescence spectroscopy by LIF was commonly applied in cancer diagnosis as well. It based on the measurement of the spectrally resolved transient decay time of laser-induced autofluorescence emission from different tissue, i.e. via detect the fluorescence lifetime to differentiate the tissue. This method is not relevant to the scattering of the detection environment and the alteration of optical system but can't identify the location of cancer [66]. Simultaneously, other

auxiliary detection methods will be performed as well to get more convincing results such as the biopsy spectra analysis [17,67].

As our eventual goal is the elimination of the need to remove these tissue samples, during transitional stage of the clinical implementation of these optical technologies, we can use *in vivo* optical method bonding with biopsy diagnosis or provide additional guidance in locating the optimum sites for biopsy. Miscellaneous organisms issue will fluoresce when irradiated with light of a certain wavelength. This kind of auto-fluorescence spectrum will change with the physical chemistry and biochemistry properties of organisms issue, fluorescence characteristic is related to the structure of organisms issue and change of metabolism. Health issue has a major difference from cancer issue on their structure and metabolism, as well as the difference of fluorescence spectrum in their mucosa. Thus, according to the fluorescence spectrum differences between healthy issue and cancer issue, we can make pointed references to conduct *in vivo* detection. In this way, the veracity of biopsy technique can be improved immensely [68]. In addition, the algorithm used in cancer detection is also an important part at the process of investigating and distinguishing the normal and abnormal issues [69]. Amounts of studies or trials based on different significant algorithms were performed as a role of vital auxiliary step. Sensitivity and specificity are two values that the algorithm always cares [70,71].

Many groups have researched the differences between normal and cancerous tissues *in vitro*. But after the tissue separating from body, the content of the fluorescence substance will change. It will lead to misdiagnosis. New methods should be developed *in vivo* for the higher reliability in clinical application. *In vivo* tumor study method is great needed in future, but a mass of trials are also demanded to a further improvement of the sensitivity and accuracy in *in vivo* optical detection.

1.4   LIF based instruments

LIF technique bases on the molecular absorption of one or several photons from the laser beam, the probed species are pumped from the ground state to the excited state. Subsequently the radiation from the excited state is emitted as transitions take place in the final state. All or part of the emitted radiation is detected [72]. The excitation spectrum means the fluorescence intensity recorded as a function of the laser wavelength, the bandwidth of the laser determinates the spectral resolution in these excitation spectra [72]. With other advantages, such as short pulse excitation, wavelength tunability, and narrow bandwidth excitation, by using different excitation wavelengths and spectra analysis techniques, the development of neoplasia, the biochemical and structural changes of epithelial tissues can manifest in tissue via the fluorescence [73,74].

In a word, the two methods we get the fluorescence sig-

nal *in vivo* are single-point and imaging measurement with an optical probe placed on or near the surface of the tissue. The intent of these systems is for *in situ*, noninvasively and in real time diagnosis and to provide diagnostic signatures. While using a small fiber-optic probe to measure the fluorescence at a single tissue site, point measurement provide a great range of spectroscopic information about the localized tissue site. In addition, a filtered video imaging technology is used to image of larger tissue surface areas. The criteria which used for evaluation of the efficacy of these researches are the sensitivity and specificity. We defined sensitivity as the percentage of diseased sites found to be abnormal by fluorescence diagnostic metric and specificity as the percentage of normal sites found to be normal by the fluorescence diagnostic metric.

Figure 2 is the LIF experimental set-up for *in vivo* cancer diagnosis. The light sources that are used to induce the fluorescence have various types such as laser, lamp, and LED. One method to distinguish lamp and laser induced fluorescence sources is that the most commonly used lamps are deuterium, tungsten or xenon. however, an argon ion (Ar^+) laser at 488 nm [75], a helium-cadmium (He-Cd) at 325 nm [76], a red diode laser at 635 nm [77], a Nd:YAG diode-pumped solid-state laser at 532 nm [78], and a helium-neon laser (He-Ne) at 633 nm [79] have usually been used as common excitation sources for LIF [80]. Generally, the fluorescence detection system is expensive and limited by the range of excitation wavelengths offered by the laser. The bulky detection system may limit the applications in portable testing and sensing, as well as diminish the benefit of miniaturization. Several attempts have been made such as coupling microchips with LEDs as the excitation source and detecting with photodiodes, yielding detection limits of approximately 25 nm for fluorescein [81–83]. The UV-blue wavelengths are quickly absorbed in tissue. In addition, growing numbers of studies use red-shifted excitation wavelengths for their increased penetration depth in tissue. It was reported that emission wavelengths producing the highest contrasts are between 400 and 480 nm while the peak is at 405 nm [84,85]. The fluorophores in this near-infrared region (about 600–1000 nm) are not as well characterized as in the UV visible spectrum, bio-
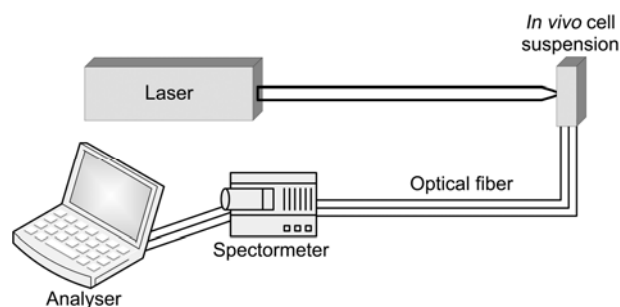

Figure 2   LIF experimental set-up for *in vivo* cancer diagnosis.

constituents such as flavins and porphyrins have been indicated as likely candidates [86]. Low power laser is directed toward biological component in LIF, such as biological fluids, single cells, cell suspensions, frozen tissue sections, and bulk tissues [87]. Till today, a large number of approaches have been studied for their ability to noninvasively diagnose tissue.

LIF probing technique has an explosive exponential growth with driving of laser technique, which has been widely applied in life science, material science, microelectronic technique fields, biology, chemistry, and clinical diagnosis in recent years.

The high sensitivity and non-invasive speciality of fluorescence spectroscopy technology, the photonasty and good sensitivity intracorporal fluorophores, the accurate and flexible measurement instruments, the appropriate and correct algorithm, make LIF a high efficient and feasible analytical technique to diversified cancers *in vivo*.

2 Applications of LIF in cancer diagnosis

Generally, there are three steps in clinical cancer diagnosis applying LIF technology, i.e. *in vitro* diagnosis, *in vivo* diagnosis and clinical application. The step of *in vivo* diagnosis is essential before using LIF for clinical cancer diagnosis. The discrimination between normal and malignant cells, systematically characterize the differences in fluorescence properties such as the fluorescence peak area, intensity variations, signal shape factor, extinction coefficient, relative quantum yield, and area ratio. After inducing by laser, the fluorescence spectroscopy and specific signal intensity give us basic information about the difference between normal tissue and abnormal tissue. For instance, many measurements show that during comparison of fluorescence spectroscopy of normal bronchial tissues and tissues with severe dysplasia or cancer, differences in the fluorescence intensity were obvious. By analysis of fluorescence or the green and red signal intensity ratio, more objective basis for the diagnosis of precancerous and cancerous lesions will be given than evaluation by eye [17]. The majority of trails have demonstrated that LIF technique has the feasibility for *in vivo* cancer diagnosis.

2.1 Colon cancer

Colorectal cancer is the third most common cancer in both men and women, 103170 cases of colon are expected to occur in 2012 on a yearly [1]. An accurate, *in situ*, and real-time diagnosis method is needed for colon cancer detection. During the past three decades, LIF was used as a high-efficiency technique to detect colon cancer. For the diagnosis of colon cancer, some difficulties make it difficult to guarantee the validity of the detection results. The complexity of fluorescence environment has great impact on the detection.

Bowel wall organization is a complex organism. After inducing by laser, the fluorescence will partly be absorbed and scattered in tissue. Thicker Mucosa has the stronger ability of absorption and attenuation fluorescent [88]. The fluorescent signals collected by auto-fluorescence spectra diagnosis system will change with the internal filtration efficacy and the microenvironment [89]. Thus, the demand to laser system and operator is very high. Only using fluorescence intensity to distinguish colon cancerous tissue or the size of the cancer tissue exists flaw due to the intensity of the laser, angle and distance of detection probe contacted with organization, complex constitute of colon tissue, and organization environmental. Those factors affect closely on the organization of the fluorescent intensity. As the calibration of laser energy is difficult to eliminate the effects of these factors, current method studying of fluorescence characteristics combined with more applications is the fluorescence intensity ratio.

Laser-induced auto fluorescence contributes to clinical diagnosis of precancerous lesions and incipient tumor with the effects of founding lesion, guiding *in vivo* diagnosis, and realizing "optical biopsy" [90–92]. It is generally acknowledged that the fluorescence peak from 460 to 505 nm is related to multiple endogenous fluorescence substances, such as NADH (excitation peak: 460 nm), FAD (520 nm), Collagen (390 nm) [92]. The change of those substances has great impact on the shape of fluorescence spectrum of the detection site [93]. Submucosa leads to auto-fluorescence of colon tissue. If mucosa incrassated, the fluorescence reduced due to the limited penetrability of laser and the increase of hemoglobin in colon issue. The thickness of healthy gastrointestinal cells mucous membrane is approximately 390 ± 100 μm, however, cancer tissue can be 800 μm or more. To healthy tissue, applying nitrogen molecular laser (wavelength: 337 nm), only 37% laser light can penetrate to mucosa. Moreover, the growth of invasive cancer tissue blocks the most light inspired on the mucous membrane obviously, so the fluorescence generated is very weak [94].

Tissue characterized by endoscopic fluorescence imaging via exogenous fluorescence was investigated to be a promising method for colon cancer detection. The fluorescence imaging system is developing to a low cost and modular way to adapt to the varying endoscope and light source configuration. Yova et al. [95] used two fluorescence probes for colon cancer diagnosis in *ex vivo* tissue sample with the development of an imaging system based on endoscopic fluorescence. Furthermore, Rhodamine B-L-leucine amide and rhodamine B-phenyl boronic acid were also used and presented high fluorescence quantum yield, good selectivity and photostability for colon cancer tissue. The distinctive binding of the rhodamine derivatives to transform colonic mucosa is a huge character to investigate the possibility of *in vivo* application [95].

Laparoscopic fluorescence diagnosis technique used in colon detection can increase the sensitivity and specificity of staging laparoscopy. Gahlen et al. [96] used 5-ALA induced Protoporphyrin IX accumulation to investigate the metastases by the fluorescence from peritoneal colon carcinoma, found that 5-ALA induced Protoporphyrin IX benefits for laparoscopic fluorescence diagnosis. Cothren et al. [97] developed a probability-based algorithm used in laser-induced fluorescence spectroscope *in vivo* to detect colonic dysplasia and evaluate that LIF has the potential to detect colonic dysplasia *in vivo* in 1996. Fluorescence spectra from normal mucosa and colonic polyps were collected during colonoscopy induced by the excitation laser of 370 nm. Tissues were classified as normal, hyperplastic and adenomatous by histologic examination. The fluorescence intensity at 460 nm and the ratio of the fluorescence intensity at 680 to 600 nm were devised with an algorithm to differentiate the tissue type. The 88% of algorithm accuracy of cases was determined, equals to the independent pathologists. Sensitivity, specificity, and positive predictive value were 90%, 95% and 90% for the detection of dysplasia.

2.2 Oral cavity cancer

Cancers affecting different anatomical sites of oral cavity now have an estimation of 22280 new cases and 15500 cases of fatality globally in 2012 [1,97]. Routinely suspicious oral lesions were mainly diagnosed by a general practitioner followed by invasive biopsy taken for a histological examination. Diagnosis and treatment of oral cavity cancers at early stages is more effective and less expensive. LIF has been proved to be highly valuable for early detection of oral cavity cancers with high sensitivity and specificity [97].

While applying LIF to detect the oral cavity cancer, it is usually postulated that the fluorescence spectrum differences from decayed and healthy dentin. The reason is mainly for the loss of mineralized tissue components along with increased presence of water in these tissues. Data are available and show that LIF can be applied successfully for the detection of pulpal remnants, pathological dentin, and microorganisms within the root canal [98].

The identification of optimal accumulation periods for 5-ALA-induced PpIX in different healthy anatomical sites of human oral cavity or different types of abnormal mucosa improves the accuracy of the clinical applications, such as photodiagnosis and tissue grading. Mallia et al. [99] demonstrated it by LIF *in vivo* with the excitation at 404 nm from a diode laser on anatomical sites of oral mucosa of 15 healthy volunteers and 15 patients, after topical application of 0.4% 5-ALA solution for 15 min. They observed that optimum time for maximum accumulation of PpIX is 90 min. In the trial, an expensive intensified CCD (ICCD) or EMCCD camera was applied to gather the fluorescence images of the entire lesion in a pony time frame, this device

could distinguish the spatial distribution of PpIX fluorescence in real time.

The algorithm also has a vital effect on the detection of oral cavity cancer *in vivo*. Gahlen et al. [96] studied with 16 patients who had cancer of oral cavity and 13 normal volunteers who had healthy oral cavity and they used the diagnostic algorithms based on spectral data acquired from LIF *in vivo*. A N_2 laser based portable fluorimeter was used to recorded *in vivo* auto-fluorescence spectra. They found the nonlinear diagnostic algorithm based on MRDF had a sensitivity of 93% and a specificity of 96% to cancer. This algorithm provided significantly improved diagnostic performance while comparing with the linear PCA based algorithm for discriminating the cancerous tissue sites of oral cancer patients, the healthy tissue, and the uninvolved tissue of the patients with cancer.

Another study was performed for the validity of dentinal fissure caries diagnosis *in vivo* through three measure methods including visual examination, bitewing radiography, and LIF with 41 Chinese young adults. Then they found that the combination of visual examination and LIF was superior to the other diagnosis methods with a sensitivity of 67% and specificity of 94% [100]. An optical device combined with LIF was performed. In this study, the LIF spectroscopy from oral and oropharyngeal issues were recorded *in vivo* at three different clinics in the research aimed at investigating the capability of this technique to discriminate between malignant and normal surrounding tissues [101]. A 600 μm optical fiber was used for guiding the laser light to the target tissue. In this trail, the fluorescence excited from the tissue was collected with the same fiber and fed to the optical multichannel analyzer. Two excitation wavelengths of 337 and 405 nm were used.

2.3 Cervical cancer

LIF has demonstrated promising results for early diagnosis and screening of cervical carcinoma. Colposcopy to get biopsies for histological examination is routinely performed, cytology smear has been applied for initial screening of cervical cancers and cervical intraepithelial neoplasia (CIN) associating with a 20%–30% false negative error rate. The application of LIF *in vivo* for the diagnosis of cervical carcinoma has a relative great sensitivity, specificity, and positive predictive value.

Ramanujam et al. [22] used *in vivo* LIF at 337 nm excitation to differentiate cervical intraepithelial neoplasia (CIN). 66 normal and 49 abnormal sites on the cervix in 28 patients were identified with a low-magnification microscope to view the cervix with reflected light. They developed a two-stage algorithm to diagnose CIN, differentiated abnormal tissues respectively with a sensitivity, specificity and positive predictive value of 92%, 90% and 88% from colposcopical normal tissues, differentiated preneoplastic and neoplastic tissues respectively with a sensitivity, specificity

and positive predictive value of 87%, 73% and 74% from non-neoplastic abnormal tissues. As tissue progress from normal to abnormal in the same patient, they found that a decrease of collagen fluorescence in the absolute contribution, an increase of reduced [NAD(P)H] fluorescence in the relative contribution, and an increase of oxyhemoglobin attenuation in the absolute contribution. There is a requirement for colposcopic identification of normal tissue prior to diagnose histologic abnormality and CIN in each patient and to the simultaneous interrogation of multiple site on the cervix allowing for the evaluation of effects of acetic acid at variable time intervals on tissue spectra following the application of variable amounts of acetic acid [22,102].

Georgakoudi et al. [103] quantify tissue biochemical changes *in vivo* with the combination of fluorescence, reflectance spectroscopic information, and the extraction of the intrinsic tissue fluorescence in the investigation on biopsy of uterine cervix. They found that the extraction of quantitative biochemical information *in vivo* from tissue, intrinsic fluorescence spectra provided a unique opportunity to study changes of the tissue without introducing excision and processing artifacts. In several of studies, a general trend is found that fluorescence from normal tissues is greater than that from abnormal tissues.

Another research was made by Ramanujam et al. [104] via developing a portable fluorimeter exciting at 337, 380 and 460 nm from 381 cervical sites from 95 patients. They used a multivariate statistical algorithm to analyze useful and extract clinical information of tissue spectra which acquired *in vivo*. Pap smear screening colposcopy was also used, they concluded that *in vivo* LIF technique has the meaning of improving the specificity of colposcopy and sensitivity of Pap smear screening. As LIF can interrogate the full thickness of the epithelium, it has other application in medical spectroscopy and gynecologic oncology when used as a detection tool of cervical precancerous.

2.4   Atherosclerosis

Nowadays, various researches on both neoplastic and atherosclerotic tissue are performed. *In vivo* LIF spectroscopy has a major impact on the detection and treatment of atherosclerosis as a non-invasive diagnostic tool that can identify diseased tissue sites in real time and in site [105].

One clinical study for the applicability of endogenous surface fluorescence spectroscopy *in vivo* to recognize atherosclerotic plaque was performed including 48 patients [106]. A 325 nm low power helium-cadmium laser was used via a flexible 200 μm optical fiber. Peak intensity, peak position, and shape index of the spectra were measured for the surface. Data were analyzed and compared with normal aorta site, a 46% decrease of peak intensity, longer peak position wavelengths and a higher shape index were demonstrated for aortic plaques. Prospective analysis of aorta and coronary spectra using an atheroma detection al-

gorithm shows a specificity of 73% for recognizing atherosclerotic sites and 100% for identifying normal sites. They demonstrated that remote human arteries with optical fibers by *in vivo* LIF spectroscopy in either a blood-filled or a bloodless environment and with a real time computer controlled algorithm is feasible, atherosclerotic can accurately discriminated from normal vascular tissue.

There exists a blood interference problem in *in vivo* application, selecting wavelengths where blood absorbs can possible be an approach to handle it. To protect the ratio from influences of the blood content, such an approach is frequently used in laser radar monitoring of atmospheric pollutants. LIF is a helpful tool to locate atherosclerotic lesion [107].

2.5   Upper gastrointestinal (GI) tract cancer

Upper gastrointestinal (GI) tract cancers are common in the Asian population. As these cancers are diagnosed usually at a late stage and often results in the failure of treatment [63,108–110]. Mayinger et al. [111] concluded that LIF spectroscopy might be a useful tool for *in vivo* detection of dysplasia and the early stage carcinoma in the upper GI tract. Lada et al. [112] explored the upper limit to temporal resolution with a microdialysis on line and LIF-CE for *in vivo* monitoring. They used it *in vivo* measurement to prove the utility of rapid sample and this system achieved multianalyte capabilities simultaneously and high temporal resolution for *in vivo* measurement which is not possible by microdiagnosis previously as the time-resolved observation of concentration changes in neurotransmitters. Rapid and a modest dialysis flow rate, on line derivation, high speed separation, and less than 10 s temporal responses were achieved.

LIF-OCT has clinical potential in identifying human gastrointestinal (GI) pathologies *in vivo*. Optical coherence tomography (OCT) is a kind of nondestructive, high resolution imaging modality, OCT has a vast array of applications such as intracoronary stenting and imaging the anterior chamber of eyes [29,113]. Image contrast is produced from backscattering of near-infrared light at optical index mismatches, achieves the resolutions of 5 to 20 μm. A cross-sectional images with short penetration depths (1 to 2 mm) was provided and can operate with either an air or water interface between imaging probe and the tissue [114]. OCT was applied in evaluation of imaging subsurface structures of human colon, duodenum, stomach, terminal ileum, rectum and esophagus *in vivo* [115–117]. The biochemical and structural complementary information provided by LIF-OCT is more sensitive and specific than either unaccompanied modality. A study of imaging neoplasms in the human cervix using OCT-LIF showed that a typical structure of scars appearing neoplastic in OCT was differentiated from cancer by the lack of abnormal LIF spectroscopy. False positives commonly produced by LIF from ab-

normally increased fluorescence in inflammatory reactions were differentiated from cancer by the lack of structural changes visualized with OCT. The combination of two modalities produced fewer false-positive results than either modality alone [115,118].

2.6 Skin cancer

Skin contains elastin, collagen, keratin, and NADH and these chromophores will contribute to the fluorescence spectrum. Non-dysplastic nevi can be distinguished from melanoma and dysplastic nevi by using LIF spectrum. LIF can also be a diagnostic tool for diagnosis of skin malignancies, which diagnosed by histological studies of skin biopsies routinely [119]. One of the studies made by Vo-Dinh et al. [120] demonstrated an application of fluorescence spectroscopy in *in vivo* diagnosis of nonmelanoma skin cancers with a high accuracy. A system consisting of a 410 nm laser and a multichannel analyzer was used. A fiber-optic probe was used for excitation of tissue and collection of fluorescence emission. Forty-nine patients participated and more than 200 measurements sites from abnormal and normal tissues were detected. They concluded that the excitation at 410 nm and applying the intensity of emission signal are highly accurate for detection of basal cell carcinoma and squamous cell carcinoma of the skin.

Laser-induced fluorescence spectroscopy (LIFS) for *in vivo* detection of skin pathologies has also been studied, although the investigation involved a small number of patients. Chromophores of the skin are expected to make a contribution to the fluorescence spectrum as well as some purely absorbing chromophores such as hemoglobin and melanin [121].

2.7 Esophagus cancer

Violetblue excitation light used in endoscopic *in vivo* measurement was also investigated with the spectra of normal mucosa, dysplastic lesions and cancer in esophagus and stomach. Mayinger et al. [64] used a special light source which is capable to deliver the excitation of tissue auto-fluorescence via endoscope. The emitted spectra were collected with a fiberoptic probe and analyzed with a spectrograph. Biopsies were taken as well for definitive classification of histopathologic status. They found that abnormal lesions were associated with special changes in emitted fluorescence spectra compared with normal mucosa; they made a conclusion that the spectrographic records were influenced on the position of the probe (angle and distance) and the intensity of the illumination.

von Holstein et al. [65] investigated seven patients with Barrett's metaplastic epithelium and oesophageal adenocarcinoma by means of LIF after injecting low dose (0.35 mg/kg bw) intravenous photofrin. Measurements were performed by LIF at characteristic wavelength immediately after resection of the oesophagus, biopsy specimens were collected as well. In addition, the fluorescence ratios of photofrin fluorescence quotient were calculated. A 15–30 locations were recorded with LIF in each surgical specimen from normal, Barrett's epithelium, and tumor tissue. By analyzing the data, adenocarcinoma shows the highest fluorescence ratios and can be distinguished from all nonmalignant tissue. Metaplastic Barrett's epithelium has a higher fluorescence ratio than normal mucosa. It was powerful evidence and proves that LIF is an effective technique used as an aid in diagnosis malignant transformation of oesophagus cancer *in vivo* during endoscopy for a real time tissue characterization.

2.8 Other applications of *in vivo* LIF in disease diagnosis

The CE-LIF technique only requires negligible sample volumes to analyze, which is especially suitable for the process of preparing functionalized nanoparticles with the properties aimed at applying for diagnostic or therapeutic purpose [122]. Microdialysis sampling provides a unique approach to the continuously biological materials sampling problem produced either *in vivo* or *in vitro* [123,124]. Microdialysis is widely used *in vivo* monitoring of extracellular neurotransmitter concentrations [125]. But it has been previously limited in great cases by its poor temporal resolution [126]. CE-LIF improved the temporal resolution of microdialysis significantly [127–130]. Parrot et al. [131] reported a high sampling rate, in the range of 5 s to 1 min by using CE-LIF for simultaneously analyzing catecholamines and amino acids in microdialysates. CE-LIF allows determination of all riboflavin vitamers far below physiological concentrations [132]. As amino acids have an important effect in many physiological processes including transmission, aging, memory, learning, neuronal survival, dendritic outgrowth, synaptic plasticity, and regress [133–136]. If excitatory amino acid system was in a state of dysfunction, many neurodebilitating diseases, such as epilepsy and Alzheimer's disease may be found [137]. Thus, this couple technique has a great meaning in disease detection.

Green fluorescence protein (GFP) also can be used to imaging of whole-body. One trial using GFP was investigated to test the efficiency of gene carriers for *in vivo* transduction. This study is aimed to determine the sensitivity and accuracy of GFP by *in vivo* investigation of tumor cells. Using a laser excitation source and band-pass filters should match to GFP specifically and constitutive tissue fluorescence emission bands. The GFP fluorescence image process need CCD camera to subtract background tissue from auto-fluorescence. Wack et al. [138] achieved 100% sensitivity and specificity for a *in vivo* investigation of pancreatic tumor after subcutaneous grafting or orthotopical implantation in nude mice pancre [139].

The clinical trial using LIF *in vivo* to detect the precancerous lesions and cancer including carcinoma *in situ* (CIS) is performed while it's difficult to be detected by white light bronchoscopy. Kusunoki et al. [17] examined 65 subjects with suspected lung cancer by the method of examining the endoscopic spectrofluorometric, analyzing pixels of LIF images composed of digital signals for the intensities of green and red and performed biopsy on 216 lesions. The sensitivity and specificity for detection of severe dysplasia or cancer using conventional bronchoscopy were 89.8% and 78.4% using LIF. In the trial, normal bronchial tissue, severe dysplasia and cancerous tissue showed different spectra. The green and red intensity of cancers on histograms of LIF images generally was greater than the ratio of metaplasia or normal bronchial wall. In addition, the endoscopic ultrasonography using LIF can show deep invasion of the bronchial wall [140].

Khosroshahi et al. [87] used a 405 nm LIF to distinguish normal and malignant bone cells and found that fluorescence intensity and the area under the spectroscopy of malignant bone cells were less than that of normal, in addition, the area ratio and shape factor were different as well. They used fluorescence sodium emission spectra. The relative quantum yield of bone cells is numerically determined.

In addition, there were also researches about noninvasive diagnosis with LIF of diabetes mellitus [141], the metabolic state of the cornea [142], *in vivo* thyroid tissue detection [143], the possible treatment of arrhythmia [105,144], possible diagnoses of transplant rejection [145]. Whether the laser beam causes damage to the myocardial tissue is lacking such studies in human heart tissue until now. If limitations are overcome, LIF may become an excellent tool for diagnoses of transplant rejection.

As an adequate analytical technique with advantages of high sensitivity, low sample consumption, short testing time, and suitable for in situ testing, LIF can be applied not only in the representative diseases above, but also some other neoplastic organ systems, such as breast and bronchus, and other systems like skin. We have screened all the typical diseases illustrating the feasibility and the high efficiency of LIF technique for *in vivo* cancer diagnosis.

3 Future development and conclusion

Continuing experimental researches have demonstrated that LIF could plausibly operate with conventional techniques such as biopsy and arteriography in a synergetic manner when it is an adjunct to standard endoscopic techniques such as laparoscopy and angioscopy. Long term clinical studies should further improve the sensitivity and specificity of LIF, especially when it is combined with imaging. This novel technique is slowly shifting to the clinician. The experience during the clinical evaluation will be an important factor of the development of this potential diagnostic tool.

Active collaboration between medical scientists, physicians, surgeons, biochemists and biophysicists also can facilitate the development of the biochemical diagnosis with LIF. Numbers of parameters which may affect the diagnosis are still under studying. Time gating of a charge-coupled device camera's intensifier can improve the signal-to-noise ratio in fluorescence imaging of cancerous lesions. The combination of fluorescence spectroscopy with endoscopy will greatly promote the early diagnosis of cancers anatomically deeper inside the human body. Flexible fibers and body core can solve the demands of a daily medical care practice. The laser induced measurement system for endogenous fluorescence spectroscopy can be applied during the routine upper endoscopy noninvasively and in real time using both video endoscopes and glass fibers. This makes it possible to obtain a map of endogenous fluorescence spectra easily.

Some fluorophores produce broad and overlapping absorption and emission spectra, it makes quantitative measurements very difficult. In addition, absorption and scattering phenomena are possible to interfere with the acquired signals from superficial tissue layers. Improvement in instrumentation and single analysis algorithms will increase the sensitivity and efficiency of LIF diagnostic techniques for cancer diagnostics. Developing new highly selective fluorophores with narrow emission bands is very important. Skin photosensitizations also need to be found. Cancer diagnosis can increase the effect in early stages on fluorescence spectroscopy with fluorescence spectroscopy [2]. To truly test the effectiveness of noninvasive diagnostics, some *in vivo* clinical trials must be performed with great significant mass of patients, but now, some of these studies are performed only in animal models. Thus, there is a great need for clinical trials on a large scale to prove the validity of these new diagnostic techniques.

In future, applications in biomedical field may develop in other different areas, such as *in-situ*, non-invasive study of fluorescent heavy metals within biofilm systems in an effective way [146]. As photosensitizer distribution heterogeneity influences of the overall photodynamic therapy response, dosimetry studies should be considered. Even single molecules can be detected and characterized by their fluorescence. It's meaningful to research single molecule technology tracking to follow the behavior of single biochemical species in a single living cell [87].

Fluorescence spectroscopy is an emerging excellent diagnostic tool for many diseases, especially in the early stage cancers. LIF is shown to be a more sensitive, high efficiency and rapid diagnostic tool compared with many routine medical diagnostic tools. This review focuses mostly on the applications of LIF, to distinguish premalignant, malignant and normal tissues in a variety of tumors, such as oral cavity, colon, cervix, esophagus, skin including some new area using this technique. Different exogenous fluorophores, endogenous fluorophores and fluorophores synthesized in the tissue are mentioned on their action principle, effect

comparison, the importance and needs for finding new auto fluorophores is discussed. The future development and application trend of LIF are presented as well in this review.

The LIF technique should be well established and characterized in human before used routinely in medical practice. There is a need for further studies and clinical trials to check the efficacy and validity of diagnostic systems on a large scale based on LIF technique. Accessing the target areas inside the human body with inflexible core bodies of instruments and fibers is difficult. Endoscopes, probes and portable devices which are used in routine medical practice should be oriented toward designing flexible systems. Natural fluorophores in body tissues and organs that can contribute to spectra need to be further studied.

The number of cancer cases increase every year, cancer have become a ticklish and deadly disease, which made so many patients lose their confidence to persist in the long and painful treatment. A significant effort has been made to find novel methods to conquer this problem especially in the early stage, therefore, trials of LIF for *in vivo* cancer diagnosis made great sense. For a better application of LIF technique to diagnose in body neoplastic tissues *in vivo*, there are lot of work need to be done in the future. A correct direction can guide us to plenty of new findings in trials

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