J Fluoresc (2008) 18:35–40
DOI 10.1007/s10895-007-0232-y

ORIGINAL PAPER

Laser-Induced Fluorescence at 488 nm Excitation for Detecting Benign and Malignant Lesions in Stomach Mucosa

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Received: 26 April 2007 / Accepted: 23 July 2007 / Published online: 17 August 2007

Abstract This work aims the detection of the histopathologic alterations of in vitro human gastric mucosa using spectral informations from laser-induced fluorescence spectroscopy (LIFS) technique with excitation at 488 nm (argon laser). A total of 108 biopsies with endoscopic diagnosis of gastritis and gastric cancer were obtained at the antral gastric region, from 35 patients with dyspeptic digestive complaints. The biopsies were collected during the endoscopic examination. On each biopsy fragment the autofluorescence spectrum was collected in two random points, through a fiber-optic catheter coupled to the excitation laser. The fluorescence emission spectra collected by the fibers were directed to the spectrograph and detected by the CCD camera. The spectra were then separated in groups (N, normal; LI, light inflammation; MI, moderated inflammation; CA, adenocarcinoma), based on the histopathology. The ratio between the emission wavelengths 550 and 600 nm was used as a diagnostic parameter. Analysis of fluorescence spectra was able to identify the normal tissue from adenocarcinoma lesions with 100% of sensibility and specificity. The ratio intensities between inflammation (light and moderated), although presented significantly statistical differences when compared to the normal mucosa, do not furnish enough sensibility and specificity for use as an identification method due to high variations. LIFS, with excitation of 488 nm, could be used in the differentiation of normal tissue and neoplasic lesions, assisting a less invasive diagnosis.

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Keywords Fluorescence spectroscopy · Stomach · Adenocarcinoma · Gastritis · Inflammation · Laser diagnosis

Introduction

Gastritis is an inflammatory process of the gastric mucosa, with wide and uniform distribution around the world. It is diagnosed essentially by the microscopical examination, and also by the endoscopic examination [1, 2]. Gastritis is usually expressed by enanthema, with or without erosions. The Sydney systematization aimed to define the endoscopic, histologic and ethiologic aspects of the disease. Due to the endoscopic alterations represent all the stomach, with focal, erosive and hemorrhagic alterations, the following diagnostics are used: (a) enanthematous pangastritis; (b) arthritis (that attempts only the antrus); (c) erosive; (d) hemorrhagic; (e) light, moderate and accentuated forms of inflammatory process [3, 4]. To the endoscopic diagnosis, it can still be added the micronodular aspect, suggestive of infection by *Helicobacter pylori* or erosive hemorrhagic, suggestive of injury by non-hormonal anti-inflammatory.

Among the malignant tumors that occur in the stomach, the carcinoma is undoubtedly the most important and most common (90 to 95% of incidence) [5]. The gastric carcinoma is a worldwide disease; however, its incidence varies widely, being particularly high in countries as Japan, Chile, Portugal and Russia, and 4 to 6 times less common in USA, Great-Britain, Canada, Australia and France. According to the Estimates of Incidence of Cancer in Brazil, there were foreseen 23,200 new cases of stomach cancer in 2006 (14,970 in men and 8,230 in women) [6].

In developed countries, the statistical data shows a decline of the incidence of gastric cancer, specifically in the USA, UK

and others Eastern European countries. High mortality is registered currently in Latin America, mainly Costa Rica, Chile and Colombia. However, the highest number of cases of stomach cancer occurs in Japan, where statistics show 780 cases per 100,000 inhabitants [6].

There are several factors that seem to affect its genesis, i.e. ambient, diet with nitrite-rich foods or cured (smoked), lack on ingestion of fresh fruits and vegetables, tobaccoism, chronic gastritis, infection by *H. pylori*, type-A blood group, familiar history. The search for a technique that could identify the disease in its early stage could save lives and costly procedures.

Many applications in Medicine are based on the ability of the light to interact with and modify the biological tissue, to be modified and also to produce therapeutical effects. Optical diagnosis could provide important information of the tissue constitution, fast and nondestructively. Since the endoscopic diagnosis depends on the physician's ability to identifying the abnormal tissue covering and surrounded by normal one, optical methods could help to increase diagnostic accuracy.

A variety of optical techniques have been proposed for distinction between benign and malignant tissue, such as the laser-induced fluorescence spectroscopy (LIFS) and the near-infrared Raman spectroscopy [7, 8]. The LIFS is a technique that shows promising in the precocious diagnosis of a variety of neoplasias, distinguishing precisely neoplastic tissue from normal one [9, 10]. The LIFS has potential to become an important tool for diagnosis of gastritis and gastric tumors. This technique is based on the sample excitation with short wavelengths (ultraviolet and blue) and the detection of the sample luminescent emission in long wavelengths (visible-red). Diagnostic is possible due to changes in the fluorophores presented in these tissues according to disease evolution and status, causing change in the fluorescent spectral information of different tissues [10, 11].

Laser spectroscopy is being used as a tool for diagnosis of atherosclerosis, colon dysplasia, esophageal and some types of lung and bladder cancers. LIFS technique presents possibility of being generated and collected remotely through fiber-optic catheters. For so, optical catheters could be introduced in endoscopes and others viewing instruments, allowing in vivo and real time analysis, without need of tissue removal [12–14].

In recent years, studies have been carried out aiming the development of diagnosis algorithms for several types of tumors, using the standards of tissue autofluorescence [12, 13, 15, 16]. These algorithms are based on the presence of characteristic prominent bands in the spectrum and calculation of the ratio between these spectral regions [10].

This work proposes the use of LIFS in the 488 nm excitation for the in vitro identification and classification of diseases of the gastric mucosa (gastritis and carcinoma) obtained from the antral region compared with normal tissues, through a spectrofluorimeter coupled to an optical fiber catheter for excitation and collection of the signal from stomach biopsy fragments. Fluorescence spectra from samples are compared with the histopathology and an identification method is implemented, based on the observed spectral differences among tissues. It is intended to collaborate with the development of a system for gastric diagnosis, helping to classify inflammation and diagnose carcinoma in real time.

Materials and methods

A total of 108 biopsy fragments from 35 patients submitted to High Digestive Endoscopy, at Gastroenterology Center of Basic Health Unit of Fernandópolis – SP – Brazil were used in this work. The patients were between 18 and 72 years old, both sexes, with dispeptic complaints, and the epigastralgy as the main one. After the approval of the Committee of Ethics in Research of UNIVAP, all patients signed a "Term of Consenting" and received detailed information about the objectives and methods of the research.

At the day of examination the patient was in jejum. Before the examination the patient received 50 drops of dimeticone (oral) and 10 mg of bromoprida (endovenous). For sedation it was used 15 mg of midazolan (endovenous) 40 min before the examination. For anesthesia of the tongue and orofaringe was used Xylocaine 2%. The routine endoscopy was done with an Olympus endoscope (GIF TYPE XQ20).

Three biopsies were collected of gastric antral region of each patient using biopsy clamps (Cook) and the collected tissue was labeled and immediately stored in liquid nitrogen cryogenic container (Cryometal, model DS18), so that did not occur morfostructural and biochemical changes in collected tissues. The samples where then carried to Laboratory of Fluorescence Spectroscopy for the accomplishment of spectroscopy. Three fragments of normal stomach were also taken to provide the spectral standard of normal tissue.

Laser-induced autofluorescence spectra were collected for each biopsy fragment as follows. The excitation laser was an argon-ion laser (Spectra Physics, model Stabilite 2017) in the wavelength 488 nm. This beam was deviated by a prism, to a convergent lens and coupled to the excitation proximal end of an optical fiber catheter (2 mm of total diameter, 250 μm the diameter of each fiber) described elsewhere [17]. Excitation laser power at the distal end was 2.0 mW. Through its central fiber, the laser beam reaches the tissue and after the laser-tissue interaction (absorption), emitted photons are caught by the collection optical fibers in the same distal end. The catheter collection proximal end was connected to the spectrograph (Chromex, model 3100) that dispersed the light

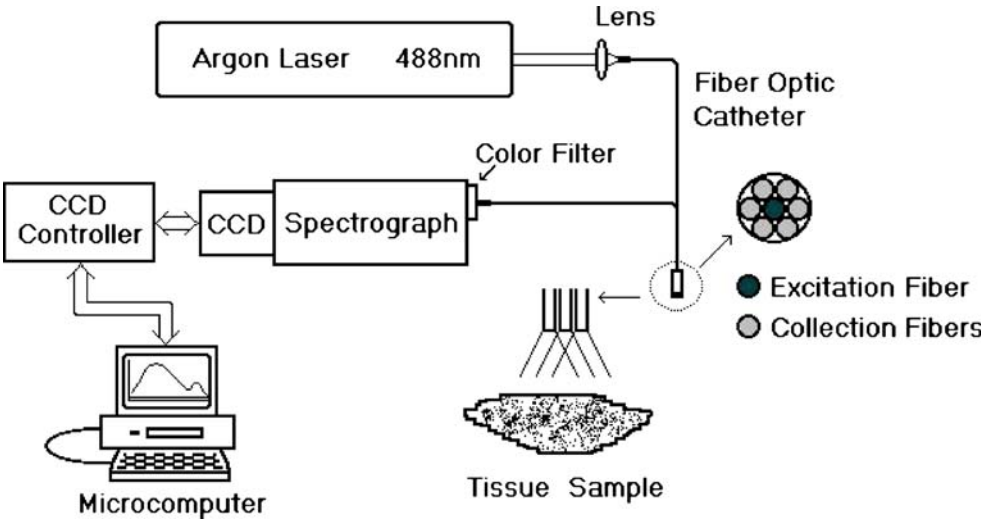

Fig. 1 Schematical diagram of the spectrofluorimeter with excitation at 488 nm (argon laser) for collection of autofluorescence spectra from gastric mucosa biopsies

in wavelengths of 490 to 700 nm, before passing through a lowpass filter with 490 nm frequency cutoff. The light was then detected by a CCD camera (Princeton Instruments, LNCCD 1024X256), and stored by a computer (IBM PC Pentium). A schematic of the system is shown in Fig. 1.

After spectroscopy, biopsies were fixed in formol 10%, the bottles were identified, labeled and submitted to histopathological examination.

Spectra were wavelength and intensity calibrated using Oriel calibration lamps (Oriel Corp, Hg–Ar spectral lamp and Tungsten lamp) and plotted using Excel software. Mean and standard deviation for some spectral regions were calculated as a part of the diagnostic algorithm.

All spectra were separated in four groups according to the type of injury found in the histopatological examination: normal tissue (N), light inflammation (LI), moderate inflammation (MI) and adenocarcinoma (CA). The autofluorescence spectra were then used to establish a criterion for differentiation based on the spectral differences among healthy and diseased mucosa. After spectral normalization by the most intense region, the average spectra for the four types of tissues were obtained, and the intensity ratio of two different wavelengths based on the analysis of mean spectrum that could give good differentiation were calculated and plotted.

For the statistical analysis of the ratio values of each tissue type compared to the other by means of ANOVA (analysis of variance) test, carried out through the Kruskal–Wallis not-parametric test, because the small number of samples in some tissue types (the normal one) recommends the use of a non-parametric test.

Results

Fluorescence spectra were taken from 108 antrogastric biopsies of 35 patients with light and moderate inflammation and adenocarcinoma alterations. Biopsies were obtained from patients with age between 18 and 72 years old, both gender. Table 1 summarizes main histopathological alterations found in all biopsies and the number of spectra taken from each tissue type. Only three fragments of normal stomach were taken to minimize the risk of such unnecessary biopsies (might cause bleeding and infection) and the disagreement of patient with longer procedure's time.

Each tissue fragment was spectroscopically analyzed with 488 nm excitation wavelength and fluorescence spectra collected in the region of 490 to 700 nm, in two random regions of the sample, getting a total of 216 fluorescence spectra. Sixteen spectra were eliminated due to low signal-to-noise ratio (lower that 5).

Spectra were separated in four groups, depending on the result of the histopathology (Table 1): six normal mucosa (N), 134 light inflammatory injury (LI), 28 moderate inflammatory injury (MI) and 32 adenocarcinoma (CA). All spectra were loaded and stored in an electronic spreadsheet (Microsoft Excel) for plotting, mean and ratio calculation. Normalized mean spectrum of each tissue type calculated using all spectra of same tissue type are plotted in Fig. 2.

Mean spectra present valleys in the region of 520 nm (intense) and 580 nm (less intense) that could be attributed

Table 1 Histopathological results of gastric mucosa lesion biopsy fragments

	Normal mucosa	Histopathological results		Carcinoma
		Light inflamation	Moderate inflamation	
Number of biopsies	1	24	7	3
Total of spectra	6	134	28	32

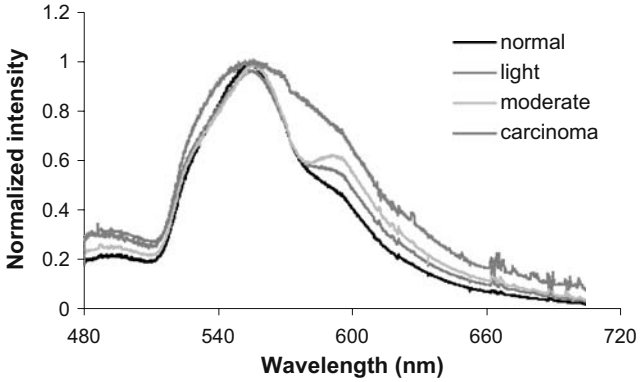

Fig. 2 Mean autofluorescence spectra from biopsies of gastric mucosa with excitation of 488 nm collected from normal tissue (N), light inflammation (LI), moderate inflammation (MI) and adenocarcinoma (CA) using optical fiber catheter

mainly to the absorption of hemoglobin from blood (Soret and Q absorption bands) [12].

It was observed that the mean spectra of different tissues (N, LI, MI and CA) present differences in the intensity profile in the region of interest for fluorescence spectroscopy (500 to 700 nm), with an increase of the spectrum intensity in the region of 600 nm following an increase of the gravity of the injury.

To develop an algorithm for disease classification based on the spectral differences found in each tissue type, it was calculated and plotted the ratio between two spectral regions (Fig. 3), and the results were tested for statistical analysis of significance. By analyzing Fig. 2, it was found that main differences can be expected in the region of 550 nm (normalized peak) compared to the one at 600 nm (less fluorescence due to blood absorption and lack of fluorophores emitting in the red for some tissues).

As mentioned before, the increased fluorescence intensity in the region of 600 nm can be observed in Fig. 3, where one can see the mean values and standard deviation of the ratio among 550 and 600 nm. This increased emission follows the degree of the disease.

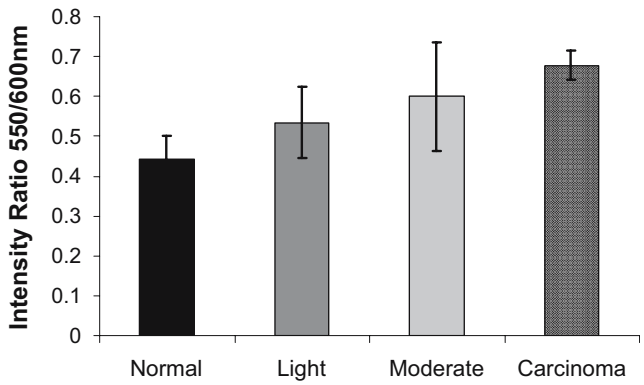

Fig. 3 Plot of mean ratio between 550 and 600 nm and standard deviation of normal mucosa (N), light inflammation (LI), moderate inflammation (MI) and carcinoma (CA)

Table 2 Results of Kruskal–Wallis statistical test applied to the ratio values of pairs of tissue types

	Significance test as a function of tissue type		
	Light	Moderate	Carcinoma
Normal	$p>0.05$[a]	$p<0.05$[b]	$p<0.001$[b]
Light	X	$p>0.05$[a]	$p<0.001$[b]
Moderate	X	X	$p<0.01$[b]

[a] $p>0.05$ is statistically non-significant
[b] $p<0.05$, $p<0.01$ and $p<0.001$ are statistically significant

ANOVA statistical test was applied to the mean ratio values of Fig. 3 to test if the ratio could give differentiation among all fragments of gastric mucosa. The variances presented statistically significant differences ($p<0.001$) among all tissues, and the use of a non-parametric, based on variance differences (Kruskal–Wallis—Table 2), comparing all columns of data between themselves was considered.

Statistical results showed that some of the values of the ratio between 550 and 600 nm present significance level higher that 0.05 (not statistically significant). The normal tissue and light inflammation do not present statistically significant differences, the same occurring between light and moderate inflammation. Normal tissue presents statistically significant differences when compared to moderate inflammation and the carcinoma, and tissue with light and moderate inflammation presents significant differences with regard to the carcinoma.

The scatter plot of the intensity ratio values of all samples used in the study is shown in Fig. 4. Although statistically significant differences had been found for tissues of type N compared with MI, and MI compared to CA, a good separation is not possible individually, without incurring into a great amount of misclassification.

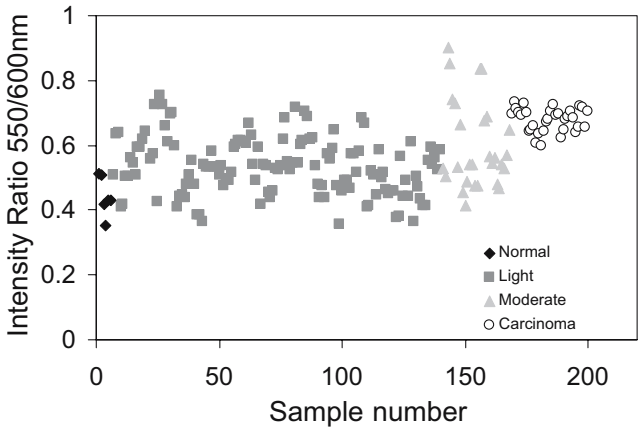

Fig. 4 Scatter plot of the intensity ratio between 550 and 600 nm from spectra of normal mucosa, light and moderate inflammation and carcinoma

The statistical result for the LI compared to CA showed high significance. Figure 5 shows its ratio value where it can be seen a good separation with high sensitivity for the CA tissue, with an empirical line drawn and separating the two groups.

Aiming to separate normal gastric mucosa tissue from carcinoma, also due to the high level of significance, the intensity ratio between 550 and 600 nm of the normal versus carcinoma were plotted in Fig. 6, and the separation line was also drawn empirically. This separation showed maximum sensitivity and specificity, since none ratio of normal spectrum coincided with the ratio of the carcinoma and vice versa.

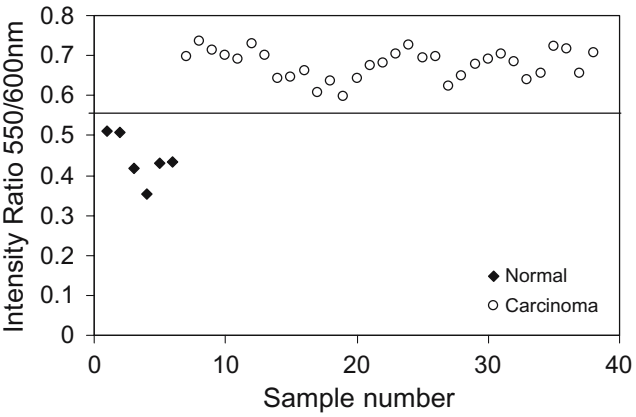

Fig. 6 Scatter plot of the intensity ratio between 550 and 600 nm from spectra of normal mucosa and carcinoma. *Empirical line* separates normal from carcinoma

Discussion

This work proposed the use of LIFS at 488 nm excitation wavelength for the identification of alterations in the gastric mucosa, specifically light and moderate inflammation and adenocarcinoma. The use of wavelength in the blue/green region, despite its lower absorption for many biological fluorophores (normally FAD/NAD and collagen), revealed satisfactory for the type of injury studied.

The developed algorithm, based on the spectral intensity ratio of two wavelengths – 550 and 600 nm – presents easiness of use and little mathematics. Selecting only few spectral features that show significant differences between tissues facilitates its employment, since an imaging spectrograph and CCD camera could be adjusted to rapidly get the intensity values for only a small spectral region.

The gastric cancer is a serious and fatal illness; however it is prevented and curable if precocity diagnosed, using high digestive endoscopy in patients under risk and with dyspeptic complaints. The high digestive endoscopy associated to fiber-optic based LIFS could be an easy, fast and safe method to identify suspicious lesions.

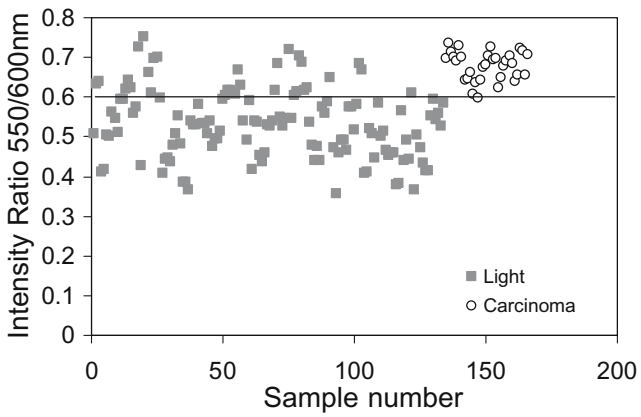

Fig. 5 Scatter plot of the intensity ratio between 550 and 600 nm from spectra of mucosa with light inflammation and carcinoma. *Empirical line* separates inflammation from carcinoma

Epithelial cells, due to the metabolic alteration and proportional to the gravity of the atypia, need greater energy transfer for its development, and this alterations could be detected through molecules in the cytoplasm, mainly the mitochondria and the activated carrying molecules, as NADH+ and FADH2 [18, 19]. It is known that the reduced NADH, oxidated FAD and collagen have fluorescent properties when excited by ultraviolet/blue light, with emission peaks at about 420 nm for collagen and 460 nm for NADH and FAD [20].

The LIFS could become a fast and effective examination method, being able to constitute an efficient diagnosis, preventing aggressive and unnecessary procedures.

The use of spectrofluorimeters with multiple excitation wavelengths, especially in wavelengths shorter than 488 nm, could make possible better diagnostic differentiation, mainly in the wavelengths that excite the autofluorescence of collagen, NADH and FAD in 400, 440 and 460 nm, respectively [19, 20]. One could accomplish research aiming to find out the ideal excitation wavelength for detecting best fluorophores for use in different illnesses that attack the stomach.

Conclusion

LIFS applied to gastric mucosa tissue in 488 nm was capable to identify the adenocarcinoma injury when compared to normal tissue, with 100% of sensitivity and specificity, using as algorithm based on the ratio between the intensities of the spectra at 550 and 600 nm. The intensity ratio for the tissue with light inflammation, when compared to the carcinoma, presented high level of significance, with a good separation and high sensitivity. The ratio for the light and moderate inflammatory tissue spectra, although present significant statistical differences

when compared to normal mucosa, did not present enough sensitivity and specificity for use as a method of tissue injury identification due to the high variation.

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