A SPECTROFLUORIMETER FOR DIAGNOSIS OF COLON DYSPLASIA

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ABSTRACT -- A new excitation-emission spectrofluorimeter with optical fiber light delivery and collection for use in rapid analysis of tissues in a clinical setting is presented. This system provides ten different excitation wavelengths, permitting collection of spectra from biological tissues in approximately. 600 ms. Fluorescence spectra were acquired in 10 patients who undergo routine endoscopic procedures for gastrointestinal disorders. Normal and adenomatous mucosa were studied with optical fiber contact probe throught the endoscope. We found that adenomas exhibit increased hemoglobin absorption compared to normal mucosa; adenoma fluorescence is less intense compared to normal tissue; and increased red fluorescence is observed in polyps. *Conclusions:* The multiexcitation provides information about optimal excitation wavelengths for the differentiation of normal and neoplasic colon and also the identity of the fluorophores which contribute to total tissue fluorescence.

Keywords: Fluorescence, *In Vivo* Diagnosis, Colonoscopy, Laser Spectroscopy

INTRODUCTION

Fluorescence spectroscopy is being explored in many laboratories for diagnosis of different diseases in human tissues. This often requires complete characterization of the absorption and fluorescence properties of normal and abnormal tissues over the entire UV-visible region. Such information can be conveniently collected and displayed in the form of an Excitation-Emission Matrix spectra (EEM) (Nelson, 1985). Up to now, EEM spectra have been acquired in commercial systems that do not take less than 1 hour for a complete set of data. Recently Oki et Maeda (1992) built a tunable dye laser for analytical spectroscopy based on a "rapid" dye cell exchanging system pumped by a high energy nitrogen laser. Therefore the required time to replace a dye cell within the next cell is about 0.6 second.

We have designed, developed and tested a Fast Excitation Emission Matrix system capable of collecting multi-excitation spectra of biological tissues in approximately 600 msecs in a reliable manner. In this arrangement, a nitrogen laser at 337 nm pumps a sequence of nine dye cuvettes. The N_2 pumping beam is also used as an excitation source, providing a total of 10 excitation

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// Trabalho recebido em 14/jul/97 e aceito em 29/nov/98 //

wavelengths, approximately uniformly spaced in the range 337 nm to 505 nm. Fluorescence spectra are collected in the range 350 nm to 700 nm by a spectrograph and a multi-channel detector. This setup also allows simultaneous measurement of diffuse reflectance from the same tissue site, by using a xenon flash-lamp, with important additional diagnostic information.

Autofluorescence spectroscopy has considerable potential for detection of colonic dysplasia since different tissues exhibit differentiation in the emission profile. Spectral data displayed in the form of EEMs provide complete characterization of the fluorophores and absorbers responsible for the observed fluorescence. EEMs are presented as fluorescence contour maps, where contour lines connect points of equal fluorescence intensity. Up to now, only *in vitro* EEMs are possible, because of the slow data acquisition time for commercial systems. This has led to *in vitro* artifacts and spectral distortion thus rendering this tool less effective. This new instrument can acquire EEMs and reflectance spectra in about 600 ms *in vivo* using fiber optic probes.

MATERIALS AND METHODS

An important component of an EEM system is the excitation source. Since gated detection on the time scale of less than 1 μs is needed for minimization of room light interference, we have selected a N_2-laser based excitation source for compactness and ease for coupling into fiber optic-based catheters. A dye laser pumped by a N_2 laser (300 μJ-pulse, 3 ns) allows the spectrofluorimeter system to operate over a wide spectral region with high laser intensity.

To predict the performance of the system, we estimated energy obtainable in each step of the light excitation path and fluorescence collection path. For the N_2 laser used in this system, with energy of about 300 μJ/pulse, a theoretical signal-to-noise ratio of 100 was calculated. For practical biological applications, it should be considered that a S/N of 100 is beyond our needs. It should be mentioned that this level of S/N ratio is obtained with a single pulse laser.

The pumping cavity design is opto-mechanically simple. It consists of a single resonant cavity (fixed cavity) which is capable of generating multiple excitation wavelengths (figure1).

The nitrogen laser pumps different dye cuvettes (Hellma Cells, QS-120) mounted on a rotating wheel which rapidly traverse the fixed cavity. Each cuvette has a different laser dye (Exciton) in which dye selection criterion is based on the good conversion efficiency when pumped with 337 nm, narrow untuned bandwidths, and emission peaks separated by 20 nm to cover the region from 360 nm to 500 nm. The system used the following dyes and respectively emission peaks: PBD, 359 nm; Exalite 384, 382 nm; Exalite 398, 397 nm; LD 425, 413 nm; Stilbene 420, 429 nm; Coumarin 440, 440 nm; Coumarin 460, 463 nm; LD 490, 485 nm and Coumarin 500, 505 nm. The measured dye emission bandwidths increase from 5 to 15 nm with increasing wavelength. Tissue absorption and fluorescence spectra are broad, and such bandwidths are not expected to affect the resulting spectral resolution.

Figure 2 shows a schematic of the full design illustrating the major components of the system. The N_2 laser is used as the excitation source. It pumps dye cuvettes mounted on a rotating wheel that rapidly traverse the cavity. Diffuse reflectance from tissue is obtained using a white light source

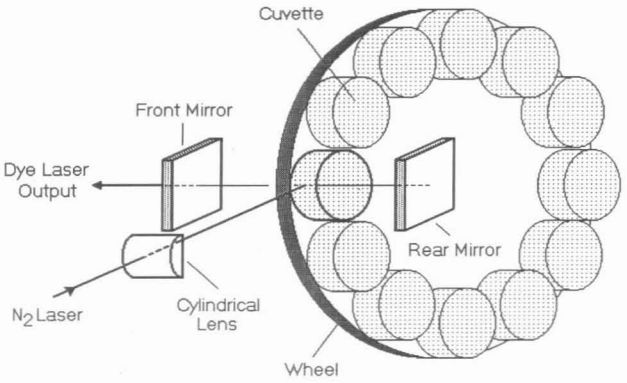

Figure 1. Nitrogen pumping dye laser configuration.

(EG&G, pulsed xenon lamp, 4J). Excitation beams from laser and white light are coupled into a 200 μm core diameter optical fiber catheter. We employ a fiber optic probe which is specially designed to provide a controlled and reproducible light delivery-collection geometry. The details are given elsewhere (Cothren *et alii*, 1986). Briefly, the distal tip of the probe consists of a central excitation fiber surrounded by an array of collection fibers (typically six) enclosed in a transparent, protective optical shield (Cothren *et alii*, 1986; Richards-Kortum *et alii*, 1989). To minimize specular reflectance the distal tip is angled by 17° from the direction normal to the optical fiber axis. The signal emitted from the tissue is collected by the probe and passed through long-pass filters (CVI Optics, color long-pass filter) to prevent scattered excitation light from reaching the detector. The filters are placed in a small wheel with 12 positions. The system uses 10 filters corresponding to the N_2 laser line and 9 dye lasers band emissions. The remaining two positions are kept empty; one is identified as "home" position and allows white light to pass for the reflectance measurements, and the other is not presently used. The proximal ends of the collection fibers are arranged linearly to form a slit at the entrance of the spectrograph (Jobin Yvon, model CP200 with 200 lines-mm). A lens is used to couple the light from the optical fiber to the spectrograph. The slit width is determined by the fiber diameter, and this corresponds to a spectral resolution of 3.5 nm. The OMA (Optical Multichannel Analyzer) detection system uses a gated intensified photodiode array (EG&G, model 1421).

A synchronization signal is produced by means of metal pins mounted on the dye wheel, in which each cuvette has it own pin. As the pins pass sequentially through an infrared sensor (emitter and detector in the same assembly), trigger signals are generated for external synchronization of the detector controller, laser and detector gate, permitting detection of respective fluorescence spectrum.

Prior to data acquisition and processing, the following calibration procedures should be performed. First, background spectra are collected to remove electronic offsets. A Hg lamp placed in front of the probe is used for wavelength calibration. The spectral response of the detection

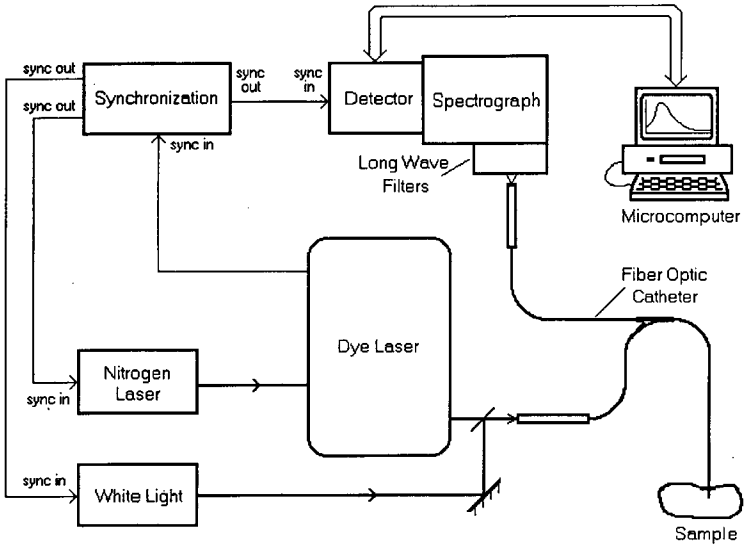

Figure 2. Block diagram of the spectrofluorimeter system. L1, L2, L3 and L4, lenses; M1 and M2, mirrors; TRIG, trigger source.

system is calibrated using a NIST traceable white light source. The optical fiber probe provides calibrated fluorescence intensities, as mentioned. However, in order to extract accurate intensity information, laser power changes due to differential photobleaching among various dyes and optical misalignment must be corrected. This is accomplished by means of a rhodamine B solution (8 g/l, ethylene glycol). The known quantum yield values of rhodamine B at various excitation wavelengths produced by the EEM system are then used to calibrate the intensities of the respective fluorescence spectra. For diffuse reflectance measurements, a suspension of $BaSO_4$ in water (20 g / 20 ml) is used to correct for the flashlamp spectral profile, and also as an intensity standard.

The spectrofluorimeter presented above, was used in clinical trial in order to obtain autofluorescence of colonic mucosal tissue and evaluate the use of fluorescence spectroscopy for early diagnosis. The experiments bellow show the performance of the described system.

CLINICAL STUDIES

Human colon tissue spectra presented bellow were obtained in endoscopic procedures to verify colon disorders. Clinical procedures were carried out in patients without severe pathologies. The patient preparation followed routine patterns for colonoscopic procedures.

Adenoma and adjacent normal sites were studied during the endoscopic procedure. To carry on this experiment an optical fiber catheter was introduced in the auxiliary channel endoscope, with its distal end lightly touching the mucosal wall. At the physician signal, the technician carried on the spectra acquisition. Fluorescence and diffuse reflectance were obtained in the polyp and normal spot. The optical catheter were substituted by a forceps introduced in the same auxiliary channel, removing a tissue fragment for histopatologic analysis. The histopatologic analysis is used in this case to validate spectroscopic results. In order to minimize the patient discomfort only three sets of spectra were performed each time. *In vivo* fluorescence spectra were acquired in a total of 10 patients. The excitation fluorescence spectra obtained with single wavelengths from normal and adenomatous colon tissue are shown in figure 3. These spectra are the same used to build the EEM maps bellow. In this case, we have ten different fluorescence spectra, each one representing one excitation wavelength laser. The view of each fluorescence spectrum and the superposition of normal-adenomatous spectra permits a better understanding between each emission spectra, identifying regions with emission differentiation and helping determine ideal excitation wavelength. This is very useful since in some cases the contour map does not show weak intensity peaks nor small variations on the fluorescence curves, as occurs in the 600-650 nm region.

RESULTS

Figure 4 shows a contour map for the colon normal mucosa. The lines in the contour map show the regions with the same fluorescence intensity, where we can observe fluorescence peaks and valleys (absorbers). In the normal tissue contour map, we observe maximum fluorescence regions in (λ_{exc} = 337 nm, λ_{em} = 390 nm), (λ_{exc} = 337 nm, λ_{em} = 460 nm) and (λ_{exc} = 460 nm, λ_{em} = 520 nm). It is also possible to observe high absorption regions, around 420 nm, parallel in both axes. This characteristic is more pronounced in the excitation axis, due to strong absorption of excitation radiation and reabsorption of emitted fluorescence.

The spectrum obtained from *in vivo* adenomatous polyp compared with normal tissue shows three folds less fluorescence intensity. This occurs mainly because the polyp has more blood irrigation than the normal mucosa. In figure 5 an adenomatous polyp contour map is shown, where we can observe fluorescence maxima in (337 nm, 460 nm) and (420 nm, 640 nm). In the same way as the normal mucosa contour map, a fluorescence minimum is observed in 420 nm, but in this case they are more pronounced and with larger bandwidth, as explained before.

Main finds can be summarized as follows: (a) adenomas exhibit three folds increased hemoglobin absorption attributable to high vascularization compared to normal mucosa; (b) adenoma fluorescence is less intense compared to normal mucosa and the peaks are mainly due to collagen, NADH and FAD (Yang *et alii*, 1995); and (c) increased red fluorescence in adenomas is assigned to porphyrin derivatives (Andersson-Engels *et alii*, 1990), which can be selectively excited with 400 nm and 510 nm. These results are promising not only for identifying dysplasia but also for analyzing underlying pathological features of tissues. Studies are under way for algorithm development based on multi-excitation fluorescence for accurate detection of dysplasia.

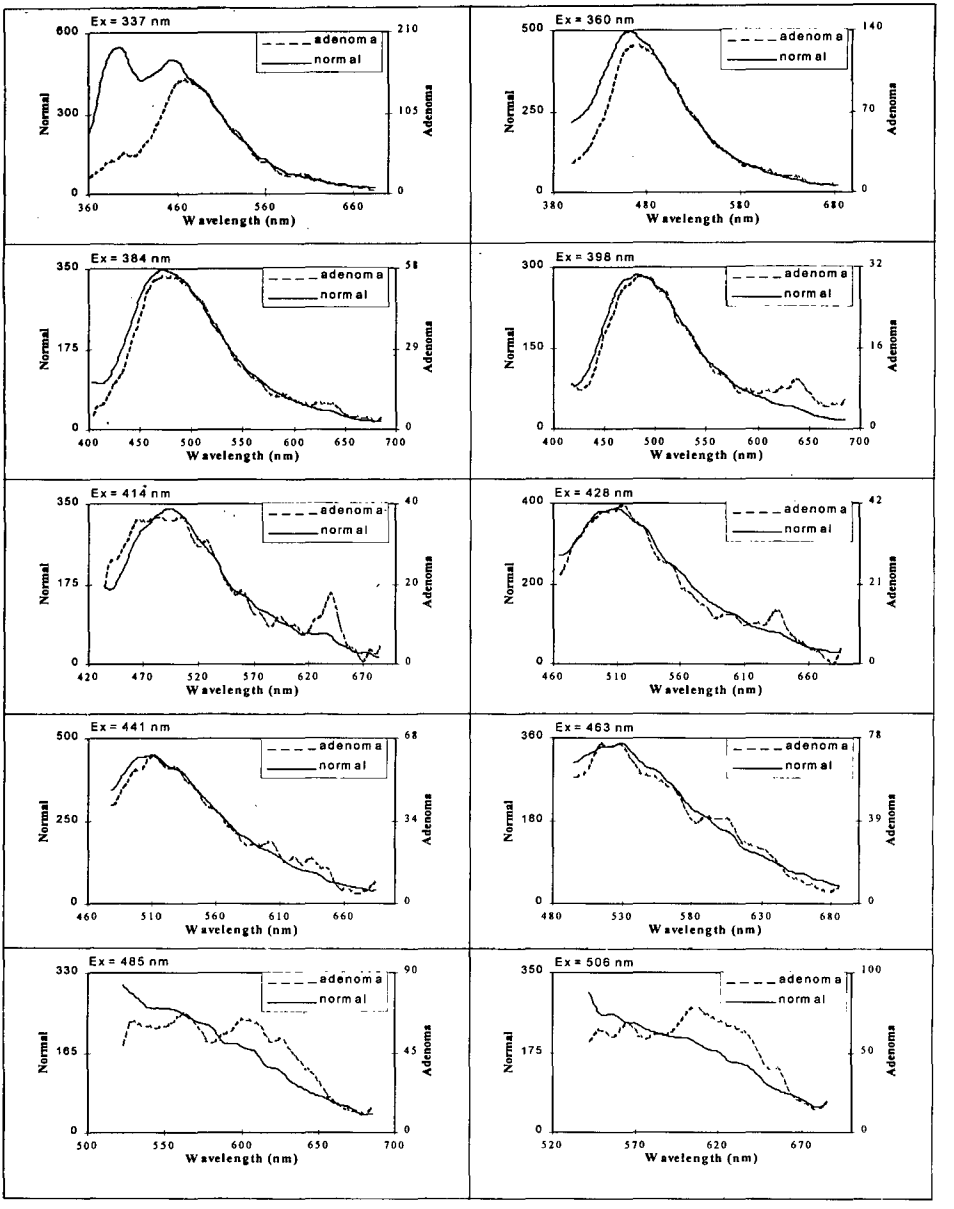

Figure 3. Set of fluorescence spectra obtained from *in vivo* colonic tissue excited by single wavelengths.

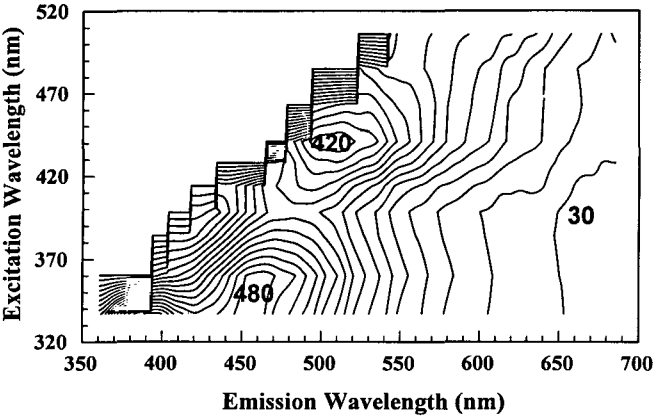

Figure 4. EEM map for normal colon mucosa obtained in clinical procedure.

The diffuse reflectance spectra for normal colon mucosa and adenomatous polyp is presented in the figure 6. Absorption bands corresponding to the "heme" group molecules can be observed, where the 420 nm band is due to Soret absorption. The two smaller bands in 540 nm and 580 nm are due to the absorption of oxi-hemoglobin Q bands (Johansson, 1993).

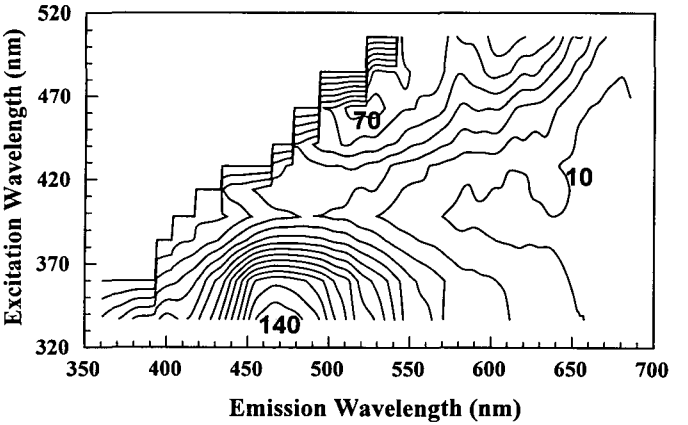

Figure 5. EEM map for adenomatous polyp obtained in clinical procedure.

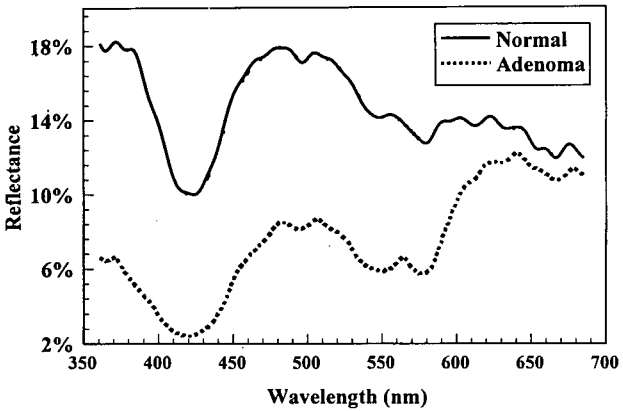

Figure 6. Diffuse reflectance from normal and adenomatous colon mucosa.

DISCUSSION

Analysis of results obtained from biological tissues using autofluorescence spectroscopy shows an important difference between normal tissue and adenomatous polyp intensity. This ratio of this intensity for the (337 nm, 460 nm) excitation-emission wavelength is around 3, and around 6 for the (460 nm, 520 nm). In polyp the occurrence of most intense blood circulation is the main responsible for the reduced intensity fluorescence, even though the biochemical variations due to morphological changes may introduce variations on the emission curves. In normal tissues, the peak corresponding to (337 nm, 460 nm) is due to the presence of fluorophore NADH, even though the collagen peak at (337 nm, 420 nm) has an important contribution to this fluorescence. In the biological tissue spectrum the collagen appears with two different bands, the band between 337 nm and 380 nm and between 337 nm and 460 nm due to emission reabsorption by the hemoglobin molecules. Polyps present lower fluorescence in part due to reabsorption by the blood and by the fact that polyps have less quantity of collagen than normal tissues. The reduction of intensity fluorescence in the band (337 nm, 460 nm) wavelength probably is due to the oxidation of NADH. The same phenomenon in the (460 nm, 520 nm) region can be explained by the reduction of the FAD which occurs in dysplasic tissues. This fluorophore emission, associated with high hemoglobin absorption induce a weak emission fluorescence in polyps.

Emission growth can be observed in polyps in the 600-680 nm region, due to the presence of porphyrins. This fact is noticed by the presence of two different peaks in (420 nm, 650 nm) and (505 nm, 650 nm), or peaks in (420 nm, 620 nm) and (420 nm, 660 nm). This emission in red region is being attributed to the protoporphyrin IX (PP IX) (Andersson-Engels *et alii*, 1990), an endogen biochemical that can be associated to an enzymatic deficiency in the heme cycle, because it is the immediate precursor of heme. This endogen porphyrin, is an important indicator of dysplasia, which associated with diffuse reflectance, can contribute significantly to identify dysplasic tissues.

The presence of hemoglobin in normal and dysplasic tissues induce raise in absorption of excitation radiation and also in the reabsorption of the emission radiation. Because heme presence in polyps is very important mainly due to the high growth rate of dysplasic tissue, spectral variations are very significant and are very important elements for spectroscopic differentiation.

Previous work (Richards-Kortum, 1990), proposed the acquisition of fluorescence contour maps of *in vitro* samples aiming to obtain ideal excitation wavelengths. This *in vitro* experiments usually present spectral distortion problems due to necrosis in the samples. Another source of distortion is due to the not very well defined excitation and collection geometry of commercial spectrofluorimeters. For best results, *in vitro* experiments should be followed by *in vivo* ones.

The determination of the ideal excitation wavelengths is important in order to optimize spectral tissue differentiation. The method is based in spectral differences obtained from different excitation wavelengths.

From our experiments, the ideal excitation wavelengths to identify normal-adenoma from human colon tissue are 360 nm, 414 nm, 441 nm and 506 nm (± 10 nm). The excitation in 360 nm and 441 nm gives a important information about the tissue absorption and the excitations in 414 and 506 are porphyrins absorption bands, exciting these bio-compounds.

CONCLUSION

In this work a new multi-excitation wavelength fluorescence spectra based on a nitrogen laser pumping a rotating dye wheel was presented. We have demonstrated that reflectance and multi-excitation fluorescence spectra can be obtained in real time. This allows for the collection of excitation-emission-matrices in vivo from biological tissues without motional artifacts. The EEMs provide information about optimal excitation wavelengths for the differentiation of normal and neoplastic colon and about the identity of the fluorophores which contribute to fluorescence tissue spectra. *In vivo* colon studies revealed that spectrum obtained from in vivo adenomatous polyps compared with normal tissue, shows three times less fluorescence intensity. We explain this difference by the fact that polyp is more irrigated than the normal mucosa, thus explaining its red color. The differentiation of normal and displastic tissues can be optimized by means of an algorithm considering several emission curves from each single excitation wavelength.

ACKNOWLEDGMENT

This work was carried out at the MIT Laser Biomedical Research Center supported by NIH P41-rr02594 grant and RHAE Program from CNPq/Brazil

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UM ESPECTROFLUORÍMETRO PARA O DIAGNÓSTICO DE DISPLASIA DE COLON

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RESUMO -- Neste trabalho é apresentado o projeto e desenvolvimento de um espectrofluorímetro portátil que utiliza um cateter de fibras ópticas para uso na rápida análise da emissão fluorescente de tecidos biológicos no ambiente hospitalar, visando a aplicação da técnica de Espectroscopia de Fluorescência Induzida por Laser (EFIL) no diagnóstico de câncer no trato gastrointestinal em estágio inicial de desenvolvimento. O sistema fornece dez comprimentos de onda diferentes, permitindo a coleta de espectros de tecidos em aproximadamente 600 ms. Os espectros de fluorescência foram adquiridos em 10 pacientes que se submeteram a procedimento de colonoscopia de rotina para verificação de desordens. As mucosas normal e adenomatosa foram estudadas com o cateter em contato com a mucosa intestinal através do endoscópio. Nós verificamos que o tecido adenomatoso apresenta aumento na absorção da hemoglobina, quando comparado com o tecido normal. A fluorescência do adenoma é menos intensa que no tecido normal, e foi observado um aumento na fluorescência na região do vermelho (650 nm) em pólipos. *Conclusões:* A multi-excitação da fluorescência fornece informação sobre o comprimento de onda ideal para a diferenciação entre tecido normal e tecido neoplásico, permitindo também a identificação dos fluoroforos que contribuem para o espectro total de fluorescência.

Palavras-chave: Colonoscopia, Diagnóstico *In-Vivo*, Espectroscopia a Laser, Fluorescência

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