SPECTROSCOPIC DIAGNOSIS OF COLONIC DYSPLASIA

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(Received 6 August 1990; accepted 13 November 1990)

Abstract—We have developed a method for defining diagnostic algorithms for pathologic conditions based on fluorescence spectroscopy. We apply this method to human colon tissue and show that fluorescence can be used to diagnose the presence or absence of colonic adenoma. This method uses fluorescence excitation-emission matrices (EEM) to identify optimal excitation regions for obtaining fluorescence emission spectra which can be used to differentiate normal and pathologic tissues. In the case of normal and adenomatous colon tissue, these were found to be: 330, 370, and 430 nm ± 10 nm. At these excitation wavelengths, emission wavelengths for use in diagnostic algorithms are identified from change difference and ratio of the spectra from normal and pathologic tissues. In colon tissue, at 570 nm excitation, 494, 480, and 680 nm were found to be useful emission wavelengths for diagnosing the presence of adenoma in vivo. The basis of colon tissue autofluorescence was investigated using EEM of pure molecules and relevant excitation-emission maxima in the literature.

INTRODUCTION

Colonoscopy is commonly used to detect adenomatous polyps, a precursor to colorectal carcinoma. Once identified, colonic adenomas are removed, either by excision or ablation, to interrupt the "adenoma–carcinoma" sequence (Muto et al., 1975). Early data suggest that aggressive removal of adenomas reduces the risk for colorectal carcinoma (Gilbertson, 1980). Colonoscopic identification of adenoma, however, is based on gross architectural and morphologic changes associated with polyp formation; thus, the technique is not specific for adenomas. Even experienced endoscopists cannot reliably differentiate colonic adenomas from non-adenomatous polyps (Norfleet et al., 1988; Chapuis et al., 1982; Kronberg and Hagl, 1985; Tedesco et al., 1982; Wayne et al., 1988), especially when dealing with lesions less than 10 mm in diameter. Therefore biopsy of all small polyps is currently recommended to guide patient management (Norfleet et al., 1988). Biopsy may add considerable time to a colonoscopic examination and does add a definite, albeit small, risk for complications such as visceral perforation or bleeding (MacRae et al., 1983).

Because colonoscopy relies on largely architectural changes to identify potential adenomatous mucosa for biopsy, it is less sensitive in clinical situations where adenomatous or dysplastic changes are flat or the normal architecture of the non-neoplastic mucosa has been disrupted. Mucosal ulcerative colitis is a clinically relevant example of this type of situation (Robbins et al., 1984). Patients with ulcerative colitis have an approximately one in three risk of developing carcinoma of the colon in their lifetime, much greater than that of the general population (Kwenter et al., 1978). Because of this risk, prophylactic colectomy is recommended to these patients (Ritchie et al., 1981). However, the impact of this procedure is severe (Bonavie et al., 1974), and not all patients elect colectomy initially. Management of those patients usually includes periodic screening for adenomatous (or dysplastic) changes, resorting to colectomy only when dysplasia is found. Current surveillance programs include regular total colonoscopy with multiple biopsies throughout the colon (Dickinson et al., 1980). However, due to the "patchy" nature of dysplasia, these programs are not completely effective. In some series, carcinoma of the bowel developed in 13 of 186 ulcerative colitis patients with a history of disease for 10 years or more, despite surveillance (Lennard-Jones et al., 1983). Thus, because of limitations in colonoscopic identification of dysplasia, the ideal result, the prevention of cancer and restriction of colectomy to patients with demonstrated precancer, has not been fully realized.

More sensitive and specific techniques for identification of colonic adenoma and dysplasia are under investigation. Many of these exploit the biochemical changes associated with colonic dysplasia, such as DNA aneuploidy (Giaretti et al., 1988), DNA hypomethylation (Goelz et al., 1985), and increased polypeptide levels (Upp et al., 1988). These biochemically based methods are promising for eventual identification of adenomatous or dysplastic foci of colonic mucosa. However, clinical implementation of these methods is currently very limited, as they are complicated, time-consuming, and cannot yet be performed in situ, necessitating biopsy.

We are developing a biochemically based, endoscope compatible method of screening for colonic
dysplasia and adenoma, which can be implemented in real time without biopsy. This method is based on fluorescence spectroscopy. It is well known that fluorescence of materials, such as human tissue, can provide information about the presence of very small amounts of biological chromophores (Campbell and Dwek, 1984; Richards-Kortum et al., 1989a). Preliminary reports have suggested that differences in the presence and concentration of biological chromophores in normal and neoplastic human colon can be measured using fluorescence spectroscopy, forming the basis of a diagnostic system for colon neoplasia (Kapadia et al., 1990; Yakshe et al., 1989; Richards-Kortum et al., 1989b; Shorracker, 1990; Cothren et al., 1990). Such fluorescence based diagnostic systems are easily implemented in vivo (Cothren et al., 1990).

However, these preliminary studies have not fully optimized the experimental parameters of fluorescence spectroscopy, such as choice of excitation wavelength. Furthermore, in these studies, only the most limited use has been made of the chemical information contained in the tissue fluorescence spectra for identifying optimal diagnostic schemes.

To more fully address these issues, we have undertaken a comprehensive study of the spectroscopic properties of normal and adenomatous colon mucosa in vitro. The purpose of this study is to survey the ultraviolet and visible spectroscopic properties of normal and dysplastic colon to determine:

1. whether the spectroscopic properties of these tissues are significantly different to permit their differentiation and
2. if so, at which excitation wavelength(s) this differentiation is best achieved.

We have utilized fluorescence excitation–emission matrices [EEM]1 to achieve these goals (Christian et al., 1981; Lehner et al., 1986). An EEM is a matrix which contains the fluorescence intensity as a function of excitation and emission wavelength. Such a matrix can be formed from a series of emission (or excitation) spectra collected for a range of excitation (emission) wavelengths.

In this paper, ratios or differences of EEM of normal and adenomatous colon are utilized to determine the excitation wavelengths most sensitive for the spectroscopic identification of colonic adenoma. At the excitation wavelengths identified in the ratio and/or difference EEM, simple empirical diagnostic algorithms were defined and tested based on features of the fluorescence emission spectra. Finally, the basis of the differences in the EEM of these tissues are discussed in terms of the biological chromophores responsible for the signal.

The approach presented here is more general than previous attempts to use fluorescence spectroscopy in the field of medical diagnostics (Kapadia et al., 1990; Yakshe et al., 1989; Shorracker, 1990). It systematically includes the important, but often overlooked, step of identifying optimal excitation wavelengths. In addition, understanding the fluorescence in terms of the tissue chromophores responsible for the signal is integral to the determination of a robust diagnostic algorithm.

**MATERIALS AND METHODS**

*Tissue selection, processing, and handling.* As a model of dysplasia accessible for in vivo studies, adenomatous polyps were obtained from resection specimens of patients with familial adenomatous polyposis. In this relatively rare disease, the mucosal surface of the colon is covered with a myriad of small adenomatous polyps (Robbins et al., 1984). The dysplasia associated with these polyps is frequently histologically identical to both the dysplasia found in sporadic adenomas, as well as that associated with mucosal ulcerative colitis (Riddell et al., 1983). All adenomas studied were less than 5 mm in greatest cross dimension and most were 1–2 mm. Normal controls were obtained from uninvolved areas in resection specimens from patients with familial adenomatous polyposis, colonic adenocarcinomas or diverticular disease. Whenever possible, matched normal controls were obtained from the same patient.

Full thickness samples of colon were snap frozen in liquid nitrogen and isopentane and stored at −70°C until study. The specimens were thawed at room temperature, kept moist with 140 mM buffered saline, pH 7.4, and mounted in a quartz cuvette for spectroscopic studies. Following study, the tissues were fixed in 4% formaldehyde solution, paraffin embedded, cut into 4 μm thick sections, and stained with hematoxylin and eosin. The resultant slides were examined by a single gastrointestinal pathologist to verify that control tissues were histologically normal and that polyps selected macroscopically were adenomas.

*Fluorescence EEM of tissue.* The spectroscopic properties of a number of samples were recorded over a wide range of UV and visible wavelengths. Fluorescence EEM were recorded for 15 colonic specimens (4 normal, 11 adenomas) from 8 patients; 3 of the normal samples were matched controls from patients with familial adenomatous polyposis, one was from a patient with colonic carcinoma or diverticular disease.

Excitation–emission matrices were collected using a standard spectrophotometer. Excitation light was incident perpendicular to the mucosal surface of the tissue which was mounted in a quartz cuvette: emission light was collected at a 23° angle with respect to the excitation beam. The instrument was modified to yield an excitation beam size of approx. 2 × 3 mm at the tissue. The spectral resolution of the excitation monochromator was 2 nm FWHM, that of the emission monochromator was 4 nm FWHM. Fluorescence intensities were calibrated daily using a standard fluorescent filter. Fluorescence intensities are reported here in arbitrary units relative to the intensity of this standard.

The EEM were constructed from a series of fluorescence emission spectra collected at excitation wavelengths (λex) varying from 250 to 500 nm in 10 nm steps. For each spectrum, fluorescence intensities were recorded at 5 nm intervals of emission wavelength (λem) over the range 10 nm greater than the excitation line, to 10 nm less than the second harmonic of the excitation line or 700 nm, whichever was lower.

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1 Abbreviations: EEM, excitation–emission matrix; λex, excitation wavelength; λem, emission wavelength; LIF, laser induced fluorescence.
All spectra have been corrected for the non-uniform spectral response of the spectrofluorometer. The wavelength dependence of the excitation power and the wavelength dependence of the detector response were both corrected for. Emission spectra were collected in order of increasing excitation wavelength, with the 350 nm excited spectrum collected first. To monitor for photobleaching, a 250 nm excited spectrum was recorded again following collection of the EEM. In all cases, deviations between the first and last 250 nm excited spectrum were less than 5%.

The EEM are presented here as fluorescence contour maps, where contour lines connect points of equal fluorescence intensity. Average EEM were determined for normal and adenomatous tissue, and the ratio and the difference of these average EEM were calculated to identify spectral regions in which fluorescence spectra were most different for these tissue types.

Laser induced fluorescence. In order to study the fluorescence of colonic mucosa in more detail at these excitation wavelengths chosen to be optimal for differentiation of normal and adenomatous tissue, a special system was developed which could easily be adapted to record and analyze tissue fluorescence spectra in vivo. The system was utilized to study 11 samples of normal colon and 16 specimens of adenomatous tissue. In these studies the system consisted of a pulsed Nd:YAG laser, and optical fiber catheter used to deliver excitation light to the tissue and collect fluorescence spectra. Tissue fluorescence was detected with a photomultiplier and a spectrum analyzer. A digital multi-channel analyzer was used to record fluorescence intensity as a function of emission wavelength.

The Nd:YAG laser provided 8.5 ns pulses of 3064 nm radiation at a repetition rate of 10 Hz. This light was frequency doubled using KDP crystals to provide 532 nm radiation. The 532 nm light was then used to pump a 1 m long cell filled with H₂ gas at pressure of 150 ps. Stimulated Raman scattering in the cell produced several Stokes and anti-Stokes frequency shifted lines; the first three anti-Stokes lines, at wavelengths of 435.7, 369.9 and 319.9 nm, were used to excite tissue fluorescence in the LIF studies of colon. Below we limit our discussion to the 369.9 nm excitation wavelength because this excitation wavelength covers the majority of the regions determined from the EEM to be sensitive to dysplasia.

The monochromatic excitation light was focused into the central fiber of a 10 fiber "shielded" optical fiber catheter. All fibers had a quartz core, and were capable of transmitting light from 300 to 700 nm efficiently. The central fiber was used to provide a 1.0 mm diameter illumination spot at the distal tip of the optical shield. This optical shield was placed in contact with tissue in order to collect fluorescence spectra. Tissue fluorescence was collected with the nine optical fibers surrounding the central fiber. These collection fibers were arranged so each fiber collected fluorescence only from the surface area of tissue directly illuminated by excitation radiation. This provided a well defined collection geometry (Keizer et al., 1989). Because spectra are collected with the probe in contact with the tissue, meaningful signal intensities can be obtained with this system both in vitro and in vivo. Intensities were calibrated with a rhodamine fluorescence standard.

The proximal tips of the collection fibers were imaged onto the entrance slit of an imaging spectograph. A long pass cutoff filter with a 50% transmission wavelength of 20 nm longer than the excitation wavelength was placed before the entrance slit, and was used to reject scattered excitation radiation. The spectograph dispersed the emission light across the face of the optical multi-channel analyzer. The spectral resolution of this system was 3 nm full width at half maximum. All data has been corrected for the non-uniform spectral response of the detection system.

To ensure that no photobleaching occurred at the excitation wavelengths used in this study, two successive spectra were recorded for all tissue samples. The total incident light needed to obtain spectra with good signal-to-noise ratio was less than 0.2 mJ/min. In all 26 cases, differences in the first and second spectra were less than 5%.

RESULTS

Fluorescence excitation emission matrices of tissue

A characterization of the UV and visible fluorescence properties of normal and adenomatous colon tissue is presented in Figs. 1 and 2. Figure 1 shows a contour map representation of the average fluorescence EEM of four normal samples. No significant differences were observed in the EEM of normal samples from patients with familial adenomatous polyposis or colonic adenocarcinoma or diverticular disease. Figure 2 shows a contour map representation of the average fluorescence EEM of 11 adenomatous samples. Three sets of linearly spaced contours are shown: 10 from 1 to 100 units; 10 from 100 to 1000 units; and 2 from 1500 to 2000 units. Although fluorescence intensity is reported in arbitrary units, the same scale of units is used for both figures so meaningful intensity comparisons can be made between the two.

Table 1 summarizes the excitation and emission maxima of the fluorescence peaks in the average fluorescence EEM of normal and adenomatous tissues. Fluorescence intensities are given for each peak in our scale of arbitrary units. Both tissue types exhibit an intense fluorescence peak at (λₑₓ, λₑₘ) = (290, 330) nm, which is similar in lineshape and intensity. Both tissues also exhibit peaks of

![Figure 1. Average EEM of four normal human colon samples. Excitation wavelength is plotted on the ordinate, emission wavelength on the abscissa. Contour lines connect points of equal fluorescence intensities. Three sets of linearly spaced contours are shown: 10 from 1 to 10 units; 10 from 10 to 100 units; and 2 from 150 to 200 units. Although fluorescence intensities are given in arbitrary units, the same scale of units is maintained throughout the paper.](image-url)
similar lineshape at or near (345, 405 nm), (460, 520 nm), (465, 555 nm) and (470, 595 nm). However, at each of these peaks, the fluorescence intensity of normal tissue is approximately twice that of adenomatous tissue. In addition, the average EEM of normal tissue shows two unique peaks at (325, 385 nm) and (315, 430 nm).

These differences are highlighted in Figs. 3 and 4, which show contour maps of the ratio and difference of the average EEM, respectively. Both the ratio and difference maps can be used to detect differences in the intensity, position or bandwidth of a peak in a tissue EEM. However, as our results will demonstrate, the ratio map is more sensitive to small differences in lineshape which are superimposed on backgrounds of similar intensity. The difference map, on the other hand, is particularly useful for detecting small absolute intensity differences in peaks with similar lineshapes.

Figure 3 shows the ratio of the average adenomatous fluorescence EEM to that of the average normal fluorescence EEM with 20 contours spaced linearly from 1.0 to 0.05. Although the fluorescence intensity of normal tissue is greater than that of adenomatous tissue for nearly all wavelengths, local maxima can be regarded as regions where adenomatous tissue exhibits additional fluorescence bands, whereas local minima represent regions where normal tissue exhibits additional fluorescence bands. This additional fluorescence can be due to differences in either tissue fluorescence quantum yield or tissue attenuation (Richards-Kortum et al., 1989a). As noted from a comparison of Figs. 1 and 2, normal tissues exhibit a unique fluorescence peak near (345, 385 nm) which is shown nicely in the ratio of the EEM as a local minimum of 0.3. In the visible region, it was noted that both tissues exhibited peaks in similar locations, with normal tissues having approximately twice the fluorescence intensity of adenomatous tissues. This is reflected in the ratio map as minima at approx. 0.4 in the visible region [(400, 480 nm) and (440, 480 nm)].

The ability of the ratio map to detect small changes on top of similar intense backgrounds is demonstrated in Fig. 3. The maximum at (350, 430 nm) in the ratio map was barely visible as an apparent shoulder at (370, 420 nm) in the average adenomatous map (arrow in Fig. 2). Also, two additional maxima not detected by comparison of the average maps are present in the ratio map at (430, 600 nm) and (430 670 nm).

Figure 4 shows the difference of the average EEM, where the normal has been subtracted from the adenomatous, with two sets of linearly spaced

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**Table 1. Excitation-emission maxima in the EEM of normal and adenomatous colon tissue**

<table>
<thead>
<tr>
<th>Maxima</th>
<th>Fluorescence intensity</th>
<th>Potential fluorophore(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\lambda_{ex}, \lambda_{em})</td>
<td>Normal</td>
<td>Adenaom</td>
</tr>
<tr>
<td>(290, 336 nm)</td>
<td>200</td>
<td>—</td>
</tr>
<tr>
<td>(325, 385 nm)</td>
<td>8.5</td>
<td>—</td>
</tr>
<tr>
<td>(315, 430 nm)</td>
<td>3.0</td>
<td>—</td>
</tr>
<tr>
<td>(345, 455 nm)</td>
<td>5.5</td>
<td>2.5</td>
</tr>
<tr>
<td>(460, 520 nm)</td>
<td>5.5</td>
<td>2.5</td>
</tr>
<tr>
<td>(465, 555 nm)</td>
<td>3.5</td>
<td>2.0</td>
</tr>
<tr>
<td>(470, 595 nm)</td>
<td>3.0</td>
<td>1.5</td>
</tr>
</tbody>
</table>

*Arbitrary units.*
fluorescence can arise due to differences in either tissue fluorescence quantum yield or tissue attenuation (Richards-Kortum et al., 1989a).

Although the largest local minimum in the difference map is located at (285, 330 nm), it represents only a small fractional change in the corresponding peak, which was approx. 200 (in arbitrary units) in the average maps of normal and adenomatous tissues. Thus, it was not a prominent feature of the ratio map.

An additional minimum is present at (330, 385 nm), which corresponds to the unique peak in the average normal tissue map at this location. Again, the peaks noted in Figs. 1 and 2 at (350, 470 nm) and (460, 520 nm) in the average maps, which were approximately two times as intense in normal as adenomatous tissue, are represented by two minima located at (370, 480 nm) and (460, 515 nm) in the difference map. The unique shoulder at (370, 420 nm) in the average adenomatous map is a fairly minor feature of the difference map, represented by a local maximum at (390, 420 nm). The maximum noted at (430, 600 nm) and (450, 670 nm) in the ratio map are not present in the difference map.

Thus, for the specific case of human colon tissue, the ratio map provides a more complete characterization of differences in the fluorescence spectra of normal and adenomatous tissues. Therefore, the average ratio map (Fig. 3), was used to choose the optimal excitation wavelengths for diagnosing the presence of adenoma in human colon tissue.† The average ratio map summarizes all spectroscopic bands where the spectroscopic properties of normal and adenomatous tissues differ. We wish to obtain the most complete set of diagnostic information with the minimum number of excitation wavelengths. A single excitation wavelength can be represented by a horizontal line on our contour map representations of EEM. Thus, we can achieve our goal graphically by drawing the minimum number of horizontal lines which intersect all minima and maxima in Fig. 3. Because these peaks are broad, this can be accomplished with only three excitation lines as illustrated by the bands in Fig. 3 centered at 330, 370, and 430 nm.

Thus, the spectroscopic properties of normal colon tissue and colonic adenoma are significantly different over a wide range of UV and visible wavelengths. The optimal excitation wavelengths for differentiating normal and adenomatous colon lie in the regions given by: 350, 370, 430 nm (each ± 10 nm).

Although these excitation regions were selected utilizing the average ratio map, these regions also intersect all the excitation-emission minima present in the average difference map (Fig. 4) except that at (285, 325). In basing our selection of excitation wavelengths on the ratio map rather than the difference map, a minimum of information has been lost.

† The ratio or difference map could also be used to choose best emission wavelength(s) in principle. However, in a clinical setting, where obtaining fluorescence spectra in a short amount of time is important, obtaining emission spectra is more practical than obtaining excitation spectra.
Laser induced fluorescence

Development of an optimal, biochemically based diagnostic algorithm is a complicated process. Many different chromophores contribute to the fluorescence and attenuation of colon tissue. Ideally, we would like to use the tissue fluorescence spectrum to extract the concentration and attenuation, or concentration and quantum yield, for each chromophore, and then base our diagnostic algorithm on these parameters (Richards-Kortum et al., 1989a). We have utilized EEM to show that chromophores which are different between normal colon and colonic adenoma can be sampled utilizing only three excitation wavelengths. Analyzing emission spectra at these excitation wavelengths in terms of individual chromophores obviously requires that we understand the nature of the chromophores. Although this is our ultimate goal, here, lacking definitive information on chromophore identity, we evaluate the performance of fluorescence diagnostics utilizing empirical diagnostic algorithms. In this paper we concentrate first on the region centered at 370 nm, as it intersects most of the peaks in the ratio map.

Figure 5 shows the average fluorescence emission spectrum of 11 samples of normal colon obtained with the spectral catheter system described above; the excitation wavelength was 369.9 nm. Again, no significant differences were observed in the 369.9 nm excited fluorescence emission spectrum of normal samples from patients with familial adenomatous polyposis or colorectal adenocarcinoma or diverticular disease. Two intense emission peaks are present near 460 and 480 nm. Valleys can be observed near 420, 540 and 580 nm. The small peak at 520 nm is an artifact due to a large decrease in the spectral response of the detection system at this wavelength.

Figure 5 also shows the average 369.9 nm excited fluorescence emission spectrum of 15 samples of adenomatous colon. Similar to the spectrum of normal colon, this spectrum shows fluorescence peaks at 460 and 500 nm, with valleys near 420, 540 and 580 nm. However, the overall intensity is significantly lower in 460 nm region. At wavelengths greater than 670 nm the fluorescence of adenomatous tissue is greater than that of normal.

The distinction in the emission spectra of these tissues are highlighted in the ratio of the two average spectra shown in Fig. 6. This ratio spectrum is characterized by four regions: a downward sloping region from 400 to 420 nm where the ratio is < 1, a flat region from 420 to 480 nm where the ratio is < 1, an upward sloping region from 480 to 650 nm where the ratio is < 1, and an upward sloping region from 650 to 700 nm where the ratio is > 1. The difference spectrum is also shown in Fig. 6 but does not provide any additional information, and thus will not be discussed here.

The downward sloping region from 400 to 420 nm indicates that the relative fluorescence intensity of the adenomas is greater than that of normal tissues. The flat region from 430 to 460 nm represents the peak at 460 nm, where the fluorescence intensity of normal tissue is greater than that of adenomatous tissue. The relatively flat region in this region indicates that the fluorescence lineshape of this peak is similar in normal and adenomatous tissue, possibly indicating that the same fluorophore is responsible for the signals in both tissue types. The upward sloping regions from 480 to 650 nm represents a region where the absolute difference in the fluorescence intensity of normal and adenomatous tissue is decreasing, and may be due to a red shift in the position of the second most intense maximum in the spectra of adenomatous tissues. Above 680 nm, the absolute fluorescence intensity in the adenoma spectrum is slightly greater than that in the normal spectrum. This difference appears to peak at 680 nm, and may be due to an additional fluorescent
Equally effective binary diagnostic algorithms could also be defined using the emission intensity at 480 nm with that at either 404 nm or 680 nm. These are shown in Figs. 7(b) and (c), respectively. Although the addition of another parameter does not improve the performance of the algorithm, in the binary scatter plots there are fewer data points which fall near the decision surface. Thus, such algorithms should have better predictive value as the size of the data set is increased. In all cases, the same adenomatous sample is incorrectly diagnosed as normal.

**DISCUSSION**

We have shown that the excitation wavelengths optimal for the spectroscopic differentiation of normal and dysplastic human colon are 230, 370 and 430 ± 10 nm. Furthermore, at 370 nm excitation, we have shown that a simple, empirical diagnostic algorithm, based on the features of the fluorescence emission spectrum, can be used to identify colonic dysplasia with a high degree of accuracy.

Although this method of algorithm development optimized several experimental parameters, it does not make use of the biochemical information which is contained in the tissue fluorescence emission spectra. Here we show that fluorescence EEM can also be used to address this issue by using the EEM presented in Figs. 1 and 2 to identify tissue fluorophores and attenuators which contribute to the tissue spectra. Potential tissue fluorophores were identified by comparing excitation/emission peaks in tissue EEM to those in EEM of individual tissue constituents as well as peaks cited in the literature (Wolffheiss and Leiner, 1985; Chung-Ho et al., 1987). Table 1 lists the local excitation/emission peaks in the colon tissue EEM as well as our preliminary assignment of tissue fluorophores to these peaks based on comparison with literature.

Note that the band positions of potential fluorophores may not exactly match the tissue band positions listed in Table 1. Several effects may be responsible for these differences. It is known that the fluorescence spectra of turbid tissues contain contributions both from fluorophores, which emit fluorescence within tissue, and attenuation, which can attenuate this emitted fluorescence due to absorption and/or scattering (Richards-Kortum et al., 1988a; Keijzer et al., 1989; Richards-Kortum et al., 1989c). Attenuation acts to alter both the observed location of the excitation/emission maxima and the line shape of individual tissue fluorophores in the multi-component tissue EEM. Similarly, the observed line shapes and positions of chromophore maxima can be altered in multi-component tissue EEM when the excitation and emission of individual chromophores closely overlap. Furthermore, the spectroscopic properties of many fluorophores are dependent on their local environment.

Species in the adenomatous tissue.

We attempted to define effective empirical diagnostic algorithms for the presence of adenoma from this data utilizing one wavelength from each of the distinct regions in the ratio spectrum. For this data set, the emission intensity at 480 nm proved to be an effective diagnostic algorithm for adenoma. Figure 7(a) is a scatter plot of the fluorescence intensity at 480 nm for all 26 samples. It indicates that a simple algorithm represented by a straight line at \( I(480) = 0.003 \) is capable of correctly diagnosing 96% of the 26 samples as normal or adenoma.
ment, changing for example as a function of concentration, pH or temperature (Lackiewicz et al., 1983). For these reasons, our identifications should be considered preliminary.

The effects of attenuation can be semi-qualitatively understood by studying the total reflectance spectra of tissue (Richards-Kortum et al., 1989c). Total reflectance spectra provide a measure of tissue attenuation. In a total reflectance spectrum, valleys indicate peaks in attenuation. Attenuation peaks act to produce valleys in the fluorescence spectra of optically thick tissue samples (Keijzer et al., 1989; Richards-Kortum et al., 1989c). As attenuation effects are important both for exciting and emitted radiation, valleys will be produced in both excitation and emission spectra. Thus, at the location of these attenuation peaks, one expects to see valleys in the tissue EEM parallel to both the excitation and emission axes.

In both normal colon and colonic adenomas, reflectance valleys are located at 270, 355, 420, 540, 575, and 635 nm (Richards-Kortum, 1990). The valleys in the total reflectance spectra can be correlated with the valleys in the average EEM shown in Figs. 1 and 2. The strongest attenuation peak at 420 nm gives rise to valleys in the tissue EEM at 420 nm along both the excitation and emission axes. Although not as prominent, valleys are also present along the emission axis at 540 and 575 nm. A small valley along the excitation axis near 355 nm can also be appreciated.

It is well known that the absorption spectrum of oxyhemoglobin exhibits peaks near 250, 350, 420, 540 and 580 nm (Van Assendelft, 1970). Thus, nearly all of the attenuation peaks noted in the total reflectance spectra of normal and adenomatous colon could be ascribed to oxyhemoglobin. The presence of oxyhemoglobin could be attributed to the vascularity of the bowel wall. It should be pointed out that previous studies of articular tissue fluorescence have noted discrepancies in the oxyhemoglobin concentration found in tissue studied in vitro and in vivo (Richards-Kortum, 1990). These in vitro studies were largely carried out with tissue obtained at autopsy, and in this situation tissues are exposed to a significant amount of hemolyzed blood, increasing their oxyhemoglobin content (Richards-Kortum, 1990). In this work, we have obtained tissues from surgical resection, thus minimizing their exposure to hemolyzed blood. It should be further noted that fluorescence EEM in this study were measured using a conventional spectrofluorometer, with a collection geometry that is not well defined. This type of system results in a significant enhancement of the reabsorption phenomenon (Richards-Kortum et al., 1989c). Thus, spectra recorded with the optical fiber catheter in vitro demonstrate a lesser degree of hemoglobin reabsorption than those recorded with the conventional fluorometer. Our preliminary results indicate that fluorescence spectra of normal colon recorded in vivo with an optical fiber catheter demonstrate a similar degree of oxyhemoglobin reabsorption to those recorded in vitro with the catheter system (Coffren et al., 1993).

The identity of potential fluorophores can now be considered. The largest peak in the tissue EEM near (290, 330 nm) has been assigned to the aromatic amino acid tryptophan, which has a maximum at (275, 350 nm) when in aqueous solution. The small difference in intensity of the tryptophan peak in normal and adenomatous tissues could be due either to a difference in the concentration of tryptophan or its environment (Bent and Hayon, 1972).

Potential fluorophores for the tissue peak at (345, 465 nm) include NADH and NADPH. These molecules function as co-enzymes in oxidation-reduction reactions, and both have an excitation/emission maximum at (350, 460 nm) in aqueous solution (Schwarz et al., 1974). It should be noted that the (345, 465 nm) peak is bounded by attenuation valleys at 420 nm along the excitation and emission axes. Thus, the precise location of its excitation-emission maximum may be significantly shifted, and our preliminary assignment of this peak to NADH/NADPH should be regarded with this caveat.

Several peaks which appear in tissue EEM are near peaks associated with fluorophores related to vitamin B<sub>1</sub> (Wolffbeis and Leiner, 1985). The peak unique to normal tissue at (315, 430 nm) is near that of 4-pyridoxic acid at (300, 430 nm). The shoulder in adenomatous tissue at (370, 420 nm) is near the reported maximum of pyridoxic acid lactone at (370, 440 nm). Although three peaks are present in the normal and adenomatous tissue EEM at (460, 530 nm), (455, 555 nm) and (470, 355 nm), they are likely due to a single peak with superimposed oxyhemoglobin attenuation valleys at 540 and 580 nm. Pyridoxal 5'-phosphate represents a potential candidate for this peak. Its largest excitation/emission maximum is at (410, 520 nm); the shift in the tissue excitation maximum could be attributed to the Soret band attenuation of oxyhemoglobin. The effects of oxyhemoglobin attenuation are reduced in the ratio map, and the peak assigned to pyridoxal 5'-phosphate is observed at (400, 480 nm). In addition, pyridoxal 5'-phosphate exhibits a second peak at (305, 385 nm), which is near the peak found at (330, 385 nm) in the normal tissue EEM.

These peaks assigned to pyridoxal 5'-phosphate could also be due fluorophores associated with the structural proteins collagen and elastin, and/or NADH or NADPH. Elastin is associated with fluorophores which exhibit fluorescence maxima at (350, 420 nm); (420, 510 nm) and (460, 520 nm), while both collagen I and collagen III are associated with fluorescence component which shows peaks at (340, 395 nm) and (330, 390 nm), respectively. This
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identification has also been suggested by Shomacker (1990). Microspectrofluorimetry studies, which are in progress, can separate contributions of extracellular and intracellular fluorescences, and may provide the definitive answer.

Finally, the peaks unique to adenomatous tissue fluorescence at (430, 600 nm) and (430, 670 nm) could be due to the presence of endogenous porphyrins. Hematoporphyrin derivative, for example, which is a mixture of several biologically relevant porphyrins, exhibits fluorescence excitation emission maxima near (400, 610 nm) and (400, 675 nm) (Chung-Ho et al., 1987).

As noted above, comparison of EEM from individual biochemical compounds with optically thick tissue EEM does not always lead to definitive fluorophore identification, because of potential difficulties created by attenuation and overlapping excitation and emission. Both of these difficulties can be overcome by modeling the tissue EEM in terms of the attenuation and fluorescence properties of individual chromophores. This will be the subject of a future communication.

It is interesting to note that the fluorescence porphyrins preliminarily assigned here have been previously associated with pre-cancerous and cancerous states. For example, the peak assigned to NADH or NADPH is twice as intense in normal tissue as in adenomatous tissue EEM. Schwartz et al. found that the absolute concentrations of NAD+ and NADH decreased 2-3 fold following murine sarcoma virus transformation in rat kidney fibroblasts (Schwartz et al., 1974). The peaks assigned to pyridoxal 5'-phosphate are also approximately twice as intense in the normal tissue as in adenomatous tissue EEM. Several workers have reported decreased levels of serum pyridoxal 5'-phosphate in cancer patients (Merrill and Henderson, 1987). The peaks assigned to porphyrins were most prominent in the adenomatous tissue EEM. An increased content of endogenous porphyrins has been noted in neoplasms of other organ systems (Yuanlong et al., 1987).

CONCLUSIONS AND FUTURE DIRECTIONS

Using an accessible in vivo model of dysplasia, familial adenomatous polyposis, we have demonstrated that 350, 370 and 430 ± 10 nm represent optimal excitation regions for detecting this dysplasia. We have developed a procedure for defining empirical diagnostic algorithms, and have shown that at 370 nm excitation, fluorescence emission spectra can be used to differentiate normal and adenomatous colon in 95% of 26 cases tested. Finally, we have made preliminary identification of the biochemical compounds which contribute to the fluorescence of normal and adenomatous colon in the UV and visible region. Further studies are necessary to confirm that these changes can be observed in vivo, and that the differences observed here between normal and adenomatous tissues are not solely based on features of tissue architecture.

Based on the results of this study we have commenced a clinical trial of fluorescence colonoscopy, to evaluate the potential of 370 nm excited emission spectra for differentiating adenomatous mucosa from normal and hyperplastic mucosa (Colhoun et al., 1990). The results of this study are encouraging; 370 nm fluorescence emission spectra were collected in real time using a fiber optic probe introduced through the biopsy channel of a conventional colonoscope. Spectra were obtained from 31 colonic adenomas, 4 hyperplastic polyps and 32 examples of normal mucosa in 20 patients. Using the method of algorithm development described here, an algorithm was defined for the precance of adenoma. This algorithm was quite similar to that shown in Fig. 7(b) and was used to correctly differentiate adenomas from normal colorectal mucosa and hyperplastic polyps in 97% of cases tested with a resulting sensitivity, specificity and positive predictive value of 100, 97 and 94%, respectively. The ability of this algorithm to differentiate hyperplastic and adenomatous polyps illustrates that spectroscopic differences between normal and adenomatous tissues are not solely based on features of tissue architecture.

A fluorescence spectroscopic diagnostic system capable of detecting dysplastic transformation could be of immense practical value. Currently, no noninvasive techniques are available to detect adenomas or other premalignant conditions in the gastrointestinal tract. The ability to distinguish adenomatous mucosa from normal and hyperplastic mucosa would save considerable time during colonoscopy as well as reduce the risk and cost of the procedure itself. More importantly, using fluorescence spectroscopy, it may be possible to construct a fiberoptic device that can "see" dysplasia in conditions where the current utility of colonoscopy is limited, such as ulcerative colitis. Using fluorescence, it may then be possible to either detect dysplasia using fluorescence alone, or at the very least to direct biopsy to areas more likely to contain dysplasia.

Acknowledgements.—The authors are grateful to members of the Laser Spectroscopy of Tissue Group at the Massachusetts Institute of Technology, and the Cleveland Clinic Foundation for continual discussions of the material presented. Support from NIH Laser Biomedical Research Center Grant No. RR00294 is also acknowledged.

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