

ULTRAVIOLET LASER-INDUCED AUTOFLUORESCENCE DISTINCTION BETWEEN MALIGNANT AND NORMAL UROTHELIAL CELLS AND TISSUES

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ABSTRACT

The aim of this study was to perform a preliminary evaluation of the diagnostic potential of laser-induced autofluorescence spectroscopy (LIAFS) for urothelial tumors using fluorescence intensity ratios at different wavelengths. After testing three laser excitation wavelengths (308, 337, and 480 nm) in normal and malignant bladder cell lines, 308 nm appeared to be the most promising wavelength since two fluorescence bands were observed at 360 and 440 nm; these were attributed to tryptophan (Trp) and reduced nicotinamide adenine dinucleotide (NADH) respectively. This study was then performed on freshly removed normal bladder and bladder tumor specimens exclusively using the 308-nm excitation wavelength. The tumor spectra, regardless of stage and grade, were very similar to the malignant cell spectra. However, a marked reduction of overall intensity was observed for carcinoma *in situ* (CIS). Normal bladder mucosa exhibited a shift of the first fluorescence band to 380 nm, indicating an overlap of Trp urothelial cell emission and collagen fluorescence derived from the lamina propria. The intensity of the NADH emission band was markedly reduced in tumor tissues compared with normal mucosa, which could indicate different redox conditions in urothelial tumors. A fluorescence intensity ratio at 360 and 440 nm can accurately discriminate normal or inflammatory mucosa from all bladder tumors, including CIS. These findings support the use of LIAFS as a new diagnostic technique for occult urothelial tumors. © 1996 Society of Photo-Optical Instrumentation Engineers.

Keywords ultraviolet laser; autofluorescence; urothelial carcinoma.

1 INTRODUCTION

The detection of urothelial carcinoma *in situ* (CIS) remains a challenge for urologists because this flat lesion may be asymptomatic and/or invisible under white light cystoscopy.¹ CIS has an important prognostic value because its presence is clearly related to tumor recurrence and the subsequent progression of cancer.^{1,2} Urinary cytology and flow cytometry of bladder washings may detect these lesions,³ but none of these methods allow precise mapping to assess the prognosis and the treatment of focal forms by a minimally invasive real-time approach.

In recent years, there has been growing interest in the use of laser-induced fluorescence to discriminate tumors from normal surrounding tissues.⁴ However, most fluorescence studies conducted in the field of urology have used hematoporphyrin derivative (HpD) and/or the commercially available product (Photofrin).^{5,6} The major drawbacks of

these exogenous dyes are their adverse cutaneous effects.⁷

Recently, Kriegmair et al. proposed the use of intravesical instillation of 5-aminolevulinic acid (ALA).⁸ ALA is a precursor in the heme biosynthetic pathway that induces intracellular accumulation of endogenous protoporphyrin IX. This detection method achieved a sensitivity of 100% without any systemic adverse effects, but with a specificity of only 68.5%. However, a median ALA exposure time of 204 min was necessary before fluorescence cystoscopy, which represents a major drawback for the patient.

Laser-induced autofluorescence spectroscopy (LIAFS) avoids administration of any parenteral or topical drug. In the early 1980s, Alfano and co-workers were the first to employ LIAFS in *in vitro* studies to differentiate normal from tumor tissues. This work was initiated on rat tissues⁹ and later extended to human breast and lung tissues,¹⁰ and more recently to the gynecological tract (uterus, cervix, ovary).¹¹ Recently, LIAFS has also been demon-

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strated to be able to distinguish bronchial, colonic, and cerebral tumors from normal tissues.¹²⁻¹⁴ The aim of our study was to compare the autofluorescence spectroscopic patterns of normal and neoplastic bladder cells and tissues in order to determine whether the spectral differences are sufficient to allow the use of this technique as a diagnostic method.

Three main endogenous fluorophores account for most of the cellular autofluorescence: tryptophan (Trp), the reduced form of nicotinamide adenine dinucleotide (NADH), and the oxidized forms of riboflavins.⁴ To allow a better assessment of the relative contributions of these fluorophores to cellular autofluorescence, three laser wavelengths were chosen: 308 nm for Trp and NADH excitation, 337 nm for NADH excitation, and 480 nm for flavin excitation.

Cell culture pellets were initially used to eliminate the spectral influence of light absorption and scattering in tissues. The optimal excitation wavelength of 308 nm for differentiation of normal and neoplastic urothelial cells was subsequently applied to freshly removed bladder surgical specimens. In this second part of our study, tissue optics and morphology were considered in the interpretation of the spectra, constituting a preliminary step to *in vivo* LIAFS.

2 MATERIALS AND METHODS

2.1 CELL CULTURES AND SURGICAL SPECIMENS

2.1.1 Cell Lines

Normal fetal bladder FHS 738 BL and human bladder transitional carcinoma cell line T24 were purchased from the American Type Culture Collection (ATCC). T24 is derived from a poorly differentiated bladder transitional cell carcinoma (TCC).¹⁵ These cells were cultured in uncoated plastic dishes in RPMI 1640 supplemented with 6% heat-inactivated fetal calf serum (FCS), glutamine (2 mM), penicillin (100 IU/ml), and streptomycin (50 μ g/ml) in a humidified atmosphere of 5% CO₂ in air.

Five samples of each cell line were spectroscopically studied. Before any fluorescence measurement, all cell suspensions (normal and T24) were washed twice with phosphate-buffered saline (PBS) to remove all fluorescent and absorbing species from the culture medium (i.e., FCS, phenol red). Each cell sample was then harvested with a cell scraper and centrifuged at 1000 rpm for 10 min ($g \approx 55$). For each spectroscopic measurement, both the supernatant (PBS) and the resulting cell pellets were then submitted to LIAFS.

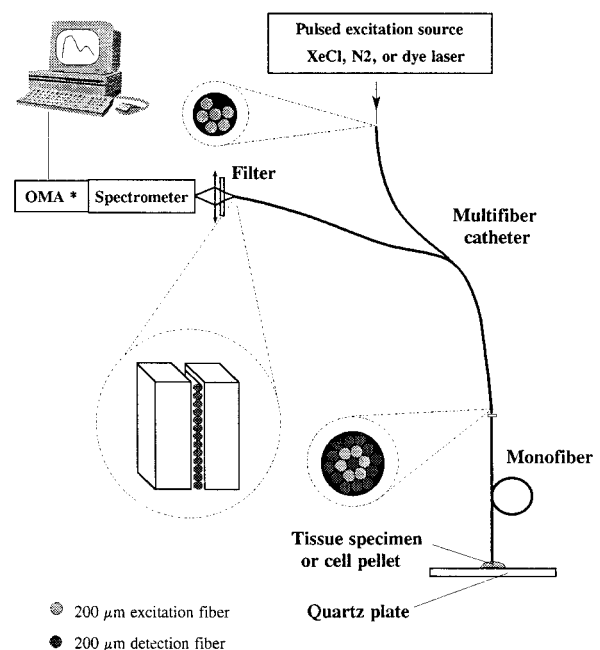
2.1.2 Surgical Specimens

A total of 21 surgical bladder specimens, including normal-appearing bladder mucosa ($n=5$);⁵ suspicious, flat, red-looking lesions ($n=7$);⁷ and papil-

lary ($n=5$)⁵ and nodular ($n=4$)⁴ bladder tumors were studied. These specimens were collected during transurethral resections and cystectomies. They were preserved at room temperature in the same culture medium as the cell cultures and were submitted to LIAFS within 1 hr. As for urothelial cell fluorescence measurements, and for the same reasons, surgical specimens were washed twice with PBS immediately before LIAFS. Conventional histological examination (fixation in 10% formalin and staining with hematoxylin and eosin as well as Masson trichrome) was performed after fluorescence studies on the surgical specimens in order to correlate the nature of the examined tissues with the spectral findings.

2.2 FLUORESCENCE EXPERIMENTAL SETUP

The LIAFS experimental setup used for this study is shown in Figure 1. Three different pulsed lasers were successively used for excitation: a homemade Xe-Cl excimer laser emitting at 308 nm (40 ns pulse duration, 1 Hz repetition rate), a nitrogen laser (Laser Science VSL 337ND) emitting at 337 nm (3 ns pulse duration, 1 Hz repetition rate), and finally a coumarin dye laser (SOPRA) pumped by the excimer laser emitting at 480 nm. A bundle composed of 6 excitation and 13 detection silica fibers (Polymicro 200/220/240 μ m, optical aperture 0.22) allowed the use of any excitation wavelength. A 1-mm core diameter silica fiber (Ensign Bickford HCSW1000) was connected to the bundle and its distal tip was placed in close contact with the sample (cell pellet or mucosal surface of each surgical specimen). The



* Optical Multichannel Analyzer

Fig. 1 Schematic diagram of LIAFS experimental setup.

excitation light energy incident on the sample at the distal tip of the 1 mm diameter fiber was for each pulse: 40 μ J at 308 nm, 14 μ J at 337 nm, and 4 μ J at 480 nm. An optimum coupling of the fluorescence light to the spectrometer input slit was obtained by a vertical arrangement of the detection fibers.

A long-wavelength pass filter was used to block scattered and reflected excitation light in front of the 75 μ m input slit of the spectrometer. For 308 nm excitation, the cutoff wavelength of the filter (345FG-01-25 ORIEL) was 345 nm. For 337 nm excitation, the cutoff wavelength of the filter (LG400 CORION) was 400 nm. For 480 nm excitation, the cutoff wavelength of the filter (LG515 CORION) was 515 nm. The fluorescence light dispersed by the spectrometer (EG&G, Princeton Applied Research, model 1235, Czerny-Turner type, grating blazed at 500 nm) was detected by an intensified diode array detector composed of 512 photodiodes interfaced to an optical multichannel analyzer (EG & G OMA III model 1461). The spectral resolution was approximately 0.6 nm per pixel. The OMA was controlled by a host computer (386 PC), which allowed data storage and spectral display. The system was adjusted to capture the whole spectrum from 320 nm to 600 nm. During spectroscopic studies, the samples were alternately placed on the same 5 mm thick fluorescence-free quartz plate. Ten spectra, each one obtained with a single pulse, were accumulated and averaged for each sample measurement. The spectra were not corrected for spectral response of the system. The very low fluorescence of PBS, probably due to impurities, was subtracted for each sample. The high sensitivity of the apparatus was demonstrated by its ability to register autofluorescence spectra of cell pellets measuring less than 1 mm.

Both the intensity and the shape of the emission spectra were examined. However, in the case of cell pellets, it was impossible to determine the exact number of cells illuminated by the fiber. Consequently, for cell cultures, no conclusions could be drawn from a comparison of absolute fluorescence intensities. In the case of surgical specimens, histological examination was performed on the very site of the previous fluorescence measurements, i.e., on the part of the tissue surface that had been in contact with the distal tip of the optical fiber. Statistical analysis of the results was performed with the Mann-Whitney test and the two sample *t*-tests. A limit of significance of $p < 0.05$ was set in both cases.

3 RESULTS

3.1 CELL CULTURE STUDY

Typical autofluorescence spectra of grossly visible pellets of normal and malignant urothelial cells, using successive excitation wavelengths of 308, 337, and 480 nm, are shown in Figure 2. The diameter of the pellets ranged from 0.5 to 2 mm. It should be stressed that two distinct fluorescence bands for

normal cells were only obtained on the 308-nm spectra, centered at 360 and 430 nm, respectively, and the intensity of the 430-nm band was twice that of the 360 nm band. At the 308 nm excitation wavelength, malignant T24 cells mainly exhibited a bright fluorescence maximum centered at 360 nm, followed by a slow decline of the emitted fluorescence [Fig. 2(a)]. At 337 and 480 nm excitation

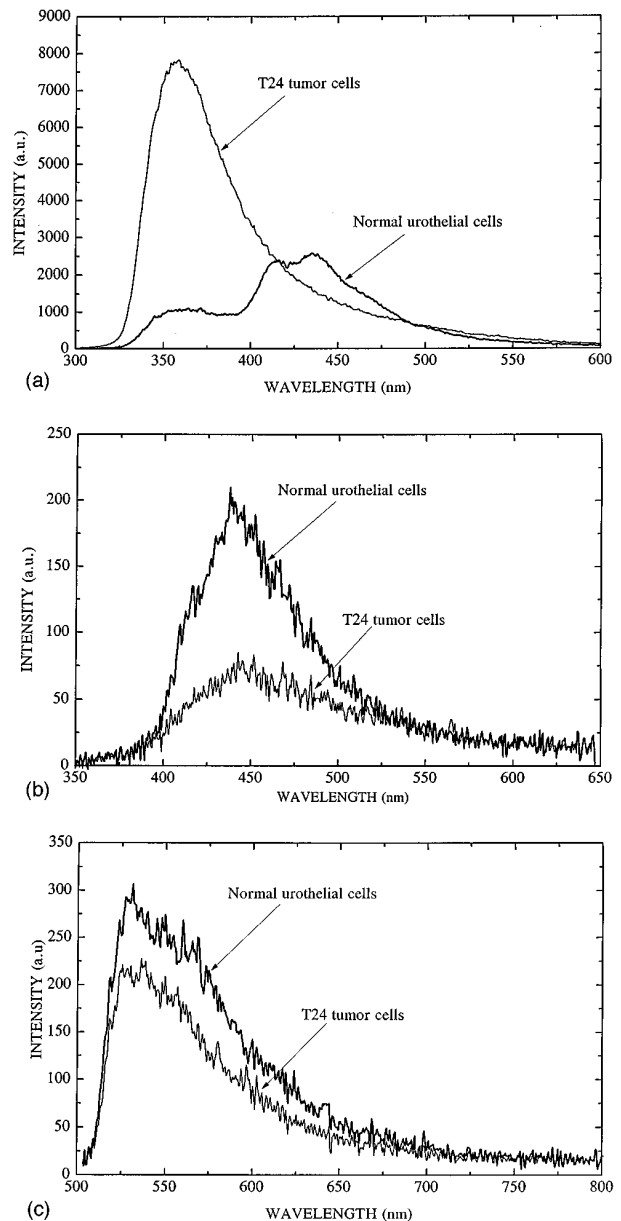


Fig. 2 Typical autofluorescence spectral shape of normal and tumor T24 urothelial cell pellets upon (a) 308, (b) 337, and (c) 480-nm excitation. The fluorescence spectra are distorted on the short wavelength side by the long-wavelength pass filters used in these experiments (cutoff wavelength at 345 nm for 308-nm excitation, 400 nm for 337-nm excitation, and 515 nm for 480-nm excitation). a.u., arbitrary units. Since the exact number of cells illuminated by the fiber was not determined, no comparison of the absolute fluorescence intensities can be made between normal and tumor cell pellets.

Table 1 Correlation of cystoscopic appearance and histological features of the specimens submitted to LIAFS *in vitro* study.

Cystoscopy/histology	Normal mucosa	Nonspecific inflammation	CIS	Ta G1	T1 G3	T2 G3	T3G3
Normal-appearing mucosa (n=5)	5						
Suspicious flat erythematous areas (n=7)		4	3				
Papillary tumors (n=5)				4	1		
Nodular tumors (n=4)					1	1	2

wavelengths, only one fluorescence band, centered at 440 and 550 nm, respectively, was observed for both normal and malignant urothelial cells [Figs. 2(b) and 2(c)]. The 308-nm excitation wavelength therefore appeared to be ideally suited for diagnostic purposes because the presence of two distinct emission bands would allow the use of fluorescence intensity ratios to differentiate normal from neoplastic urothelial tissues. This method has the advantage of providing a dimensionless function in which both fluorescence intensities are equally influenced by variations of experimental parameters such as excitation energy and detection efficiency.

3.2 LIAFS STUDY OF BLADDER TISSUE SPECIMENS USING THE 308 NM EXCITATION WAVELENGTH

Table 1 summarizes the correlation between the cystoscopic appearance and the histological features of the bladder specimens submitted to our *in vitro* LIAFS study. Figure 3 shows typical fluorescence spectra of normal bladder mucosa and of a deep-muscle invasive, poorly differentiated T3 G3 bladder tumor. The spectra of the visible bladder tumor specimens studied, regardless of their stage and grade, were very similar to this T3 G3 spec-

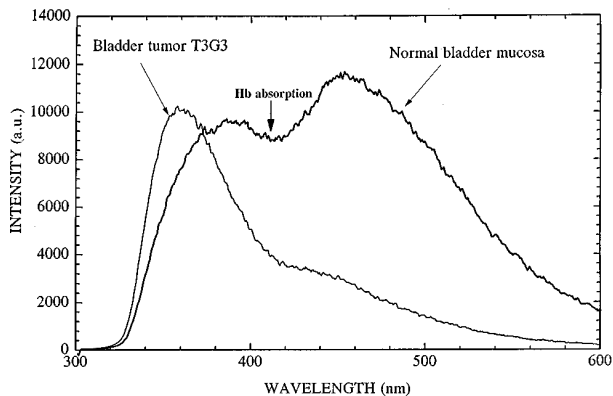


Fig. 3 Typical *in vitro* autofluorescence spectra of normal bladder mucosa and of a muscle-invasive bladder tumor T3 G3, upon 308-nm excitation. Again, the fluorescence spectra are distorted on the short wavelength side by the long-wavelength pass filter at 345 nm.

trum. Normal bladder mucosa typically displayed two distinct fluorescence bands with respective peaks at 380 and 450 nm and separated by a valley centered at 420 nm. The 450-nm band was slightly more intense than the 380-nm band and was followed by a rapid decline of the signal until 600 nm. Typical spectra of nonspecific inflammatory bladder mucosa were not very different from those of normal mucosa, but the 450 nm band was often less intense.

The tumor spectra typically had an appearance very similar to the T24 malignant cell spectra, since a very sharp and intense fluorescence maximum centered at 360 nm was observed. However, a deflection point appeared at 420 nm followed by a weak fluorescence maximum at 440 nm. Figure 4 shows a CIS spectrum compared with a T3 G3 solid tumor spectrum. It should be noted that the overall fluorescence intensity of CIS was significantly reduced. Interestingly, using a higher gain ($\times 5$), the shape of the CIS spectrum was very similar to that of the tumor spectrum. Table 2 presents, for each histological group studied (i.e., normal bladder mucosa, nonspecific inflammatory bladder mucosa, bladder tumors including CIS), the average intensities I_{360} at 360 nm and I_{440} at 440 nm, and their ratio $R=I_{360}/I_{440}$, together with the corresponding stan-

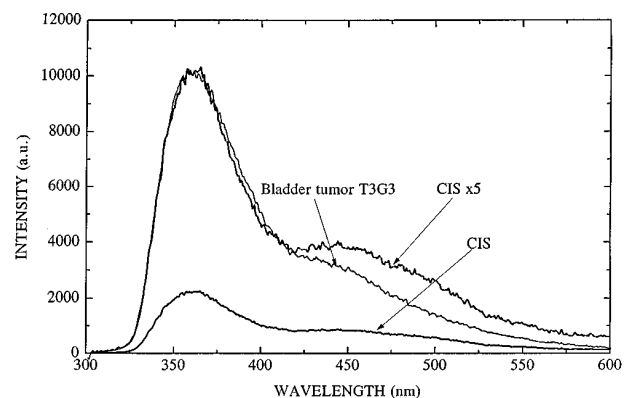


Fig. 4 Typical *in vitro* autofluorescence spectrum of a urothelial carcinoma *in situ* (CIS) compared with a T3 G3 bladder tumor spectrum, upon 308-nm excitation. The autofluorescence of the CIS obtained by increasing the gain of the detector ($\times 5$) is displayed.

Table 2 Mean fluorescence intensities in arbitrary units (a.u.) at 360 nm (I_{360}) and 440 nm (I_{440}) and their ratio $R=I_{360}/I_{440}$. Values are displayed with standard deviations. Excitation was performed at 308 nm. CIS specimens are included in bladder tumors group. Statistical analysis of the results was performed with the Mann-Whitney test and the two sample *t*-tests. A limit of significance of $p<0.05$ was set in both cases.

	I_{360} (a.u.)	I_{440} (a.u.)	R
Normal bladder mucosa (n=5)	7525±4660	8771±4617	0.85±0.29
Nonspecific inflammatory mucosa (n=4)	4494±3350	4455±5154	1.31±0.54
Bladder tumors (n=12)	6122±2686	2141±995 ^a	2.9±0.62 ^{a,b}

Note: ^a Significantly different from normal bladder mucosa; ^b Significantly different from nonspecific inflammatory mucosa.

standard deviations. A significant difference was observed between the absolute fluorescence intensities of normal mucosa and bladder tumors at 440 nm ($p<0.05$). In comparison with normal mucosa, the mean fluorescence intensity reduction at 440 nm observed for bladder tumors was 75% (range 25 to 90%). However, at this wavelength, no statistically significant difference was observed between the absolute fluorescence intensities of nonspecific inflammatory mucosa and bladder tumors. In contrast, a significant difference in *R* ratio was always observed between normal or nonspecific inflammatory mucosa and bladder tumors ($p<0.05$) (Table 2 and Fig. 5).

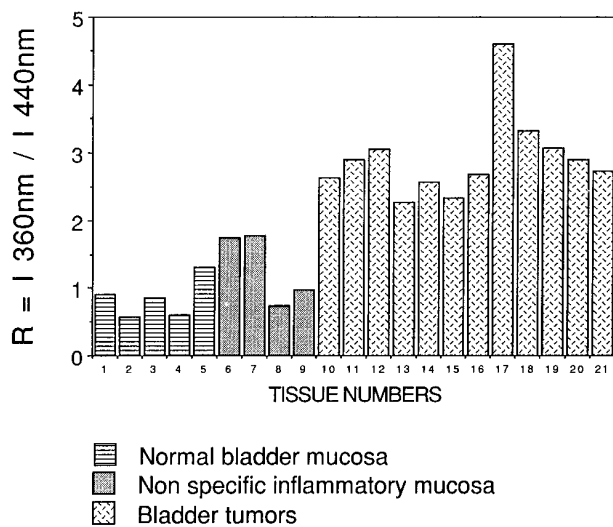


Fig. 5 Histogram for ratio $R=I_{360}/I_{440}$ values of the 21 specimens studied with LIAFS. CIS specimens (numbers 10, 11 12) are included in the bladder tumors group.

4 DISCUSSION

LIAFS has been investigated in neoplastic tissues and atherosclerotic arteries by a number of workers in order to evaluate its diagnostic potential.¹⁶ This technique could prove to be useful in urology for real-time early detection of urothelial CIS because this lesion may present a normal cystoscopic appearance or nonspecific inflammatory changes.^{1,2} However, many problems must be resolved before LIAFS can be used clinically for this purpose.

This *in vitro* study had two objectives: (1) to assess the ability of LIAFS to discriminate normal from neoplastic urothelial cells in cell pellets, and (2) to compare the cell culture spectra with the *in vitro* tissue spectra, in order to evaluate the contribution of the morphology and optical properties (absorption and scattering)¹⁷ of the urothelial specimens studied. The first part of our study was performed on normal and neoplastic urothelial cell pellets. An auto-fluorescence signal could be detected for each of the three excitation wavelengths.

At the 308 nm excitation wavelength, two distinct fluorescence bands were observed centered at 360 and 430 nm, respectively. In cell pellets, the 360 nm emission band displayed by normal as well as neoplastic cells was probably almost entirely due to tryptophan (Trp) because protein absorption for wavelengths between 295 and 310 nm is primarily due to Trp, which accounts for about 90% of total protein fluorescence.^{18,19} In our experiments, Trp peak fluorescence was artificially red shifted from 350 to 360 nm by the 345-nm-long wavelength pass filter.

The second fluorescence band, centered at about 430 nm, was clearly observed only in normal cell spectra and was more intense than the 360 nm band [Fig. 2(a)]. Previous studies indicate that this fluorescence band can be attributed to expression of the reduced form of the respiratory chain coenzyme, nicotinamide adenine dinucleotide.²⁰ Absorption and emission maxima of NADH are 340 and 450 nm, respectively.²⁰

Using either 337 or 480 nm laser excitation, we observed a single fluorescence band for all cell pellets. At 337 nm excitation, this emission band was centered at about 440 nm and could be attributed to NADH because the absorption of this fluorophore is maximal at this excitation wavelength.²⁰ At 480 nm excitation, this maximum was centered at about 550 nm, probably corresponding to expression of oxidized flavoproteins.²¹ Riboflavins (flavin mononucleotide and flavin adenine dinucleotide) absorb light in the visible range around 450 nm and emit a green fluorescence peak located at 530 nm.²² According to Benson, the emission of flavins covalently bound to proteins (i.e., flavoproteins) is red shifted by 10 to 30 nm over that of free flavins.²¹ This LIAFS study of cultured cells showed that, among the three different excitation wavelengths tested, 308 nm appeared to be optimal for discrimi-

nating normal cells from neoplastic urothelial cells, since it simultaneously provided the fluorescence emission of two distinct fluorophores (i.e., Trp and NADH). We therefore selected 308 nm as the excitation wavelength for the subsequent *in vitro* LIAFS study on surgical bladder specimens. At 308 nm, the fluorescence profile of normal bladder mucosa was quite different from that of normal urothelial cell pellets. The first emission band of normal bladder tissue was shifted toward the visible range, with a maximum at about 380 nm. This observation could indicate the contribution of another fluorophore, most likely collagen, for which the excitation and emission maxima are situated at 335 and 390 nm, respectively.¹³ The maximum observed at 380 nm could then result from overlap of Trp and collagen emissions. This collagen fluorescence expression in normal bladder mucosa is probably derived from collagen fibrils of the lamina propria supporting the urothelium. At 308 nm, the depth of tissue penetration is on the order of 100 μm .²³ As the normal urothelium thickness of a full bladder is estimated to be less than 100 μm ,²⁴ the LIAFS signal in normal bladder mucosa cannot be entirely due to urothelium, but must also include a contribution from underlying collagen fibrils. This observation may explain the shift of the first fluorescence band toward the visible range in normal bladder mucosa. A second important characteristic of the normal mucosa spectrum at 308 nm was the presence of a valley between the two fluorescence bands with a minimum at 420 nm. This valley is consistent with absorption of fluorescent light attributable to hemoglobin, and could be explained by trauma to the mucosa at the time of removal.^{13,25} Since the intensity of the second fluorescence maximum at about 450 nm, displayed by normal mucosa, is higher than the intensity of the 380-nm peak, this maximum cannot be entirely interpreted as an artificial structure due to the presence of a hemoglobin absorption valley on the first fluorescence band. Therefore the major part of this 450 nm band is probably due to the expression of NADH, as in the cell culture system.²⁰

The spectrum of all visible bladder tumors, regardless of their stage and grade, was dramatically different from that of normal bladder mucosa. Bladder tumors displayed a sharp, bright maximum at 360 nm corresponding to Trp emission.^{18,19} The deflection point at 420 nm could correspond to hemoglobin absorption and the second very weak band at 440 nm could express a low emission from NADH.²⁰ Furthermore one can notice, in Table 2, that absolute fluorescence intensities at 440 nm can statistically differentiate normal mucosa from bladder tumors, which was not the case for the 360 nm absolute intensities. This markedly reduced NADH fluorescence in bladder tumors could reflect different redox conditions since the oxidized form of this coenzyme is weakly fluorescent.²⁰ The absence of significant collagen fluorescence in the spectra of

visible bladder tumors could be explained by a thickened urothelium,¹ preventing the excimer laser light from reaching the lamina propria. Using 337 nm excitation, Schomacker et al. also found a decreased collagen fluorescence in colonic polyps compared to normal mucosa. They attributed this phenomenon to the mucosal thickening in the polyps.¹³ Similar results were observed in the upper aerodigestive tract by Kolli et al., who reported a correlation between the increased epithelial thickness present in tumors and the decrease of the collagen fluorescence signal exhibited by these tumors.²⁶

Remarkably, the fluorescence pattern of CIS, although globally decreased in intensity, had a spectral shape very similar to that of any visible bladder tumor. As in visible tumors, a minimal contribution of collagen fluorescence was observed in the CIS spectra. This could be ascribed to an increased size of poorly differentiated urothelial cells and/or an increased number of cell layers.^{1,2} The dramatic spectral differences displayed between normal urothelial mucosa and any bladder tumor specimens, including CIS, therefore appear to be due to different fluorophore contents as well as different morphologies, inducing different collagen expressions.

Absolute fluorescence intensities at 440 nm failed to statistically differentiate nonspecific inflammatory mucosa from bladder tumors. Nevertheless, the presence of two fluorescence bands in both normal and neoplastic bladder specimens meant that the absolute intensity ratio at two characteristic wavelengths (i.e., 360 and 440 nm) could be used for this purpose. Andersson et al.,⁴ using contrasting dyes, and Alfano et al., using native tissue fluorescence,¹⁰ considered that this ratio method eliminated variations of experimental parameters such as excitation light energy and efficiency of the detection system. Such experimental drawbacks could explain the high values for standard deviations observed in our study for fluorescence intensities at 360 and 440 nm. Das et al. also recommended ratioing of the intensities at two wavelengths equally absorbed by blood, to minimize the effects of tissue light absorption.²⁷ Following the recommendations of Das et al., we have considered the fluorescence intensity ratio at two selected wavelengths almost equally absorbed by Hb (i.e., 360 and 440 nm). Since the scattering of light over the ultraviolet and visible range remains relatively constant,²⁷ the reduction of fluorescence intensity by both absorption and scattering at 360 and 440 nm would be about the same. The difference in intensity ratios at these two wavelengths could then be attributed to the difference in the fluorescence yield from the native fluorophores at these two wavelengths (i.e., Trp, collagen, and NADH).

Our *R* ratio, representing a dimensionless function, provided a very clear distinction between nor-

mal or nonspecific inflammatory mucosa and bladder tumors, including CIS ($p < 0.05$). Using a diagnostic criterion defined by a cutoff value of 2, 100% of the tumors, including CIS, would be discriminated from normal or inflammatory bladder mucosa (Figure 5).

In conclusion, our LIAFS study on the cell culture system helped us to select an optimal wavelength (308 nm) to obtain maximum information from a single emission spectrum. This information concerns the expression of two distinct fluorophores (i.e., Trp and NADH). Our subsequent LIAFS study on bladder tissue specimens using 308 nm for excitation indicated the probable expression of collagen in normal bladder mucosa, absorption of fluorescent light by hemoglobin at 420 nm in all specimens, and a reduced NADH fluorescence in bladder tumors, including CIS.

Two characteristic wavelengths (360 and 440 nm) at which blood absorption and light scattering are equal, were chosen to establish a dimensionless ratio. This ratio allowed a clear distinction between normal bladder mucosa and all bladder tumor specimens, including CIS. A clinical study is currently under way to confirm these preliminary results, which could support the use, in the near future, of LIAFS as a new diagnostic tool for the detection of occult urothelial neoplastic lesions.

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