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

Maurice Anidjar,† Olivier Cussenot,† Sigrid Avrillier,‡ Dominique Ettori,‡
Jean Marie Villette,* Jean Fiet,* Pierre Teillac, and Alain Le Duc†
†Hôpital Saint-Louis, Département d’Urologie, Paris, France; ‡Université Paris XIII, Laboratoire de
Physique des Lasers, Villetaneuse, France; *Hôpital Saint-Louis, Laboratoire de Biologie
Hormonale, Paris, France
(Paper JBO-056 received Dec. 10, 1995; revised manuscript received Mar. 6, 1996; accepted for publication Mar. 13, 1996.)

ABSTRACT
The aim of this study was to perform a preliminary evaluation of the diagnostic potential of laser-induced
autofluorescence spectroscopy (LI-AFS) 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 LI-AFS as a new diagnostic tech-

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 un-
der white light cystoscopy.1 CIS has an important
prognostic value because its presence is clearly re-
lated to tumor recurrence and the subsequent pro-
gression of cancer.1,2 Urinary cytology and flow cy-
tometry 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 ap-
proach.

In recent years, there has been growing interest in
the use of laser-induced fluorescence to discrimi-
nate 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 avail-
able 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 in-
travesical instillation of 5-aminolevulinic acid
(ALA).8 ALA is a precursor in the heme biosyn-
thetic pathway that induces intracellular accumula-
tion of endogenous protoporphyrin IX. This detec-
tion 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 (LI-
AFS) 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, cer-
vix, ovary).17 Recently, LIAFS has also been demon-

Address all correspondence to Maurice Anidjar. Fax: 331 42 49 96 17.
strated to be able to distinguish bronchial, colonic, and cerebral tumors from normal tissues. 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. 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). 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 µg/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 ≈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), suspicious, flat, red-looking lesions (n = 7), papillary (n = 5) and nodular (n = 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 (Lasertechnology VSL 337ND) emitting at 337 nm (3 nm 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 µm, 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).
excitation light energy incident on the sample at the
distal tip of the 1 mm diameter fiber was for each pulse: 40 \mu J at 308 nm, 14 \mu J at 337 nm, and 4 \mu 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 \mu 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 exci-
tation, 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 approxi-
mately 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 ad-
justed 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 accu-
culated and averaged for each sample measure-
ment. 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 appa-
ratus was demonstrated by its ability to register auto-
fluorescence 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. Conse-
quently, for cell cultures, no conclusions could be
drawn from a comparison of absolute fluorescence
intensities. In the case of surgical specimens, histo-
logical 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 con-
tact 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, us-
ing 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 wave-
length, malignant T24 cells mainly exhibited a
bright fluorescence maximum centered at 360 nm,
followed by a slow decline of the emitted fluores-
cence [Fig. 2(a)]. At 337 and 480 nm excitation

![Figure 2](https://www.spiedigitallibrary.org/journals/Journal-of-Biomedical-Optics)
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 spectrum. 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 (∗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-

<table>
<thead>
<tr>
<th>Cystoscopy/histology</th>
<th>Normal mucosa</th>
<th>Nonspecific inflammation</th>
<th>CIS</th>
<th>Ta G1</th>
<th>T1 G3</th>
<th>T2 G3</th>
<th>T3 G3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal-appearing mucosa (n=5)</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Suspicious flat erythematous areas (n=7)</td>
<td>4</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Papillary tumors (n=5)</td>
<td></td>
<td></td>
<td>4</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nodular tumors (n=4)</td>
<td></td>
<td></td>
<td>1</td>
<td>1</td>
<td>2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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.

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 (∗5) is displayed.
Table 2  Mean fluorescence intensities in arbitrary units (a.u.) at 360 (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.

<table>
<thead>
<tr>
<th></th>
<th>I_{360} [a.u.]</th>
<th>I_{440} [a.u.]</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal bladder mucosa</td>
<td>7525±4660</td>
<td>8771±4617</td>
<td>0.85±0.29</td>
</tr>
<tr>
<td>Nonspecific inflammatory mucosa (n=4)</td>
<td>4494±3350</td>
<td>4455±5154</td>
<td>1.31±0.54</td>
</tr>
<tr>
<td>Bladder tumors (n=12)</td>
<td>6122±2686</td>
<td>2141±995</td>
<td>2.9±0.62^a,b</td>
</tr>
</tbody>
</table>

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

4 DISCUSSION

LIAFS has been investigated in neoplastic tissues and atherosclerotic arteries by a number of workers in order to evaluate its diagnostic potential. 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. 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) 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. 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. Previous studies indicate that this fluorescence band can be attributed to expression of the reduced form of the respiratory chain coenzyme, nicotinamide adenine dinucleotide. Absorption and emission maxima of NADH are 340 and 450 nm, respectively.

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. At 480 nm excitation, this maximum was centered at about 550 nm, probably corresponding to expression of oxidized flavoproteins. 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. 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.

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. 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. As the normal urothelium thickness of a full bladder is estimated to be less than 100 μm, 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. 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.

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. 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. 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. The absence of significant collagen fluorescence in the spectra of visible bladder tumors could be explained by a thickened urothelium, 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. 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.

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. 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. using contrast dyes, and Alfano et al., using native tissue fluorescence, 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. 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, 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-

ANIDJAR ET AL.
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.

REFERENCES
15. J. Bubenik, M. Baraesova, V. Vlkicky, Jakoubrova, H. Saini-
19. E. A. Burstein, N. S. Vedenkina, and M. N. Ivkova, “Fluo-