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Abstract. Fluorescence cystoscopy enhances detection of early bladder cancer. Water used to inflate the bladder during the procedure rapidly contains urine, which may contain fluorochromes. This frequently degrades fluorescence images. Samples of bladder washout fluid (BWF) or urine were collected (15 subjects). We studied their fluorescence properties and assessed changes induced by pH (4 to 9) and temperature (15°C to 41°C). A typical fluorescence spectrum of BWF features a main peak (excitation/emission: 320/420 nm, FWHM = 50/100 nm) and a weaker (5% to 20% of main peak intensity), secondary peak (excitation/emission: 455/525 nm, FWHM = 80/50 nm). Interpatient fluctuations of fluorescence intensity are observed. Fluorescence intensity decreases when temperature increases (max 30%) or pH values vary (max 25%). Neither approach is compatible with clinical settings. Fluorescence lifetime measurements suggest that 4-pyridoxic acid/riboflavin is the most likely molecule responsible for urine's main/secondary fluorescence peak. Our measurements give an insight into the spectroscopy of the detrimental background fluorescence. This should be included in the optical design of fluorescence cystoscopes. We estimate that restricting the excitation range from 370–430 nm to 395–415 nm would reduce the BWF background by a factor 2. [DOI: 10.1117/1.JBO.19.9.097002]

Keywords: photodetection; fluorescence spectroscopy; background fluorescence; urinary bladder.

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1 Introduction

Fluorescence cystoscopy has been developed to enhance the detection and to improve the therapeutic outcome of early bladder cancer. Its efficiency has been demonstrated in both cases.[1–3] Nowadays, it is mainly performed by detecting the red (610 to 720 nm) fluorescence of photoactivable porphyrins (PAPs), including protoporphyrin IX (PpIX) (for the purpose of this paper, "PPIX" will be used throughout as a proxy for all PaPs, of which PPIX is the most widely studied), produced in the cancerous cells of the bladder wall after instillation of 50 ml of a precursor solution,[4] usually hexylaminolevulinate (Hexvix® is the commercial name, Photocure, Oslo, Norway).[5] Hexvix® has been approved and covered by health care for many years in various countries, including the EU and the USA, where its counterpart is called Cysview®, for indications of known or suspected bladder cancers. In the EU, hexylaminolevulinate fluorescence cystoscopy (HAL-FC) has been recommended for more than a decade by the European Association of Urology for the detection of bladder cancer in patients with known or suspected bladder cancer, based on screening cystoscopy or positive urine cytology. According to the present guidelines, HAL-FC should be used as an adjunct to standard white-light cystoscopy (WLC) as a guide for taking biopsies. The abundance of data that supports the use of HAL for this indication has been summarized recently.[6,7] After 1 to 2 h, strong, red PPIX fluorescence is selectively produced in early cancerous lesions, which can be detected using blue–violet excitation light (typically between 380 and 435 nm). The resulting images often have a blue–green color for healthy tissues, while the early cancerous lesions appear in red. Depending on the instrumentation considered, the blue–green appearance of healthy tissues is due to the detection of a small amount of backscattered blue–violet fluorescence excitation light and/ or blue–green tissue autofluorescence.[7]

Fluorescence cystoscopy responds to an important medical need since it has been estimated that more than 10 to 20% of bladder tumors are overlooked in conventional WLC, even in the case of experienced operators.[8] Moreover, incomplete resections of undetected and detected tumors can result in unwanted recurrences.[1–3] Furthermore, Hexvix® itself significantly improved the pre-existing fluorescence cystoscopy procedure based on 5-aminolevulinic acid by providing a notable increase of PPIX fluorescence intensity with a lower instillation concentration and shorter instillation time, while preserving the high specificity.[7,9]

To perform a fluorescence cystoscopy, about 150 ml of water is needed to inflate the bladder after its drainage to remove the Hexvix® solution. During the cystoscopy itself, the kidneys produce urine with an average flow of 1 to 2 ml/ min. Hence the water instilled during the fluorescence cystoscopy rapidly contains a non-negligible amount of urine. This bladder washout fluid (BWF) must be periodically flushed during the cystoscopy to maintain an acceptable image quality during fluorescence cystoscopy. If the BWF absorbs or scatters relevant photons, or is fluorescent, the fluorescence cystoscopy images will be degraded.

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Unfortunately, the spectral design of most commercially available instrumentation for fluorescence cystoscopy does not take the optical properties of the BWF fluorescence into consideration. Hence, depending on the spectral design of these devices, the fluorescence background generated by the BWF or the absorption/scattering properties of the BWF may contribute to the limited performances of fluorescence cystoscopy. As such, our measurements give an insight into the exact spectroscopy of the detrimental background fluorescence, an element that we suggest should be included in the optical design of future generations of fluorescence cystoscopes.

Several fluorophores are potentially at the origin of the fluorescence of BWF[10] (see Fig. 1), but their exact source and relative contribution to it are poorly understood. It is, however, accepted that the main peak of fluorescence is from 4-pyridoxic acid (excitation at 317 nm, emission at 420 to 425 nm).[11]

The main purpose of this study is to identify the optimal spectral conditions to be used for fluorescence cystoscopy to minimize the perturbations resulting from the BWF fluorescence. Consequently, absorption and fluorescence spectroscopy measurements of urine and of the BWF collected during the drainage of the bladder have been performed.

A second goal was to limit disruption of this procedure due to BWF flushing: currently, the best way to minimize the disruption of fluorescence cystoscopy due to the BWF is to empty the bladder and fill it with clean water again. Because the clean water used to fill the bladder is soon laced with fluorescent urine, the procedure to flush and refill has to be repeated several times during the course of the exam, which is time-consuming and suboptimal. There are systems with continuous flows, but these are unusual in most clinical settings.

Finally, it was also of interest to determine if simple approaches like changing the pH or the temperature can reduce or modify the spectroscopy and intensity of the BWF luminescence and still be compatible with clinical settings.

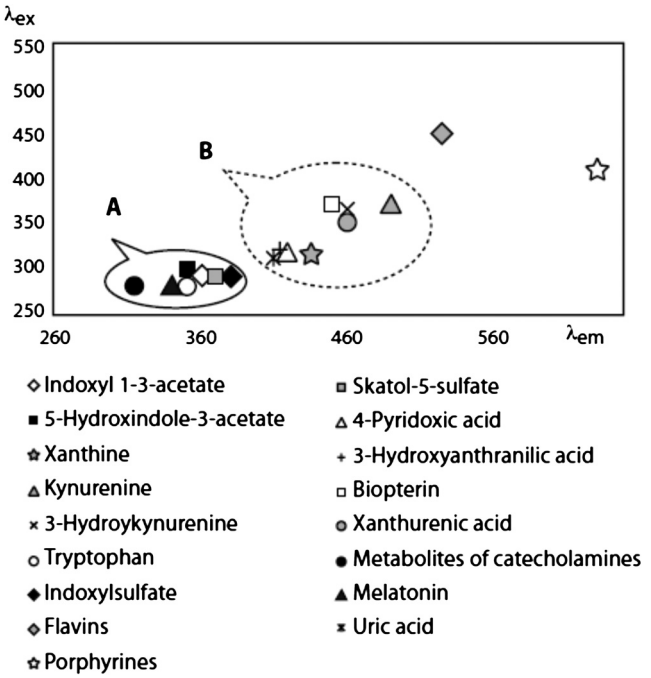

Fig. 1 Localization of urine fluorophores maxima in the excitation–emission matrix (EEM). Group A represents the highest contribution to the whole urine fluorescence. Group B is characteristic with four to six times lower intensity than group A (adapted from Ref. 10, with permission from Taylor & Francis Inc.)

2 Materials and Methods

2.1 *Patients, BWF, and Urine Samples*

BWF samples have been obtained from nine patients [seven males, two females, mean age: 70 years old (32 to 78 years old)] subject to fluorescence cystoscopy performed at the Urology Center of the Lausanne's University Hospital (CHUV). This study was performed in compliance with the local regulation and rules of the local Ethics Committee of the CHUV university Hospital. BWF samples were obtained during the course of the cystoscopy, before transurethral resection of the bladder (TURB) and before the cytological procedure.

After drainage of the Hexvix® solution, the bladder was filled with purified water (Aqua, B. Braun, Melsungen, Germany) or NaCl solution during fluorescence and/or WLC to ensure good visualization of the bladder mucosa. One sample per patient (typically 15 ml) of the resulting BWF was collected during the second drainage of the bladder. Depending on the progress of the medical intervention, the water solution remained in the bladder about 6 min (range: 3 to 10 min). Therefore, the urine concentration in the BWF varied (estimated range based on the typical urine secretion) from about 1% to 7% between patients. No spectral difference was evidenced in correlation with the liquid used to fill the bladder during cystoscopy (Acqua or saline solution).

Six samples of pure urine were also collected from healthy volunteers (three males, three females), mean age: 39 years old (24 to 69 years old) in order to assess the spectral effects induced by pH and temperature changes, as described in more detail below.

2.2 *Sample Preparation*

All BWF and urine samples were stored at 4°C in the dark between collection and the spectral measurements. The typical time interval between collection and measurement was 2 h. Some samples were measured up to 15 days after collection to ensure that no aging effects were affecting the spectral properties by examining the spectral features of the samples at regular intervals after collection. No significant differences were observed (data not shown).

For the fluorescence spectroscopy measurements, ~3.5 ml of noncentrifuged BWF and urine samples were withdrawn and investigated at room temperature. Since the optical density of urine or the BWF in the UV can possibly impact our fluorescence measurements due to the "inner-filter effect" (IFE),[12] we diluted each urine sample with purified water (Aqua) until it reached an optical density smaller than 1 at 350 nm (typical dilution for urine samples 1:25) before measuring their fluorescence properties. Indeed, we established in the context of a preliminary separate study (data not shown) that samples with optical densities smaller than this value are not subjected to this IFE. In the case of points taken with samples with OD <1 at 350 nm, no correlation could be evidenced between OD and fluorescence intensity. This is not entirely surprising since BWF and diluted urine are a complex mixing of nonfluorescent and fluorescent molecules, absorbing in the range that we measured, and whose relative contributions and spectral characteristics are poorly understood.

2.3 *Spectroscopy*

The samples were placed in a 3.5 ml quartz cuvette (Hellma OS, Müllheim, Germany). The fluorescence was analyzed with a fully calibrated spectrofluorometer (LS-50B, Perkin Elmer, Waltham, Massachusetts) and the results are presented in the form of an excitation–emission matrix (EEM), as well as fluorescence emission spectra. The excitation wavelengths ranged from 320 to 445 nm in 5 nm steps, and the emission wavelengths ranged from 350 to 800 nm. Fluorescence was collected with an angle of 90 deg. To account for background fluorescence, fluorescence properties of NaCl 0.9% and Aqua have also been measured, confirming that these solvents are not fluorescent at the wavelengths of interest.

Absorption spectroscopy was performed between 300 and 800 nm with a spectrophotometer (Cary 500 UV/VIS/NIR, Varian, Palo Alto, California). Samples were analyzed in a 1 cm "optical path length" quartz cuvette (Hellma OS).

The fluorescence data were postprocessed with an in-house MATLAB algorithm that combined all the emission spectra at several excitation wavelengths in one EEM.

2.4 *Temperature Dependence*

The temperature dependence of the fluorescence spectroscopy was performed by positioning the cuvette containing the solution in a thermostabilized holder whose temperature was Peltier-cooled (Cary PCB-150, Varian); the Peltier-cooling system was connected to a spectrofluorometer FluoroLog 3-22 (Horiba Jobin Yvon, Longjumeau, France). The temperature influence was evaluated using six diluted urine samples: three urine samples came from male volunteers and three from female volunteers. Four different temperatures were tested: 15°C–25°C to 35°C–41°C (precision: ±1°C).

2.5 *pH Dependence*

The pH experiments were conducted starting with the urine samples of the six volunteers (three males and three females). The pH measured after the urine dilution with purified water was taken as a baseline. It ranged from 5.9 to 7.1. The baseline pH was varied by adding 0.1 ml of buffer concentrated solutions at pH 4 or pH 9 (Metrohm AG, Herisau, Switzerland). The pH was measured using a pH meter (CG-825 Schott, Mainz, Germany) and a 178-mm micro pH electrode (Metrohm AG). The pH meter was calibrated before each measurement. Calibration was performed with two standard buffer solutions at pH 4 and 9 (Metrohm AG).

2.6 *Fluorescence Lifetime Measurements*

The fluorescence lifetime of diluted urine (urine concentration: 1.9%) and pure riboflavin (Riboflavin 5′-monophosphate Sodium Salt Dihydrate, Fluka, St. Louis, Missouri) was measured with a spectrofluorometer FluoroLog 3-22 (Horiba Jobin Yvon). The concentration used to prepare an aqueous solution of riboflavin was chosen according to reported parameters for pH 7.[13]

The measurement of lifetime was carried out at excitation wavelength 320 nm (NanoLED, Horiba Jobin Yvon), pulse width <1.2 ns, and emission was detected at 420 ± 10 nm. For the secondary peak, the excitation wavelength was set at 405 nm (NanoLED, Horiba Jobin Yvon), the pulse width 200 ps, and emission was detected at 530 ± 10 nm.

Table 1 Absorption of BWF samples at 350 nm.

Patient	Absorption at 350 nm
Patient 1	0.47
Patient 2	0.75
Patient 3	2.15
Patient 4	2.24
Patient 5	2.57
Patient 6	0.35
Patient 7	0.14
Patient 8	0.77
Patient 9	0.35

3 Results

Within the BWF samples collected during the clinical procedure, samples with an optical density of 350 nm higher than 1 (3 out of 9) were not taken into consideration for identification of the excitation and emission wavelengths of the main peak. This is because optical densities higher than 1 are known to induce a bias through the IFE, as mentioned above (Table 1). They were nonetheless taken into account for the investigation of the relative fluorescence intensity fluctuations of the secondary peak because these were assessed in another (less absorbed) spectral range, which does not correspond to the absorption range of the main absorbing species of BWF. The measurements of fluorescence intensity fluctuations of the main peak were carried out at its maximum.

Absorption spectra were measured between 300 and 800 nm. A typical absorption curve of a BWF sample collected during fluorescence cystoscopy is shown in Fig. 2. It shows an optical density lower than 1 at 350 nm. Urine's absorption properties are mainly attributed to Urobilin, a nonfluorescent molecule and the final degradation product of heme.

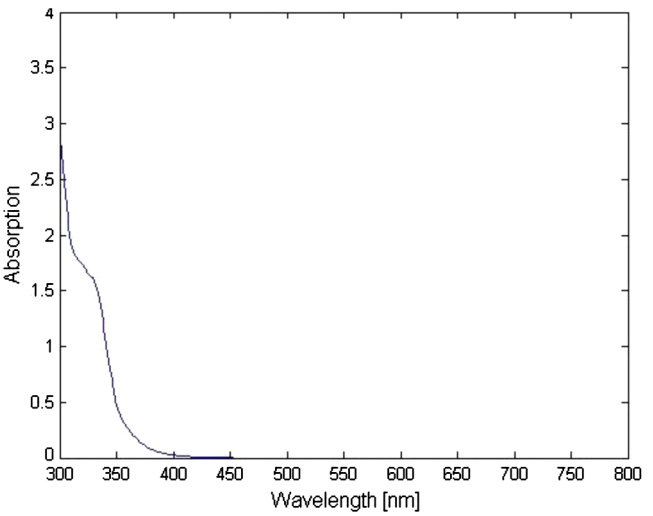

Fig. 2 Typical bladder washout fluid (BWF) absorption spectrum showing absorption <1 at 350 nm.

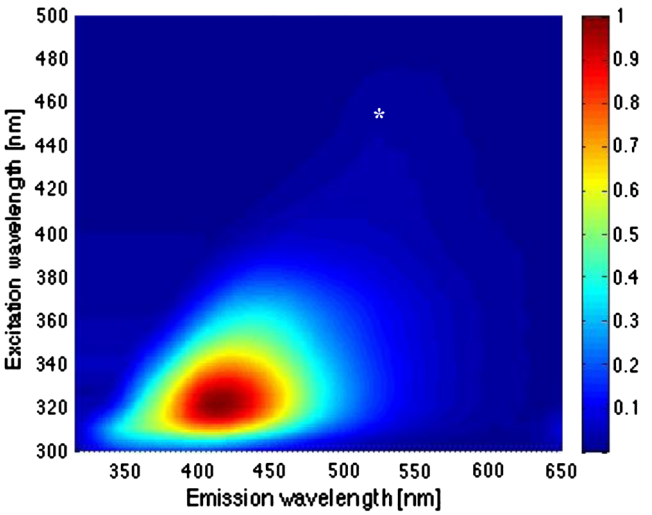

Fig. 3 Typical EEM of BWF fluorescence showing two peaks (ex.: 300 to 500 nm; em.: 330 to 650 nm). The secondary peak is weaker, and its intensity relative to the main peak is variable. In order to minimize possible confusion to locate this secondary peak, we have identified the location of its maximum with a white star.

In a preliminary, separate study, initial samples were collected after TURB or during the urothelial cell collection for cytological diagnosis. However, it was observed that the presence of blood or urothelial cells in the solutions could affect the identification of the BWF's spectral properties (data not shown). For this reason, the BWF samples described in this paper were only collected before TURB and before the cytological procedure.

An EEM of BWF is given in Fig. 3, chosen among 46 EEMs because it was closest to the average of all normalized EEMs, thus the most representative of a typical EEM. It consists of a main peak (excitation wavelength: 320 nm, FWHM = 50 nm; emission wavelength: 420 nm, FWHM = 100 nm) and of a weaker, secondary peak (excitation wavelength: 455 nm, FWHM = 80 nm; emission wavelength: 525 nm, FWHM = 50 nm, whose intensity relative to the main peak is variable (between 5 and 20%).

It is worth noting from the spectra of the nine BWF samples that there is a significant interpatient fluctuation of fluorescence intensity: the maximal fluorescence intensity of the main peak varied by a factor of up to fourfold. The fluctuations of the second peak were calculated only relative to the main peak due to the fact that the second peak was at times too weak to be accurately measured.

3.1 *Temperature Dependence*

Figure 4 shows the effect of modifying the temperature of diluted urine within a specific range (15°C to 41°C). The temperature dependence was studied using either an ascending or descending temperature scale, showing that no significant difference can be noted between the two protocols (data not shown).

It can be observed that an increase of temperature induces a decrease in fluorescence intensity. For the temperature range closest to physiological temperatures, a fluorescence intensity decrease of about 10% is observed with a temperature increase from 35°C to 41°C, whereas from 14°C to 35°C, the fluorescence intensity decreases by about 18%. The main peak position in the EEM is not affected by the temperature variation (data not shown).

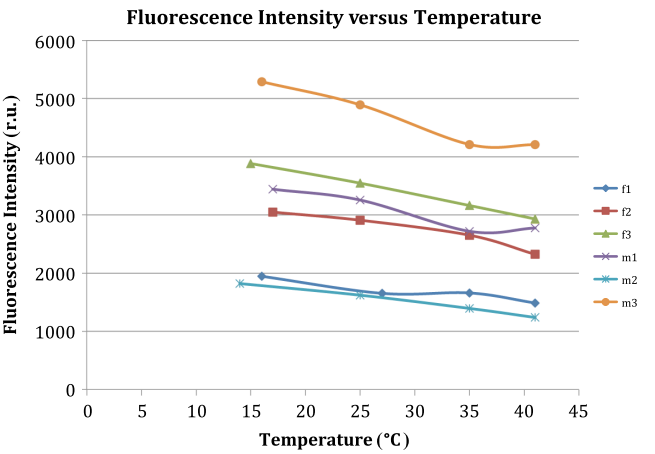

Fig. 4 Fluorescence Intensity of the main peak (at the wavelengths corresponding to the maximum) of BWF fluorescence emission as a function of the temperature of six diluted urine samples collected from three male and three female volunteers.

3.2 *pH Dependence*

Figure 5 shows the correlation between the fluorescence intensity of the main fluorescence peak and the pH value of the sample. When the pH value deviates from the baseline values (pH = 5.9 to 7.1), fluorescence intensity decreases. In the acidic range (from pH = 4 to the baseline), the fluorescence decrease is about 17% and in the alkaline range (from the baseline to pH = 9), it corresponds to about 12%. The excitation and emission wavelengths of the main fluorescence peak did not show a measurable pH dependence (data not shown).

4 Discussion

Urine is a complex biological mixture of chemical compounds, some of which are fluorescent. Therefore, it is not straightforward to attribute the BWF fluorescence to a specific molecule because many fluorophores in urine have similar spectral characteristics and they affect each other, which impacts their

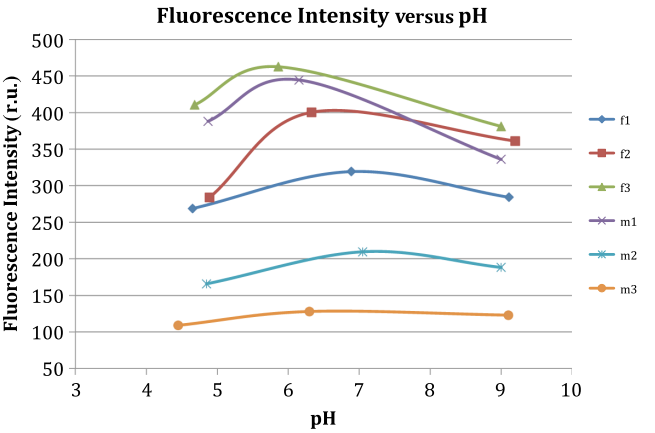

Fig. 5 Fluorescence Intensity of the main peak (at the wavelengths corresponding to the maximum) of BWF fluorescence emission as a function of the pH of six diluted urine samples collected from three male and three female volunteers.

spectral properties.[10,11] Generally speaking, it should be noted that our study is preliminary in nature and only aimed at charting the issue of BWF fluorescence to address the underlying issue of suboptimal conditions for fluorescence cystoscopies. This sets it apart from many similar studies that were carried out on pure human urine. We approached the questions at hand from the clinician's perspective, and chose to work on BWF, much closer to the reality of the clinical settings than pure urine. This aspect is one of the interesting features of our study: whereas other groups studied pure human urine, we carried out measurements on BWF, taken directly in the clinical context during Hexvix® fluorescence cystoscopy.

Our measurements explore the excitation and emission aspects of the spectroscopy of BWF and urine. This is an important aspect for fluorescence cystoscopy as the method relies on maximal contrast between lesions and background. It is clear from this study that minimizing the BWF luminescence with an excitation and emission spectral design that is optimized for PPIX will result in much better performances. As such, our measurements give an insight into the exact spectroscopy of the detrimental background fluorescence, an element that we suggest should be included in the optical design of future generations of fluorescence cystoscopes. It could be as simple a modification as, for example, the excitation and emission filters. Although this perturbation is observed with all commercially available systems, this spectral optimization will certainly be particularly valuable for systems detecting tissular native fluorescence (autofluorescence) images in the blue–green region, instead of a blue–violet backscattering image. This is due to the fact that the brightness of the former is smaller than that of the latter by about 1 order of magnitude.[14] The commercial availability of violet LEDs also opens new fields of optimization for fluorescence cystoscopy hardware. Among many advantages, these light sources have a higher spectral power (in W/nm) than the medical filtered Xe lamps. Therefore, more illumination power could be delivered in a narrower spectral band, thus minimizing the illumination range. Currently, fluorescence cystoscopies are typically performed using an excitation light ranging between 370 and 430 nm, and a long-pass filter (cut-on wavelength: 450 nm) rejects the excitation. Since this spectral design excites and detects a significant portion of the BWF, a much better excitation wavelength range would be between 395 and 415 nm. An initial estimate is that restricting the excitation range from 370–430 nm to 395–415 nm would reduce the BWF background by a factor of 2. These conclusions only apply to environments devoid of blood, a strong absorber of blue/violet light. If blood were found in the BWF (as it is the case when fluorescence cystoscopy is used to monitor/delineate resections), the optimal excitation wavelength range would be slightly red shifted.

The secondary peak fluorescence intensity is between 5% and 20% relative to the main peak. The reasons for the secondary peak intensity variations still need to be explored and assessed, as, for example, a study of the influence of hypervitaminic diet or specific food items/drugs consumption on the BWF fluorescence. A similar study on a larger pool of patients would support our initial results, in particular if there is a need to more accurately identify the molecules or food components responsible for the BWF fluorescence.

One should, however, note that general variations in BWF fluorescence intensity can be linked to elements such as time during which BWF stays in the bladder (the longer the time, the higher the concentration of fluorescent urine in the BWF), the quantity of fluorophores in urine produced by the patient, and the individual metabolism of each patient. We did not find any gender correlation. While it is impossible to fully standardize diet in the days prior to a cystoscopy, one could imagine that guidelines could be released for patients who are to undergo a cystoscopy, precluding them from consuming some foods containing OTC tablets [i.e., vitamin supplements, and in particular those containing Vitamin B2 (Riboflavin)] in the days preceding their exam.

Our set of BWF samples showed a main emission peak at excitation wavelength: 320 nm (FWHM = 50 nm) and emission wavelength: 420 nm (FWHM = 100 nm). They also showed a weaker, secondary peak at excitation wavelength: 455 nm (FWHM = 80 nm) and emission wavelength: 525 nm (FWHM = 50 nm); this is similar to what has been described by Kusnir et al. for pure human urine.[10] The position of the fluorescence peak we observed in the EEM of diluted urine is also in good agreement with Perinchery et al.,[15] who studied the spectral features of undiluted urine samples from healthy individuals and from those with urinary tract infection; they showed the presence of an emission peak around 440 ± 15 nm when the samples were excited at 355 ± 15 nm. Due to the different aims of their study, they used undiluted urine samples. It is reported that this could cause IFEs,[12] which may explain the red shift of the peak they measured compared to what we observed in our results.

According to Leiner et al., the blue–green urine fluorescence is associated with the presence of two main groups of fluorophores.[11] Most of them are metabolites of tryptophan.[10] The main emission peak is most likely due to 4-pyridoxic acid (with a fluorescence peak at 317 nm/420 nm (excitation wavelength/emission wavelength), a catabolite of vitamin B6.[16,17] 3-Hydroxynthranilic acid (320 nm/415 nm), xanthine (315 nm/435 nm), and neopterin (353 nm/438 nm) also likely contribute to this peak.[11,15,17] Leiner et al. also observed secondary peaks at (450 nm/525 nm; 465 nm/525 nm; 420 nm/525 nm) and they attributed them to the presence of riboflavin or one of its urinary metabolites or a combination thereof.[11] Riboflavin has its excitation maximum at around 450 nm and emission at around 525 nm.[18] In our study, the secondary peak was observed in virtually all samples, but its intensity relative to the main peak varied between 5% and 20% of the main peak intensity.

The temperature dependence of the fluorescence is well described in the literature.[19–22] Zaman et al. already demonstrated a decrease in fluorescence intensity as tissue temperature increased.[22] A decrease of fluorescence such as the one we observed on BWF could be of interest in a quest to minimize BWF fluorescence. Our observations show that this reduction is small and appears at temperatures too high to be of clinical use (i.e., 41°C). Furthermore, it is unclear whether such a temperature increase would also impact the other fluorescent species present during the procedure and possibly be detrimental to the cystoscopy.

The properties of many urine fluorophores present strong pH dependence.[13,16,23,24] Pyridoxinic compounds, likely to be responsible for the main fluorescence peak we observe, are sensitive to pH, since they may exist in different ionization states.[16] Our study confirmed this pH dependence, but only to a limited extent. Again, this would limit the applicability of changing the pH for clinical purposes. Generally speaking, while a

temperature or pH modification sounds like an easy way to impact the fluorescence of BWF, neither approach (raising the temperature to 41°C or modifying the pH to 8.5 or 4) would be feasible in most clinical settings for obvious safety reasons. In any event, our measurements show that the decrease of fluorescence would not be so important since it would not allow a significant improvement of the blurring of the image.

To confirm our findings, we carried out fluorescence lifetime measurements. They suggest that 4-pyridoxic acid is responsible for BWF main fluorescence. This is in good agreement with previous works.[11,16,17,25] The fluorescence lifetime measurement of urine, using an excitation at 320 nm, showed a biexponential decay function yielding the following fitted parameters: $\tau_1 = 3.2$ ns (relative amplitude 26.18); $\tau_2 = 8.7$ ns (relative amplitude 73.82); $\chi^2 = 1.24$. This converges with previous work in suggesting that 4-pyridoxic acid, whose fluorescence lifetime is 8.4 ns (in water at pH = 7),[26] is the most likely molecule responsible for urine's fluorescence.

5 Conclusion

Our study explores the issue of fluorescence of the BWF, primarily to optimize the spectral design of photodynamic detection systems. Currently, fluorescence cystoscopies are performed using a spectroscopy that excites and detects a significant proportion of the BWF fluorescence, thus requiring frequent emptying of the bladder. Our measurements give an insight into the exact spectroscopy of the detrimental background fluorescence, an element that we suggest should be included in the optical design of future generations of fluorescence cystoscopes. An initial estimate is that restricting the excitation range from 370–430 nm to 395–415 nm would reduce the BWF background by a factor of 2.

In addition, our study provides useful information for the identification of the fluorochromes that are responsible for the BWF fluorescence. The studies we have performed to determine if simple approaches compatible with the clinical context can decrease the fluorescence intensity or change the fluorescence spectroscopy of the BWF prove that we can decrease this fluorescence intensity by changing the temperature or modifying the pH, but also showed that this is not compatible with clinical settings.

Acknowledgments

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