Analytical Methods

PAPER

View Article Online
View Journal | View Issue

Cite this: *Anal. Methods*, 2016, **8**, 4991

Methodologies for bladder cancer detection with Raman based urine cytology†

Laura T. Kerr,[a] Thérése M. Lynn,[b] Ivor M. Cullen,[c] Padraig J. Daly,[c] Nigam Shah,[d] Shirley O'Dea,[b] Alison Malkin[e] and Bryan M. Hennelly*[a]

Bladder cancer has the highest recurrence rate of any cancer. The American Urological Association recommends cystoscopic surveillance every 3–6 months for 3 years, and at least once a year thereafter, particularly for high-risk patients; however, cystoscopy is invasive, expensive, and is not without insignificant morbidity for the patient. Urine cytology is often used as an adjunct to cystoscopy; however, it has a low sensitivity in detecting low grade bladder cancers. Recent studies have investigated the application of Raman micro-spectroscopy for the detection of bladder cancer *via* urine cytology, and it has been demonstrated to significantly improve the diagnostic sensitivity of urine cytology for low grade bladder cancer under ideal experimental conditions. In this paper we attempt to move Raman micro-spectroscopy a step closer to the clinic by systematically examining the potential of this technology to classify low and high grade bladder cancer cell lines under the stringent clinical conditions that can be expected in the standard pathology laboratory, in terms of consumables, protocols, and instrumentation. We show that the use of glass slides, traditional fixing agents, lengthy exposure to urine, red blood cell lysing agents, as well as common cell deposition methods, do not significantly impact on the diagnostic potential of Raman based urine cytology. This study suggests that urine samples prepared with the ThinPrep® UroCyte™ method and analysed with Raman micro-spectroscopy could provide a useful alternative to cystoscopy for long term bladder cancer surveillance.

Received 16th December 2015
Accepted 29th May 2016

DOI: 10.1039/c5ay03300d

www.rsc.org/methods

1 Introduction

Bladder cancer, otherwise known as urothelial carcinoma (UC), is the seventh most common cancer in the UK, with approximately 10 000 people being diagnosed every year, the majority of which are males. UC has the highest recurrence rate of any cancer, and it has been reported as the most expensive malignancy from diagnosis to death for health care systems, costing the NHS in the UK approximately £ 55 million a year.[1–3] Urine cytology coupled with cystoscopic examination is the standard for bladder cancer diagnostics. However, cystoscopy is invasive, expensive, and may miss some flat lesions. Approximately 75% of patients present with superficial disease (Ta or T1), known as non-muscle invasive bladder cancer (NMIBC), and the majority of new diagnoses do not require a radical cystectomy with urinary diversion. These patients are managed with an endoscopic procedure *via* the urethra, known as a trans-urethral resection of the bladder tumour (TURBT), which 'scrapes' the tumour from the lining of the bladder, ensuring that the deep margins of the tumour are completely resected, and the surrounding abnormal or dysplastic mucosa is adequately fulgurated.

Voided urine cytology is a useful non-invasive adjunct in the diagnosis of UC. Examination of voided urine for exfoliated cells has high sensitivity in high grade tumours, but low sensitivity in low grade tumours.[4] Diagnostic delays lead to delayed management of the cancer with resultant poorer outcomes for the patient. Therefore, early detection of urothelial lesions is important in order to optimise patient treatment, reduce costs, and to improve patient anxiety.

Of particular concern for public health systems internationally is the approach to surveillance for patients previously diagnosed and treated for NMIBC. This particular subset of UC has a high rate of recurrence, and therefore, these patients remain on costly invasive cystoscopic surveillance programmes for the remainder of their lives. Previous research has been carried out into the usefulness of urinary cytology *versus* cystoscopy in the follow-up of NMIBC.[5,6] However, urinary cytology is limited by its low sensitivity for low grade tumours, and whilst urinary biomarkers have produced better results for detecting these tumours, they are still unable to detect half of

[a]*Department of Electronic Engineering, Maynooth University, Maynooth, Co Kildare, Ireland. E-mail: bryanh@cs.nuim.ie; lauratheresekerr@gmail.com*
[b]*Institute of Immunology, Maynooth University, Maynooth, Co Kildare, Ireland*
[c]*Department of Colorectal/Urologic Surgery, University Hospital Waterford, Waterford, Ireland*
[d]*Histology Department, University Hospital Waterford, Waterford, Ireland*
[e]*School of Biological Science, Dublin Institute of Technology, Ireland*

† Electronic supplementary information (ESI) available. See DOI: 10.1039/c5ay03300d

the low grade tumours that have been identified by cystoscopy.[5,7] According to current knowledge, no urinary marker can replace cystoscopy during follow-up, or help to lower cystoscopic frequency in a routine fashion.[8]

1.1 Standard urine cytology

Urinary cytology is usually requested for patients with unexplained hematuria, irritative voiding symptoms, patients suspected of bladder cancer, and patients monitored for bladder tumour recurrence. The predominant cellular component of urine cytology is normal urothelial cells, which can vary greatly in numbers, sizes, and shapes. Inflammation, infective pathogens, and blood components are also found across many samples. The process of isolating, and identifying, urothelial tumour cells within a voided urine sample can be challenging, particularly for low grade UC.

Cytologically, high grade UC is relatively easy to diagnose due to the presence of anaplastic cells. These cells have high nucleus to cytoplasm (N/C) ratios; the nuclei are often eccentric with large irregular nucleoli. On the other hand, low grade UC is more difficult to diagnose; these tumours are cytologically characterised by increased cellularity, and the presence of an increased number of urothelial clusters that may or may not be papillary. The cells in these clusters have high N/C ratios with nuclei bulging out of the cytoplasm. Carcinoma *in situ* (CIS) is a flat lesion, and is the precursor to most invasive urothelial cancers. Cytology plays an important role in the detection of urothelial CIS since these lesions may be multifocal and not visualised on cystoscopy. These cells are large with high N/C ratios.[9] Other types of urothelial malignancies that may be present include squamous carcinoma, adenocarcinoma, small cell carcinoma, lymphoma, or metastatic tumour cells from different neoplastic primaries.

The specificity of cytology is greater than 90% and the sensitivity is ~80% for high grade UC; however, the sensitivity is 20–50% for low grade UC.[10] The main reason for this is due to the fact that low grade tumour cells have a similar cytomorphology to normal urothelial cells, and the observation of increased cellularity or papillary fragments, which are associated with UC, may instead be related to lithiasis, infection, or urinary tract instrumentation. The cohesive nature of low grade tumours may also result in less cells being shed into the urine.[2]

Urinary cytology is useful, particularly as an adjunct to cystoscopy, when a high grade malignancy or CIS is present. Positive voided urinary cytology can indicate a urothelial tumour anywhere in the urinary tract; negative cytology, however, does not exclude the presence of a tumour. Cytological interpretation is highly user dependent.[11] Evaluation can be hampered by low cellular yield, urinary tract infections, stones, recent intravesical instrumentation, or instillations, but in experienced hands specificity exceeds 90%.[12]

1.2 Raman based urine cytology

Raman micro-spectroscopy is a powerful technique for the identification and classification of cancer cells and tissues. Raman spectroscopy is based on the inelastic scattering of light, and occurs when incident laser photons interact with molecular bonds in the sample, resulting in emitted photons of a different energy, which can be used to identify biomolecular changes within cells as they progress from a healthy to a cancerous state. In recent years, the applications of Raman micro-spectroscopy, whereby a Raman spectroscopy system is integrated with a microscope, to biological cells has increased, with many reporting the application of Raman micro-spectroscopy to analyse and accurately classify cervical, bladder, and oral cytological samples.[10,13–15]

Multivariate statistical analysis is often applied to Raman spectroscopic data for classification. This involves the application of pattern recognition techniques, such as Principal Components Analysis (PCA) or Linear Discriminant Analysis (LDA), in order to identify subtle changes across datasets that can be used to accurately differentiate between different pathological groups and subgroups.[16]

Recent studies based on the application of Raman micro-spectroscopy to urine cytology have shown good results for analysing and classifying disease in bladder cells. In 2008, Harvey *et al.*[17] used Raman optical tweezers to trap and analyze both live and chemically fixed bladder cell lines, and were able to differentiate between normal and cancerous cells. In 2009, Harvey *et al.* continued to develop this application by performing a comparison between SurePath™ (Becton Dickinson, US) and formalin fixing agents, as well as monitoring the effect of urine exposure times on bladder cell lines.[18] They discovered spectral contamination present in the spectra recorded from SurePath fixed cells, which was not found in the formalin group. The authors also compared unfixed cells that were exposed to urine for a range of time points from 15 min to 12 h, and based on a PC-LDA model, it was reported that, in general, the prediction values did not deteriorate over 12 h. In 2011, Canetta *et al.*[19] applied modulated Raman micro-spectroscopy to bladder cell lines that were exposed to urine fixed with PreservCyt™ (0234004, Hologic, Screenlink Healthcare, UK). Based on PCA of the modulated data, they were able to distinguish between two cell lines with >80% sensitivity and specificity after 6 h exposure to urine.

Shaprio *et al.*[20] were the first group to apply Raman micro-spectroscopy to fresh epithelial cells from human urine samples. Voided urine samples were obtained from 340 patients, and Raman spectra were recorded from unfixed cells on aluminium slides, and based only on the 1584 cm^{-1} Raman peak, they reported classification between healthy and cancerous cells with >90% sensitivity and specificity.

1.3 Experimental motivation

The objective of this paper is to investigate if Raman micro-spectroscopy can successfully classify low and high grade cell lines under the stringent conditions imposed by a typical pathological laboratory. In order to achieve this, four experiments that systematically examine the performance of Raman based urine cytology for cell classification across a range of parameters are proposed.

A comparison between air dried, formalin fixed, and PreservCyt fixed UC cells is the basis of our first experiment. The impact of formalin fixation has previously been investigated

with Raman micro-spectroscopy,[21] however, to the best of our knowledge, PreservCyt has never been previously compared with air-dried and formalin fixed cells with Raman micro-spectroscopy. This initial study allows for the identification of biomolecular differences that are introduced to the UC cell samples by these fixation methods, and provides information on the impact these changes have on the capacity to classify between low and high grade UC cell lines. In order to minimise the number of variables in this initial experiment, these cells were not exposed to urine.

An important consideration is the length of time that cells can remain within the urine solution before Raman based classification becomes unreliable. It has previously been shown that it is possible to distinguish between different cell types after exposure to urine using Raman micro-spectroscopy.[18–20] However, it should be noted that these previous studies dealt with cells that were exposed to urine for relatively short periods of time. In order to analyse urine samples from a clinic, it is impractical to design a Raman study based around fresh, unfixed samples; this is due to the rapid deterioration of cells in urine, and the fact that recording all cells on a slide using Raman micro-spectroscopy is typically a time-consuming process. To overcome this problem, standard urine cytology involves the addition of a preserving agent into the urine collection vial, with cells being fixed again after the urine solution has been decanted.[2,22–24] However, the impact of these agents on the recorded spectra, and the capability of multivariate statistical algorithms to classify different cell types after fixing, must be considered. Therefore, the second experiment deals with the impact of urine on cells over 72 h following PreservCyt fixation, with 72 h representing the maximum expected time frame for the transfer of a urine sample from the clinic to the lab for analysis.

Urine cytology is usually performed with a liquid based processing technique such as SurePath or ThinPrep. These systems are designed to prepare uniform monolayer cells onto glass slides with minimal cell debris or blood residue in the background. SurePath is a density gradient based cell enrichment process that fixes cells with an ethanol based solution, whereas ThinPrep is a filter based cell concentration technique that uses the methanol based solutions PreservCyt and CytoLyt™ (0236004, Hologic, Screenlink Healthcare, UK).[25] ThinPrep UroCyte is the standard method used for the preparation of urine samples with the ThinPrep 2000 (T2) machine.

The third investigation in this study involves preparing urine/cell solutions with the ThinPrep UroCyte method for Raman micro-spectroscopy. There are two subgroups within this experiment: (i) application of only PreservCyt, and (ii) application of both PreservCyt and CytoLyt; this enables a make direct comparisons of both methods, and the impact of CytoLyt on UC cells can be identified in isolation.

Hematuria is the most common symptom present in patients diagnosed with UC, occurring in approximately 90% of cases.[26] Whilst hematuria may be intermittent for patients, it is important to consider the impact of red blood cells in urine when analysing these samples using Raman micro-spectroscopy. Red blood cells are known to produce a large signal that can often swamp the weak Raman spectrum obtained from epithelial cells. Urine solutions that contain blood can be treated with red blood cell lysing agents such as the combination of H_2O_2, ethanol, and industrial methylated spirits as reported by Bonnier *et al.*,[13] or CytoLyt, which is part of the standard ThinPrep cytology method. In the final experiment, the impact of both scant and frank hematuria on Raman spectra of UC cell lines is investigated. Samples are processed using the ThinPrep UroCyte method, in conjunction with CytoLyt in order to lyse any red blood cells that may be present.

2 Methods

2.1 Sample preparation

2.1.1 Cell culture. Bladder cell lines T24 (high grade UC) and RT112 (low grade UC) were obtained from Cell Lines Service (CLS GmBH, Germany) and were cultured in 1 : 1 mixture of DMEM and Hams-F12 medium supplemented with 5% fetal bovine serum and 2 mM L-glutamine. Flasks were maintained in a humidified atmosphere with 5% CO_2 at 37 °C. When both cell lines reached between 80–100% confluency, the culture medium was removed, and the cells were rinsed with sterile PBS. Trypsin–EDTA (0.5%) was added to the flask, which was incubated at 37 °C until the cells had completely detached (not exceeding 15 min). An equal volume of 5% serum-containing medium was added to the flask to neutralise the trypsin enzyme. The contents of the flask were transferred into a sterilin container and centrifuged (1200 rpm, 5 min). The supernatant was removed, and the cell pellet was resuspended in fresh medium. This solution was centrifuged, the medium was decanted, and the cells were resuspended in 1 ml PBS. This step was repeated and the cell pellets were then processed as explained later in Sections 3.1.2–3.1.6.

2.1.2 Fixing agents. In order to compare between air dried, formalin fixed, and PreservCyt fixed cells, the samples were prepared as follows:

(A) Air dried: the cell pellet was suspended in 2 ml PBS, followed by centrifugation. The PBS was decanted, and the cell pellet was resuspended in 1 ml PBS. 30 μl of this cell suspension was dropped onto a glass substrate and left to air dry at room temperature for 2–3 hours.

(B) Formalin fixation: 10% neutral buffered formalin (HT501128, Sigma Aldrich, US) was passed through a 0.2 μm filter (Minisart filters, Sigma Aldrich, US) in order to remove any large salt crystals present in the solution. The cell pellet was suspended in 2 ml of formalin and left for 10 min at room temperature. The solution was centrifuged, decanted, and the cells were resuspended in 2 ml PBS. The resulting solution was again centrifuged, decanted, and the cells were resuspended in 1 ml PBS. 30 μl of this cell suspension was dropped onto a glass substrate and left to air dry at room temperature for 2–3 hours.

(C) PreservCyt fixation: the cell pellet was suspended in 20 ml of PreservCyt and left for 15 min at room temperature. The solution was centrifuged, decanted, and the cells were resuspended in 2 ml PBS. This solution was centrifuged, decanted, and the cells were resuspended in 1 ml PBS. 30 μl of this cell suspension was dropped on to a glass substrate, and left to air dry at room temperature for 2–3 hours.

2.1.3 Urine exposure times. In order to prepare urine samples containing UC cells, the cell pellet was suspended in 10 ml artificial urine (AU-001, Biopanda Diagnostics, UK) and 5 ml PreservCyt. These samples were stored in the fridge for 5 h, 24 h, and 72 h respectively. Samples were then centrifuged, decanted, and the cell pellet was resuspended in 20 ml PreservCyt and left at room temperature for 15 min. The resulting solution was centrifuged, decanted, and the cell pellet was resuspended in 1 ml PBS. A final centrifugation step was carried out, followed by decantation, and resuspension in 50 μl PBS. This entire volume was then dropped onto a glass substrate and left to air dry at room temperature for 2–3 hours.

2.1.4 ThinPrep UroCyte. In order to prepare urine samples containing UC cells with the ThinPrep UroCyte method, the cell pellet was suspended in 10 ml artificial urine and 5 ml PreservCyt, and stored in a fridge for 24 h. Samples were then centrifuged and the supernatant decanted. In order to compare the differences between cells exposed only to PreservCyt, and those exposed to both PreservCyt and CytoLyt, the samples were prepared as follows:

(A) Two or three drops of the cell pellet was suspended in a 20 ml PreservCyt vial and left at room temperature for 15 min. The vial was inserted into the T2 and the cells were transferred onto a ThinPrep glass slide.

(B) The cell pellet was vortexed and resuspended in 30 ml CytoLyt. The solution was centrifuged and decanted, and two or three drops of the cell pellet were resuspended in a 20 ml PreservCyt vial and left at room temperature for 15 min. The vial was inserted in the T2 system and the cells were transferred onto a ThinPrep glass slide.

2.1.5 Hematuria. In order to compare the impact of scant hematuria and frank hematuria present in urine samples, UC cells were suspended in 10 ml artificial urine, 5 ml PreservCyt and either 0.5 ml whole blood (creating a pink solution to represent scant hematuria), or 3 ml whole blood (producing a deep red solution representing frank hematuria). All samples were centrifuged and decanted. The cell pellet was vortexed and resuspended in 30 ml CytoLyt. The resulting solution was centrifuged, decanted, and two or three drops of the cell pellet was resuspended in a 20 ml PreservCyt vial and left at room temperature for 15 min. The vial was inserted in the T2 system, and the cells were transferred onto a ThinPrep glass slide.

2.2 Raman spectral acquisition

A custom-built Raman micro-spectroscopy system was employed for all measurements in this study, as shown in Fig. 1. This system consists of a 150 mW laser with 532 nm wavelength laser (Laser Quantum, Cheshire, UK; Torus), spectrograph (Andor Technology, Belfast, UK; Shamrock 500) operating with a 600 lines per mm grating, and a cooled CCD camera (Andor Technology, Belfast, UK; DU420A-BR-DD) operating at −80 °C, with spectra recorded using the Andor Solis software system. All measurements were recorded using a 50×/0.8 Olympus UMPlanFl microscope objective and a 100 μm confocal aperture. The confocal aperture (CA) is used to ensure that the light reaching the spectrograph has originated from a specific three dimensional location within the biological sample. The confocal aperture serves to provide a spatial resolution of ~3 μm, and helps to reduce the background signal emanating from the sample substrate and from optical elements in the Raman system. The long pass filter (Semrock, US; LP03-532RU-25) and dichroic beamsplitter (Semrock, US; LPD-01-532RS) were chosen to filter out the laser wavelength from reaching the spectrograph, while transmitting the longer Raman scattered wavelengths. The dichroic short pass filter (Edmund Optics, US; 69-202) reflects all wavelengths greater than 500 nm while allowing shorter wavelengths from the halogen lamp to pass through, which permits imaging of the sample of the digital camera (Basler, Germany; acA2000-340km). Spectra were recorded within the 600–1800 cm^{-1} range with an acquisition time of 5 s each. Two spectra were recorded from the same location within the nucleus of 50 cells from each slide.

2.3 Data processing

The recording of two spectra facilitates the removal of cosmic rays as described by James *et al.*[27] Following this, an extended multiplicative signal correction (EMSC) algorithm was applied to remove the glass signal and the slowly varying baseline from each Raman spectrum.[28–30] This algorithm computes a background signal made up of an N order polynomial (to remove the baseline signal), and a weighted glass signal (recorded from a clean glass slide). The EMSC algorithm applied a least squares fit to (i) a reference Raman spectrum, (ii) the glass signal, and (iii) an N order polynomial. The weight of (i) and (ii), as well as the coefficients of the polynomial are returned by the EMSC algorithm. The reference spectrum provides a basis for all other spectra to be fitted; the reference spectrum chosen here is based on the mean of a dataset of T24 cells recorded on CaF_2. In order to remove any potential bias, the same reference spectrum was

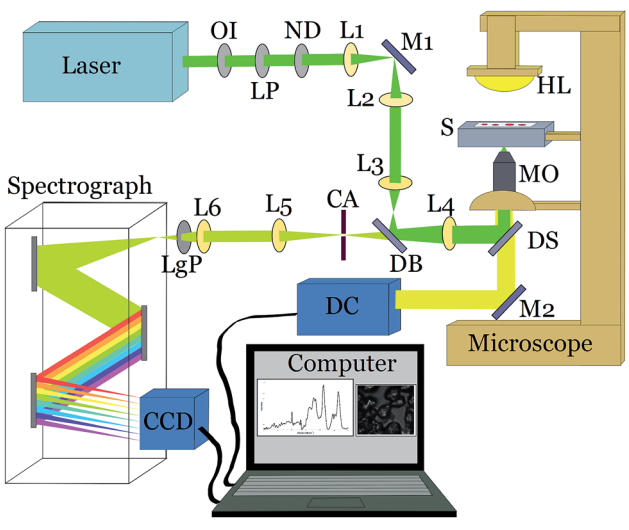

Fig. 1 Schematic of the conventional Raman micro-spectroscopy set-up used in this study. OI, optical isolator; LP, line pass filter; ND, neutral density filter; L, lens; M, mirror; DB, dichroic beamsplitter; DS, dichroic short pass filter; MO, microscope objective; HL, halogen lamp; CA, confocal aperture; LgP, long pass filter; DC, digital camera.

used for all experiments in this study. We have found that similar results are achieved using an alternative reference spectrum based on another epithelial cell type recorded on CaF_2. Following a least squares determination of the "best fit" for a given spectrum, components (ii) and (iii) are subtracted from the raw spectrum. The value of N is dataset dependent, with higher order polynomials required for accurate modelling of the baseline signal across some datasets. It has been shown elsewhere that the use of high values of N (up to $N = 7$) does not result in over-fitting with EMSC.[28] For this study, a 1st order polynomial was used in the EMSC subtraction algorithm for all datasets, except for the case of T24 and RT112 formalin fixed on glass, which had a particularly strong baseline signal due to experimental parameters on that given day; in this case N was chosen to be 7. Fig. 2 demonstrates the results of the EMSC algorithm when applied to raw Raman spectra for the removal of the simultaneous glass and baseline signals. Additionally, all spectra were smoothed with a Savitzky–Golay filter ($k = 5$; $w = 7$).

In order to compare the spectra recorded across the various parameters discussed in this paper, and in order to determine the impact of these parameters on the classification of both cell lines, a combination of PCA and LDA was applied to the two groups in each of the four experiments. The sensitivity and specificity values were determined based on a leave-one-out cross validation method, as described in more detail elsewhere.[31,32]

2.4 Haematoxylin & eosin (H&E) staining

Following Raman spectral acquisition, all samples prepared with the ThinPrep UroCyte method were H&E stained.[33] This was achieved by placing the slides into 95% alcohol for 10 min, and then washed with water. Slides were placed into Harris haematoxylin (Sigma Aldrich, US) for 5 min, and then transferred into water before being submerged into 1% acid–alcohol for 1 s, washed with water, and placed into water for 5 min to 'blue' the haematoxylin. Slides were placed into 1% eosin (Sigma Aldrich, US) for 3 min in order to counterstain the cytoplasm, following which the slides were submerged in water for 1 s, 95% alcohol for 1 s, and 100% alcohol for 1 s. The slides were placed into 100% alcohol for 5 min, and were then transferred into a bath of xylene (Sigma Aldrich, US) for 3 min, and a second bath of xylene for a further 3 min. Finally, the slides were removed and a coverslip applied with DPX mounting medium (Thermo Fisher Scientific, Ireland) and were left to dry. Following staining, cells were examined under a photomicroscope, with a 40× microscope objective, for changes relating to different stages of cancer such as abnormal cellular shape and size, abnormal nuclei, and cellular arrangement/distribution. The amount of residual blood cells present on the slide from hematuria contaminated samples was also monitored. Alternatively, slides could have been stained with Papanicolaou (Pap) if this method was preferred.

3 Results

3.1 EMSC background subtraction

Fig. 2 demonstrates the application of the EMSC algorithm for the removal of the glass signal from Raman spectra,[34] along with the slowly varying baseline signal for both T24 and RT112 datasets, based on one reference spectrum. As shown here, the glass signal, present within 1050–1150 cm^{-1} region has been

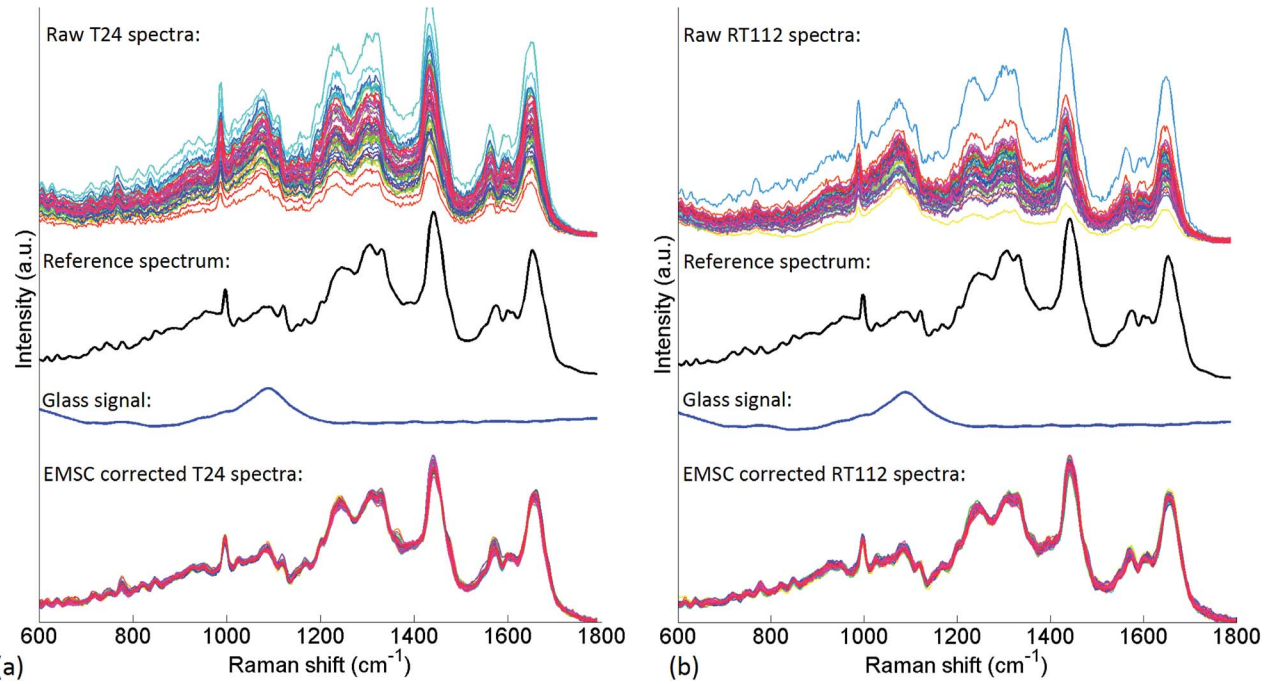

Fig. 2 A demonstration of the EMSC algorithm applied for the removal of the glass and baseline signals from Raman spectra; (a) raw and EMSC corrected T24 cells following PreservCyt fixation; (b) raw and EMSC corrected RT112 cells following PreservCyt fixation. The same glass signal and reference spectrum are applied to both datasets, with the reference spectrum based on the mean of T24 cells recorded on CaF_2.

reduced significantly, in addition to a reduction in the variance seen across each dataset.

3.2 Fixing agents

The spectra shown in Fig. 3 represent the mean Raman spectra recorded from T24 UC cells for the cases of air dried, formalin fixed, and PreservCyt fixed respectively. Raman spectra of formalin fixed cells were found to be most similar to air dried cell spectra with no significant differences observed. PreservCyt fixed cells were found to have differences in the intensity of some peaks within the fingerprint region; an increase can be seen in the peaks at 1093 cm^{-1} and 1250 cm^{-1}, which relate to PO_2 stretching (DNA/RNA), and a decrease in peaks at 750 cm^{-1}, 1310 cm^{-1}, 1340 cm^{-1}, and 1580 cm^{-1}, corresponding to tryptophan, guanine, adenine, and bending modes of phenylalanine.[35] All T24 and RT112 spectra for each fixing method are shown in the ESI.†

For each fixation method, a PC-LDA training model was implemented based on a leave-one-out cross validation in order to classify T24 and RT112 UC cells, with associated PCA scores and coefficients available in the ESI.† The resulting sensitivities and specificities were 100% and 98.0% for air dried cells, 98.0% and 100% for formalin fixed cells, and 100% and 100% for PreservCyt fixed cells. These classification values indicate that the three fixing agents measured here have no significant impact on the ability of PC-LDA to classify spectra across both cell types. This demonstrates that PreservCyt fixation of cells, a common preservation method used across clinics today, can be integrated into Raman micro-spectroscopy. Interestingly, as shown in the ESI,† the PC scores for PreservCyt cells are most similar to air-dried cells, which shows that although PreservCyt fixation changes the spectra more significantly than formalin fixation, it appears to more accurately preserve the spectral differences that are seen between T24 and RT112 after air-drying.

3.3 Urine exposure times

The associated mean spectra for both cell lines following urine exposure for 5 h, 24 h, and 72 h are shown in Fig. 4; individual cell spectra can be found in the ESI.† For each exposure time, a PC-LDA training model was implemented based on a leave-one-out cross validation, and the corresponding sensitivities and specificities are listed in Table 1, where it can be seen that it is still possible to accurately differentiate between both cell lines after 72 h (PCA scores and coefficients are available in the ESI†). Interestingly, when the data from all three time durations is combined, the classification accuracies remain above 90%, which indicates that PreservCyt can preserve cells in urine for up to 3 days without significantly altering the cell biochemistry or the diagnostic potential of the method.

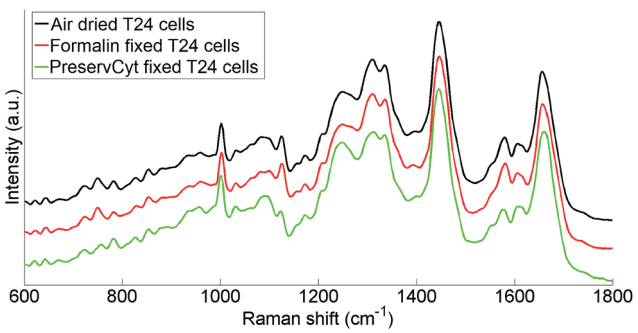

Fig. 3 Mean spectrum of air dried T24 cells (black), formalin fixed T24 cells (red), and PreservCyt fixed T24 cells (green). All cells were drop-dry deposited onto glass slides.

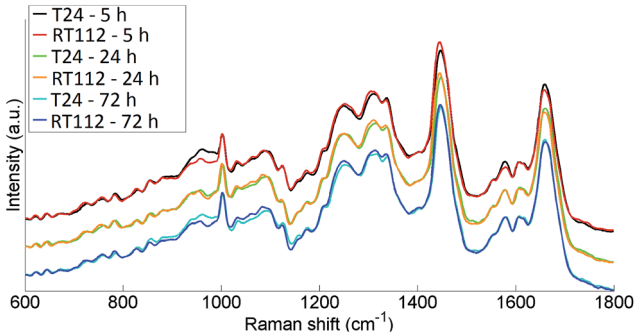

Fig. 4 Mean spectrum of T24 and RT112 UC cells after exposure to urine and PreservCyt for 5 h, 24 h and 72 h; all cells were drop-dry deposited onto glass slides.

3.4 ThinPrep UroCyte

Fig. 5 shows the mean spectra recorded from T24 and RT112 UC cells after exposure to urine and PreservCyt for 24 h, with and without the addition of the red blood cell lysing agent CytoLyt, followed by ThinPrep processing. Here, it can be seen that CytoLyt has no significant impact on UC cells, and no spectral contributions were observed from cells that were exposed to

Table 1 Sensitivity and specificity values for T24 and RT112 cell lines after urine exposure based on a PC-LDA model with leave-one-out cross validation, for the drop-dry and ThinPrep deposition methods

	Sens.	Spec.
Drop-dry method		
T24 – 5 h urine exposure	97.9%	94.2%
RT112 – 5 h urine exposure	94.2%	97.9%
T24 – 24 h urine exposure	97.9%	96.1%
RT112 – 24 h urine exposure	96.1%	97.9%
T24 – 72 h urine exposure	100%	100%
RT112 – 72 h urine exposure	100%	100%
T24 – combined urine exposure times	91.5%	93.2%
RT112 – combined urine exposure times	93.2%	91.5%
ThinPrep method		
T24 – 24 h urine – PreservCyt only	94.3%	100%
RT112 – 24 h urine – PreservCyt only	100%	94.3%
T24 – 24 h urine – PreservCyt and CytoLyt	96.0%	96.0%
RT112 – 24 h urine – PreservCyt and CytoLyt	96.0%	96.0%
T24 – both ThinPrep samples	88.4%	99.0%
RT112 – both ThinPrep samples	99.0%	88.4%

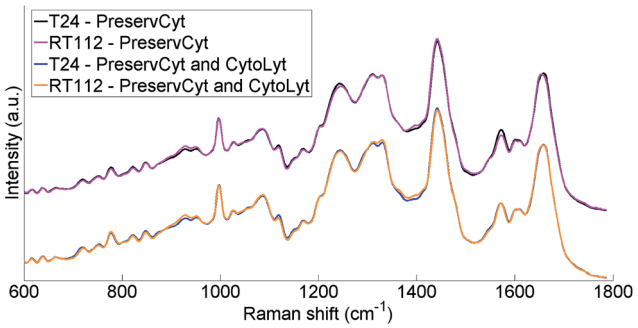

Fig. 5 Mean spectra of T24 and RT112 UC cells after exposure to urine and PreservCyt for 24 h, followed by ThinPrep processing with and without the addition of the red blood cell lysing agent CytoLyt.

CytoLyt compared with those exposed to PreservCyt alone. The spectra recorded from ThinPrep processed slides were more consistent than those measured with the drop-dry method, *i.e.* a smaller standard deviation was measured across the spectra from both T24 and RT112 (see ESI†). It is possible that this is due to the uniform monolayer deposition method employed by the T2 machine compared to the random distribution of cells that can accumulate on the slide after the drop-dry method.

A PC-LDA model was generated based on a leave-one-out cross validation method for both of these methods individually and combined, and the results are displayed in Table 1, with PCA results available in the ESI.† The sensitivities and specificities found are greater than 88%.

Fig. 6 shows the images obtained from these slides after H&E staining (after Raman spectral acquisition); distinct differences can be seen between both cell lines in terms of cellular shape and size, along with more abnormal nuclei present in T24 UC cells, as expected. The cells do not appear to have been photodamaged by the laser, which indicates that a cytopathologist should still be able to perform standard urine cytology diagnostics on slides that have been stained following Raman spectral acquisition, thus allowing for both Raman and standard urine cytology to be performed on the same slide.

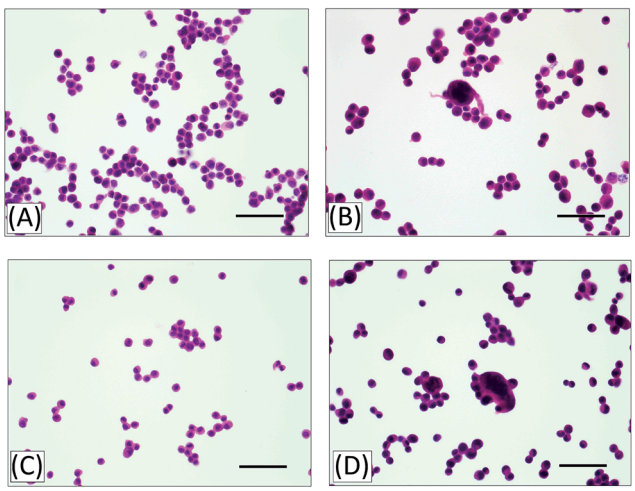

Fig. 6 Images obtained from H&E stained UC cells after Raman spectral analysis, prepared with the ThinPrep UroCyte method. (A) RT112 cells and (B) T24 cells without CytoLyt; (C) RT112 and (D) T24 cells with CytoLyt. Scale bar = 100 μm.

3.5 Hematuria

Spectra recorded from cells obtained from ThinPrep processed urine samples replicating scant and frank hematuria are shown in Fig. 7. Here, it can be seen that cells obtained from a urine sample with scant hematuria appear to be free from any contamination; this is due to the effective removal of many of the red blood cells present in the sample using CytoLyt. However, for the frank hematuria sample, several additional peaks have been observed that correspond to the presence of residual components of blood cells, a further breakdown of the peaks associated with blood cells can be found elsewhere.[36] This result indicates that samples that have relatively high blood cell concentrations are not suitable for diagnostics with Raman micro-spectroscopy, although further investigation into additional CytoLyt washes, or the use of H_2O_2 as suggested by Bonnier *et al.*,[13] could possibly help to remove residual blood from these samples.

Fig. 8 shows the H&E stained images obtained from both samples containing scant and frank hematuria respectively. Here, small regions of blood remain visible within the scant hematuria sample, but the majority of UC cells in this sample appear to be isolated from the blood regions. The frank

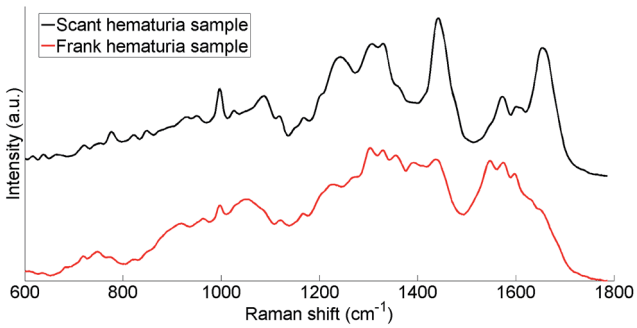

Fig. 7 Mean spectrum of T24 and RT112 UC cells after exposure to urine and PreservCyt for 24 h with the addition of blood to replicate scant and frank hematuria. All samples were ThinPrep processed with the red blood cell lysing agent CytoLyt.

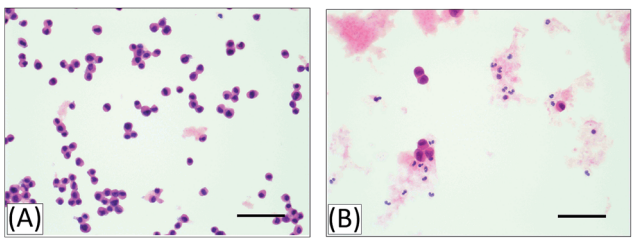

Fig. 8 H&E stained images of (A) RT112 cells from a scant hematuria urine sample, and (B) T24 cells from a frank hematuria urine sample. Both samples were processed with CytoLyt and the ThinPrep method. Scale bar = 100 μm.

hematuria sample, however, has a low UC cell yield, and contains large regions of blood contamination. It is possible that this is due to large amounts of blood cell components gathering on the ThinPrep filter, resulting in less space for the UC cells. This demonstrates the importance of removing blood cells with a lysing agent as well as applying additional washes to further remove this contamination.

4 Conclusion

In this paper the ability of Raman micro-spectroscopy to discriminate between low and high grade bladder cancer cell lines with a high degree of accuracy has been demonstrated, even within the practical constraints of a typical pathology laboratory. Four separate experiments were conducted that validate the performance of Raman based classification when applied as an adjunct to the standard practice of urine cytology.

In the first experiment, it was concluded that the Raman spectra obtained from cells following fixation, using the commercial methanol based fixative, PreservCyt, are not significantly different to those recorded from air dried cells and formalin fixed cells, with negligible differences in statistical classification accuracies observed. A PCA based analysis revealed that the statistical variation between the two datasets was better preserved by PreservCyt fixation when compared with air dried cells, despite the fact that PreservCyt introduces greater alteration of the spectra compared with formalin. This is an important result; not only does it demonstrate that Raman micro-spectroscopy can easily be adopted into the standard urine cytology protocol within a clinical setting, but it also provides an alternative to the use of formalin, a common fixative of choice in Raman based cell studies,[21,22,24] which is a known carcinogen.[37] While it has been shown by Harvey *et al.*[18] that the alcohol based fixative SurePath could be used as part of the BD SurePath liquid-based Pap test, SurePath produced a large signal within the fingerprint region, which makes PreservCyt a preferable fixative for urine cytology samples.

In the second experiment, it was shown that UC cells can be exposed to urine for up to 3 days in the presence of PreservCyt without significantly altering the cellular biochemistry. The ability to accurately classify between two cell lines after exposure to urine for 5 h, 24 h, and 72 h has not been significantly affected (see Table 1). Interestingly, the results in Table 1 indicate that the sensitivities and specificities increase slightly with longer urine exposure times; however, is more likely to be related to minute changes in the system's alignment or sample preparation methods rather than an improved accuracy that corresponds directly to urine exposure times. Therefore, the combined sensitivities and specificities (91.5% and 93.2% respectively) are a better representation of this method. This result indicates that it may be possible to transport urine samples from a clinic into a Raman lab within a realistic time frame. It is impractical to consider analysing urine samples that have not been exposed to a preservative unless the samples will be processed within a few hours of urine collection due to the rapid deterioration of cells in urine.[38]

Additionally, in a clinical setting, the urine collection method is important to avoid unwanted contaminants from entering into the sample such as bacteria, fungal infections, and other genital contamination. Therefore, it is typically recommended that patients follow the "clean catch", "mid-stream" method. The urine sample should then to be stored in a 2 : 1 ratio with PreservCyt to avoid further cell degradation.

In the third experiment, it was demonstrated that the ThinPrep UroCyte method can be applied in combination with Raman micro-spectroscopy resulting in spectra that have a smaller standard deviation than that obtained from cells deposited onto a slide *via* the drop-dry method. This is most likely due to the uniform monolayer deposition method employed by ThinPrep, as seen in Fig. 5. ThinPrep also provides a cleaner background, increased cellularity, better preservation, and facilitates easier and faster preparation.[25]

The two chemicals employed by the ThinPrep UroCyte method have little impact on the Raman spectra obtained, and no significant impact in diagnostic classification results were observed. Uniform cellular distribution resulted in smaller deviations in the background signal when compared to the spectra obtained from cells deposited onto a slide *via* the drop-dry method. Cells did not require any washing steps after fixation in PreservCyt with ThinPrep due to its filter based properties, whereas drop-dried cells needed further washing with PBS in order to avoid spectral contamination from the PreservCyt solution. Additional washing steps could result in a reversal of the fixation process of PreservCyt.

The sensitivity and specificity values calculated for ThinPrep prepared samples, as shown in Table 1, are greater than 88%. Whilst these results are slightly lower than those found for the drop-dry method, ThinPrep offers many other advantages that make it a preferable deposition method for Raman micro-spectroscopy. These advantages include ease of preparation, which is particularly important in a busy clinic, the inclusion of red blood cell lysing agents, and a monolayer distribution of cells across the slide where one focal depth is sufficient to analyse most of the cells on the slide.

In the final experiment, the impact of hematuria on the spectra recorded from UC cells obtained from a urine sample was investigated. Approximately 90% of patients diagnosed with bladder cancer will present with hematuria. Therefore, it is inevitable that Raman spectra will have to be recorded from patients with blood in their urine. The amount of blood will vary from patient to patient, resulting in urine of a mild pink colour (scant hematuria) to deep red (frank hematuria), with the most severe cases resembling the colour of coffee. In this experiment it was shown that urothelial cells can be easily separated from blood in scant hematuria samples using ThinPrep with CytoLyt, as can be seen in Fig. 8(A). However, it is more difficult to fully remove red blood cells from frank hematuria samples, resulting in urothelial cells being deposited on to the slide in very close proximity to regions of blood residue (Fig. 8(B)). The consequence of this is that the Raman spectra obtained from UC cells are contaminated by spectral peaks associated with blood. Additional CytoLyt washes should help to further eliminate this problem, but it may be necessary to wait for the patient to

provide a sample at a later date when their levels of hematuria have lowered, *e.g.* after an antibiotic for infection, or medication for kidney stones, *etc.*

There are additional factors associated with urine cytology samples which have not been considered here, such as the ability to identify UC cells that are randomly distributed across a slide containing healthy urothelial cells, squamous and glandular cells, renal tubular cells and casts, inflammation, or infection. It must also be noted that in this paper, and in previous literature on Raman based urine cytology, the number of cells to be recorded from each slide has not been considered in great detail. Shapiro *et al.* reported recording only five cells per slide; however, since it is possible for the majority of urothelial cells in a typical sample to be non-cancerous, it is unknown how these five cells were chosen or identified.[20] An important problem with standard urine cytology is the inability to differentiate between healthy cells and low grade cancer cells under a microscope, and this task becomes more difficult when the cells are unstained, as required by Raman micro-spectroscopy. Therefore, one reasonable approach may be to record spectra from every available urothelial cell on the slide, and to monitor if any of these cells are classified as cancerous by the trained statistical algorithm. If a cell is identified as such, the corresponding patient could then be flagged for further investigation. Alternatively, it may be possible to train image processing algorithms to identify cells of interest.

Similarly, the number of patients that should be used to accurately train a classifier needs to be further examined. Beleites *et al.* reported that a sample size of 75–100 is typically needed to verify the accuracy of a classifier.[39] However, it is difficult to train a classifier from urine cytology samples due the low sensitivities associated with the standard urine cytology method; thus, training of algorithms based on biopsy and tissue samples with a known pathological status may be preferable.

In summary, the combination of Raman micro-spectroscopy and the ThinPrep UroCyte method provides an ideal platform to replace cystoscopy for bladder cancer surveillance, particularly for high-risk patients who otherwise would require frequent repeat cystoscopic procedures. Raman based urine cytology provides all of the advantages associated with standard urine cytology, *i.e.* ease of procurement, non-invasive and low cost, as well as the advantages of high sensitivity and specificity typically associated with cystoscopy. This technique would help to significantly lower the financial burden associated with bladder cancer surveillance for health care systems worldwide, as well as improving the patient's quality of life.

Acknowledgements

This research was conducted with the financial support of Science Foundation Ireland (SFI) under Grant Number 11/SIRG/I2140. Ethical approval for this study was granted by Maynooth University's Ethics Committee No. BSREC-2015-015. The blood sample used within this study was obtained from a residual hospital sample that was irreversibly anonymised; all experiments were performed in compliance with National guidelines (Safety, Health and Welfare at Work (Biological Agents) Regulations, Ireland; S.I. No. 572 of 2013).

References

1 V. Sangar, N. Ragavan, S. Matanhelia, M. Watson and R. Blades, *BJU Int.*, 2005, **95**, 59–63.
2 P. Sullivan, J. Chan, M. Levin and J. Rao, *Am. J. Transl. Res.*, 2010, **2**, 412–440.
3 K. Sievert, B. Amend, U. Nagele, D. Schilling, J. Bedke, M. Horstmann, J. Hennenlotter, S. Kruck and A. Stenzl, *World J. Urol.*, 2009, **27**, 295–300.
4 G. Koss, D. Dietch, R. Ramanathan and A. Sherman, *Acta Cytol.*, 1985, **29**, 810–816.
5 B. Van Rhijn, H. van der Poel and T. van der Kwast, *Eur. Urol., Suppl.*, 2009, **8**, 536–541.
6 V. Yutkin, B. Nisman and D. Pode, *Expert Rev. Anticancer Ther.*, 2010, **10**, 787–790.
7 B. Van Rhijn, H. van der Poel and T. van der Kwast, *Eur. Urol.*, 2005, **47**, 736–748.
8 A. Kamat, J. Karam, H. Grossman, A. Kader, M. Munsell and C. Dinney, *BJU Int.*, 2011, **108**, 1119–1123.
9 R. Ashfaq, *ThinPrep Non-Gyn Morphology Reference Atlas: Urinary Cytology*, Mass: Cytyc, Matlborough, 2005.
10 L. T. Kerr, K. Domijan, I. Cullen and B. M. Hennelly, *Photonics Lasers Med.*, 2016, **3**(3), 193–224.
11 M. Raitanen, R. Aine, E. Rintala, J. Kallio, T. Juusela and F. Group, *Eur. Urol.*, 2002, **41**, 284–289.
12 V. Loseshwar, R. Habuchi, H. Grossman, W. Murphy, S. Hautmann, G. Hemstreet, A. Bono, R. Getzenberg, P. Goebell, B. Schmitz-Drager, J. Schalken, Y. Fradet, M. Marberger, E. Messing and M. Droller, *Urology*, 2005, **66**, 35–63.
13 F. Bonnier, D. Traynor, P. Kearney, C. Clarke, P. Knief, C. Martin, J. O'Leary, H. Byrne and F. Lyng, *Anal. Methods*, 2014, **6**, 7831–7841.
14 L. Carvalho, F. Bonnier, K. O'Callaghan, J. O'Sullivan, S. Flint, H. Byrne and F. Lyng, *Exp. Mol. Pathol.*, 2015, **98**, 502–509.
15 G. Clemens, J. Hands, K. Dorling and M. Baker, *Analyst*, 2014, **139**, 4411–4444.
16 H. J. Byrne, P. Knief, M. E. Keating and F. Bonnier, *Chem. Soc. Rev.*, 2016, **45**, 1865–1878.
17 T. Harvey, C. Hughes, A. Ward, E. Gazi, E. Faria, N. Clarke, M. Brown, R. Snook and P. Gardner, *Proceedings of the XXI International Conference on Raman Spectroscopy*, 2008, pp. 1015–1017.
18 T. Harvey, C. Hughes, A. Ward, E. Faria, A. Henderson, N. Clarke, M. Brown, R. Snook and P. Gardner, *J. Biophotonics*, 2009, **2**, 47–69.
19 E. Canetta, M. Mazilu, A. De Luca, A. Carruthers, K. Dholakia, S. Neilson, H. Sargeant, T. Briscoe, C. Herrington and A. Riches, *J. Biomed. Opt.*, 2011, **16**, 037002.
20 A. Shapiro, O. Gofrit, G. Pizov, J. Cohen and J. Maier, *Eur. Urol.*, 2011, **59**, 106–112.

21 A. Meade, C. Clarke, F. Draux, G. Sockalingum, M. Manfait, F. Lyng and H. Byrne, *Anal. Bioanal. Chem.*, 2010, **396**, 1781–1791.
22 M. Mariani, P. Lampen, J. Popp, B. Wood and V. Deckert, *Analyst*, 2009, **134**, 1154–1161.
23 J. Kiernan, *Microsc. Today*, 2000, **00–1**, 8–12.
24 F. Draux, C. Gobinet, J. Sule-Suso, A. Trussardi, M. Manfait, P. Jeannesson and G. Sockalingum, *Anal. Bioanal. Chem.*, 2010, **397**, 2727–2737.
25 L. Koss and R. Hoda, *Koss's Cytology of the Urinary Tract with Histopathologic Correlations*, ed. L. G. Koss, 2012.
26 E. Pasin, D. Josephson, A. Mitra, R. Cote and J. Stein, *Reviews in Urology*, 2008, **10**, 31–43.
27 T. James, M. Schlosser, R. Lewis, S. Fischer, B. Bornschein and H. Telle, *Appl. Spectrosc.*, 2013, **67**, 949–959.
28 N. Afseth and A. Kohler, *Chemom. Intell. Lab. Syst.*, 2012, **117**, 92–99.
29 H. Martens and E. Stark, *J. Pharm. Biomed. Anal.*, 1991, **9**, 625–635.
30 K. Liland, A. Kohler and N. Afseth, *J. Raman Spectrosc.*, 2016, DOI: 10.1002/jrs.4886.
31 L. Reisner, A. Cao and A. Pandya, *Chemom. Intell. Lab. Syst.*, 2011, **105**, 83–90.
32 R. Gautam, S. Vanga, F. Ariese and S. Umapathy, *EPJ Tech. Instrum.*, 2015, **2**, 1–38.
33 A. Fischer, K. Jacobson, J. Rose and R. Zeller, *Cold Spring Harb. Protoc.*, 2008, **5**, 4986.
34 L. Kerr, H. Byrne and B. Hennelly, *Anal. Methods*, 2015, **7**, 5041–5052.
35 F. Lyng, D. Traynor, I. Ramos, F. Bonnier and H. Byrne, *Anal. Bioanal. Chem.*, 2015, **407**, 8279–8289.
36 A. Bankpur, E. Zachariah, S. Cihdangil, M. Valiathan and D. Mathur, *PLoS One*, 2010, **5**, 10427.
37 M. Blackwell, H. Kang, A. Thomas and P. Infante, *Am. Ind. Hyg. Assoc. J.*, 1981, **42**, A34.
38 H. Ahmed and M. Tom, *Oman Med. J.*, 2011, **26**, 14–18.
39 C. Beleites, U. Neugebauer, T. Bocklitz, C. Krafft and J. Popp, *Anal. Chim. Acta*, 2013, **760**, 25–33.