FULL ARTICLE

Classification of fixed urological cells using Raman tweezers

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In this paper we report on preliminary investigations into using Raman tweezers to classify urological cell lines. This builds on earlier work within the group, whereby Raman tweezer methodologies were developed, and the application of this technique to differentiate between live prostate cancer (CaP) and bladder cells lines (PC-3 and MGH-U1 respectively) was demonstrated.

In this present study we analysed chemically fixed cells using two different fixative methods; SurePathTM (a commercial available liquid based cytology media) and 4% v/v formalin/PBS fixatives. The study has been expanded from our previous live cell study to include the androgen sensitive CaP cell line LNCaP, primary benign prostate hyperplasia (BPH) cells as well as primary urethral cells. Raman light from the cells was collected using a 514.5 nm Ar-ion laser excitation source in back-scattering configuration mode.

Principal component-linear discriminate analysis (PC-LDA) models of resulting cell spectra were generated and these were validated using a blind comparison. Sensitivities and specificities of >72% and 90% respectively,

for SurePath fixed cells, and >93% and 98% respectively for 4% v/v formalin/PBS fixed cells was achieved. The higher prediction results for the formalin fixed cells can be attributed to a better signal-to-noise ratio for spectra obtained from these cells.

Following on from this work, urological cell lines were exposed to urine for up to 12 hours to determine the effect of urine on the ability to classify these cells. Results indicate that urine has no detrimental effect on prediction results.

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1. Introduction

Prostate cancer (CaP) is the most commonly diagnosed cancer amongst men, with a mortality rate second only to lung cancer [1]. Current pre-operative tests for detecting cancer include serum prostate-specific antigen (PSA) measurement and the findings of digital rectal examination (DRE), which are recommended in the USA for men over 50 years of age with a life expectancy of greater than 10 years [2].

Prostate-specific antigen (PSA) is a glycoprotein that is secreted by the secretory cells of the prostate gland [3]. Elevated levels of PSA in the blood serum can be attributed to cancer, but the test suffers from low specificity and low sensitivity; results have shown that for patients with a positive PSA test, only 22% have a definitive cancer diagnosis following biopsy [4]. The low specificity results from the fact that elevated PSA levels can also arise due to a benign tumour or inflammation caused by a viral infection [5]. It is also known that PSA levels generally increase with age of healthy men. The consequence of this low specificity is a high rate of false positive diagnoses which leads to unnecessary biopsies and anxiety for the patients. Also, the low sensitivity results in a significant proportion of men who have prostate cancer exhibiting low PSA values and thus not being diagnosed [6].

A study by Concato *et al.* [7] has questioned the effectiveness of the PSA screening method and suggested that mortality rates were not improved by the use of PSA. Furthermore, a study of 267 men with localised prostate cancer found that while the PSA test is an important tool for monitoring cancer progression it provides little information on the malignant nature of the tumour and thus is a poor predictor of lethal cancer [8]. The potential of a tumour to metastasize is closely linked to mortality, as it is the formation of secondary tumours that is the cause of mortality within patients suffering from CaP. The median life expectancy for patients suffering from secondary prostate cancer in the bone is about 18 months [9].

Due to the shortcomings highlighted for both the PSA and DRE tests there is a need to develop complementary tests that are both specific and sensitive to prostate cancer, and which would reduce the rate of unnecessary biopsies. Currently the PCA3 (DD3) test is at the forefront of CaP diagnosis [10, 11]. This is a genetic test for CaP, which tests for the prostate cancer antigen-3 gene (PCA3), which is overexpressed in cancer. However, alternative methods to the PCA3 test, which could potentially enhance current diagnostic methods might involve the spectroscopic detection and analysis of prostate cells in urine, after they have been shed into the urine by prostate massage [12, 13]. In essence, this method would be a form of urine cytology, which is currently used for the diagnosis of TCC (transitional cell carcinoma) in the urinary tract [14].

Non-invasive, *in-vitro* Fourier transform infrared spectroscopy (FTIR) and Raman spectroscopy are powerful bio-analytical tools which have shown great potential for the identification, classification and diagnosis of cancer cells and tissue [15–18]. The transition of a normal cell to its diseased state is accompanied by changes in a variety of biomolecules, such as nucleic acids, carbohydrates and proteins which can be simultaneously and indiscriminately probed by FTIR and Raman spectroscopy. This yields spectral signatures that enable differentiation between normal and cancerous cell. Moreover, research has shown that it is not only possible to distinguish between cancerous and non-cancerous prostate epithelial tissues based on FTIR spectra, but that it is also possible to correlate the spectra with the Gleason grade [19, 20] (the histopathological gold standard for grading malignant prostate tissue following biopsy [21]). FTIR-photoacoustic spectroscopy [22] and Raman microspectroscopy [23] have also been successfully used to distinguish different metastatic CaP cell lines.

Our goal is to develop a high-throughput diagnostic method for prostate cancer that is less invasive than the current methods, by analysing prostate cells present in urine, after they have been shed into the urine by prostate massage. Ultimately, a micro-fluidic high-throughput delivery system is envisaged, which would be suitable for laser-based spectroscopy, but which would not be suitable for analysis by FTIR. To this end, in our earlier Raman tweezers study we developed Raman tweezers methodologies, which have been applied to study micro-organisms [24–28], blood cells [29–32] and malignant epithelial cells [33–35], for the analysis of live single-prostate and single-bladder cells in liquid media [36]. In this proof of principle study, the Raman tweezer system facilitates a reproducible and reliable measurement position, free from any interferences that could originate from a substrate. However, in the future, it may be possible to eliminate the optical trap completely.

Analysis by Raman tweezers involves trapping a single-cell in the waist of a tightly focused laser beam and collecting and recording the resultant Raman light [36]. The ability to analyse single-cells is of great importance as the number of cells in a urine sample is expected to be low and hence a single-cell technique would be ideal.

In our previous study we trapped and analysed live PC-3 cells (a malignant prostate cell line [37] from the bone marrow) and MGH-U1 (a malignant bladder cell line [38]) using both back-scattering and forward-scattering configurations of the Raman tweezers containing a 514.5 nm laser for Raman excitation. Principal Component Analysis (PCA) of the resulting spectra demonstrated that it was possible to discriminate between the two cell lines using either configuration.

In the present paper we report on significant progress to that work. This paper is broadly divided into two main parts; a study concentrating on building diagnostic models from SurePath and formalin fixed cell spectra and a study ascertaining the effect of urine on the diagnostic performance. We analysed an extended range of cells which included not only the prostate cells, PC-3 and the bladder cells MGH-U1 cells used in our earlier study, but also other prostate malignant cells, *i.e.* LNCaP (a malignant prostate cell line from the lymph node [39]), BPH (primary benign prostate cells) and PNT2-C2 (normal prostate cell line transfected by SV40 virus [40]), as well as primary urethral cells. These cells were chosen to represent the types of cells that might be present in urine after a prostate massage. The epithelial cell count in a urine sample could include highly invasive CaP cells, poorly invasive CaP cells, BPH cells and healthy cells, as well as cells from other organs such as the bladder, kidney and urethra. It was therefore important to include these cells in the study.

In this study the cells were chemically fixed before analysis, and thus were not analysed live. In a clinical setting cells are often fixed to preserve them until they are analysed, as analysis is not usually immediate, and often is carried out in a lab situated elsewhere. Fixation preserves the biochemical and structural integrity of cells and tissues, with minimal alteration to biochemical composition and structure of the cell [41]. It is likely that cells obtained from urine to be used in a future test would have to be fixed before analysis. Fixing the cells would not only allow cells to be stored before analysis without degradation occurring, but would also significantly reduce any possible photo-induced damage from the laser and eliminate any stress response from the cell to any photochemical oxidative damage. The reduction in photo-induced damage for fixed cells was investigated by Lam *et al.*, who looked at time-lapse fluorescence microscopy of HeLa cells [42]. The researchers found a reduction in the green fluorescent protein (GFP) fluorescence signal (over 250 seconds) from the nucleoplasmic regions of live HeLa cells, caused by photo-bleaching as a result of the fluorophores' loss of ability to fluoresce due to photon-induced chemical damage. However no loss of signal was observed for paraformaldehyde fixed HeLa cells over the same time period, thus indicating no photon-induced chemical damage.

The reduction in photo-induced damage and stress responses for fixed cells compared with live is particularly important for diagnostic purposes, where the aim is to classify cells. The different cell lines used in this current study will only exhibit very subtle biochemical differences, and thus high laser powers and/or long exposure times are required to obtain the signal-to-noise necessary to distinguish very small differences in spectra.

The fixation methods used were the formalin and alcohol fixation methods. Formalin reacts with primary and secondary amine groups of proteins to form chemically stable methylene bridges; this is referred to as cross-linking [41]. Alcohol acts as a fixative by displacing water from proteins, thus altering the internal hydrogen bonding environment; this stabilises the secondary but denatures the tertiary structure [41]. Alcohol fixatives also tend to solubilise lipids, but fix carbohydrates. In this study the SurePath preservative was used as the alcohol fixative, which consists of an aqueous solution of ethanol (24% v/v), isopropanol (24% v/v) and methanol (1% v/v). This is now commonly used as a liquid based cytology (LBC) media for the preservation of cervical cells obtained from cervix examinations. PC-LDA models were generated using the spectra of cells that have been cultured in growth media and fixed and analysed in a fixative media. Principal Components Analysis is a commonly used unsupervised chemometric method, which reduces the high dimensionality of Raman spectra to a few principal components (PCs). The aim of PCA is to capture as much of the natural variance in the spectra in a small number of PCs [43]. In contrast, Linear Discriminant Analysis (LDA) is a supervised method which seeks to optimise separation between groups of data, but minimise separation within groups [44]. As such it classifies spectra into well defined groups or categories, acting as a predictive model for unknown samples. To prevent over-fitting of the LDA model, PCs are used as variables rather than wavenumbers, a method known as PC-LDA [44].

Because a future cytology test is expected to involve the recovery and subsequent analysis of cells from urine; it was important to determine the applicability of such a test on such samples. Thus, we analysed the cells after exposure to urine to determine if this would have an effect on the classification and discrimination of the cells. The cells exposed to urine were also classified using the PC-LDA method, and the diagnostic capability of this method was determined by blind validation.

2. Material and methods

2.1 Cell preparation

2.1.1 SurePath and formalin fixation experiments

LNCaP and PNT2-C2 cells were cultured in RPMI 1640 containing 10% fetal calf serum (FCS) and 2 mM L-glutamine. PC-3 and MGH-U1 cells were

cultured in Ham's F12 containing 7% FCS and 2 mM L-glutamine. BPH epithelial cells were isolated from primary prostate tissue, collected from patients with full ethical consent undergoing transurethral resection for bladder outflow obstruction. BPH cells were cultured in Keratinocyte-Serum Free Media (K-SFM) containing 2 mM L-glutamine. Healthy urethral cells obtained, with full ethical consent, from two patients suffering from renal cancer and one patient suffering from bladder cancer were also cultured in Keratinocyte-Serum Free Media (K-SFM) containing 2 mM L-glutamine. All reagents used for culturing were purchased from Sigma-Aldrich UK apart from K-SFM which was purchased from Invitrogen. All commercial cell lines were obtained from Invitrogen.

Once 70–80% confluent, cells were detached by trypsinisation and harvested by centrifugation at 1500 rpm. The cells were then resuspended in fixative, either SurePath or 4% v/v formalin/PBS. SurePath fixed cells were stored at 2–4 °C until required for analysis, whilst the formalin fixed cells were stored at ambient temperature. For reproducibility purposes, cells of at least two different passages were used.

2.1.2 Urine exposure experiment

BPH, LNCaP, PC-3 and MGH-U1 cell lines were cultured in their appropriate media, as described in section 2.2.1. Cells that were 70–80% confluent were detached by trypsinisation. An aliquot of the cells to be used as the control was isolated and the cells harvested by centrifugation at 1500 RPM and fixed with 4% v/v formalin/PBS. The remaining cells were harvested by centrifugation and then resuspended in artificial urine (BioPanda Diagnostics, UK). For each cell line ten aliquots of the cells in urine suspension were taken. For each aliquot cells were left in urine for one of the following times: 15, 30, 45 minutes, 1, 2, 4, 6, 8, 10 and 12 hours. The urine was then neutralised with PBS and the cells harvested by centrifuging at 1500 RPM. The cells were then resuspended in 4% formalin/PBS. All samples (11 per cell line) were subsequently stored at ambient temperature prior to analysis.

2.2 Raman tweezers setup and analysis

2.2.1 SurePath fixed cell experiment

A diagram of the Raman tweezers configuration used is shown in Figure 1(a). Initial investigations re-

vealed that scattering of the laser beam from the cells resulted in them being pushed upwards above the focal point and eventually escaping the trap. To prevent this, a secondary, counter-propagating 1064 nm Nd:YAG beam was directed from above the cell via a microscope condenser. This provided an opposing radiation pressure, which was sufficient to balance the optical forces and retain the cell in the trap. A 50 µm pin-hole between the sample and the detector was included, which reduced the magnitude of Raman signal contributions from the background media.

Cells were trapped and analysed using the 514.5 nm Ar-ion laser with an output power of 200 mW. This was attenuated by optics in the laser pathway and measured to be 32.6 mW at the cell sample chamber. The counter-propagating 1064 nm beam was positioned above the cell with an output power of 250 mW. Laser light from the 1064 nm beam is significantly outside the spectral bandwidth collected for the Raman shifted light (520–630 nm) that it has no impact on the acquired spectra. Raman spectra were acquired over 1 minute. A background spectrum without a cell in the trap was taken every 1–5 cell spectra. The focus was not changed in between taking backgrounds. In total 100 MGH-U1, 104 BPH, 104 LNCaP, 115 PC-3 and 110 PNT2-C2 cells were analysed.

2.2.2 Formalin fixed cell experiment

A diagram of the Raman tweezers configuration used for formalin fixed cells is shown in Figure 1(b). The counter-propagating beam, used for the SurePath cells was not required as cells were retained in the single beam gradient trap. The reflective dichroic edge filter used in previous work and above to direct the light into the microscope and reflect Raman light was replaced with a long-pass Raman filter transmission dichroic edge (Semrock, Razoredge LPD01-514). This reflects 514.5 nm light, but transmits Stokes-shifted Raman light. The setup results in a higher percentage of laser output power reaching the sample, and improved efficiency of Raman light reaching the detector from the sample. Because of the weaker background from formalin media compared with SurePath media there was no requirement for the pin-hole to be included in the setup.

Cells were trapped and analysed using the 514.5 nm Ar-ion laser with an output power of 400 mW. This was measured to be 100 mW at the cell. Raman spectra of cells and backgrounds were acquired over a 1 minute period, with backgrounds taken every 1–5 cell spectra. In total 135 different fixed cells were analysed for each cell type.

Figure 1 Optical setup for SurePath fixed cells (a) and formalin fixed cells (b). The SurePath setup includes a counter-propagating Nd:YAG laser.

2.2.3 Urine exposure experiment

The same Raman tweezer optical setup and spectral collection procedure were used as for the formalin fixed cell experiment. However, BPH cells exposed to urine for 12 hours could not be trapped, so analysis took place with the cells on the surface. A minimum of 65 spectra were obtained for each time point, apart from BPH (30 minutes), because the sample did not contain enough cells for analysis and BPH (4 hours and 8 hours), in which 16 and 32 cells were analysed respectively.

2.3 Data processing

2.3.1 SurePath and formalin experiment

2.3.1.1 Background subtraction

For each spectrum obtained from SurePath cells, the background contribution (*i.e.* SurePath media) was removed by a weighted subtraction of the background. This was necessary because a straight 1:1 subtraction gave a large negative peak at 880 cm^{-1} .

Figure 2 (online colour at: www.biophotonics-journal.org)
(a) Cell and SurePath spectrum (i), SurePath spectrum (ii), 1:1 subtraction (iii), weighted subtraction (iv). (b) PCA plot of 30 spectra subjected to subtraction by three different operators.

Whilst optically trapped, the cell (diameter of about 25 microns) occupies a significant volume at the focus of the laser. Thus, when the cell is displaced a relatively greater signal is acquired from the background media than when the cell is trapped. To perform the weighted subtraction, a correction factor was adjusted so that the 880 cm⁻¹ peak was brought up to the baseline. Typically subtraction factors of 0.8–0.9 were required.

Figure 2(a) displays a raw spectrum (i), SurePath spectrum (ii), a straight 1:1 subtraction (iii) and a weighted subtraction (iv).

The subjectivity of this subtraction process was tested by running the process on 30 different PNT2-C2 spectra by three different operators, with each operator given the same 30 spectra. A PCA plot of the 90 spectra (Figure 2(b)) shows that each spectrum processed by the three different operators could not be differentiated, which suggests that the process is not adversely subjective.

Figure 3 Raw spectrum (i), formalin spectrum (ii) and formalin subtracted spectrum (iii).

The spectra obtained for all formalin fixed cells were corrected by a straight 1:1 background subtraction, as in our previous work for live cell spectra [36], as the contribution from formalin was negligible. Figure 3 shows the spectrum of a cell and formalin (i), formalin alone (ii) and formalin subtracted spectrum (iii).

2.3.1.2 Quality assessment

Prior to full chemometric analysis, quality assessment of the spectra was carried out. Spectra from each cell line were subjected to extended multiplicative scattering correction (EMSC), [45] vector normalisation and mean centring and then followed by PCA. All analysis was performed in MATLAB 7.0.1 (Mathworks, Inc.). The spectra were quality assessed by calculation of the mean and standard deviation of the scores on PC1 and PC2 respectively for each PCA plot. Spectra with scores greater than 1 standard deviation on either PC were rejected, and not used for chemometric analysis. This method of spectral quality control was based on a procedure outlined in [46], in which PCA was used to screen pixels (relating to individual spectra) from FTIR maps.

Following quality assessment of the spectra obtained for SurePath fixed cells, 83 MGH-U1, 84 BPH, 93 LNCaP, 102 PC-3 and 93 PNT2-C2 spectra remained for the full chemometric analysis. After the quality assessment of the formalin fixed cells: 127 BPH, 129 LNCaP, 132 PC-3, 132 PNT2-C2, 131 MGH-U1 and 127 urethral spectra remained. The average rejection rate for SurePath fixed cells (14.6%) was greater than for formalin fixed cells (3.7%). An examination of the rejected SurePath spectra suggests that these spectra were rejected on the basis of poor signal to noise ratios and/or errors in background subtractions. This was

caused by the raw spectra (prior to subtraction) containing relatively little contribution from the cell compared with spectra that had passed. These were likely to be obtained from cells which were not sufficiently well trapped. Therefore the subtracted spectrum would have very poor SNR, or the spectrum appearance would appear abnormal due to background subtraction errors. In general, the strength of the Raman signal was lower for the spectra that had failed than spectra that had passed.

2.3.1.3 Multivariate chemometric analysis

Following the quality assessment, all the spectra that had passed were identified from the full data set. These were separated from the spectra that had failed. The successful spectra were then subjected to EMSC, vector normalisation and mean centring. This was followed by PCA and then PC-LDA using MATLAB for PCA and SPSS Release 14 (SPSS, Inc.) for PC-LDA. Different cell line combinations were examined, which is discussed in more detail in the Results and Discussion section.

2.3.2 Urine exposure experiment

All spectra were subjected to the same formalin background subtraction regime as outlined in section 2.3.1.1. PCA was carried out on each time point for each cell line (EMSC, vector normalisation and mean centring). The spectra were then subjected to the quality assessment procedure with a rejection rate of 20%.

PC-LDA was carried out, incorporating all four cell lines for each time point using the spectra that passed the quality assessment test, apart for 30 minutes and 8 hour time points where BPH was excluded (due to lack of data).

3. Results and discussion

3.1 SurePath and formalin fixation studies

3.1.1 Cell sizes

Table 1 lists the mean cell diameters (measured from an optical image obtained from every trapped cell) of the analysed SurePath and formalin fixed cells. PC-3 and MGH-U1 cells fixed in SurePath are ca. 5 μm larger than when they are live, *i.e.* unfixed [36]. This could be explained by the fact that the cells swell up when the alcohol breaches the cellular membrane. For SurePath fixed cells a one way-ANOVA (Analysis of Variance) (at the 0.05 level) shows that BPH and MGH-U1 cell sizes are not significantly different, but the sizes of all other cells are significantly different from each other. In the case of formalin fixed cells, MGH-U1 and urethral cells are not significantly different, while the sizes of all the other cells are significantly different. These observations are important when determining if PC-LDA classifications, reported latter in this paper can be attributed to cell size. Histograms showing the distribution of cell diameters are shown in Figure 4, together with normal distribution curves. These results show that, in the same way as for live cells [36], no bias towards trapping smaller cells was introduced. Histograms (b), (e), (g) and (k) appear to show a log-normal distribution, similar to that observed for live MGH-U1 cells.

3.1.2 Raman spectra

The mean and vector normalised spectra for both SurePath and formalin fixed cells are shown in Figure 5(a) and (b) respectively. For SurePath fixed cells the number of spectra contributing to each mean spectrum was (mean standard deviation in normalised counts in brackets): 84 BPH (0.0037),

Table 1 Mean cell diameters and respective standard deviations (SD) for SurePath and formalin fixed cells.

Cell Type	SurePath fixed cells		Formalin fixed cells	
	Diameter (μm)	SD	Diameter (μm)	SD
BPH	24.3	3.5	24.4	2.6
LNCaP	25.5	3.8	23.7	3.5
PC-3	31.0	6.3	27.6	4.9
PNT2-C2	28.3	3.8	26.4	4.3
MGH-U1	24.0	4.4	19.3	3.0
Urethral	N/A	N/A	18.7	2.8

Figure 4 (online colour at: www.biophotonics-journal.org) Histograms showing distribution of cell size diameters for SurePath fixed cells: BPH (a), LNCaP (b), PC-3 (c), PNT2-C2 (d), and MGH-U1 (e), and formalin fixed cells: BPH (f), LNCaP (g), PC-3 (h), PNT2-C2 (i), MGH-U1 (j) and urethral (k). A normal distribution curve is included for each histogram.

93 LNCaP (0.0049), 102 PC-3 (0.0030), 93 PNT2-C2 (0.0027) and 83 MGH-U1 (0.0035). The standard deviations were calculated as the mean of all the standard deviations obtained for each wavenumber. For formalin cells the number of spectra contributing to each mean spectrum was: 127 BPH (0.0033), 129 LNCaP (0.0036), 132 PC-3 (0.0033), 132 PNT2-

C2 (0.0034), 131 MGH-U1 (0.0039) and 127 urethral (0.0068).

The standard deviations for both SurePath and formalin fixed cells are in the order of 10 times lower than those obtained for live cells [36]. This is in agreement with the work of Jess *et al.* [47], who found lower variance for fixed cells compared with

Figure 5 (online colour at: www.biophotonics-journal.org) Mean spectra from SurePath fixed cells **(a)** and formalin fixed cells **(b)** (all spectra off-set).

live cells. A possible explanation for the observed higher standard deviation in the analysis of live cells might be that the biochemistry of the live cells changes when exposed to PBS, in which they were suspended when analysed. This would result in higher spectral variance than for fixed cells. The larger variability in the spectra obtained for the formalin fixed urethral cells, compared to the other formalin fixed cells, might be explained by the fact that these primary cells were from three different patients.

3.1.3 Classification of SurePath fixed cells

3.1.3.1 PC-LDA diagnostic modelling

Spectra obtained from SurePath fixed cells were subjected to supervised PC-LDA to classify them and determine the diagnostic capability of this method. For this study, three different cell line combinations were examined:

- All cell lines included (*i.e.* BPH, LNCaP, PC-3, PNT2-C2 and MGH-U1).
- Four cell lines not including PNT2-C2 (*i.e.* BPH, LNCaP, PC-3, and MGH-U1).

→ Four cell lines not including MGH-U1 (*i.e.* BPH, LNCaP, PC-3, and PNT2-C2).

For each of the cell combinations a PC-LDA training model was constructed using 60 randomly selected spectra per cell line. Thirty PCs from each of the PCA plots (accounting for about 97% of total variance in each case, with PC1, PC2 and PC3 accounting on average for 65.3%, 8.9% and 5.5% of the variance, respectively) were then imported into the LDA algorithm. The resulting PC-LDA training set templates are shown in Figure 6(a–c). In the case of the four cell line models good clustering of each cell line using the first three LDFs is clearly observed. As for the five cell model (*i.e.* BPH, LNCaP, PC-3, PNT2-C2 and MGH-U1) clustering is apparent, although this is more difficult to visualise.

Each PC-LDA diagnostic classifier was then validated with the remaining spectra, not used for training by projecting the spectra blind into the classifier. The number of spectra used for blind testing was as follows: BPH 24, LNCaP 33, PC-3 42, PNT2-C2 33 and MGH-U1 23. Table 2 shows the sensitivities and specificities achieved for each of the PC-LDA classifiers. The values from Table 2 can be used to calculate false positives and false negatives, and subsequently the likelihood ratios (LR) [48], which are tabulated in Table 3.

Table 2 Sensitivity and specificity values achieved by each of the three PC-LDA diagnostic classifier models.

	Cell Line				
	BPH	LNCaP	PC-3	PNT2-C2	MGH-U1
Sensitivity %	79.2	78.8	95.1	72.7	93.8
Specificity %	99.1	97.8	95.7	94.5	92.1
Sensitivity %	93.8	90.2	72.7		91.7
Specificity %	97.1	94.5	98.2		96.4
Sensitivity %	88.3	95.7	75.8	87.9	
Specificity %	98.3	97.1	91.7	91.1

Figure 6 (online colour at: www.biophotonics-journal.org) PC-LDA plots obtained from Sure-Path fixed cells for BPH/LNCaP/PC-3/PNT2-C2/MGH-U1 (a), BPH/LNCaP/PC-3/MGH-U1 (b) and BPH/LNCaP/PC-3/PNT2-C2 (c).

The prediction parameters from Tables 2 and 3 are often used in the clinical environment to express the performance of diagnostic models. However, in this present study we are not dealing with actual patients with or without cancer, but rather cell lines. Therefore, when patients and disease are mentioned in the following text we are actually referring to cell lines, but to put our study into a clinical context we use the terms “patient” and “disease”.

Sensitivity measures the proportion of patients giving positive results who actually have the disease. Specificity measures the proportion of patients giving negative results who do not have disease [48]. Clearly in these cases a high value indicates good diagnostic performance. In this particular case sensitivity is expressed as the proportion of correctly classified cells.

Table 3 Likelihood ratios for positive (LR^+) and negative test results (LR^-).

	Cell Line				
	BPH	LNCaP	PC-3	PNT2-C2	MGH-U1
LR^+	88.0	35.8	22.1	13.2	11.9
LR^-	0.21	0.22	0.05	0.29	0.07
LR^+	32.3	16.4	40.4		25.5
LR^-	0.06	0.10	0.28		0.09
LR^+	51.9	33.0	9.1	9.9	
LR^-	0.12	0.04	0.26	0.13	

To explain the likelihood ratios, reported in Table 3 one can consider the case of LNCaP for the five cell line model. This gives a LR^+ of 35.8, which means that if the patient has a positive test for LNCaP (*i.e.* CaP) then he is 35.8 times more likely to have the disease than a patient with a negative test for CaP. Clearly the greater the LR^+ value, which is calculated by dividing sensitivity by the false positive rate, the better the test is at diagnosing CaP, with values of 10 considered to be the lower threshold limit for a good diagnostic test [48]. We now turn our attention to LR^- , which can be explained in terms of probability; how much the odds of having BPH decrease when the test result is negative. LR^- is calculated by dividing the false negative rate by specificity. The smaller this value the better the test is at excluding CaP, with values of 0.1 considered to be the upper threshold limit for a good diagnostic test.

Observation of Table 3 shows all three models are generally useful diagnostic tests. Taking all three models together, 2 out of 13 cell lines would fail the LR^+ threshold test (PC-3 and PNT2-C2 for the BPH/LNCaP/PC-3/PNT2-C2 model). The LR^- values gives slightly less good results, with 5 out of 13 cells failing the threshold test.

The process of randomly choosing training and testing sets from the whole data set was repeated a total of three times to ensure no bias was unintentionally introduced. Examination of the results obtained showed a high degree of similarity in the sensitivities and specificities between the three different

sets (results not shown). Therefore it can be concluded that the training and test sets which are represented herein were chosen completely randomly with no unintentional bias.

3.1.3.2 Loading plot analysis

Loadings can be used to determine variables and hence biochemical features that are responsible for discrimination between cell lines. Mathematically, loadings are the cosine of the angle between the PC and the original variable [49]. Multiplying the original PCA loadings by the discriminate function coefficients obtained during the PC-LDA calculations allows for the wavenumbers responsible for discrimination observed in the PC-LDA plots to be determined. Such an approach has been used by Fletcher *et al.* [50] and Baker *et al.* [51].

The PC-LDA plot incorporating BPH/PC-3/LNCAp/MGH-U1 spectra (Figure 6(b)) shows good clustering of the four cell line types. Key features of this plot include the following:

- MGH-U1 spectra are well separated from BPH and PC-3/LNCAp spectra on LDF1, and are positively correlated to LDF1. This function does not separate PC-3 from LNCAp spectra, but separates BPH from LNCAp/PC-3 spectra. Therefore LDF1 can be considered to discriminate between bladder and prostate cells and between benign and malignant prostate cells.
- LDF2 separates non-malignant BPH cells from the cancerous PC-3, LNCAp and MGH-U1 cells, with BPH spectra being positively correlated to LDF2. This function does not separate the three malignant cell types.

→ LDF3 separates PC-3 from LNCAp, with PC-3 spectra being positively correlated to LDF3.

Loading plots for LDF1–3 are shown in Figure 7, with tentative band assignments given in Table 4A–C [52–58].

Key Features from LDF1 (Table 4A) – Positive loadings are mainly attributed to lipid and nucleic acid moieties, whilst negative loadings are largely attributed to proteins (refer back to Figure 6(b)). Given that MGH-U1 spectra are positively loaded on LDF1, lipid and nucleic acids are likely to be more abundant in MGH-U1 cells. Proteins are likely to be more abundant in malignant prostate cells. This is in contrast to what was found using live cells. In this case proteins and nucleic acids were found to be more abundant in MGH-U1 than PC-3 cells, whilst lipids were more abundant in PC-3 cells [36]. This may reflect differences in the role that the fixative reagent plays on the cell biochemistry between cell types.

Key Features from LDF2 (Table 4B) – Nucleic acids moieties give largely negative loadings, and so are likely to be more abundant in the malignant cells than BPH, because BPH spectra are positive on LDF2. On the other hand carbohydrate groups give positive loadings, so are likely to be most abundant in BPH. These findings could reflect the greater metabolic turnover for malignant cells compared with BPH, requiring an increase in carbohydrate consumption for ATP generation [19].

Key Features from LDF3 (Table 4C) – Lipids and carbohydrates give largely positive loadings, so are likely to be more abundant in PC-3 than LNCAp, because PC-3 spectra are positive on LDF3. Differences in biochemistry between LNCAp and PC-3 might be a consequence of differences in the nu-

Table 4A Raman band assignments for LDF1 (Figure 6(a)), tabulating key peaks.

Band	Wavenumber (cm ⁻¹)	Tentative Raman band assignment
	+ve	
1	733	Phosphatidylserine (lipids)
2	792	$\nu_{\text{sym}}\text{PO}_2^-$ (nucleic acids)
3	840	Glucose bands
4	879	Trp/hydroxyproline, SurePath contamination?
5	1019	$\nu\text{C-O}$ ribose (nucleic acids)
6	1058	νPO_2^- , C–O vibration (nucleic acids), chain C–C (lipids)
7	1089	$\nu_{\text{sym}}\text{PO}_2^-$ (nucleic acids)
8	1142	$\nu\text{C-O}$ (lipids)
9	1302	CH ₂ twist/wag/def (lipids)
10	1378	$\delta_{\text{sym}}\text{CH}_3$ symmetric (lipids)
11	1440	CH ₂ /CH ₃ def, CH def, ester bands (lipids)
12	1459	DNA, δCH_2 (nucleic acids)
13	1493	DNA bands
14	1580	$\nu\text{C-C}$ (lipids)
15	1739	$\nu\text{C=O}$ stretch, (lipids)
	–ve	
16	759	Trp ring breathing (proteins)
17	935	$\nu\text{C-C}$ (proteins)
18	1000	Phe (proteins)
19	1031	Phe, C–H in plane bending (proteins)
20	1126	C–N (proteins), C–C stretch (lipids)
21	1154	C–C and C–N stretch (proteins)
22	1171	Tyr, Phe, C–H bend (proteins)
23–26	1204–1319	Amide III + CH ₂ wag vibrations from Gly, Pro, Cys
28	1555	Amide II, Trp, N–H bend (proteins)
29–31	1605–1666	Amide I bands

cleus-to-cytoplasm (N/C) ratio and cell size; as the proportions of nucleus and cytoplasm probed by the laser is likely to vary with these factors. Lipids and carbohydrates are largely distributed within the cytoplasm and, in the case of lipids cell membrane [59]. Therefore differences in these lipid and carbohydrate amounts between PC-3 and LNCaP may be a consequence of differences in the N/C ratio between these cells. A similar argument to explain discrimination of live PC-3 and MGH-U1 cells was postulated in [36].

The difference in lipid amounts between LNCaP and PC-3 may not be due to actual intrinsic biochemistry, but rather due to differences in the effect the fixative has on the cells. Alcohol fixatives act on cells by creating holes in the membrane, which allow entry into the cell. Alcohols do not generally fix lipids and the majority will be leached out of the cell. If the behaviour of the fixative towards PC-3 and LNCaP cells is slightly different then this may be re-

Table 4B Raman band assignments for LDF2 (Figure 6(b)), tabulating key peaks.

Band	Wavenumber (cm ⁻¹)	Tentative Raman band assignment
	+ve	
1	854	$\nu\text{C-C}$ of Tyr and Pro (proteins), carbohydrate bands
2	879	Hydroxyproline, Trp, (SurePath contamination?)
3	902	C–O–C skeletal modes (carbohydrate bands)
4	938	Pro, hydroxyproline (proteins), carbohydrate bands
5	1002	Phe (proteins)
6	1030–1041	C–H and C–N bend, Phe (proteins), carbohydrates
7	1109	Carbohydrate bands
8	1125–1134	$\nu\text{C-C}$ (lipids), C–N stretch from Phe (proteins)
10	1409	CO ₂ ⁻ (lipids)
11	1457	DNA
12	1605–1625	Protein bands
13	1678	β -sheet of amide I
	–ve	
14	731	Phosphatidylserine (lipids)
15	783	T, C, U ring breathing, phosphodiester (nucleic acid)
16	1000	Phe (possibly also NADH)
17	1148	PO ₄ ³⁻ (nucleic acids)
18	1184–1204	C, G, A (nucleic acids), amide III + CH ₂ wag Gly and Pro
19	1221	β sheet of Amide III
20	1252	Amide III
21	1297	CH ₂ def and CH twist (lipids)
22	1336	A, G, C–H def, amide III, CH ₂ wag (protein)
23	1375	T, A, G (nucleic acids)
25	1439	CH ₂ def (lipids)
26–28	1484–1574	G, A, C (nucleic acids), amide II

flected in differences in lipid concentrations. In addition to the effects of the alcohol fixative, it is possible that using different growth media might alter the lipid amounts in PC-3 and LNCaP cells. Both cell lines have FCS in their media which contain lipids but the Ham's F12 media, used for PC-3 cells also contains 0.084 mg/l linoleic acid unlike RPMI 1640. However this is unlikely to be a discriminatory factor since PC-3 and MGH-U1 can be discriminated, but both were grown in the same media.

With regard to nucleic acids, there is a positive loading at 1061–1078 cm⁻¹ which can be assigned to the PO₂⁻ moiety of RNA (although lipids might also contribute to this feature). Dovbeshko *et al.* report that there is an increase in this contribution for tumour cells compared with normal cells [57]. This also

Table 4C Raman band assignments for LDF3 (Figure 6(c)), tabulating key peaks.

Band	Wavenumber (cm ⁻¹)	Tentative Raman band assignment
+ve		
1	722	A, DNA
2	745	T ring breathing mode (nucleic acids)
4	846	C–O–C skeletal modes (carbohydrates)
5	1000	Phe (possibly NADH)
6	1025	Carbohydrate bands
7	1061–1078	C–C stretch (lipids), PO ₂ ⁻ stretch (nucleic acids)
8	1120	C–C band of ribose (nucleic acids)
9	1151	Carbohydrate bands
10	1168	ν C=O and ν C–OH (lipids)
11	1201	RNA contributions, Amide III, CH ₂ wag
12	1221	T, A (nucleic acids), amide III
13	1297	CH ₂ def (lipids)
14	1414	ν CH ₂
15	1440	CH ₂ def, CH ₃ def, δ CH ₂ , ester groups (all lipids)
16	1549	Trp (proteins)
17	1602	Phe (proteins), C (nucleic acids)
18	1655	T, G, C (nucleic acids)
19	1739	Ester bands
–ve		
20	789	PO ₂ ⁻ (nucleic acids)
21	882	Try (proteins)
22	941	C–C residue of Pro (proteins)
23	1002	Phe (proteins)
24	1044	C–O and ν C–N (proteins)
25	1092	ν PO ₂ ⁻ stretch (nucleic acids)
26	1232–1255	Amide III, CH ₂ wag, ν PO ₂ ⁻ (nucleic acids)
27	1344	δ CH
28	1380	ν_{symm} CO ₂ ⁻ (lipids)
29	1490	DNA
30	1580	δ C=C of Phe (proteins)
31	1678	Amide I

coincides with the appearance of a positive peak at 1120 cm⁻¹. Dovbeshko *et al.* suggest that these changes for tumour cells are as a result of spatial changes in the position and organisation of the phosphate groups in the RNA helix.

3.1.4 Classification of formalin fixed cells

3.1.4.1 PC-LDA diagnostic modelling

For this study using formalin fixed cells three different cell line combinations were examined:

- Four cell types not including PNT2-C2 and urethral cells (*i.e.* BPH, LNCaP, PC3 and MGH-U1). This model was investigated for the SurePath fixed cells, hence allowing a comparison to be made.
- Four cells types not including LNCaP and PNT2-C2 (*i.e.* BPH, PC-3, MGH-U1 and urethral). This situation could represent a real cell composition of urine after prostate massage, although an important caveat is that PC-3 or a similar invasive cell might have metastasised from the primary tumour, so might not be present in urine to a great extent.
- All six cell types included (*i.e.* BPH, LNCaP, PC3, PNT2-C2, MGH-U1 and urethral).

In a similar method to the SurePath spectra, PCA was initially carried out on the data, followed by PC-LDA using the first 30 PCs (PC1, PC2 and PC3 accounted on average for 44.3%, 18.8% and 8.6% of the variance, respectively). PC-LDA training plot templates of 80 spectra per cell line was generated (Figure 8(a–c)). This was followed by blind testing using the remaining spectra

The PC-LDA plots for BPH/LNCaP/PC-3/MGH-U1 (Figure 8(a)) and BPH/PC-3/MGH-U1/urethral (Figure 8(b)) models show good separation of the four cell types using two LDFs alone. This compares with SurePath fixed cells, in which three LDFs were required to separate fully BPH/LNCaP/PC-3 and MGH-U1. When all six cell types are included some clustering is observed using the first three LDFs (Figure 8(c)).

Sensitivities and specificities for all three PC-LDA classifiers are shown in Table 5 and LR⁺ values in Table 6. The number of spectra used for validation was: BPH 37, LNCaP 49, PC-3 52, PNT2-C2 52, MGH-U1 51 and urethral 47.

Prediction results for the BPH/PC-3/MGH-U1/urethral and BPH/LNCaP/PC-3/MGH-U1 classifiers are very high, with specificities in three cases for the former of 100%. Even the model incorporating 6 cell types generally gave high prediction rates.

Examination of Tables 5 and 6 shows that specificity values of 100% gives undefined LR⁺ values (marked as “–” in Table 6), because this calculation involves division by zero. In this case the LR⁺ has reached infinity, which is the ideal value of LR⁺. Sensitivity values of 100% give LR⁻ values of zero, *i.e.* the probability of having the disease when the test is negative is zero. In a similar vein to a LR⁺ value of infinity, this is the ideal case. Observation of Table 6 shows that all LR⁺ and LR⁻ values are ≥ 10 and ≤ 0.1 respectively, thus passing the test for a good diagnostic classifier [48].

Whilst these results are very promising, in a real clinical setting the operator recording the spectra would not know the identity of the blind-test spectra.

Figure 8 (online colour at: www.biophotonics-journal.org) PC-LDA plots obtained from formalin fixed cells for BPH/LNCaP/PC-3/MGH-U1 (a), BPH/PC-3/MGH-U1/urethral (b) and BPH/LNCaP/PC-3/PNT2-C2/MGH-U1/urethral (c).

This is in contrast to the blind-tests carried out in this study, in which the operator is in possession of the spectra identity. To overcome this situation and to further increase confidence that these results might be useful in a diagnostic setting, a double-blind test was carried out, in which neither the PC-LDA algorithm nor the operator knew the identity of the cells analysed. The operator analysed cells, followed by data analysis without knowing the cell identity. The double-blind spectra were projected into the BPH/PC-3/MGH-U1/urethral classifier model and classification scores obtained. Each double-blind spectral data set (15 spectra per cell line) was projected into the BPH/PC-3/MGH-U1/urethral PC-

LDA model one data set at a time. Each data set clustered into different clusters with 100% sensitivity, which allowed the identity of the spectra to be realised. This was compared against the known identity of the cells, held by a second operator. All four cell lines of unknown identity were correctly assigned. This result significantly enhances confidence in the Raman tweezers method generally, and specifically in the BPH/PC-3/MGH-U1/urethral model as a potential diagnostic tool. However, there is a caveat to this promising double-blind result; the double-blind spectra are of cells of the same type that are used in the model. In a real clinical setting the cells will probably be different than the ones in the model.

Table 5 Sensitivity and specificity values achieved by the PC-LDA models for formalin fixed cells.

	Cell Line					
	BPH	LNCaP	PC-3	PNT2	MGH-U1	Urethral
Sensitivity %	97.8	93.5	96.0		98.0	
Specificity %	99.3	99.2	99.3		97.9	
Sensitivity %	100		96.1		96.1	100
Specificity %	96.2		100		100	100
Sensitivity %	100	93.5	98.0	99.5	88.2	97.8
Specificity %	99.2	99.6	99.6	98.4	98.1	98.1

Table 6 LRs calculated from values in Table 4.

	Cell Line					
	BPH	LNCaP	PC-3	PNT2-C2	MGH-U1	Urethral
LR ⁺	139.7	116.9	137.1		46.7	
LR [−]	0.02	0.007	0.04		0.02	
LR ⁺	26.3		–		–	–
LR [−]	0.0		0.04		0.04	0.0
LR ⁺	125	233.8	245	62.2	46.4	51.5
LR [−]	0.0	0.07	0.07	0.01	0.12	0.02

3.1.4.2 Loading plot analysis

The PC-LDA plot of the BPH/PC-3/MGH-U1/urethral model displays good clustering of the four cell types using just the first two LDFs (see Figure 8(b)). The PC-LDA plot shows the following:

→ LDF1 separates the healthy urethral cells (negative scores) from malignant PC-3 and BPH (positive scores).

Table 7A Raman band assignments for LDF1 (Figure 9(a)), tabulating key peaks.

Band	Wavenumber (cm ^{−1})	Tentative Raman band assignment
	+ve	
1	705–731	Cholesterol, phospholipids, DNA, A
2	785	Ring breathing modes of U, C, T, PO ₂ [−] (nucleic acids)
3	1093	PO ₂ [−] , DNA (nucleic acids)
4	1193	C, G (nucleic acids)
5	1240	RNA, <i>v</i> _{asym} PO ₂ [−] (nucleic acids), amide III
6	1332	Phospholipids, DNA, G (nucleic acids)
7	1376	T, A, G, (nucleic acids), δ CH ₃ (lipids)
8	1487	G, A, G (nucleic acids)
9	1582	C=C bend, Phe (proteins)
10	1667	Amide I
	−ve	
11	864	RNA
12	908	Formalin contamination?
13	934	Pro, val, (proteins), carbohydrates
14	1000	Phe
15	1029	<i>v</i> O–CH ₃ (carbohydrates)
16	1078	C–C, <i>v</i> C–O (lipids), PO ₂ [−] (nucleic acids)
17	1124	<i>v</i> C–C (lipids)
18	1152	Carbohydrates bands
19	1453	<i>v</i> CH ₂ /CH ₃ def (lipids) δ C–H (proteins)
20	1649	<i>v</i> C=C and <i>v</i> C=O (L), amide I
21	1739	Ester groups (lipids)

→ LDF2 separates PC-3, MGH-U1 and BPH. LDF2 therefore separates cells based on their disease and tissue of origin. Malignant PC-3 spectra have negative scores, whilst non-malignant BPH spectra have positive scores.

Loading plots for LDF1–2 are shown in Figure 9, with band assignments given in Table 7A–B [52–58].

Key Features from LDF1 (Table 7A) – Because urethral cells have negative scores, negative moieties corresponding to negative peaks are prominent in these cells (refer back to Figure 8(b)). From the loading assignments it can be seen that lipid and carbohydrate groups tend to be prominent in urethral cells. On the other hand nucleic acid moieties are

Table 7B Raman band assignments for LDF2 (Figure 9(b)), tabulating key peaks.

Band	Wavenumber (cm ^{−1})	Tentative Raman band assignment
	+ve	
1	869	C–O–C (polysaccharides), Pro (proteins)
2	906	Disaccharides, possible formalin contamination peaks
3	931	Skeletal C–C, α -helix, carbohydrates
4	1029	<i>v</i> O–CH ₃ (carbohydrates)
5	1062	<i>v</i> C–O, <i>v</i> C–C (lipids), carbohydrates
6	1078	<i>v</i> C–O, <i>v</i> C–C/ δ C–C (lipids), phospholipids, PO ₂ [−]
7	1127	C–C skeletal stretch (lipids), C–O bend (disaccharides)
8	1150	Carbohydrate bands
9	1281	Amide III and CH ₂ wag vibrations from Gly and Pro backbone
10	1299	CH ₂ def (lipids)
12	1441	CH/CH ₂ /CH ₃ def, CH ₂ scissors and δ CH ₃ (lipids)
13	1540	Amide carbonyl groups
	1603	C=C in plane bending of Phe and Tyr (proteins)
15	1649	Amide I
16	1736	C=O ester (lipids)
	−ve	
17	733	A (NA), Phosphatidylserine (lipids)
18	787	PO ₂ [−] , C, U, T (nucleic acids)
19	1093	<i>v</i> _{symm} PO ₂ [−] (nucleic acids)
20	1242	<i>v</i> _{asym} PO ₂ [−] (nucleic acids), amide III (β -sheet/coils)
21	1340	G (nucleic acids)
22	1379	T, A, G (nucleic acids)
23	1489	DNA, (possible formalin contamination?)
24	1510	A, C (nucleic acids)
25	1579	Nucleic acids contributions
26	1677	Amide I

Figure 9 (online colour at: www.biophotonics-journal.org) Loading plots for LDF1 (a) and LDF2 (b). Colours relate to assignments given in Table 7A–B.

prominent in PC-3 and/or BPH cells. One explanation for this could be as an indirect consequence of the relative cell sizes. Urethral cells are generally smaller than BPH and PC-3 cells (refer back to Table 1). The surface area (cell membrane) to volume ratio for these urethral cells will therefore be larger than for BPH and PC-3 cells. This might be reflected in a greater abundance of lipids, particularly membrane lipids for urethral cells. However, geometric factors will only partially explain the abundance of lipids in urethral cells, and other factors such as the concentration of lipids in the cytoplasm may also play a role.

Differences in amounts of nucleic acids and carbohydrates between urethral and PC-3/BPH cells could be due to differences in the N/C ratio. Because the main proportions of nucleic acids are located in the nucleus and carbohydrates are distributed within the cytoplasm, it could be postulated that the BPH and PC-3 cells have a higher N/C ratio than for urethral cells. A similar argument was made for SurePath and for live cells [36].

Key Features from LDF2 (Table 7B) – From the loading assignments, discrimination between PC-3 and LNCaP on LDF2 is clearly due to differences in lipids, carbohydrates and nucleic acids. Moieties assigned to positive loads are more prominent in BPH than PC-3. These are on the whole lipids and carbohydrates. However, nucleic acid moieties are largely negative loadings, and so can be assigned to PC-3 cells. Therefore, an anti-correlation exists between the malignant PC-3 and non-malignant BPH in the relative concentrations of nucleic acids and biochemicals required for ATP generation for metabolic activity, such as glycogen and lipids. The relationship has been seen in a number of previous studies [19, 60], and is partly due to the greater metabolic turnover for malignant cells. Therefore a greater consumption of glycogen and lipids are required to generate ATP, with subsequent reduction in their concentration. Another reason could be due to the low concentration of zinc in malignant prostate cells

[61]. Zinc plays a role in the regulation of the citric acid cycle, with a reduction resulting in a continuation of this cycle. This results in over-production of ATP, resulting in the increase in need for glycogen. At the same time nucleic acid concentrations increase due to the increase in cellular activity of the malignant cell. Stone *et al.* report that DNA content increases in both prostate and bladder tissue when increasing in malignancy, most probably due to an increase in the N/C ratio for malignant tissue [62].

Also differences between PC-3 and BPH can be attributed to the fact that BPH cells have the normal number of chromosomes whereas PC-3 have significant duplication and actually have nearly a triplicate genome with an average of 62 chromosomes per nucleus [37].

Another interesting observation from this loading plot is that amide I peaks, 15 and 26 are anti-correlated to each other, with the negative loading at the higher wavenumber. This could suggest that the secondary structure in urethral cells largely comprises of turns or random coils, while for PC-3 and BPH cells the α -helix dominates [63].

3.1.5 Comparisons between SurePath and formalin fixed cells

Observation of the sensitivities, specificities and LR results for the BPH/LNCaP/PC-3/MGH-U1 models shows better diagnostic performance for the formalin fixed cells compared with SurePath cells (refer back to Tables 2, 3, 5 and 6).

The better diagnostic performance of the PC-LDA model for formalin than for SurePath cells is clearly demonstrated by plotting a version of a receiver operating characteristic (ROC) curve (Figure 10). This curve is commonly used to test the performance of a diagnostic model, and plots false positive rate (1-specificity) vs true positive rate (sensitivity) [64–66]. A good diagnostic model is

Figure 10 (online colour at: www.biophotonics-journal.org) ROC curve for SurePath and formalin fixed cells.

characterised by a sensitivity close to 1 and 1-specificity close to zero, and thus the closer to the top left corner the data lays the better the performance [64, 66]. Figure 10 shows that the formalin fixed data is closer to the top left corner than SurePath fixed data, which demonstrates better performance for the formalin fixed model.

The superior diagnostic performance for formalin fixed cells is likely to be due to better spectral quality rather than indicative of intrinsic biochemical differences between the SurePath and formalin fixed cells. However the latter cannot be completely ruled out until further investigations are carried out. The signal quality was determined by measuring the mean SNR for both SurePath and formalin fixed spectra. The SNR was quantified by measuring the root-mean-square (RMS) noise in the 1780 to 1800 cm^{-1} region (which is free from any bands) and the maximum height of the amide I band (at about 1655 cm^{-1}). The amide I peak height was divided by the RMS to obtain the SNR value. Mean SNR results of 170 and 207 for SurePath and formalin spectra respectively were obtained. By employing a one-way ANOVA at a confidence level of 95% these means were found to be statistically different.

The greater SNR for the formalin cells is primarily due to a higher laser power at the cells (100 mW compared with 32.6 mW for SurePath fixed cells). Examination of the spectral noise suggests that it is likely to be mostly additive and shot noise, but not multiplicative noise. Therefore an increase in signal reaching the CCD due to a greater power flux would result in a higher SNR.

For the SurePath cells it was ascertained that 32.3 mW was the maximum power that could be used, because of the problems associated with the cell escaping the trap. However, for formalin cells this problem did not arise so a higher power could be used. Therefore the better prediction rates for formalin fixed cells is indirectly related to the fixation type used.

3.2 The effect of urine on urological cell classification

3.2.1 Issues with cell trapping

When analysing the cells that had been exposed to urine, it was observed that a proportion of cells that were exposed to urine had a tendency to trap above the focal point, thus appearing slightly out of focus. This was not the case for every cell, but the effect became more prevalent as exposure time increased. This effect was most noticeable for the BPH and LNCaP cells. For these cells, as exposure to urine increased beyond about 8 hours, a significant proportion were being pushed so far above the focal point that they were completely escaping the trap. A similar effect was observed for the SurePath fixed cells (refer back to section 2.2.1). Only cells that would stay in the trap could therefore be analysed.

The precise reason for a cell exposed to urine having a tendency to be pushed upwards in the trap is unknown. However, the fundamental cause is likely to be an imbalance in the two forces that act on a particle at the focal point of the optical trap; the scattering and gradient forces, caused by a change in the refractive index of the cell. For a particle (e.g. a cell) to be trapped the refractive index of the particle has to be greater than that of the medium in which it is suspended in. However, whilst the scattering force that acts on the particle to push it away from the trap increases with the refractive index of the particle, the gradient force, which pulls the particle into the trap increases with refractive index only up to a point and then decreases again. Hence, at a particular value of the refractive index, which is higher than that of the medium, the scattering force is greater than the gradient force and the particle is not trapped [67]. It could be speculated that this may be due to dehydration of the cell, due to osmosis. This might occur if, as expected urine is hypertonic with respect to the cell. The artificial urine was found to have an osmolarity of 440 mOsm/kg, compared to 283–296 mOsm/kg for the cell culture media, and consequently the cells. Thus, the artificial urine is hyperosmotic in relation to the cells. Although, it does not necessarily follow that the artificial urine is hypertonic in relation to the cells (tonicity is determined by solutes that cannot penetrate the membrane, while osmolarity is the concentration of all solutes in a solution) since the differences in the osmolarity is considerable, it could be inferred that the urine is hypertonic. This would cause water to diffuse out of the cell into a medium of higher osmotic pressure (resulting from higher solute concentration).

Furthermore, it could be surmised that a change in refractive index of the cell might be as a result of

the effect that the urine might have on the cell membrane; *i.e.* the membrane could be compromised due to abnormal osmotic pressures. When the cells are placed in 4% formalin/PBS, more fixative could enter cells which have a compromised membrane at a greater rate. This could change the degree and rate of fixation of the intercellular components [41], which could affect the refractive index. An increase in the amount of scattering from the cell might also occur, thus increasing the scattering force, resulting in a subsequent reduction in trapping efficiency. It could be surmised that urine will have a lesser effect on the bladder cell membrane than the prostate cell membrane. Prostate cells have limited contact with urine, but urethral cells and bladder cells (MGH-U1) are in either constant or regular contact with urine of different concentrations (depending on the hydration of the body) and therefore will have an adapted cell membrane to cope with the urine unlike the prostate cells. During data collection it was observed that the MGH-U1 cells trapped better than the prostate cells at extended urine exposure times.

The reduction in trapping efficiency and subsequent pushing of cells above the focal point prob-

ably explains the high proportion of cells being rejected during the quality assessment procedure (20% rejection rate). Because the cells are not in the focal point of the trap, the signal intensity reaching the detector will not be optimised, thus leading to a poorer SNR. Problems with under or over background subtractions as a result of the cell not being correctly situated in the trap may also be the cause of a high number of rejected spectra.

3.2.2 Classification results

PC-LDA was carried out for MGH-U1/BPH/LNCaP/PC-3 spectra obtained from cells exposed to urine, with PC-LDA models created for each exposure time period. Figure 11 shows the variation in sensitivities, specificities and overall model accuracies over a 12 hour period. Overall results are lower than those reported in section 3.1.4, due to the fact that the ratio of PCs to number of spectra per group is lower. The smaller number of PCs was chosen to avoid the risk of the PC-LDA models being over-fitted, since the models are cross-validated rather

Figure 11 (online colour at: www.biophotonics-journal.org) Graphs showing variation in sensitivity (a and b), specificity (c and d) and accuracy (e and f) obtained for cells exposed to urine for up to 12 hours and first two hours expanded, respectively.

than blind tested. An over-fitted model might be recognised to a lesser extent using internal cross-validation rather than blind-testing [68].

Examination of the plots in Figure 11 highlights the first two hours of exposure in urine to be of significant interest. Observations of plots for sensitivities, specificities and accuracy reveal an increase in prediction rates over the first 30 minutes. It could be postulated that this could be due to a stress response of the cells. A stress response is the reaction of the cell to abnormal conditions and environments [69]. Cells are likely to find urine a hostile environment due to abnormal osmotic pressures and pH as well as exposure to potential toxic salts and metabolites (e.g. uric acid) [70]. Kültz reports that the fundamental motivation of a stress response is the protection of the cells' biochemical integrity during the initial stages of exposure to the adverse environment [69]. With regards to cells exposed to urine, this could relate to the first 30 minutes.

Of particular interest to this study is the finding by Kültz [69], that hypertonic stress causes protein instability and DNA damage. It is possible that urine is hypertonic in relation to the internal cell environment, thus causing these stresses. As a response to hypertonic stress, cells undergo growth arrest by introducing cell cycle checkpoints, an increase in DNA repair and the removal of macromolecular debris. This results in the up-regulation of several proteins (GADD45 and GA153) to induce growth arrest and for DNA and chromatin repair [71, 72].

Consequently, as a response to stress, there is likely to be change in the urological cells' biochemistry when initially exposed to urine. It could be postulated that the four cell lines up-regulate either different stress-related proteins or different amounts. This would result in an increase in biochemical difference between the cell lines when first exposed to urine, which could explain the increase in the prediction rates. It is likely that the bladder cell line, MGH-U1 will exhibit less of a stress response than the CaP cell lines. This is because bladder cells in the body are regularly exposed to urine, while for prostate cells urine exposure will be more limited. However, an important caveat to this is the fact that MGH-U1 is a cell line and not a primary cell. Therefore the cells would have progressed through many passages, with the possibility that any phenotypic resistance to urine might have subsequently been lost, or significantly reduced.

Between 30 and 45–60 minutes there is a drop in prediction rates, which could be due to a reduction in the rate of the production of the stress-response proteins after the cells have become accustomed to their environment. It is noted that, apart from PC-3, the prediction rates are now similar to what they were before urine exposure, suggesting that the cells' biochemistry could have returned to near their initial state.

From 1 hour onwards, undulating features are observed in the prediction rates, until 12 hours when values start to coalesce (*i.e.* come together). These features could be attributed to the natural variance within each data set. Such variance could be caused by the inherent biochemical heterogeneity that exists within a population of cells. Alternatively, for any given time-point a proportion of cells might have undergone apoptosis. It would be expected that the proportion of dead cells increases as exposure to urine increases. This factor is therefore likely to be responsible for some of the observed variance.

However, despite these undulating features reported over the 12 hours, the prediction values have not deteriorated over this time. Therefore, it can be concluded from these results, urine has little affect on the ability to classify these cell types.

4. Conclusions

In this paper we have demonstrated the ability of Raman tweezers combined with supervised chemometric analysis to discriminate between cancer and non-cancer cell lines and between different types of cancer cell with a high degree of accuracy. Furthermore, we have demonstrated that the diagnostic performance following formalin fixation was better than for the alcohol based SurePath. This can be directly linked to the fact that the experimental methodologies and Raman tweezer setups used for these two methods were different. The overall effect of these different approaches meant that more Raman light was reaching the CCD detector for formalin fixed cells than for SurePath cells. This resulted in a higher SNR for the formalin fixed cells, with a subsequent improvement in diagnostic performance.

It can therefore be concluded that the effect that the SurePath has on the cells (*i.e.* in terms of the cells escaping the trap) has an indirect consequence on the diagnostic performance. This, coupled with the problems of analysing the cells in SurePath, such as the requirement for a pin-hole (thus reducing Raman light), background subtraction issues and the logistics of using a second laser beam, makes the formalin fixation method a more attractive option.

Results presented here suggest that cell size has no direct effect on the classification, which was the conclusion reached for our previous live cell study [36]. For example in this present study separation was observed between cell lines, with statistically the same mean cell diameter (for example, SurePath fixed MGH-U1 and BPH cells, and formalin fixed MGH-U1 and BPH cells). However, it is possible that cell size may indirectly influence classification, since proportions of the nucleus and cytoplasm probed will vary with cell size. In this paper we have

comprehensively analysed loading plots to determine possible biochemical features responsible for the classification. Whilst these do not provide definitive answers and are in no way quantitative they do go some way in justifying the observed classifications.

With regards to the urine exposure experiment, results have demonstrated that exposure of urological cell lines for 12 hours has no detrimental effect on the ability to accurately classify them. Therefore, cells can be exposed to urine for up to 12 hours, and still be accurately classified.

Despite these promising results exposing cells in urine does present some logistical issues. Exposure to urine results in a reduction of trapping efficiencies. Spectral quality deteriorates with reduced trapping efficiencies resulting in a rejection rate of about 20%, which is greater than that for cells not exposed to urine. Therefore, for future work a greater number of cells might need to be analysed to take into account the potential number of rejected spectra. Alternatively a counter-propagating beam, similar to the one used for the trapping of SurePath cells, could be introduced. However, this is undesirable as it increases the complexity of the experiment, and would reduce the turn-over rate of analysed cells.

Due to these trapping problems, the maximum exposure time of cells in urine may well depend on trapping efficiency, rather than the ability to classify them.

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