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The clinical Transferability of Raman Micro-Spectroscopic Systems for Cervical Cytopathology

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The clinical transferability of Raman micro-spectroscopic systems for cervical cytopathology

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ABSTRACT

The clinical potential for Raman microscopic systems is well established for early diagnosis via cytology. Although Raman systems offer a complementary diagnostic tool providing molecular information, it is not yet utilised substantially in clinics. A few challenges for the clinical implementation of Raman spectroscopy are system and user variability. In this study, we asked how much variability occurs due to different Raman systems or users. To address these questions, we measured the same set of cells using two different Raman microscopes and by two different users. And classification models were generated using multivariate partial least squares discriminant analysis (PLS-DA) and analysed for clinical implementation. Raman spectra were measured from single exfoliated cells (*n=400*) from ThinPrep samples with negative cytology (*n=10*) and high-grade cytology (*n=10*). Raman spectra were acquired from the same set of cells via two identical HORIBA Jobin Yvon XploRA™ systems (Villeneuve d'Ascq, France), as well as two different users. The Raman data was subjected to PLS-DA and cross-validated via leave-one-patient out. The study's findings suggest that the data acquired from the two Raman systems are 99% identical. However, the observed classification accuracy for the data obtained by user-1 was 92%, whereas by user-2 was 99%.

Keywords: Cervical Cancer, Raman micro-spectroscopy, Principal component analysis, Cancer screening

1. INTRODUCTION

A major global challenge-cervical cancer is the fourth most common female cancer for incidence and mortality in the world [1]. According to 2020 estimates, there are a total of 604,000 new cases and 342,000 deaths globally [2], [3], and over 100 million people have been fully vaccinated with Gardasil worldwide [4]. The World Health Organisation (WHO) Director-General has announced a call for action to eliminate cervical cancer and has emphasized a scale-up of cervical cancer screening and HPV vaccination [5]. The WHO recommends that by 2030, European nations should reach at least 90% HPV vaccination for both genders by the age of 15 years[5]. However, HPV vaccination does not protect against all high-risk HPV subtypes, therefore excellent quality screening programmes are still crucial to prevent cervical cancer in this new HPV vaccination era [6]–[8].

Optical spectroscopic techniques are well established and are promising for the characterization of biomaterials, due to their molecular specificity. Several previous studies on cells have shown the potential of mid-infrared and Raman spectroscopy for cervical cytopathology[9]–[12].

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A cervical cytopathology study via Raman micro-spectroscopy has demonstrated that Raman spectroscopy could detect the presence of high-risk HPV in cytology samples[13]. As well as, for the first time Raman spectroscopy can discriminate between normal and abnormal cell pellets with ~80% after the removal of blood from specimens [14]. This was extended, where Raman analysis of cervical and oral exfoliated cell pellets showed the main discriminating features were DNA and protein and improved classification was achieved when average spectra from each sample were used to overcome intra-sample heterogeneity (84% cervical and 86% oral cancer) compared to all spectra from each sample (77% cervical and 82% oral cancer).

As compared to previous work, a new method was presented for recording Raman spectra from cervical cytology samples prepared as single exfoliated cells using the ThinPrep liquid-based cytology method [15], where DNA/RNA content in Raman spectral features of the cells contributed to the discrimination of spectra from normal and high-grade cytology samples, this pre-treatment method was later adapted by adding hydrogen peroxide directly to the vial before slide preparation to Thinprep specimens with excessive blood contamination (blood scale index 2-3) [16]. Later multiple studies in this area to improve the Raman spectral cytopathology methods were published including a study explaining a detailed protocol for Raman spectral cytopathology via ThinPrep liquid-based cytology samples prepared onto glass slides [17]–[23]. However, there are a few unaddressed questions, such as how much variability occurs due to different Raman systems or users. To address these questions, we measured the same set of cells using two different identical Raman microscopes and by two different users, and the finding of the study are discussed in this manuscript.

2. METHODS AND MATERIALS

2.1 Sample preparation: Following ethical approval (REC-20-173), cervical smear samples (*n=20*) were obtained, of which 10 specimens were normal whereas 10 specimens were CIN 3 (cervical squamous intraepithelial neoplasia 3). The standard cytological test was conducted to determine the cytopathological grade of specimens, Whereas DNA and mRNA HPV-positive specimens were determined by HPV Cobas 4800 test and Aptima HPV test, respectively. The normal specimens were cytologically graded normal and HPV DNA and mRNA negative, whereas abnormal specimens were cytologically graded CIN-3 and were HPV DNA and mRNA positive. The ThinPrep® slides were prepared using ThinPrep® 2000 processor by Hologic Inc.

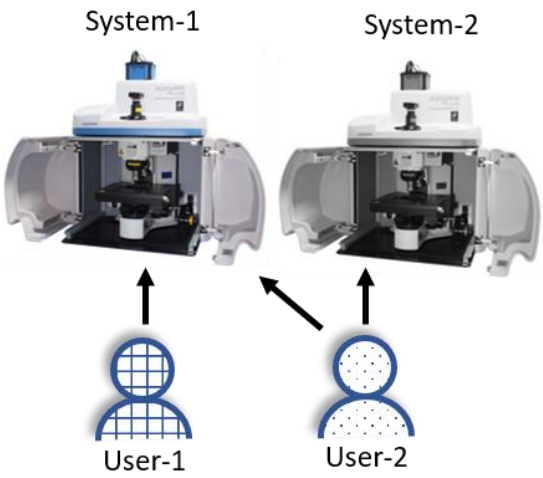


Figure 1 Experimental procedure

2.2 Raman micro-spectroscopy: We measured the same set of cells using two different Raman microscopes (**Figures 1 and 2-a**) and by two different users**.** Raman spectra were acquired using two different identical HORIBA Jobin Yvon XploRA^{TM} systems with a 532nm diode laser source at 100% power which gave a laser power of 8 mW at the 100X objective. The XploRA^{TM} systems were calibrated every day to obtain maximum signal intensity using silicon and the National Institute of Standards and Technology (NIST) was measured to check the CCD response. The software LabSpec 6.0 from HORIBA Scientific was used to view and manage Raman spectra. The spectral measurement parameter and system setting were kept the same during the experiments. The spectra were recorded in the 200-3500 cm^{-1} range, 30 seconds acquisition time with two accumulations. To obtain white-light images, a light source Olympus TH4-200 and a camera for images were used (Optical Microscope Olympus IX51/TH4-200, 2022), and the TANGO from Märzhäuser Wetzlar was used to control the sample stage. Each ThinPrep® slide has the word "ThinPrep®" imprinted at the top of the glass slide, we mapped the location of the cells by using the centre dot from the letter 'i' in the word ThinPrep® was set to 0-0 XY coordinate. It allowed us to use it as a reference point on each slide, and label XY coordinates for target cells to be stored in a file, this file can be used in other XploRA^{TM} systems to find the exact location of the measured cells.

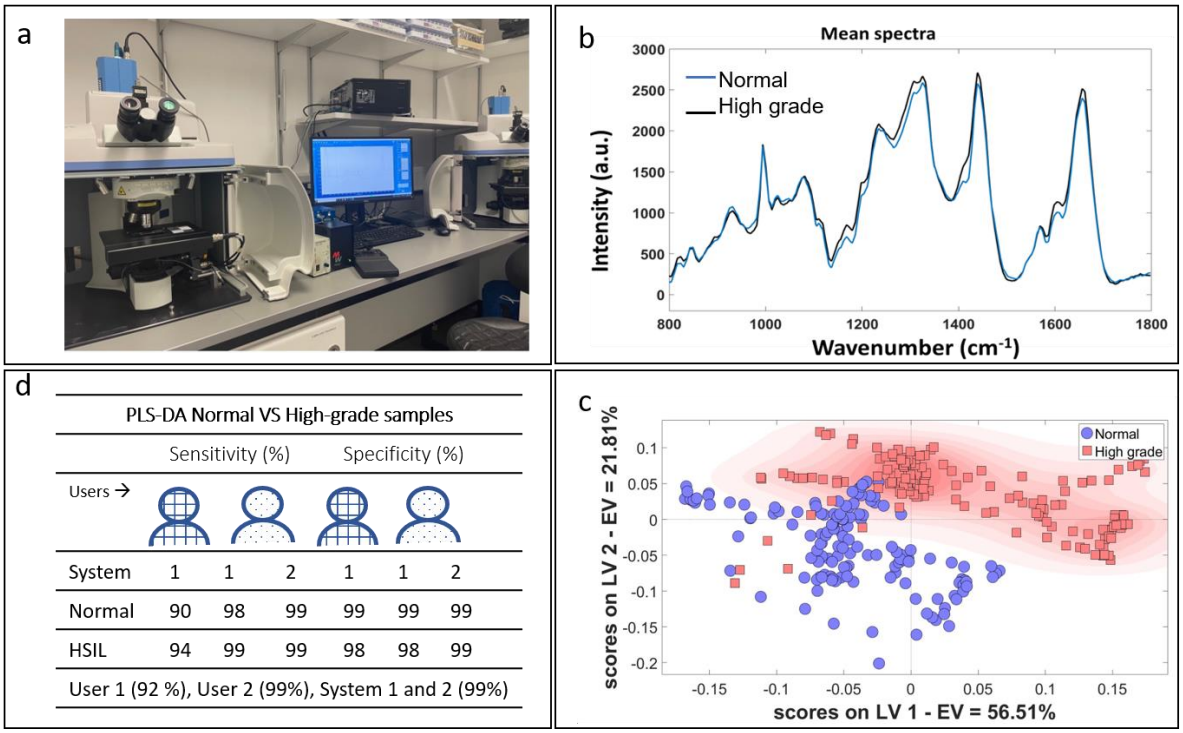
2.3 Data Analysis: The specific scripts developed and adapted in our laboratory for the pre-processing and uploading of data were carried out using MATLAB [Mathworks, CA, USA]. All the data were pre-processed and analyzed using

MATLAB, data pre-processing included smoothing (Savitzky-Golay K=5, K=13), baseline correction (rubber band) and followed vector normalization. We also carried out the removal of glass contamination from the spectra using a non-negative least-squares (NNLS) method [23]. The data was mean-centred and subjected to partial least square discriminant analysis (PLS-DA) in MATLAB environment and using the PLS-DA toolbox.

3. RESULTS AND DISCUSSION

The mean Raman spectra of normal and high-grade samples in the spectral range 800-1800 cm^{-1} are shown in **Figure 2-b**, major differences were noted at 1670 cm^{-1} (amide-I), 1450 cm^{-1} (δCH_2), 1238 cm^{-1} (amide III). The observations were consistent with earlier studies[18], [21]. PLS-DA classification models were created and the representative scatter plot is shown in Figure 2-c. The classification accuracy in terms of sensitivity and specificity of detecting normal and high-grade cervical cancer cells was tested using PLS-DA, as shown in the confusion matrix (**Figure 2-d**).

In our study, user-1 was not very experienced in using the Raman microscope and was trained for a few days, before the start of experiments, and user-2 was an experienced user. User-1 created the location map on the ThinPrep® slide, and these location map files were used by user-2 to identify the same measured cells, and user-2 used the same location map file on Raman system-2 to measure the same set of cells a third time. This was done, to reduce the variability in the data.



System	1	1	2	1	1	2
Normal	90	98	99	99	99	99
HSIL	94	99	99	98	98	99

User 1 (92 %), User 2 (99%), System 1 and 2 (99%)

Figure 2 (a) Raman systems used in experiments (b) Mean Raman spectra of normal and high-grade cervical cancer cells (c) Scatter plot for partial least squares discriminant analysis (PLS-DA) (d) confusion matrix for PLS-DA normal versus high-grade CIN-3 specimens.

We observed that PLS-DA, exhibited the same specificity between different users and systems, whereas we observed more differences in the sensitivity of two different users as compared to the system variability (**Figure 2d**). Previous studies have an emphasis on the same standardized pre-processing step to ensure the clinical transferability of Raman spectroscopy data in different experimental and instrumentation conditions [24]. In our pilot study, we kept the same pre-processing steps, experimental conditions, and similar systems, but user variability showed to affect the PLS-DA models. In this study, the data acquired from the two different Raman systems are 99% identical, exhibiting strong

proof of clinical transferability for cervical cancer cytopathology, but it also suggests good user training is necessary before the clinical implementation.

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