

2015-3

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Recommended Citation

Lyng, F. et al. (2015) Vibrational Microspectroscopy for Cancer Screening, *Applied Sciences*, 5, 23-35 (2015)

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Review

Vibrational Microspectroscopy for Cancer Screening

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Academic Editor: Ken Ledingham

Received: 7 December 2014 / Accepted: 10 February 2015 / Published: 13 February 2015

Abstract: Vibrational spectroscopy analyses vibrations within a molecule and can be used to characterise a molecular structure. Raman spectroscopy is one of the vibrational spectroscopic techniques, in which incident radiation is used to induce vibrations in the molecules of a sample, and the scattered radiation may be used to characterise the sample in a rapid and non-destructive manner. Infrared (IR) spectroscopy is a complementary vibrational spectroscopic technique based on the absorption of IR radiation by the sample. Molecules absorb specific frequencies of the incident light which are characteristic of their structure. IR and Raman spectroscopy are sensitive to subtle biochemical changes occurring at the molecular level allowing spectral variations corresponding to disease onset to be detected. Over the past 15 years, there have been numerous reports demonstrating the potential of IR and Raman spectroscopy together with multivariate statistical analysis techniques for the detection of a variety of cancers including, breast, lung, brain, colon, oral, oesophageal, prostate and cervical cancer. This paper discusses the recent advances and the future perspectives in relation to cancer screening applications, focussing on cervical and oral cancer.

Keywords: optical diagnosis; lasers; Raman spectroscopy; FTIR spectroscopy; cervical cancer; oral cancer; screening; cytology

1. Introduction

Cancer is a leading cause of disease worldwide with 14.1 million new cases, 8.2 million cancer deaths and 32.6 million people living with cancer (within five years of diagnosis) in 2012 [1]. Many cancers develop through pre-malignant stages which allow screening programmes to be implemented to detect cellular changes before invasive cancer develops. A good example is cervical cancer screening using the Papanicolaou (Pap) test which involves microscopic examination of cells exfoliated from the cervix.

Cytology is the study of disease at the cellular level and involves the preservation, staining and microscopic examination of cells that have been extracted from the body. Cytology can be used for practically any tissue type to detect abnormal cells. The principle of cytology is based on the fact that cancer cells lose their adhesive properties early in the disease process and can thus be easily exfoliated from tissue. An advantage of cytology is that sample collection is generally simple, rapid and painless.

Cytological screening aims to detect pre-cancer and cancer in a healthy population. In such populations, disease prevalence is relatively low and achieving a high test sensitivity is critical (see Figure 1). The aim of screening is to correctly identify as many cases of disease (positives) as possible while minimising incorrect identification of disease in individuals who do not have the disease (false positives) and negatives in individuals who do have the disease (false negatives). A false positive would result in a patient possibly undergoing unnecessary treatment while a false negative would result in a patient remaining undiagnosed and possibly progressing to cancer.

There is an unmet clinical need for new methods to aid cytologists in the early detection of pre-cancer. These methods ideally should be rapid, non-destructive, require minimal sample preparation and mostly importantly should not be subjective.

Sensitivity	Specificity
<ul style="list-style-type: none"> • a measure of the ability of the screening test to identify positives • expressed as the percentage of positives correctly identified by the test out of the total number of positives actually tested 	<ul style="list-style-type: none"> • a measure of the ability of the screening test to identify negatives • expressed as the percentage of negatives correctly identified by the test out of the total number of negatives actually tested

Figure 1. Definition of sensitivity and specificity.

2. Vibrational Spectroscopy

Vibrational spectroscopy analyses vibrations within a molecule and the spectrum of vibrational energies or frequencies (in wavenumbers, cm^{-1}) can be used to characterise a molecular structure. Every molecule has a unique spectrum which allows identification of the molecular compound and its abundance in a sample [2].

The exact energy required to excite a molecular vibration depends on the masses of the atoms involved in the vibration and the type of chemical bonds between these atoms and may be influenced by molecular structure, molecular interactions and the chemical microenvironment of the molecule.

Vibrational energies fall within the mid Infrared (IR) region of the electromagnetic spectrum and are commonly probed using IR absorption spectroscopy. IR spectroscopy is based on the absorption of infrared radiation by the sample and the fact that molecules absorb specific frequencies of the incident light which are characteristic of their structure.

A typical cellular FTIR spectrum is shown in Figure 2, together with a list of tentative spectral assignments in Table 1 [3]. The spectrum shown in Figure 2 can be divided into two regions, the high wavenumber region from 2500–4000 cm^{-1} , corresponding to absorptions due to O-H, N-H, C-H stretching vibrations, and the fingerprint region from 900–1800 cm^{-1} , which consists of many overlapping bands (see Table 1), and is characteristic of the sample.

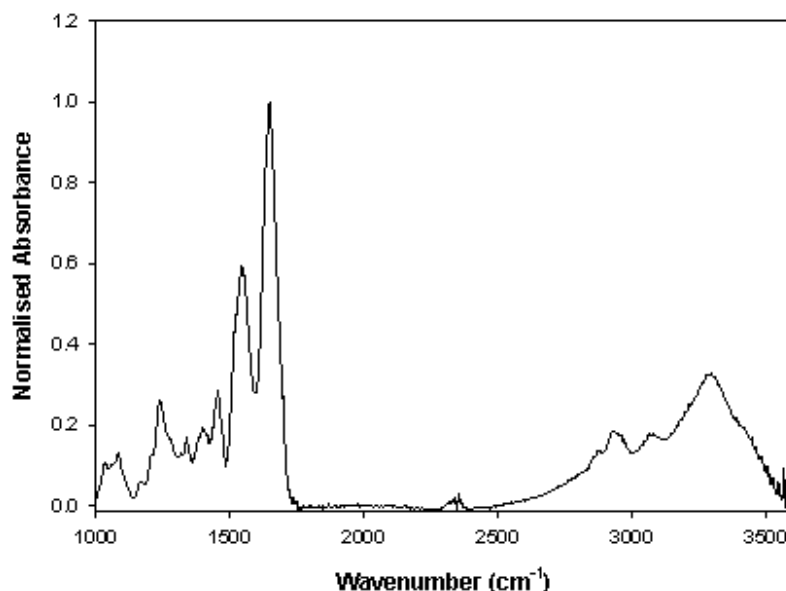


Figure 2. Typical FTIR spectrum from a population of cells.

Table 1. Tentative peak assignments [3] for typical FTIR cellular spectrum shown in Figure 2.

Wavenumber (cm^{-1})	FTIR Peak Assignments
1036	C-C skeletal stretching (proteins)
1080	PO_2 symmetric stretching (DNA/RNA)
1150	C-C and C-O stretching (proteins)
1220–1280	PO_2 asymmetric stretching (DNA/RNA), Amide III (C-N stretching, N-H bending, proteins)
1310	CH_2 stretching (phospholipids)
1450–1470	CH_3 asymmetric stretching (proteins, lipids)
1520–1550	Amide II (N-H bending, C-N stretching, proteins)
1620–1700	Amide I (C=O stretching, C-N stretching and N-H bending, proteins)
2850	CH_2 symmetric stretching (lipids)
2920	CH_2 asymmetric stretching (lipids)
2960	CH_3 asymmetric stretching (lipids and proteins)
3290	NH asymmetric stretching (proteins)
3400	OH stretching (water)

Raman spectroscopy is an optical method based on inelastic light scattering. The sample is illuminated by monochromatic laser light and interactions between the incident photons and molecules in the sample result in scattering of the light. The coupling of the light generates vibrations within the material, which are again characteristic of the chemical structure, and the energy of the scattered light is reduced by an amount equal to the energy of the vibrational energy. Thus, the positions, relative intensities and shapes of the bands in a Raman spectrum carry detailed information about the molecular composition of the sample.

A typical cellular Raman spectrum is shown in Figure 3, together with a list of tentative spectral assignments in Table 2 [4]. As for Figure 2, the spectrum can be divided into two regions, the high wavenumber region, from 2500–3500 cm^{-1} , and the fingerprint region, from 400–1800 cm^{-1} .

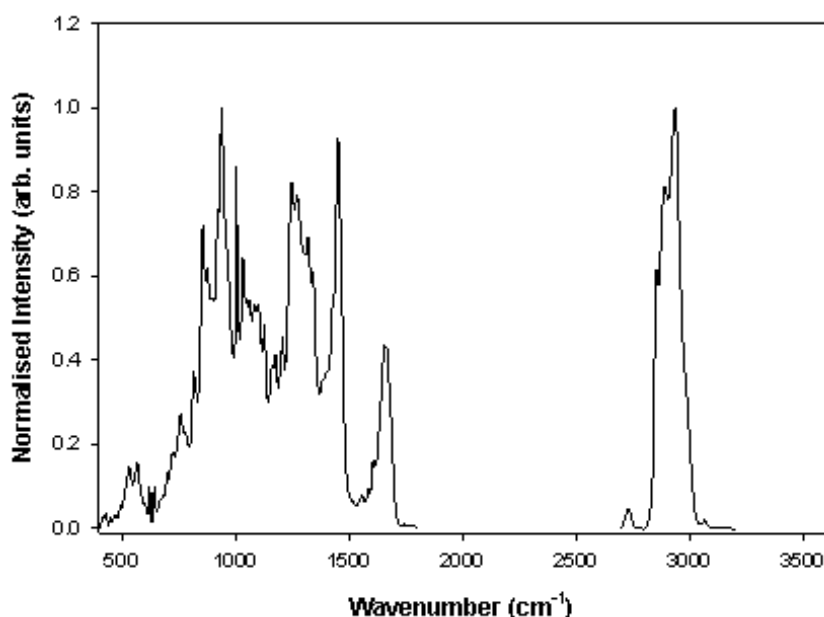


Figure 3. Typical cellular Raman spectrum.

Table 2. Tentative peak assignments [4] for typical Raman cellular spectrum shown in Figure 3.

Wavenumber (cm^{-1})	Raman Peak Assignments
621	C-C twisting mode of Phenylalanine (proteins)
642	C-C twisting mode of Tyrosine and Phenylalanine
670	Thymine, Guanine (DNA/RNA)
720	C-N stretching in Adenine and lipids
750	symmetric breathing of Tryptophan (protein)
782	Uracil, Thymine, Cytosine (ring breathing modes in the DNA/RNA)
827	PO_2 stretching in DNA, Tyrosine
854	Ring breathing in Tyrosine and Proline (proteins)
935	C-C stretching mode of Proline and Valine
1003	C-C aromatic ring stretching in Phenylalanine
1030	C-H bending mode in Phenylalanine, C-N stretching in proteins
1080	PO_2 symmetric stretching (DNA/RNA)
1085	C-O stretching

Table 2. Cont.

Wavenumber (cm ⁻¹)	Raman Peak Assignments
1130	C-N stretching in proteins; C-O stretching in carbohydrates
1155	C-C and C-N stretching of proteins/lipids
1175	C-H in plane bending mode of Tyrosine and Phenylalanine; Cytosine, Guanine
1208	C-C ₆ H ₅ stretching mode in Tryptophan, Phenylalanine
1220–1280	Amide III (C-N stretching, N-H bending, proteins), PO ₂ asymmetric stretching (DNA/RNA)
1311	CH ₃ /CH ₂ twisting mode of collagen and lipid
1340	Guanine (DNA/RNA), CH def. in proteins and carbohydrates
1430–1460	CH (CH ₂) bending mode in proteins and lipids
1485	Amide II (N-H bending, C-N stretching, proteins); Adenine, Guanine (DNA/RNA)
1580	Adenine, Guanine (DNA/RNA); C=C bending mode of Phenylalanine
1615	C=C Phenylalanine, Tyrosine and Tryptophan
1620–1700	Amide I (C=O stretching, C-N stretching and N-H bending, proteins)
2890	CH ₂ symmetric stretching (lipids)
2940	CH ₂ and CH ₃ symmetric stretching (lipids)
2980	CH ₃ symmetric stretching (lipids)

Raman and IR spectroscopy are complementary techniques. IR spectroscopy involves the absorption of radiation whereas Raman spectroscopy is a scattering technique, whereby the incident radiation couples with the vibrating polarisation of the molecule and generates or annihilates a vibration. These different underlying mechanisms give rise to the complementarity of the two techniques and they each have different selection rules. For a vibration to be active in IR spectroscopy, a change in dipole is required, whereas a change in polarisability is required for a vibration to be Raman active. Vibrations of asymmetric, polar bonds tend to be strong in IR spectra, whereas symmetric, nonpolar groups are strong in Raman spectra. For example, O-H vibrations of water are very strong in IR spectra, whereas they are relatively weak in Raman spectra. This feature means that Raman spectroscopy may be a more suitable technique for biological applications. Another difference is that Raman spectroscopy can be employed in the UV, visible or near IR regions of the electromagnetic spectrum whereas IR spectroscopy monitors the absorption of IR radiation. Therefore, Raman spectroscopy has a higher spatial resolution (<1 μm for Raman, $\sim 5\text{--}10\ \mu\text{m}$ for IR). Raman spectroscopy is, however, inherently weak with only a small percentage of photons undergoing Raman scattering ($\sim 10^{-6}$). Longer spectral acquisition times are therefore needed to achieve adequate signal to noise in Raman compared to IR spectroscopy.

IR and Raman spectra of biomedical samples such as cells and tissues are a superposition of all the contributions from each individual biochemical component such as DNA, RNA, proteins, lipids and carbohydrates. Thus vibrational spectroscopy methods can provide a molecular diagnosis by providing a “biochemical fingerprint” of the complete genome, proteome and metabolome of the cell or tissue [5]. This can be provided in a rapid, label free, non-destructive manner allowing additional analyses to be performed subsequently, such as staining, immunocytochemistry *etc.* Standardised methods and procedures for collecting IR spectra and images from biological samples have recently been described [6].

In recent years, Raman and FTIR spectroscopy have been used in the detection of a variety of cancers, including breast, prostate, oesophageal, colon, lung, oral and cervical cancer, and excellent sensitivity and specificity values have been reported [7–9].

For the purposes of this review, cervical and oral cancers will be focussed on as these tissues are readily accessible and amenable to cytological screening.

3. Cervical Cancer

Cervical cancer is the fourth most common cancer in women worldwide with an estimated 528,000 new cases and 266,000 deaths in 2012 [1]. The mortality associated with cervical cancer can be reduced if this disease is detected at the early stages of development or at the pre-malignant state (cervical intraepithelial neoplasia, CIN).

Currently, a Papanicolaou test is used to screen for CIN and cervical cancer in the general female population. The Papanicolaou test (also called Pap smear, Pap test, cervical smear, or smear test) is a screening test used to detect cancer and pre-cancer in the ectocervix. A spatula or cytobrush is used to collect the cells from the cervix and after placing in liquid fixative, the cells are then collected on a filter and transferred to a slide and stained. The cells are examined for abnormalities under the microscope by highly trained personnel. The Pap test is an effective, widely used method for early detection of cervical cancer and pre-cancer. Despite a high specificity of 95%–98%, the sensitivity of the Pap test is reported to vary greatly from 74% to 96% [10]. Low grade dysplasia or CIN 1 can spontaneously regress without leading to cervical cancer. However some CIN 1 lesions progress to moderate (CIN 2) and subsequently severe dysplasia (CIN 3), finally progressing to invasive cancer [11]. An abnormal Pap smear is followed by colposcopy, biopsy and histological confirmation of the diagnosis. This involves the visual examination of histological sections by highly trained personnel. The grading characteristics are quite subjective and pre-malignancy may not be visually perceptible at all.

Persistent infection with high-risk human papillomavirus (HPV) (e.g., HPV types 16, 18) is accepted as the major cause for the development of cervical pre-cancer and cancer [12]. HPV testing has recently been introduced in parallel with Pap smear screening for safe and cost-effective lengthening of the cervical screening interval due to its high-negative predictive values [13]. The HPV DNA test identifies high-risk HPV oncogene expression. However, this test is expensive, time-consuming and requires experienced technicians. Over the last decade, prophylactic HPV vaccines have been developed but despite the introduction of these vaccines, there is still a need for regular cervical screening, as the vaccines do not protect against all oncogenic HPV types. Additionally, some women may not benefit from the vaccines if there is a pre-existing HPV infection or they do not receive the complete number of doses (three doses over a 6-month period). After vaccination, women must still get routine Pap tests which can detect abnormal cervical growth regardless of what HPV type causes it to develop.

The first investigations of cervical exfoliated cells using IR spectroscopy were in the early 1990's by Wong and co-workers. They published a number of papers showing significant differences between normal, pre-cancer and cancer samples [14–17].

These initial studies recorded spectra from cell pellets rather than from individual cells and a number of confounding factors such as polymorphs, endocervical columnar cells, metaplastic cells, cervical mucus and debris were subsequently identified [18]. Further investigations by other groups identified similar confounding factors such as inflammation, metaplasia, hormonal changes, metabolic activity, blood, mucus [19–23].

However, an important early study by Cohenford and Rigas [24] showed that the FTIR spectra of morphologically normal exfoliated cells from women with dysplasia or cancer exhibit extensive IR spectroscopic changes. This finding was later confirmed by Schubert *et al.* [25] who showed spectral changes in cytologically normal cells, most likely due to HPV infection.

Walsh *et al.* [26] employed attenuated total reflection (ATR) FTIR spectroscopy to show spectral differences between normal and low grade and high grade dysplasia cytology samples. ATR-FTIR spectroscopy involves the IR beam being directed through a high refractive index crystal, typically diamond, germanium or zinc, where it undergoes total internal reflection generating an evanescent waveform that interacts with the biological sample at the surface of the crystal. Interestingly, a recent ATR—FTIR study has shown that cervical pre-cancer is more accurately identified when histology rather than cytology is used as the gold standard to classify the samples [27].

ATR-FTIR spectroscopy has also been shown to be capable of distinguishing between high risk HPV infections in women with low grade and high grade dysplasia [28]. Interestingly, spectral differences were also found between women in their 20s and in their 30s in samples of the same cytological grade and HPV type, most likely because of a persistent HPV infection. Schubert *et al.* [25] also showed that FTIR spectroscopy could discriminate cervical exfoliated cells based on their HPV status. In addition, a number of studies have used cervical cell lines as model systems to show the ability of vibrational spectroscopy (both IR and Raman) to differentiate cells based on HPV status [28–33].

In contrast to the extensive work on exfoliated cervical cells using IR spectroscopy, there are relatively few studies using Raman spectroscopy, most likely because of the issues with confounding factors.

Vargis *et al.* [33] showed that Raman spectroscopy could classify HPV-positive and -negative cytology samples with an accuracy of 98.5%. A study by Rubina *et al.* [34] used Raman spectroscopy to distinguish between normal and cervical cancer cytology samples. As the spectra of cervical cancer samples were dominated by blood features, cytology samples were treated with red blood cell lysis buffer prior to Raman acquisition. A classification accuracy of ~80% was obtained. Both of these studies used cell pellets rather than recording Raman spectra from individual cells and this probably resulted in the relatively low classification accuracy in the study by Rubina *et al.* due to sample heterogeneity.

A recent study by Bonnier *et al.* [35] addressed many of the issues involved in recording Raman spectra from ThinPrep cervical cytology samples. A new method based on pre-treatment of the slides with hydrogen peroxide to clear blood residue contamination before Raman recording was shown to minimise variability within the data sets. This resulted in the collection of highly reproducible data with clear discrimination between negative cytology and CIN cytology. Importantly, all data was recorded on glass ThinPrep slides which are currently used for clinical cervical cytology rather than spectroscopic substrates such as MirrIR or calcium fluoride substrates which are commonly used for research purposes and, although they reduce the presence of confounding contributions of the substrate, are considerably more expensive.

4. Oral Cancer

Oral cancer is defined as squamous cell carcinoma of the lip, oral cavity and oropharynx, and is the sixth most common malignancy in the world [36]. Patients with oral squamous cell carcinoma (OSCC) often have a poor outcome even though advances in treatment have been made. The 5 year survival

rate is approximately 80% for early stage disease but only approximately 20% for late stage disease [37] and patients often present with advanced disease. Multiple primary tumours are common and second primary tumours are the most common cause of death in patients with early stage disease [38].

OSCC is a multi causal disease and risk factors include lifestyle habits (tobacco exposure and alcohol consumption), dietary factors, occupational activity, socioeconomic status, exposure to external agents, and genetic susceptibility [39,40]. Most cases of oral cancer occur in patients of 60 years or over and the risk increases with advancing age.

The gold standard method for diagnosis of oral cancer and pre-malignant lesions is clinical examination and histopathological evaluation of biopsied tissue [41]. Conventional visual and tactile examination (CVTE) is used to detect OSCC and pre-malignant oral lesions in a screening setting but oral pre-malignant lesions and some early OSCC can be difficult to distinguish from inflammatory lesions. Most lesions are white patches known as leukoplakia and only approx. 4% of these lesions progress to cancer. CVTE cannot distinguish between these lesions and non-progressive lesions. Biopsy is followed by histopathology, where the excised tissue is processed, cut into sections and mounted on a glass slide for examination by a pathologist. Grading of tumours depends on the visual assessment of tissue architecture and characteristics of individual cells. The grading characteristics used are highly subjective and early proliferation/pre-malignancy may not be apparent morphologically. In addition, it can be difficult to discriminate between reactive/inflammatory lesions and pre-cancer lesions.

A number of screening and diagnostic aids for early detection and diagnosis of oral cancer and pre-cancer have become available over the last decade including brush biopsy, toluidine blue vital staining, post-acetic acid rinse tissue reflectance and tissue autofluorescence. Some of these techniques show promise but as yet none has been definitively proven to perform better than conventional visual examination [42]. Well designed clinical studies are needed where the techniques are directly compared to the diagnostic gold standard, scalpel biopsy and histopathology.

Early detection of cancer greatly increases the chances for successful treatment. There is a significant clinical need for new techniques that can detect pre-malignant changes that may progress to cancer. Oral cancer progression is not as clear cut as, for example, in cervical cancer in which there is a well-defined progression from mild to moderate to severe dysplasia (CIN 1, 2 and 3). The majority of histologically pre-malignant oral lesions do not progress to cancer at all and mild dysplasia can progress to cancer as readily as severe dysplasia. In a long term follow up of 236 patients with oral lesions, Holmstrup *et al.* [43] found that the degree of dysplasia was not a predictor of malignant potential of the lesions. The inability of conventional histopathology to predict which lesions will progress to cancer underlines the need for new techniques or new technologies to address this clinical need.

While a number of Raman spectroscopy studies have been carried out on oral tissues [44–46], to date, only a limited number of IR studies have focused on oral exfoliated cells.

Papamarkakis *et al.* [47] showed that oral cells could be classified according to anatomical region with cells from cheek, palate and gums being spectrally similar whereas cells from the tongue and from the floor of the mouth were spectrally distinct due to an abundance of keratin and collagen respectively. It was further shown that samples from patients with reactive atypical changes or malignancy associated changes were more spectrally similar to a sample from a patient with squamous cell carcinoma than to samples from healthy volunteers [47]. A further IR study from the same group [5,48] showed that spectra from squamous cells collected from the tongues of healthy volunteers could be discriminated

from squamous cells collected from the tongues of patients with oral disease, mostly dysplastic and cancer cases, using unsupervised principal components analysis. An artificial neural network was trained to automatically distinguish the clinical cases from the normal cases and sensitivity and specificity values of 96% and 94.3% were obtained. Interestingly, these results were obtained from morphologically normal cells, the majority of cells present in exfoliated cell samples. This indicates that biochemical changes can be detected by IR spectroscopy before morphological changes are apparent.

5. Future Perspectives

Vibrational spectroscopy shows great potential as a label free, high sensitivity and specificity test for cervical and oral cancer screening. The respective techniques of Raman and IR spectroscopy each has its own potential advantages for clinical applications (reviewed in [49]), with Raman spectroscopy having a higher spatial resolution, minimal sample preparation, and minimal interference from water and FTIR spectroscopy having a higher signal to noise ratio allowing faster acquisition times. ATR-FTIR spectroscopy can achieve higher spatial resolution and can allow measurement from aqueous samples. Recent advances in these techniques have included surface enhanced Raman spectroscopy (SERS), which allows significant enhancement of the Raman signal through close proximity/adsorption onto a roughened metal surface, a colloidal solution or a roughened electrode, tip enhanced Raman spectroscopy (TERS) which combines the high spatial resolution of atomic force microscopy with SERS, coherent anti-Stokes Raman scattering (CARS) and stimulated Raman scattering (SRS) (reviewed in [50]) which allow rapid real time imaging of single cells, and the development of high brightness quantum cascade lasers to improve acquisition times for IR imaging. For cytology applications, a key consideration is that these spectroscopic techniques could fit into the current workflow. Both Raman and IR spectroscopy can be operated in microscopic mode and, with automated spectral acquisition, these techniques could be similar to the Thinprep Imager (Hologic) or the Focal Point GS Imaging system (BD), which use image processing algorithms to automatically review liquid based cytology Thinprep and SurePath slides. Both Raman and IR spectroscopy are reagent free, so costs should be favourable when compared to the imaging systems or to manual scoring where personnel costs could be reduced. A recent study [35] has shown that glass ThinPrep slides can be used for Raman spectral recording whereas spectroscopic substrates, such as MirrIR or calcium fluoride substrates, are used for IR spectroscopy and although they reduce the presence of confounding contributions of the substrate, they are considerably more expensive than the glass slides used in the cytology laboratory.

However, much work still remains before these techniques could be translated into standard clinical practice [51]. Many studies have used relatively small sample sizes and as such may be biased. Screening populations are heavily weighted towards negative results with only a small percentage of positive results so validation in large multi centre studies is needed using real world cytology samples from screening populations. Large scale clinical trials are needed to obtain the large volumes of data necessary for the development of robust classification algorithms and histopathology rather than cytology should be used to classify the samples.

If spectroscopy can be shown to be as good as, or better than, the gold standard histopathology, then there is great potential for these techniques to be used as an alternative or an adjunct to the current cytology screening methods. The advantages would be higher accuracy, higher throughput

and reduced workload for the cytologist/pathologist and higher accuracy and chance of earlier detection for the patient.

Acknowledgments

The authors acknowledge funding from Enterprise Ireland co-funded by the European Regional Development Fund (ERDF) and Ireland's EU Structural Funds Programme 2007–2013, CF2011 1045, Science Foundation Ireland, 12/IP/1494, and Dublin Institute of Technology Fiosraigh Research Excellence Award.

Author Contributions

F.M.L. conceived the idea for the manuscript and wrote the manuscript with input from I.R., O.I. and H.J.B.

Conflicts of Interest

The authors declare no conflict of interest.

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