Clinical Spectroscopy: Lost in Translation?

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Abstract

This focal point article discusses the developments of biomedical Raman and infrared spectroscopy, and the recent strive towards being a recognised clinical tool for various applications. The promise of vibrational spectroscopy in the field of biomedical science, alongside the development of computational methods for spectral analysis, has driven a plethora of proof-of-concept studies which convey the potential of various spectroscopic approaches. Here we report a brief review of the literature published over the past few decades, with a focus on the current technical, clinical and economic barriers to translation, namely the limitations of many of these early studies, the lack of understanding of clinical pathways, health technology assessments, regulatory approval, clinical feasibility and funding applications. The field of biomedical vibrational spectroscopy must acknowledge and overcome these hurdles in order to achieve clinical efficacy. Current prospects have been overviewed with comment on the advised future direction of spectroscopic technologies, with the aspiration that many of these innovative approaches can ultimately reach the frontier of medical diagnostics and other clinical applications.

Introduction

The use of vibrational spectroscopy, namely Raman and infrared (IR), has been well proven over the past 50+ years, showing its abilities to accurately characterise clinical and biological specimens and provide useful information on the disease status of the specimens. Accuracy and potential of the technique have been the foundation of a vast amount of global research targeted towards solving issues within disease identification, analysis, prognosis and therapy for human good which has enabled strides forward in measurement techniques, technologies and our fundamental understanding of the interaction of electromagnetic radiation with matter.

In 1997, Jackson et al. published a review entitled "Infrared Spectroscopy: a new frontier in medicine" with a call to action: "As we enter the second half of the nineties, one of the major challenges for biological infrared spectroscopists is to transfer the knowledge we have gained from studies on isolate molecules to the complex world of medicine. That it is possible to meet this challenge is suggested by comparison with the development of other biophysical techniques, such as magnetic resonance spectroscopy and imaging, which have already found their place in medical research and practice" [1].

Magnetic resonance (MR) spectroscopy and magnetic resonance imaging (MRI) are valuable comparators for clinical vibrational spectroscopy. MRI is a non-invasive method of mapping the internal structure and certain functions within the body [2]. The technique uses radio frequency radiation in the presence of carefully controlled magnetic fields in order to provide this information. MRI has evolved from being a technique with great potential, to one that has become the primary diagnostic investigation for many medical complications [3]. In fact, in 1971 Raymond Damadian suggested that MR relaxation times could be used to detect cancerous tissue [4]. Lauterbur published his work in Nature in 1973, on how to use NMR to obtain images in macroscopic dimensions [5]. The first human images were able to be taken in 1977 [6], and since then MRI has become a primary medical tool. Off the back of their ground-breaking research in the 1970s, the 2003 Nobel Prize in Physiology or Medicine was jointly awarded to Lauterbur and Mansfield for their discoveries concerning magnetic resonance imaging [7] which was by then fully utilised in the clinic.

A web of knowledge search for publications including the terms Fourier transform infrared (FTIR) or Raman when combined with 'clinical', 'biology' or 'medicine', with a date range between 1960 and 2020, is illustrated in Figure 1. The exponential increase in publications over

time clearly demonstrates the evolution of a rapidly expanding research field, acknowledging the first related publication in 1978.

<Insert Figure 1 here>

In light of this excellent body of research combined with the concerted global effort to translate spectroscopy to the clinic, and the comparison with MRI in the 1990s, we have to consider and evaluate the reasons why vibrational spectroscopy is yet to become an acknowledged clinical tool. Various spectroscopic societies have been formed in the past few decades, paving the way for many annual and biannual conferences for experts in the field to monitor and discuss the necessities to achieve clinical translation.

A Faraday Discussion is a prestigious conference series organised by the Royal Society of Chemistry, UK. These meetings are targeted at fast-growing areas of the physical sciences, to act as a record of international opinion in the area and uniquely publish the debates alongside the discussion papers. A Faraday Discussion for a subject area is awarded after a written proposal and aims to drive that area forward through scientific discourse. Our research area has been the subject of two Faraday Discussions. In 2004, Jackson gave the introductory lecture at a Faraday Discussion entitled "From biomolecules to biodiagnostics: Spectroscopy does it all" and highlighted in his paper reasons for the apparent slow penetration of optical diagnostics into clinical practice [8]. Specifically, the causes for lack of penetration were highlighted as:

1) Biological spectroscopy is not progressing quickly, rather the published work is more of the same and a lack of imagination from researchers;
2) Quality of studies compromised by a "publish or perish" culture;
3) Quality of studies compromised by lack of statistical power;
4) Lack of health economic evidence of potential impact for financial viability;
5) No published long-term, multi-site, multi-instrument studies that validate optical technology for clinical use.

In the 2016 Faraday Discussion, Petrich gave the closing lecture entitled "Translating vibrational spectroscopy into clinical applications – vision or reality?", highlighting that there has been progress in clinical application and that biomedical vibrational spectroscopy has reached a pivotal point at which questions like diagnostic value, therapeutic consequence and financial viability are becoming paramount [9]. Petrich concluded by focusing upon a series of questions that we need to answer from a healthcare standpoint:

1) What is the unmet need?
2) What precisely is the diagnostic value?
3) What is the therapeutic consequence?
4) Will this application be financially viable?

In a 2015 paper which was linked to SPEC2014, Byrne et al. addressed the question "Quo Vadis?" (where are you going?) for the next generation of spectropathology [10]. This article was based on discussion around the specific topics of: (i) What are the most achievable, strategic target applications?; (ii) What are the technical challenges, and how can they be addressed?; and (iii) What are the challenges to implementation (legislative, clinical trials etc.), and how can they be addressed? The SPEC conference series, which began in 2000, is now considered the flagship of the International Society for Clinical Spectroscopy (CLIRSPEC). The authors also highlighted the need to:

1) Enable a more significant engagement between the broad range of stakeholders (academia, industry and clinical);
2) Establishing and promoting standard operating procedures (SOPs), for measurement and data handling protocols;
3) More targeted engagement with the medical community undertaken to establish strategic target applications and performance levels for minimal viable solutions;
4) Funding needed to conduct large-scale, multi-lab and -instrument intercomparisons and ultimately clinical trials.

The establishment of various peer-reviewed journals in the field – such as the Journal of Biophotonics, Translational Biophotonics and Clinical Spectroscopy – has provided a valuable platform for spectroscopic researchers to showcase their potential for clinical translation. Furthermore, it is worth noting the formation of multinational networks, namely CLIRSPEC and Raman4Clinics. CLIRSPEC is a non-profit organisation, founded in 2015, that aims to act

as a platform for these individuals, teams and organisations wishing to promote the translation for spectroscopy into the clinical environment [11]. The society covers a wide range of spectroscopy techniques with the overarching aim of the collaboration to improve patient diagnosis and prognosis [11]. Raman4Clinics, again founded in 2015, pools European expertise to progress in the field of novel, label-free and rapid technologies based on a wide variety of Raman spectroscopy for the clinical diagnostics of body fluids, bacteria, cells and tissues [12]. Raman4Clinics is a European platform that combines six working groups and numerous partners aiming for clinical diagnostics of biological fluids, bacteria, cells and tissues utilising a wide range of Raman spectroscopy techniques [12]. Each working group is designed to target separate aims including therapeutic drug monitoring, identification of microbial pathogens, cancer cell monitoring, histopathology, in vivo assessment and outreach to improve public and industry awareness. Raman4Clinics combines the use of non-destructive and tuneable Raman spectroscopy with microscopic techniques for mapping, as well as fibre optic probes for minimal invasive endoscopies, as described in their 'European Cooperation in the field of Scientific and Technical Research' (COST) action plan, published in 2014 and completed in 2018 [13].

The process of technology transfer from the laboratory bench is of course not simple, rather a multifaceted challenge that requires expertise from a wide range of different disciplines. An article published by Morris et al. highlights that several studies have converge around an average time lag of 17 years from research evidence to move through to clinical practice [14]. However, the authors warned that these studies use different metrics and a greater understanding of the reasons behind the time-lag would be beneficial for all, including investment decisions. A conceptual matrix for measuring and understanding the time lags suggested by Hanney et al. is shown in Figure 2 below [15].

<Insert Figure 2 here>

The current state of the translation field is described by Figure 2, where most of the research performed within clinical spectroscopy is still on the discovery research track concerned with "most relevant discovery research". The field has managed to generate some level of investigation within the 'human research and review' tracks, yet there is very little research

within our discipline that could be considered in the 'clinical and health service public policy development' tracks. We are now at the beginning of the third decade of the 21st Century and vibrational spectroscopy is still not currently used as a standard clinical tool. This focal point review will discuss and assess recent progress in the area, in light of the highlighted needs from the position papers described above.

Proof-of-concept Studies for Biomedical Applications

Tissues & Cells

Over the years, applications of vibrational spectroscopy techniques have been tested and developed as potential tools for disease diagnostics due to their high specificity and ability to cover a broad range of molecular species [16–18]. Many diseases lead to changes at a molecular level [19–21], whether that be a change in the chemical compositional or molecular structures within biofluids, tissues or cells, making spectroscopic techniques ideal for the detection of many different disease states [22–25].

In the field of oncology, tissue specimens are often judged by a pathologist to determine the stage and grade of a tumour. Typically, this is an extensive and subjective process involving assessment of the morphological features, requiring histological preparations, and does not utilise biochemical information at a cellular level [26–28]. However, vibrational spectroscopy techniques provide a biochemical fingerprint of the sample and several proof-of-concept studies have shown the ability of these techniques to assess and diagnose tissue samples faster than conventional methods [29–34].

Gajjar et al. utilised glioma brain tissue enabled discrimination between glioma subtypes using both attenuated total reflection Fourier transform infrared (ATR-FTIR) and Raman spectroscopies [35]. Spectral biomarkers were identified using both modalities, of most prominence was a lipid-to-protein ratio, with a notable decreasing trend recorded with the progression of gliomas from healthy tissue to most severe. Several other ratios exploiting different chemical biomarkers were also identified in each of the modalities to help determine between glioma subtypes. Results obtained in this study were comparable to those from histopathology staining techniques, yet on a much quicker timescale.

A number of early studies investigated vibrational spectroscopy as a tool for aiding the detection of cervical cancer. This area has since expanded dramatically with more recent studies probing the spectral response of cervical cancer cell lines to human papillomavirus [36–38]. Furthermore, cells exfoliated from oral tissues have also been shown to be effective in the diagnosis of oropharyngeal and laryngeal cancers [39]. In a bid to develop a diagnostic tool for lung cancer, FTIR analysis of sputum cell pellets created from 50 patients' sputum samples was published by Lewis et al. [40]. The FTIR measurements of the cell pellets revealed significant

differences between the IR spectrum of samples from patients suffering with lung cancer and healthy controls. In particular, tumour cells showed elevated signals of proteins, glycogen and nucleic acids which led to their identification from controls by multivariate analysis methods, with accuracies >92%. A number of aspects of spectrocytology make it well suited to the clinic; samples are generally easy to obtain and the opportunity to adjunct onto existing screening programmes make it an attractive target for translation. However, there remains no clear progression towards a clinical setting.

For the analysis of tissue and cell samples, IR spectroscopies can be combined with microscopy techniques offering label-free and rapid mapping of large areas of biopsies, enabling high sensitivity and admirable spatial resolution (~1-10 μm). IR spectral histopathology collects IR spectra across thousands of pixels allowing a 'hyperspectral datacube' to be built for each tissue specimen. One study employing the use of artificial neural network (ANN) machine learning algorithms, highlighted the power of IR microscopy for the classification of lung cancer [41]. An accuracy of 96.8% was achieved in identifying cancerous vs non-cancerous tissues. Additionally, small cell lung carcinomas, squamous cell carcinomas and adenocarcinomas were also classified with an average accuracy of 95%. In an attempt to better understand lung cancer, an FTIR microspectroscopy study of lung cancer cells was carried out to assess their response to chemotherapy cancer treatment [42]. Addition of the chemotherapy drug showed clear indication of a halt in the cancer cell proliferation, in the form of a ratio associated with carbohydrates moieties and glycogen. The increase in this ratio was shown to be linearly correlated with the cells' survival after treatment, presenting a means of monitoring cancer cells in vitro.

Like many cancers, treatment outcomes for colon cancer are critically dependant on the accuracy of the final histopathology diagnosis. An IR microscopy study of 77 patients' colon samples showed differentiation of normal and malignant features with very promising results [27]. This study highlighted the potential of a complementary objective biochemical diagnostic approach allowing a reduction in human involvement, thus speeding up the diagnosis process. Furthermore, an FTIR microscopy study of colon tissues combined with random forest (RF) classification has identified particular IR regions of interest, pertaining to CH stretching mode of lipids and the phosphate asymmetric stretch of nucleic acids, for the differentiation between cancerous and non-cancerous colon samples with accuracies greater than 90% [43].

Combining an FTIR microspectroscopic system with a synchrotron light source for a broadband high brightness allows high spatial resolution images to be obtained, suitable for a single cell measurement. The application of this technique has shown significant potential to help understand disease formation and progression [44–46]. This synchrotron technique has been employed using the ATR configuration and was found to be able to identify a single malaria infected red blood cell (RBC) [45]. Chemical characterisation of the infected cells showed a decrease in haemoglobin levels in infected cells as well as the presence of haemoglobin by-products. ATR-FTIR spectroscopy studies led by Wood et al. have also shown successful detection of RBCs infected with malaria [45,47–50]. One study in particular quantifies the parasite even in its early stages with an absolute detection limit of 1 infected RBC per microlitre of blood [50].

Delineating tumour tissue from healthy tissue during surgery is challenging, thus accurate tumour margins can be difficult to obtain for some cancers. Often adjuvant therapies or further imaging and surgeries are needed before the entire tumour is removed. Due to its compatibility with fibres and low water signal, Raman spectroscopy based intra-operative imaging techniques have been developed, allowing rapid assessment of tumour margins and help assist surgeons in real time [51,52]. This provides *in situ* analysis of tissues allowing excision of only the cancerous tumour, leaving as much healthy tissue as possible. Maximal safe resection decreases the likelihood of further surgeries to completely excise the tumour and generally improves prognosis for the patients. The ability to differentiate between cancerous and non-cancerous bladder tissue has been illustrated by using a compact fibre-based Raman probe imaging system [53]. The study led by Cordero et al. facilitates real time evaluation of bladder tissue, enabling rapid analysis and diagnosis. Prediction models using linear discriminant analysis coupled with partial least squares (PLS-DA) resulted in a sensitivity and specificity of 92% and 93%, respectively. Furthermore, through biochemical characterisations in the form of peak assignments can be made to also determine the bladder tumour grade, allowing a more accurate treatment plan and prognosis to be made. Kong et al. examined the potential of a Raman-based intra-operative evaluation of tumour excision, during breast conserving surgery (Figure 3) [54]. The tumour regions were discriminated from the healthy tissue structures based on increased concentration of nucleic acids and reduced concentration of collagen and fat. The authors concluded that the technique has the potential to detect mammary carcinoma in tissue

and assess resection margins, as well as rapid intra-operative assessment of sentinel axillary lymph node biopsies during breast cancer surgery.

<Insert Figure 3 here>

Other techniques have been developed to utilise Raman spectroscopy in a bid to obtain accurate diagnoses from needle biopsies. It is widely known that multiple needle biopsies are necessary to obtain quality tissue for an accurate conclusion of disease state, and patients are at increasing risk of infections if they have to undergo multiple biopsies. Targeted in vivo Raman methods have been developed to guide surgeries, in order to acquire biopsies at the correct tissue location and/or to maximise cancer resection, providing cancer characterisation *in situ* [55–58]. An in vivo study involving 19 patients with gliomas between grades II-IV has highlighted the potential of an intra-operative Raman probe during brain surgery to successfully detect cancer tissue from non-cancerous tissue [55]. Following spectroscopic interrogation, a tissue biopsy was collected from the same area and evaluated by a neuropathologist. Based on a threshold of 60% of cells showing signs of cancer, high values of accuracy were produced using a support vector machine (SVM) approach, with 141 spectral features for optimum discrimination. The spectroscopic data can be collected instantaneously and offers significant advantage over pathology reports. Raman probes have also been utilised to assess and compare in vivo Raman spectra of skin legions and normal healthy skin tissue without the need for a tissue biopsy [28]. Suspected non-melanoma skin cancer, scar tissue and healthy skin were identified with a balanced accuracy of 95%, highlighting the potential for Raman spectroscopy to guide tumour resection, allowing clean margins to be obtained in real time without the need for additional surgeries.

Biofluids

Despite the wealth of information provided via tissue and cell interrogation, one of the major drawbacks is the need for surgery or other invasive procedures to extract sample biopsies for diagnosis and prognosis information. In light of this, the use of biofluids in vibrational spectroscopy has seen numerous publications in recent years and provides a minimally invasive, rapid and label-free technique to analyse biological samples [59–64]. Biofluids, such as blood, urine and saliva, are particularly attractive for diagnostic studies as they are easily accessible [63,65].

In recent years advanced analytical techniques, in particular machine learning algorithms, are being utilised with spectroscopy due to their ability to recognise patterns and changes in the broad spectral fingerprint associated with disease [66–73]. An FTIR study of 389 patients with acute chest pain was shown to differentiate patient with acute myocardial infarction from those with minor chest pains of other origins, allowing therapeutic attention to be given to those in need of it most [74]. This IR transflection mode study led by Petrich et al. conceives a diagnostic pattern recognition method that has the potential to diagnose within the first 6 hours after the onset of chest pain. The spectral results were collated rapidly and were found to be comparable to routine cardiac laboratory markers on the same dataset.

The IR analysis of whole blood samples and their derivatives have been used for the detection of Hepatitis B and C viruses, human immunodeficiency virus (HIV) and malaria by a research group in Australia [47–49,75,76]. In a study utilising blood films on glass slides and ATR-FTIR spectroscopy, the detection of the malaria parasite was detected with specificity and sensitivity of 98% and 70%, respectively. Additionally, the concentrations of both glucose and urea were also detected. This is important as with the progression of the malaria disease, the demand for glucose by the virus is high and blood glucose levels tend to fall, causing hypoglycaemia. In severe causes of malaria, the virus can interfere with renal function resulting in high levels of urea in the blood compared with healthy controls, therefore testing urea levels can help determine progression of the disease. The use of ATR-FTIR here allows the rapid and simultaneous quantification of multiple analytes in a single measurement [47]. Similarly, the detection of Hepatitis C at varying levels of infection has been characterised using Raman spectroscopy of patient blood plasma samples [77]. Current diagnostic tests for this virus utilise polymerase chain reaction (PCR) or enzyme-linked immunosorbent assays (ELISA) which are costly, laborious methods and they tend to require specific reagents. The use of Raman spectroscopy offers a rapid and label-free method of diagnosis, detecting the biochemical changes associated with Hepatitis C.

Huntington's Disease (HD) is an incurable and inherited neurological disorder leading to progressive motor and cognitive decline. Despite many attempts for the diagnosis of HD using specific blood biomarkers, no single biomarker has shown to be reliable in the conclusive diagnosis or disease progression of HD. However, the use of Raman spectroscopy by Huefner et al. has allowed identification of HD patients from healthy controls using serum [78].

Additionally, the use of surface enhanced Raman spectroscopy (SERS) offered a higher degree of specificity when monitoring disease progression, which may enable diagnosis at very early stages.

Many proof-of-principle oncology studies using biofluids have established the use of IR spectroscopies for the detection of cancers and progression of the disease. For example, survival rates for brain cancers are low with a ten-year survival rate of only 15%, which is primarily due to the non-specific symptoms and thus delay to diagnosis. As a result of this, most patients with brain cancer present as an emergency with scans often showing advanced progression of disease. We have previously demonstrated the capability of IR spectroscopy utilising ATR mode to diagnose brain cancer and further classify these brain tumours into sub-types [79–83]. Utilising serum samples from a total of 433 patients, of which 122 did not have cancer, successful classification of cancer *versus* non cancer was achieved with sensitivity and specificity as high as 97.1% and 95.1%, respectively. Furthermore, the severity of tumour grade could also be identified with high sensitivities and specificities. A further study combining the SVM classification technique with ATR-FTIR spectroscopy has seen the identification of brain cancer *versus* non cancer on a larger study containing 724 patients. The results obtained an average sensitivity and specificity of 93.2% and 92% on a per patient basis [82]. Further study on this patient cohort tested and compared different machine learning algorithms combined with class balancing techniques, which all resulted in similar sensitivity and specificity values, highlighting the potential for biofluid spectroscopy to aid in distinguishing between brain tumour types [81]. Thus, the rapid diagnosis via serum spectroscopy holds advantageous over current clinical diagnostic techniques, as patients typically require multiple brain scans to determine the presence and severity of a brain tumour, which can be overwhelming for the patient and very costly to the health services.

Current biomarker detection technologies for early-stage ovarian cancer are ineffective and the disease can often go undetected until it has reached its later stages, sparking the need for alternative early detection methodologies. Through ATR-FTIR spectroscopy, patient plasma samples were diagnosed as cancerous or healthy with 96% of patients identified correctly [84]. Additional ATR-FTIR studies concluded that the ovarian cancer patient plasma samples could be separated into disease stage with high sensitivity and specificity (>91%) [68]. A different study assessed the ability of classification algorithms combined with ATR-FTIR spectra of urine to diagnose patients with endometrial cancer or ovarian cancer from healthy controls [85].

Using SVM with principal component analysis (PCA) for feature selection, accuracies greater than 92% were obtained.

Backhaus et al. have demonstrated the use of transflection and transmission mode serum spectroscopy combined with cluster analysis and ANN analysis to distinguish from breast cancer patients and healthy controls. Current breast cancer diagnoses in clinics are often complicated, time consuming and subjective. The use of IR spectroscopy offers a rapid and simple means to diagnosis, and as described in this study extremely high sensitivities and specificities (>95%) are attainable. Spectral comparisons with 11 other diseases, covering a total of 3119 patients, assured spectral changes observed were in fact from breast cancer [86]. Similarly, for gastric cancer the process of diagnosis is uncomfortable for patients and is often dependant on the judgement of the pathologist. However, Sheng et al. have shown the ability of FTIR spectroscopy to diagnose gastric cancer from healthy controls, by using a ratio of amplitudes provided by the stretching vibrations of RNA and DNA found in serum [87]. Despite testing a very small patient cohort the method achieved encouraging results, with 12 blind samples leading to a sensitivity of 85.7% and specificity of 100%.

The minimal water signal detected via Raman spectroscopy makes it highly appropriate for biofluid analysis. Mahmood et al. have shown the ability of Raman spectroscopy combined with multivariate analysis in identifying patients infected by the dengue virus [88]. Distinct features in the Raman spectra have been assigned to different immunoglobulin proteins and have been able to successfully monitor disease progression. Results attained were comparable to the outcomes achieved using ELISA and PCR techniques, yet the spectroscopic method is much faster with lower associated costs. Using similar analytical techniques, Sahu et al. have predicted the recurrence of oral cancers from serum collected before and after surgery [89]. Average classification efficiencies of 81% and 82% using principal component discriminant analysis of patient serum samples collected before and after surgery, respectively. The ability to predict relapse would undoubtedly benefit patients.

Colorectal cancers have also been classified against control samples using patient serum, with a Raman-based approach coupled with PCA providing accuracies as high as 88%. In the spectra from those with colorectal cancer, bands associated with tryptophan, phenylalanine and beta-carotene were found to exhibit a lower Raman intensity [90]. This study demonstrates the

advantage of spectroscopy, not only allowing detection of disease but also identifying specific blood-based biomarkers which could aid with the understanding of the disease.

Arguably one of the major advantages of spectroscopic analysis is the ability to measure multiple analytes in a single non-destructive measurement. Ultimately, gaining more information on a patient's health on a much faster timescale when compared to techniques currently in use in clinics (e.g. ELISA) is admirable. The detection and quantification of 8 major constituents of serum – total protein, glucose, cholesterol, high and low density lipoproteins, triglycerides, urea and uric acid – have been made from a study containing 247 patients by comparing Raman and IR spectroscopies [91,92]. In order to make these measurements in the current clinical environment, multiple measurements would be required, which would necessitate a significantly larger sample volume when compared with spectroscopic studies of biofluids. Not only do spectroscopic techniques require minute sample volumes, but also less sample preparation and they are not dependant on availability of reagents, thus have much lower associated costs.

Advances in technology have also enabled strides toward high-throughput analysis and large scale trials, making vibrational spectroscopy techniques more applicable for healthcare settings [82,93–95]. The development of disposable internal reflection elements (IREs) for ATR-FTIR modalities combined with an automated slide indexing unit allows accurate movement of the IRE across the spectrometer for high-throughput analysis [82]. Additionally, robotically spotting of serum samples onto a 384 multi-well titre plates combined with automated FTIR has also been established allowing high-throughput spectroscopic analysis of serum [95]. As a result of the many proof-of-concept applications of vibrational spectroscopy for disease detection, suggested protocols for sample preparation and analysis of biological materials using FTIR spectroscopies have been published [96,97]. Despite the wealth of evidence from many studies around the world, there still appears to be various obstacles to overcome before it can be translated to a healthcare setting.

<u>Barriers to Translation</u>

As already discussed, there has been an abundance of diagnostic studies in the field of biomedical vibrational spectroscopy in the last few decades signifying its potential clinical utility [98–105]. Although, the transition from research to routine clinical testing has faced several major technological challenges, as well as the challenges presented from current patient pathways and the persuasion of clinicians on the reliability of a new diagnostic test.

Technical Challenges

Due to a lack of standardisation, the majority of the variance in clinical data occurs within the pre-analytical phase. A recent study reported that most of the inconsistencies (~61%) are introduced prior to carrying out the analysis [106]. There are several pre-analytical steps that can affect sample integrity if they have not been performed correctly.

One problem with the spectroscopic analysis of tissue is that heterogeneity within the specimen is not unusual, where tissue sections contain both tumour and normal tissues or several different grades of tumour [107]. The best alternative to making in vivo measurements is the use of fresh tissue samples, however it can be difficult to acquire the necessary ethical approval as well as finding suitable volunteers, thus currently formalin fixation and paraffin embedded (FFPE) tissue is the preferred source for the histological examination of tissue sections. Use of FFPE tissues is admirable for early studies – for both Raman and FTIR analysis – mainly due to the ease of collection from biobanks and the ability to reanalyse if required, though it may not be directly comparable to fresh tissue since there can be contributions to the signal from some of the chemicals used in the FFPE and dewaxing (paraffin removal) processes [108,109]. The formalin or ethanol – used in the dewaxing process – induces coagulation of the globular proteins present in the cytoplasm, which can result in the loss of structural integrity of potentially important organelles. Furthermore, the use of ethanol can precipitate lipid molecules that are not preserved through the primary fixation step. Preparation of cells for spectral analysis can also encounter some technical challenges, mostly based on the chemical fixation and drying processes. Cells are naturally present in a hydrated form, whereby water molecules are bound to macromolecules which contributes to their structural integrity and function [110]. Many studies initially focused on an air-drying process to dehydrate the cells directly onto substrates, however the this can result in delocalization of biomolecules as a result of large surface tension forces associated with passing the water–air interface. Another fundamental aspect of sample preparation that can influence cellular biochemistry is the surface on which they are grown.

The surface can induce changes in cell adhesion and motility, in their proliferation and differentiation and in gene expression [110]. To overcome the confounding factors of the drying process, the cells must be appropriately fixed by means of chemical fixation methods. Unfortunately, all of the currently employed fixation approaches can still affect the resulting Raman or FTIR spectra. The main vibrational bands that are known to be modified by the current methods of tissue and cell processing are outlined and summarised in Table I.

Table I – Summary of affected bands in Raman and FTIR spectroscopy through currently employed tissue and cell processing methods [109–115].

		Raman bands affected	FTIR bands affected
Tissue processing	*Cryopreservation*	Decreased intensity in the 1002, 1447 and 1637cm^{-1} bands	Shifting of Amide I and II bands, and reduction in overall intensity
	Chemical fixation	Change in appearance of 1490cm^{-1} and reduction in intensity of Amide I	Shift in Amide I/II bands by ~10cm^{-1}
	Paraffin embedding	Paraffin signals at ~1063, ~1296, ~1441cm^{-1} and contributions from xylene ~1002, 1032, 1203cm^{-1}	~1465cm^{-1} and bands between 2850-2950cm^{-1} altered
Cell Processing	*Air drying/desiccation*	Loss of features between 1253-1127cm^{-1} and	-
	Formalin/critical point drying	Loss of features between 1600-1500 and 1448-1127cm^{-1}; various protein, nucleic acid and lipid bands affected	Loss of ester lipid peak due to acetone and ethanol (~1750cm^{-1})
	Glutaraldehyde– osmium tetroxide and critical point drying	-	Decreased intensity of 1000cm^{-1} and less resolved lipid band ~1750cm^{-1}
	Methanol:acetic acid (3:1)	Various nucleic acid (<1000cm^{-1}, 1060-1095cm^{-1}), protein (1668-1661, 1450, 1339-1305, 1176cm^{-1}) and lipid 1447-1453, 1339-1247, ~1065cm-1) bands disturbed	-
	Acetone	-	Diminished cellular lipid bands (1740, 2925cm^{-1}) and alterations to the Amide I/II bands

With regards to biofluid analysis, the quality of a blood sample can be distorted by variables that occur during the blood collection; fragile veins, insufficient sample mixing and interruption of blood flow during collection can all result in the sample clotting, or activation of the coagulation cascade which could lead to abnormal clotting results [116,117]. Biospecimens must be stored in mechanical freezers at extremely low temperatures to prevent degradation and the destruction of important biomolecules, yet the temperature and duration of time that blood samples are stored are currently not standardised [118–121]. The number of freeze-thaw

cycles should also be minimised, in order to avoid alterations in protein concentrations and potential discrepancies in clinical tests [122]. The integrity of a blood sample can also be compromised by molecular contamination through fractionation methods, which in turn can affect the spectroscopic signature [123,124]. Sample preparation can also influence the reproducibility of spectral data, as well as intra- and inter-operator variation. Standardising the preparation of biofluids is not a simple task, as the analysed sample state will be dependent on the available equipment and/or the project requirements. When analysing biofluids through FTIR spectroscopy, liquid drops are often dehydrated onto a substrate due to the spectral interference of water [125–128]. On the other hand, aqueous samples are well suited to Raman spectroscopy since water is a weak Raman scatterer, hence it is less common for biofluids to be dehydrated prior to Raman measurements [129]. We have published various studies in the last decade exploring these types of pre-analytical factors that seem to inhibit the clinical progression of FTIR spectroscopy, as well as computational methods to overcome some of those barriers [130–134]. To date, there are no pre-analytical research studies published in the literature relating to a Raman-based approach, yet this may be a requirement for clinical Raman spectroscopy to reach its full potential.

There are certain analytical and post-analytical factors that can cause issues, albeit less common. The operation of instruments from various manufacturers can occasionally cause discrepancies, making it difficult to reliably compare and contrast spectra from different spectrometers. For spectroscopic analysis of tissue, we have technical limitations like lengthy acquisition times for imaging; signal detection; the difficulty of making quantitative measurements of tissue composition in vivo, and ambient light effects, as well as several considerations in data handling – such as spectral pre-processing, feature extraction and tissue classification [135]. Likewise, other analytical issues can stem from altering the collection parameters, such as the sampling mode, number of scans and spectral resolution. Similarly, post-analytical variation may be attributed to the plethora of available data analysis packages and computational analysis software, containing several types of basic statistical analysis, multivariate analysis and machine learning algorithms. Research groups can utilise various types of software and analysis to process their data in order to achieve the best results for their dataset, thus the output and statistics may not be directly comparable even within the same field of expertise.

An interesting overview of factors slowing down progression of clinical applications of FTIR imaging was described by Goormaghtigh et al., which focused on the variation in pre-analytical conditions, spectrometer settings and collection parameters [136]. Furthermore, some recommendations on these spectral histopathology imaging parameters – such as tissue section thickness, air purging, pixel size and data pre-processing – were suggested by the authors to try and drive further discussion on the topic, and ultimately determine a final consensus for routine clinical analyses of tissue specimens.

A later publication from Santos and colleagues reviewed the actual clinical needs in oncology that could potentially be addressed by Raman spectroscopy [137]. Yet again, the concluding remarks emphasised that most of the applications addressed in proof-of-principle studies are still far from clinical adoption and commercialisation. The need for the consistency of protocols is evident, and has been nicely put by Dey et al. in their recent review article, as without standardisation they state that: "we are comparing apples to oranges and may be stuck with numbers that are not comparable from one research group to the other, thus leading nowhere" [138].

Clinical Issues

There are also several clinical issues that must be considered. Defining the clinical question and unmet need is generally the first step in translational research for the treatment or diagnosis of a disease or clinical problem [139]. The unmet need should be fully explored, and the diagnostic pathway must be carefully mapped out in order to understand the issues that need to be resolved [140]. An important aspect of clinical research is the close interaction between clinicians and experts in the field [141]. Coupling this clinical question to an appropriate technique or technology is the next consideration. This may require technical developments in order to best approach and suit the clinical need, with automation often being a desirable feature of any new intervention [142].

Another common issue is that many proof-of-concept studies involve relatively small patient cohorts, and this can over-estimate the significance of the results as they are not entirely representative of larger populations [143]. Sample numbers are often a limiting factor in the development of new technologies since the recruitment of sample specimens from patients through clinical studies can often be difficult and take several months or years, leading to many studies with limited cohorts. Even when some funding is secured for the purchasing of

biospecimens, biobanks and tissue banks may not have the preferred disease and/or control samples in order to conduct the study – for example, due to the scarcity of a particular disease/demographics data making many samples unsuitable for the purpose of the study. A noticeable pattern in the literature is an alarming low number of patient numbers used to establish early utility, which can often pose questions on the reliability of the results. The use of machine learning algorithms to classify between patient sets is heavily influenced by the number of subjects fed into the training and testing of these computational models [144]. Despite the availability of various sampling methods that can overcome class imbalance in training and testing sets [145], they often struggle with small datasets.

Likewise, the majority of these studies are based on retrospective samples, and these cohorts can be susceptible to bias as the disease states of the patients are already known. Hence, when progressing on to a prospective study, it is common to observe a lesser diagnostic performance [146]. These effects are commonly described as *'spectrum bias'*, which accounts for the variation in test performance for prediction, screening and disease diagnostics among different population subgroups. For example, a test that has been developed in a population with higher disease prevalence, will normally have a lower sensitivity and higher specificity when applied in a population with lower prevalence of disease [147]. Likewise, symptomatic patients who do not have the disease become more prevalent in prospective studies, so it can be more difficult to successfully detect the disease in these cohorts. Thus, determining the true clinical utility may only be possible through studies conducted in the targeted clinical environment.

Furthermore, there is not yet a widely recognised threshold for the statistical performance for diagnostic tests – in terms of the minimum accuracy, sensitivity, specificity, area under the curve (AUC) for receiving operating characteristic (ROC) curves etc. – that would be an acceptable clinical value. Likewise, it is unclear which parameter between sensitivity and specificity is preferred to assess the diagnostic potential. For example, several studies endeavour to maximise sensitivity (>90%) by sacrificing specificity (~50%), and conversely, others appear to tailor their machine learning algorithms to enhance specificity, which in turn can reduce the sensitivity. There is also inconsistency with how patient training and testing population splits are determined then reported. Instead of ensuring the spectral data from one individual patient cannot be in both the training and testing datasets, some studies have spectra from one patient in both sets, which can overestimate their diagnostic ability [148]. Similarly, there should be several train/test splits by utilising resampling methods to guarantee different

patients are being tested on, yet it is not uncommon only to see only one train/test split, with the results being reported from single classification models. Moreover, the proportion of patients in the testing set can be variable across studies, e.g. a 70:30 split (70% training and 30% testing) or a 60:40 split. These factors may be dependent on the desired application, but a more standardised approach would undoubtedly enable a more efficient clinical translation.

Health Economic and Regulatory Barriers

In addition to scientific and clinical development, there is also the commercial element. Intellectual property (IP) applications, difficulty in securing funding and international markets can often represent a further barrier to translation. There is often a lack of concrete understanding of what the unmet clinical needs are, and the limitations of the current gold standard techniques [10]. As already discussed, the standardisation of sample preparation and measurement protocols is inadequate, and with a wide array of data pre-processing and analysis techniques to choose from, the lack of consistency in the vibrational spectroscopy field could be detrimental for potential funding and inhibit progress [149]. There are many challenges that new technologies face when trying to protect their IP. Patent applications are very expensive and will often face renewal fees. Currently, there is no single patent that covers IP worldwide, thus it is difficult to protect IP in the multiple jurisdictions around the world, and the lack of a more universal patent system leaves small innovating companies in fear of potential global competitors [150]. The development of a start-up or spin-out company may appear to be the ideal pathway to commercialisation [151,152]. This can be a daunting task for many researchers; hence it is crucial to seek business advice and discover the approach to successful IP strategies [153].

There are currently two major hurdles placed in the path to successful translation; 1) approval from regulatory agencies for marketing and 2) the acceptance from health technology assessment (HTA) agencies who determine which technologies are made available in the public health system. Regulatory agencies are responsible for ensuring new medicines and medical devices are safe, such as the Food and Drug Administration (FDA) in the USA, the European Medicines Agency (EMA) in the EU, and the Medicines and Healthcare products Regulatory Agency (MHRA) in the UK. These agencies ensure the manufacturer of new interventions follow the appropriate conformity assessment procedure, which is essential for products to attain CE marking and comply with relevant health, safety and environmental requirements [154]. Criteria for successful acceptance require statistically verified clinical trials to prove medical

utility, but also clear understanding of the current diagnostic pathways in order to determine the economic and clinical impact of new technologies. For successful translation of such techniques, it is vital to examine the existing competition, define where in the current pathway the proposed 'diagnostic test' would be best suited, and undertake an economic evaluation to establish the potential cost and health benefits for the health services.

Health economics concerns how the health services allocates its funds [155]. Health economists commonly undertake an *economic evaluation,* which analyses alternative courses of action - in terms of both their *costs* and *consequences* – often in comparison to current practices [156,157]. Health economists will often carry out a *cost-effectiveness analysis* (CEA), which is a comparison of costs in monetary units with outcomes in quantitative non-monetary terms, with respect to the best currently available treatment, strategy or intervention [158]. The benefits of an intervention can be portrayed by variety of different outcomes, such as the differences in quality adjusted life years (QALY) incorporates the impact on both the quantity and quality of life of a proposed intervention [159]. In the UK, the National Institute for Health and Care Excellence (NICE) make decisions regarding whether a new technology will be funded within the UK's National Health Service (NHS), and this is informed by the evidence of cost-effectiveness. Economic evaluation and CEA have been progressively employed in the last few decades to examine the feasibility of novel pharmaceuticals, drugs, biomarkers and medical devices [160]. Notably, the first health economic assessment (HEA) of a new technology relating to vibrational spectroscopy was issued recently in the BMJ Open. In 2018, Gray et al. published a pre-trial HEA for the introduction of a serum-based spectroscopic blood test into the clinic [161]. The proposed test was for the diagnosis of brain tumours, which utilises ATR-FTIR spectroscopy to detect the biosignature of the disease from a patient's blood serum [162,163]. The current pathway for brain tumour diagnosis consists of magnetic resonance imaging (MRI) or computed tomography (CT) imaging followed by surgical biopsy and histopathology. MRI and CT imaging are considerably expensive: a single patient costs the NHS around ~£300 for imaging alone [161]. A reliable blood serum test at the primary care setting could fast track these patients into the diagnostic pathway much quicker, whilst reducing the number of unnecessary brain scans, saving the health services on avoidable spending. A further economic evaluation recently looked at updating this early economic model with the results from a prospective clinical diagnostic accuracy study [164], and also explored the effect of brain tumour type predictions on the patient outcomes and healthcare costs. The authors concluded even after the

inclusion of the prospective clinical data, the technology still has the potential to be cost-effective in both primary and secondary care [165].

This is only one example of a health economic assessment for a new spectroscopic intervention, but there is a real lack of CEAs in the literature that relate to the clinical translation of spectroscopic techniques for other applications. These types of analyses are essential before proof-of-concept studies can progress into the next phase of determining their clinical utility. Ultimately, biomedical vibrational spectroscopists who are eager to successfully translate their research must consider the following: the unmet needs of the current diagnostic pathway/their target application; how their results compare to the current gold standard methods; the costing of their new technology or intervention; and the benefits to the health services and/or population.

<u>Where we are now?</u>

Raman

Vibrational spectroscopy has been at the forefront of translational clinical research for many years now. In 2015, Stevens et al. reviewed the development of fibre optic Raman probe instrumentation for clinical applications [166]. Numerous factors were considered for the potential translation into clinical workflow ensuring materials and design were compatible for the target outcomes. This review concluded that in vivo clinical diagnostics, surgical targeting and treatment monitoring using Raman probe technologies could be the way forward for numerous clinical applications [166]. In the same year, a Raman-based approach utilising a handheld probe for live, local detection of cancer cells in the human brain was established by Jermyn et al. (Figure 4) [167]. Using this probe intra-operatively, the authors successfully differentiated normal brain from dense cancer and normal brain invaded by cancer cells. This Raman-based probe enabled detection of the previously undetectable diffusely invasive brain cancer cells in various glioma grades. The authors later published a similar *in situ* intra-operative approach, with a more generic cancer detection system that combines intrinsic fluorescence spectroscopy, diffuse reflectance spectroscopy, and Raman spectroscopy [168]. Using this multimodal optical cancer detection system, Jermyn et al. highlighted brain, lung, colon, and skin cancers could be detected *in situ* during surgery with an accuracy, sensitivity, and specificity of 97%, 100%, and 93%, respectively. A more recent study by Livermore et al. demonstrated the ability of Raman spectroscopy to aid neurosurgeons in determining personalised treatment during surgery, by targeting specific glioma subtypes *in situ* [169]. Knowledge on the genetic subtype of the patient's glioma at the time of surgery would allow the surgeon to tailor their strategy for the best possible prognosis. These types of intra-operative technologies have the potential to classify cell populations in real time, making it an ideal guide for surgical resection and decision-making.

<Insert Figure 4 here>

An article by Krafft et al. focused on developments with coherent Raman scattering (CRS) imaging techniques outlining the need for translation to hospitals while discussing the unmet needs in biomedicine, clinical diagnosis and healthcare applications [170]. The paper also outlined the extensive amount of literature within the field of CRS microscopy over the last twenty years and the need for networks such as the International Society for Clinical Spectroscopy (CLIRSPEC) and Raman4Clinics in order to push the clinical translation through collaborative efforts. Pence and Mahadevan-Jansen continued the discussion of utilising Raman spectroscopy for clinical applications, citing several modalities of Raman scattering, including: Raman imaging, high wavenumber region (HWN) Raman, spatially offset Raman spectroscopy (SORS) as well as surface enhanced Raman spectroscopy (SERS) [171]. They also noted that the most common clinical target is cancer with all research groups fundamentally having the same goals: i) to improve early detection with high sensitivity and specificity, ii) understanding/monitoring the disease processes and iii) easy access to the target organ being as non-invasive to the patient as possible.

More specific research involves the use of HWN Raman spectroscopy to investigate the diagnostic capabilities of tumours by comparison of water, protein and lipid content in surrounding healthy tissue. Although this idea was displayed by Garcia-Flores et al. back in 2001 by identifying differences in water signals for breast tumours in rat models [172], it has only recently been adapted to explore human breast tissue combined with fibre optic probes for intra-operative margin analysis (IMA) [173]. Similarly, Puppels' research group in the Netherlands – who had previously published distinctions between oral cavity squamous cell carcinoma (OCSCC) and healthy tissue using HWN Raman spectroscopy [174] – recently developed a fibre optic needle probe to improve the accuracy of specimen-driven intra-operative assessment of resection margins (IOARM) [175]. Other recent literature of particular interest includes publications by Avram et al. who developed a liquid biopsy for diagnosing colorectal cancer with SERS [176], Nicolson et al. who reported the first in vivo study of surface enhanced SORS for brain cancer imaging [177], and Du et al. who reported a serum-based Raman spectroscopy analysis of the hepatitis B virus [178].

The translation from academic research to the clinic environment requires collaborations between researchers, clinicians, industrial partners, and funding bodies. Currently there are numerous national and international research initiatives surrounding Raman spectroscopy

techniques, including the aforementioned CLIRSPEC and Raman4Clinics, as well as the Coherent Raman Imaging for the Molecular Study of the Origin of Diseases (CRIMSON).

CRIMSON involves collaborative efforts from numerous research groups, partners and enterprises [179]. It is funded through the European Union's Horizon 2020 research and innovation programme and lasts from December 2020 for 42 months. The primary aim of CRIMSON is to understand cellular origin of diseases by the development of a vibrational spectroscopy-based cell/tissue imaging system. The focus of the project involves CRS combined with microscope/endoscope for inter- and intra-cellular processes. The European consortium involves collaborative efforts from research centres including Politecnico di Milano (Italy), Leibniz Institute of Photonic Technology e.V and IF-CNRS (Germany); biomedical partners such as Istituto Nazionale dei Tumori (Italy), Institut National de la Santé Et de la Recherche Médicale (France) and Jena University Hospital (Germany); as well as small and medium-sized enterprises, Active Fiber Systems GmbH (Germany), Lightcore Technologies (France), Cambridge Raman Imaging Ltd (UK) and 3rdPlace (Italy) [180]. Through this trans-disciplinary collaboration CRIMSON aims to provide solutions to numerous limitations currently found with CRS applications, including the ability to detect within the fingerprint region, use of multiple vibrational frequencies and the development of a low-cost, compact laser system. Validation of the new imaging system will focus on cancer with a clear cellular origin, targeting autophagic processes, interactions between cancer and immune cells, and senescence [179].

The Raman Nanotheranostics (RaNT) research programme is currently developing a novel all-in-one technology to diagnose and treat cancers with a single device [181]. The project is led by Prof. Nick Stone at the University of Exeter, collaborating with scientists at the University of Cambridge, the UCL School of Pharmacy and the Science and Technology Facilities Council Rutherford Appleton Laboratories, all based in the UK. The Engineering and Physical Sciences Research Council (EPSRC) awarded RaNT a £5.7 million grant in 2018 for a 5-year period, and the team is focused on reducing the need for cancer surgery by providing rapid, pain-free detection and therapy, right at the patient bedside.

Companies such as RiverD International B.V. (Netherlands) provide commercially available Raman spectroscopy instrumentation, and they are specifically targeted towards quantitative in vivo skin analysis [182]. However, they do also supply custom-designed microscope accessories

for near-IR Raman microspectroscopy. The skin analysis can be widely utilised for occupational skin exposure, trans-dermal drug delivery monitoring and also developing, optimising and testing topical products [183]. The accessories available to purchase can provide countless opportunities for analysis of cells and tissues using near-infrared Raman spectroscopy for diagnostic applications.

Two collaborative large-scale cross-laboratory studies have recently been published by members of Raman4Clinics, off the back of the COST action plan [184,185]. In the study by Guo et al.*,* Raman spectral data from 15 different institutes was contrasted and compared to determine potential intra- and inter-operability [184]. The authors concluded by recommending a number of future actions, such as a standardised spectrometer calibration approach for manufacturers, development of SOPs for instrument verification, calibration and performance, and encouraging researchers to make their data and findings openly available to establish larger databases.

Infrared

There have also been several exciting developments within the IR clinical spectroscopy community and the technique shows great promise for translation into diverse healthcare pathways. For biofluid applications, ClinSpec Diagnostics Ltd. (ClinSpec Dx) recently demonstrated the clinical potential of a disposable silicon ATR-FTIR test for serum-based cancer diagnostics, with discrimination of brain cancer and non-cancer control samples with sensitivity and specificity of 93.2% and 92.8% respectively in a retrospective study [81,186]. ClinSpec Dx further participated in a prospective clinical validation study comprised of 385 blinded patients where the technology showed high diagnostic capability in a clinical environment, with sensitivity and specificity of 81% and 80% respectively [164]. As already discussed, the overwhelming majority of research efforts have reported clinical test performance based on retrospective patient studies. The transition to prospective studies is an important step towards the realisation of IR tests in the clinic, as it provides technological validation within clinical pathways and establishes analytical confidence with clinical end users. Ultimately, the execution of the initial health economic evaluation by Gray et al. was instrumental in defining key test parameters in the research and development phase of the technology, as well as demonstrating a strong economic argument for clinical adoption to healthcare decision-makers and potential investors [161]. Thus far, few health economic evaluations have been published on IR technologies despite the plethora of promising clinical

research. These studies must be strongly considered by the field going forward to initiate conversations with key decision-makers and health technology assessment agencies.

Biotech Resources, developed by Professor Wood's and Dr Heraud's research team in collaboration with the Monash University Centre for Biospectroscopy, is another emblematic example of how IR technologies are on the cusp of clinical adoption for biofluid diagnostics. Biotech Resources have developed a low-cost, simple point-of-care test based on ATR-FTIR, for analysis of blood and its derivatives for a diverse range of clinical test applications. Biotech Resources have shown significant clinical potential of their technology for malaria diagnostics [187–190], and have recently conducted feasibility studies in real clinical environments in Papa New Guinea and Thailand [191]. Recent test results of 318 suspected malaria patients at four regional clinics in Thailand demonstrated high test sensitivity and specificity of 92% and 97% respectively [188]. The point-of-care system, which analysed spectroscopic measurements on a cloud-based system, and subsequently delivered clinical test results to a mobile phone or computer, highlights the accessibility of the technology for mass screening of malaria in resource limited countries. As well as detection of parasites, they have shown the clinical utility of their spectroscopic approach for identification of bacterial and fungal pathogens [192,193], and hepatitis viruses [75]. In fact, Biotech Resources have recently been awarded an investment from the Australian Government's $35 million BioMedTech Horizons program, for development of a rapid spectroscopic test for direct detection of bacterial and fungal pathogens that cause sepsis in patients from whole blood [194]. Additionally, the spin-out company are developing a rapid antibiotic susceptibility test capable of detecting resistance to numerous antibiotics in under two hours from positive blood cultures [195]. Ultimately, the identification of parasites, viruses, bacteria and fungi is enabled by detection of specific biochemical signatures that correspond to distinct vibrational modes associated with nucleic acid, lipid and protein constituents in blood, and highlights the breadth of possibilities of IR spectroscopy for clinical biofluid diagnostics in the future.

Recent commercialisation of IR spectroscopy for disease diagnostics is further evident from developments at Glyconics, who are an IR diagnostics company in the UK developing proprietary handheld FTIR devices for analysis of diverse samples to enable point-of-care diagnostics for multiple conditions [196]. Initially, they wish to target chronic obstructive pulmonary disease (COPD) and diabetes mellitus. Recently, they completed a licensing agreement with Ghent University (Belgium) for worldwide exclusivity and future collaboration

on IR spectroscopy technologies that include determination of protein glycation in fingernails for diagnosis and continued monitoring of diabetes mellitus [197–199]. Very recently, Glyconics were also recipient of a UK Research and Innovation grant as part of the Global Challenges Research Fund, to study the clinical feasibility of implementing IR technologies for diabetic screening in developing countries, which re-iterates the clinical potential of IR spectroscopy for resource limited settings.

The clinical potential of IR spectroscopy further extends to analysis of biological tissue and represents an attractive future alternative to histopathology for cancer diagnostics in clinical laboratories. The projection of IR light onto tissue sections has several analytical benefits and would permit simultaneous characterisation of different biomolecules in a non-destructive, low-cost manner without the need for expensive and time-consuming staining and labelling procedures [200]. Furthermore, the acquisition of spectroscopic information would allow for a complete biochemical fingerprint of tissue samples, as oppose to exclusively identifying tissue architecture and protein biomarkers with antibodies using immunohistopathology [201]. Thus far, spectral histopathology has demonstrated considerable diagnostic potential for numerous malignancies, including lung, colorectal adenoma, melanoma and breast cancers [202–206]. However, one aspect of spectral histopathology that has previously restricted clinical translation concerns the limited acquisition time to scan entire tissue samples.

Typically, spectral histopathology utilises focal plane array detectors that may take several hours to days to complete scanning of an individual tissue sample and requires significant computational capabilities to store tens of thousands of collected spectra [207]. This is undoubtedly an advancement on previous single point mode analysis with single element detectors, but still has significantly reduced sample throughput compared to conventional histopathology and is not conducive to busy clinical laboratories. Nevertheless, recent technological advances and different sampling formats have emerged that make spectral histopathology more attractive for routine clinical adoption. The emergence of quantum cascade lasers (QCL's) represents an important advancement for clinical translation of spectral histopathology and permits measurement of discrete frequencies pertinent to biological discrimination between sample types, rather than measuring all wavenumbers of an entire IR spectrum [208,209]. This has significant implications for acquisition time, allowing tissue samples to be analysed within minutes to hours [210]. Recent adoption of tissue micro-arrays (TMA's) in spectral histopathology further extends time advantages compared to previous approaches of

fixing tissue sections to calcium or barium fluoride substrates, or low emissivity slides. The high-brilliance of QCL's permits spectroscopic interrogation with increased signal-to-noise and diffraction limited resolution, which enables improved analysis of sub-cellular details in tissue for enhanced diagnostic capabilities [211]. Lastly, adoption of room-temperature QCL's negates the need for cooling with liquid nitrogen and allows simplification of instrumental design, which undoubtedly makes spectral histopathology more attractive for clinical translation in future years [212,213].

The recent adoption of these new approaches for spectral histopathology and their subsequent integration with conventional histopathology instrumentation is perhaps best underlined through the seminal work of the Bhargava group, based in Illinois [214]. The authors recently demonstrated an all-digital, 'stain-less' staining hybrid platform that combined wide-field visible microscopy with a QCL IR light source and tissue micro-arrays for analysis of breast cancer tissue. The study highlighted that the integration of IR spectroscopy and machine learning with ubiquitous optical microscopy offered the possibility of label-free molecular characterisation of tissue in an easy to use, accessible format within clinical laboratories, and further underlines the importance of designing IR technologies that are suitable and compatible with current clinical workflows.

<Insert Figure 5 here>

Conclusion

It is clear that both Raman and IR spectroscopy hold great promise for diverse clinical applications and there now needs to be a concerted effort alongside clinicians, instrument developers, commercial enterprises, health economists and healthcare decision makers to ensure the technology reaches its full potential and translates to the clinic. Typically, commercial spectroscopic and imaging systems are built for research laboratories and not for medical diagnostics, thus many of the currently available analytical instruments are not necessarily compatible with clinical workflows. Many proof-of-concept studies are performed within academia on a 3-4 years' timescale which is not realistic when standardisation of protocols, methodologies, validations and translation requires a much greater timescale. The

question of "How good is good enough?", is still discussed within the community however the numerous on-going collaborations between researchers, clinicians and enterprises provides hope for translation of spectroscopy into the clinical environment within the coming years [10,149].

In order to further aid translation and realisation of patient benefit we need to revisit and update the requirements suggested by many researchers over the years. Whilst it should be noted that with time and progress of the field these propositions may change, it is the opinion of the authors that to overcome the initial barriers to translation, we should be focusing on:

1) A standardised protocol for samples preparation or further studies on the pre-analytical factors that affect a particular sample preparation.

2) Performing research projects and studies with sufficient sample numbers and appropriate statistical analysis techniques so valid conclusions can be drawn. If a study only has a small number of samples in each disease and/or healthy group, then there needs to be a valid reason provided for this and a recognition of the impact upon results reported by the authors of the study. As reviewers of current peer-reviewed papers we can uphold this.

3) Results should be reported correctly and the requirements for reporting should be standardised. This can be something the field decides upon in the future, but a good starting point would be to review and begin to include the requirements of the Standard for Reporting of Diagnostic Accuracy Studies (STARD) statement [215] (long-standing approach to accurate reporting of diagnostics studies and as such has been accepted by multiple journals and researchers alike).

4) Show evidence that the level of performance has some economic benefit to the area, within which the authors are aiming to translate the research to. An understanding of the limitations of the proposed test / clinical environment in terms of how the test would be used, what the workflow would be and the overall economics of what is required. This would start to focus studies on the correct applications. Of course, there are many examples of research that have not had a target application or appreciation of this that have made a huge impact in medicine, but in order to maximise the probability of success, we feel that this is necessary.

5) Start to prepare for, and engage in, large scale trials of the technology in order to truly show clinical effectiveness. This must be proven in a symptomatic population with correct symptomatic controls in order to establish accurate measures of sensitivity and specificity, highlighting the potential issues of comorbidities.

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<u>Figure Legend</u>

Figure 1 – Evolution of publications from a web of knowledge search for "FTIR" and ("clinical" or "biology" or "medicine") or "Raman" and ("clinical" or "biology" or "medicine") from 1978-2020.

Figure 2 – Conceptual framework for visualising translation of biomedical research. Adapted from ref. [15] with permission from Springer Nature.

Figure 3 – Example of assigning Raman spectra to tissue structures and ductal carcinoma assigned by two-step k-means clustering: (a) and (b) samples with invasive ductal carcinoma, (c) normal breast tissue. Typical centroid Raman spectra are shown for the clusters indicated by arrows: focus of invasive ductal carcinoma (red arrows), tumour-surrounding inflammatory stroma (green arrows), lobules and ducts (blue arrows), stroma (black arrows) and fat (orange arrows). Reproduced from ref. [54] under a Creative Commons Attribution 3.0 licence.

Figure 4 – The handheld contact fibre optic probe for Raman spectroscopy utilised by Jermyn et al.: (a) Experimental setup diagram with the 785-nm near-IR laser and the high-resolution charge-coupled device spectroscopic detector used with the Raman fibre optic probe; BP, band-pass; LP, long-pass. (b) The probe was used to interrogate brain tissue during surgery. A schematic diagram illustrates the excitation of different molecular species, such as cholesterol and DNA, to produce the Raman spectra of cancer versus normal brain tissue. A simple molecular vibrational mode is conceptually depicted (individual atoms in blue and green) interacting with the laser light (in red) to produce Raman scattering (in purple). Reproduced from ref. [167] with permission from AAAS.

Figure 5 – Infrared-optical hybrid (IR-OH) microscopy imaging of breast tissue and spectral cell type recognition. (A) Haematoxylin and eosin image of a breast tissue microarray section; (B) IR-OH absorption of an adjacent, unstained tissue section at 1550 cm^{-1}; (C) A four-class model (blood, epithelium, stroma, and other) allows rapid tissue component visualization based on five IR bands; (D) Epithelial classification (five-class model) permits both histologic cellular identification and recognition of cancer based on seven IR bands; (E) the ROC curves related to the classes in D; (F) the AUC of the ROC curve increases with number of IR frequencies used for classification; (G and H) Representative class spectra obtained with IR-OH and FT-IR imaging, respectively. Adapted from ref. [214] under the PNAS License to Publish.
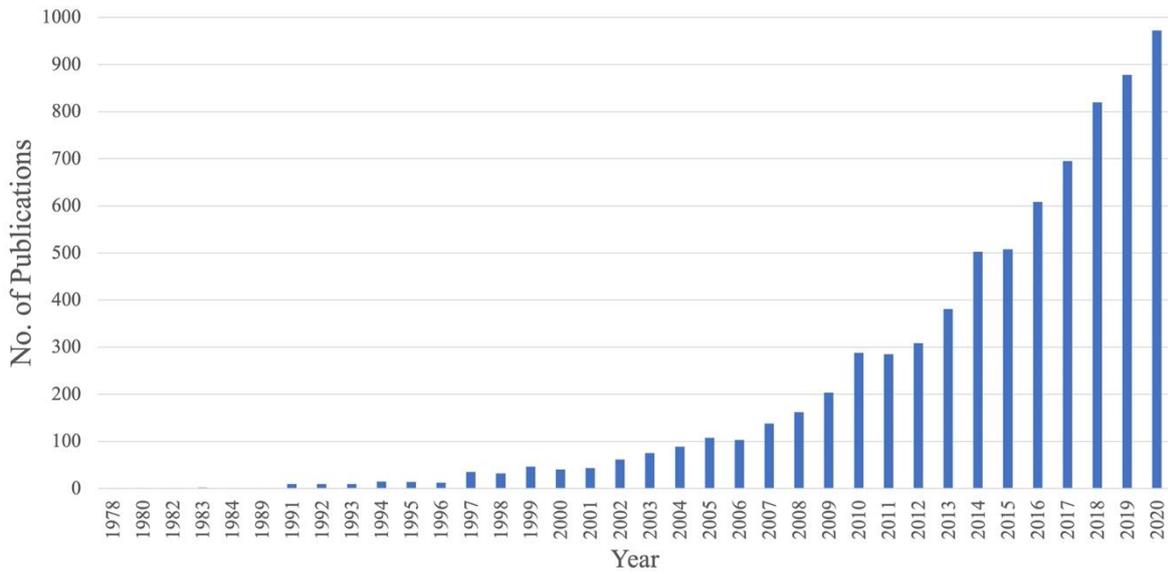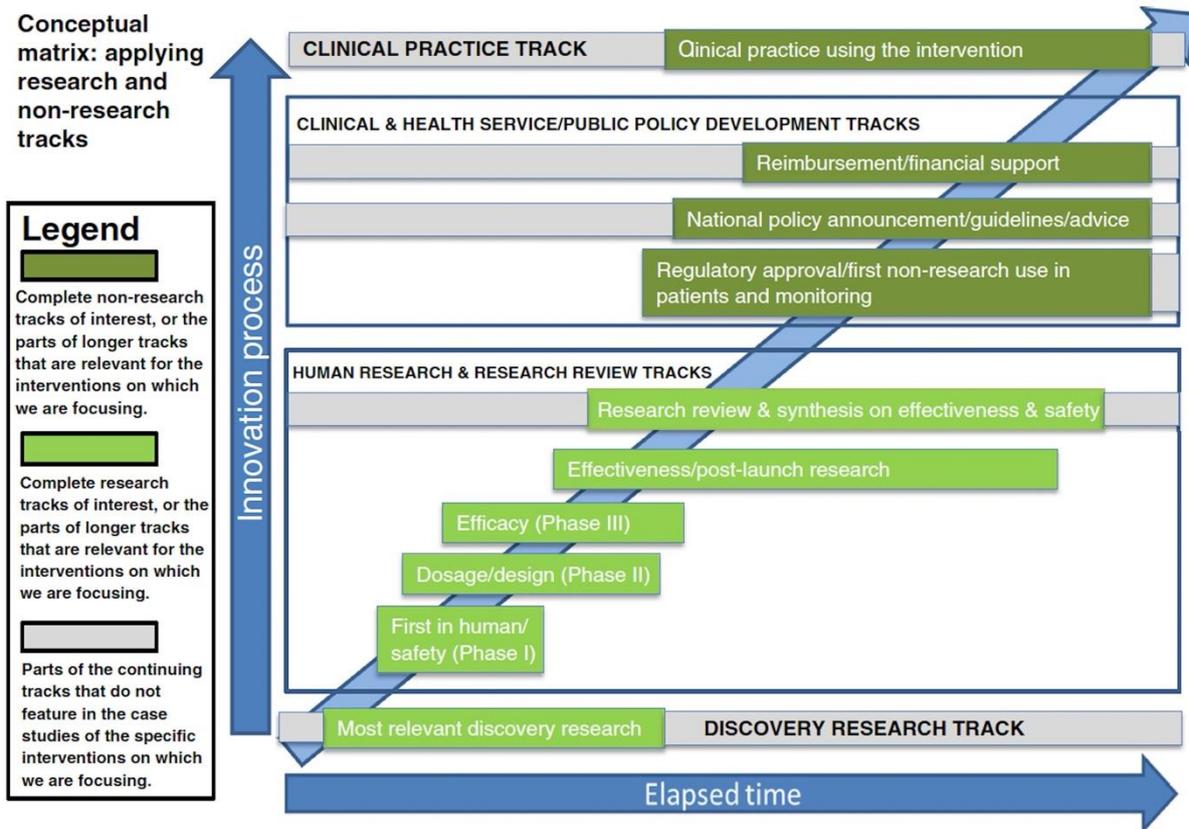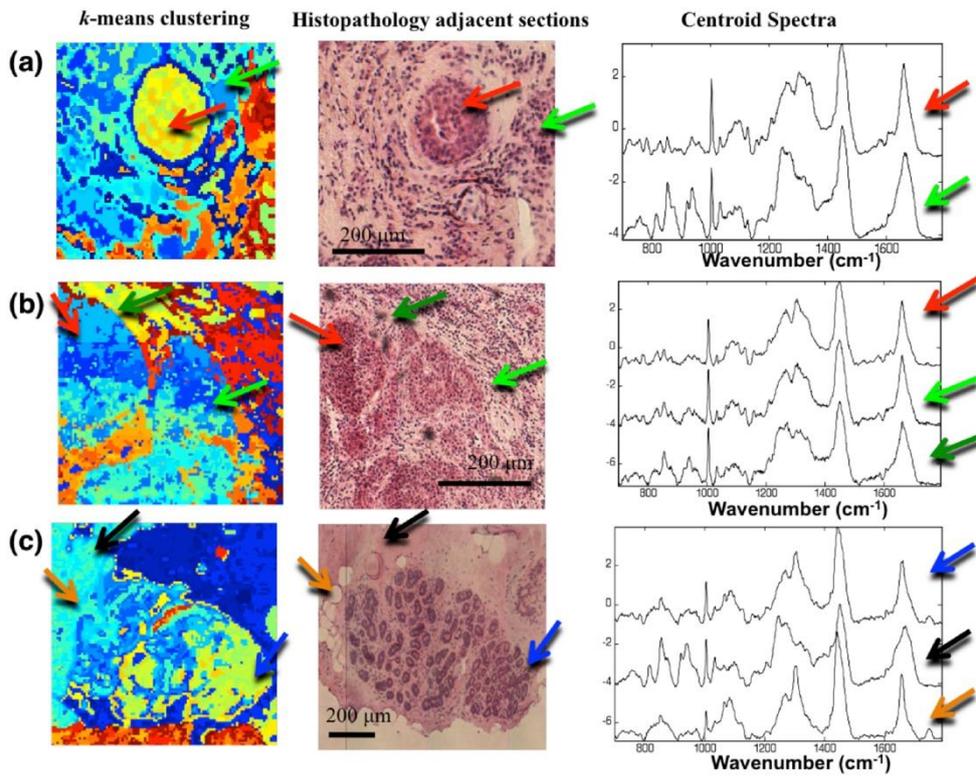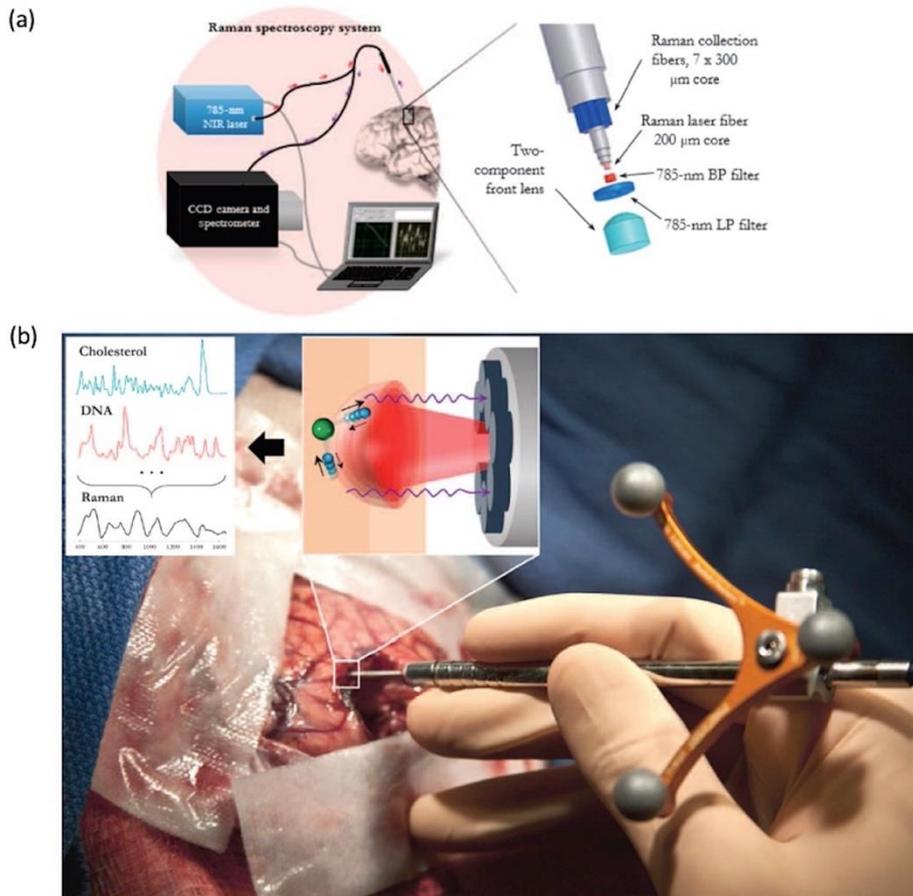
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