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Complete List of Authors:	Byrne, Hugh; Focas Institute, Dublin Institute of Technology Baranska, Malgorzata; Jagiellonian University, Chemistry Puppels, Gerwin; RiverD International B.V., Stone, Nick; University of Exeter, College of Engineering, Mathematics and Physical Sciences Wood, Bayden; Monash University, Department of Chemistry Gough, Kathleen; University of Manitoba, Chemistry Lasch, Peter; RKI, ZBS 6 Heraud, Philip; Monash University, Monash Immunology and Stem Cell Laboratories Sulé-Suso, Josep; 2Cancer Centre, City General Hospital,, University Hospital of North Staffordshire, Newcastle Rd, Sockalingum, Ganesh; Universite de Reims, Unite Médian, CNRS UMR 7369-MEDyC

Spectropathology for the Next Generation: Quo Vadis?

Hugh J. Byrne[1]*, Malgorzata Baranska[2], Gerwin J. Puppels[3], Nick Stone[4], Bayden Wood[5], Kathleen M. Gough[6], Peter Lasch[7], Phil Heraud[8], Josep Sulé-Suso[9], Ganesh D. Sockalingum[10]

1. FOCAS Research Institute, Dublin Institute of Technology, Kevin Street, Dublin 8, Ireland
2. Faculty of Chemistry, Jagiellonian University, Ingardena 3, Krakow, Poland.
3. RiverD International B.V., Marconsitraat 16, 3029 AK Rotterdam, the Netherlands & Erasmus-University Medical Center, Center for Optical Diagnostics & Therapy, Dept of Dermatology Rotterdam, the Netherlands.
4. Biomedical Spectroscopy Lab, School of Physics, College of Engineering, Mathematics and Physical Sciences, University of Exeter, Stoker Road, Exeter, EX4 4QL, UK.
5. Centre for Biospectroscopy and School of Chemistry, Monash University, Clayton, Victoria 3800, Australia.
6. Department of Chemistry, University of Manitoba, Canada
7. Robert Koch-Institut, Center for Biological Threats and Special Pathogens: Proteomics and Spectroscopy(ZBS6), 13353 Berlin, Nordufer 20, Germany.
8. Centre for Biospectroscopy and School of Chemistry, Monash University, Victoria 3800, Australia
9. Institute for Science & Technology in Medicine, Keele University, Stoke-on-Trent, ST4 7QB, UK
10. Université de Reims Champagne-Ardenne, MéDIAN-Biophotonique et Technologies pour la Santé, UFR de Pharmacie, 51096 Reims Cedex, France.

Abstract

Although the potential of vibrational spectroscopy for biomedical applications has been well demonstrated, translation into clinical practice has been relatively slow. This perspective assesses the challenges facing the field and the potential way forward. While many technological challenges have been addressed to date, considerable effort is still required to gain acceptance of the techniques among the medical community, standardize protocols, extend to clinically relevant scale, and ultimately assess the health economics underlying clinical deployment. National and international research networks can contribute much to technology development and standardization. Ultimately, large-scale funding is required to engage in clinical trials and instrument development.

Keywords: Vibrational Spectroscopy, Raman Spectroscopy, Infrared spectroscopy, Translational Research, Clinical applications

***Correspondence:** hugh.byrne@dit.ie

Introduction

Disease diagnostics have long relied on visual differences in tissue appearance, aided in modern histopathology and cytology by optical stains and microscopic technologies. However, such approaches are based on changes in tissue and cell morphology, often apparent only at the later stages of disease development, rather than the underlying biochemical changes associated with disease onset or aetiology. Optical techniques for routine screening are particularly suitable for more accessible anatomical sites such as mouth and throat, skin and cervix, but although optical techniques such as colposcopy for cervical or conventional oral examination for oral cancer are routinely used, they have low sensitivity and specificity. They rely on the trained eye of a clinical specialist, and consensus between such specialists can be low, particularly in the early stages of disease onset [1]. Spectroscopic techniques are based on changes in underlying biochemical structure and therefore potentially offer a more objective analysis, which is therefore automatable and adaptable to routine screening. In this context, fluorescence spectroscopic techniques have received considerable attention, although, for a label free technology, analysis is based only on endogenous fluorophores such as collagen, elastin, keratin, riboflavin porphyrin and NADH. Emission bands are broad and overlapping, reducing the specificities of such techniques. The use of UV excitation also limits the sampling depth in tissue. As an alternative, vibrational spectroscopy, both infrared absorption and Raman scattering, offers many potential advantages, as it provides a spectroscopic signature of all molecular constituents of the sample, and as such a complex fingerprint which can be used to uniquely identify a compound, collection of compounds, or subtle changes to it or them.

The potential of vibrational spectroscopy for biomedical applications has been well established through many proof of concept studies over the past decades [2-5]. Due to its unique fingerprinting capability, vibrational spectroscopy can play a significant role in histopathology, cytology, biopsy targeting, surgical targets, treatment monitoring and drug studies. Application scenarios could include single shot measurement of targeted nuclei for cytological screening, or in imaging and/or mapping larger areas for diagnostics or high content analysis, *in vivo*, *ex vivo* or *in vitro*. However, translation into the clinical environment has been slow, and it is appropriate at this stage to assess and evaluate questions such as (i) What are the most achievable, strategic target applications, (ii) What are the technical challenges, and how can they be addressed (iii) What are the challenges to implementation (legislative, clinical trials etc.), and how can they be addressed. This perspective considers such questions under the subheadings of (i) Translational research into *in vivo* clinical applications (ii) *Ex vivo* tissue biopsies, body fluids and cytological samples for diagnostics and disease studies (iii) *In vitro* cell culture and 3D models for Research and Medical applications.

(i) Translational research into *in vivo* clinical applications

As an optical based technology, vibrational spectroscopy is easily adaptable to *in vivo* disease diagnostics and monitoring applications, ranging from intraoperative assessment of auxiliary lymph nodes using Raman spectroscopy [6] to IR photoacoustic dermal screening [7]. Significant development of Raman fibre probes for *in vivo* diagnostics and intraoperative patient monitoring has been achieved in recent years [8]. In the latter context, identification of tumour margins has been highlighted as a potentially a significant aid to surgeons. This has been shown in various forms using fibres or microscope based approaches.

FTIR probes for surface analysis and or evanescent wave analysis of fluids are available [9], although these are clearly limited if lesions of interest are to be found more than a few microns below the surface.

Skin is the most accessible organ and therefore should be a strategic target. In addition to diagnostic applications [10], spectroscopy could provide significant guidance for monitoring and optimising transdermal drug delivery [11], as well as understanding dermal toxicity from external agents such as nanoparticles, chemicals and radiation [12].

The question should therefore be posed, in terms of *In vivo* clinical applications – Is the vision right? If the performance of the technology is optimised, and applications demonstrated with appropriate large scale studies, will there be uptake in a clinical environment? There is a need to balance the drive for technology development from the research community with the needs of the clinical environment. Are there technological solutions looking for a problem or clinical problems looking for a solution?

In this context, it is important to engage with the medical community to establish firstly the demand for the technological solutions, and secondly, what is the Minimal Viable Solution. Researchers strive for both fast acquisition rates and high quality spectral data, but in a surgical environment, time is of the essence, and so a fast, simple spectroscopic modality, which is cheaper, more cost effective but has lower sensitivity and specificity may be a viable solution. This poses the question as to how much of a compromise can be made in terms of performance. While values approaching 100% have been quoted for laboratory based studies, is 80% sensitivity/ specificity sufficient in a clinical setting? The required performance levels will depend on the specific application, (Tissue type, screening/biopsy targeting/margin assessment) and therefore it is difficult to establish a generic Minimal

Viable Solution. It is, however, crucial to establish what the realistic and actual current gold standard performance is for a number of specific strategic applications such that spectroscopic performance can be directly compared and evaluated with these reference diagnostic techniques. Realistic evaluations and comparisons of variability and costs are also imperative. This is something that was demonstrated by providing Kappa statistics of the performance of the technique versus a panel of expert pathologists [1].

Most research studies to date have been conducted on general purpose research instruments and there is a need to develop and optimise commercial products to be trialled and validated. In this context, reproducibility of systems is a vital technical challenge to be addressed, as is transferability of datasets between systems. There is currently a huge gap between the research community and commercial/industrial partners. Big medical diagnostic companies need to be on board and engaged in the drive towards strategic and targeted technological development.

Critically, while the research environment has demands on demonstrating reproducibility of data, the demands of clinical deployment are substantially more rigorous. Studies must therefore be extended to use of clinically appropriate scale and statistical analysis, to be considered meaningful. The statistical and ethical plan needs to be outlined for each study, which should include significant sample sizes (~150 patients) and blind datasets, while outcomes should detail sensitivity, specificity, AUC etc.

To date, the proof of concept has been adequately demonstrated, but there is a need to develop large (randomised controlled) clinical trials for the technology to be adopted by the clinical community. However, such trails carry considerable cost implications, taking into

account implementation costs and the cost of developing further studies; the cost of (randomized) clinical trials and the cost of bringing something to market.

The field of diagnostic applications of spectroscopy could potentially learn from the successes and failures of other technologies in the clinical field and engaging with large transnational organisations such as the European Clinical Research Infrastructures Network (ECRIN - http://www.ecrin.org/) may help guide the strategic development.

Funding is limited to translate and develop technologies across the so called 'valley of death' to full *in vivo* clinical studies and implementation. Financing of development on such a scale would require the support and commitment of National and International funders, the medical community, instrument manufacturers and private funders. In order to justify substantial funding, Health Economics need to be more critically explored and addressed, and realistic business plans composed. In this context, identification of strategic target applications may be crucial.

There is also a need to raise awareness of optical diagnostics within, and better engage, the medical community, through conferences like SPEC. This includes surgeons, oncologists, medical physics, Chief Executives of hospitals for local implementation, national boards e.g. NICE in UK. This can be done through personal/local contacts, but also through advertisement in relevant clinical publications. There is a need to create curiosity and demand among the clinical community, but the talks at SPEC can be technically detailed and inaccessible for clinicians. It is important to adopt clinical language: power analysis, sensitivity/specificity, ROCs, patient benefit. Presentations need to be applications focused and clinical sessions should be held at the weekend to facilitate attendance by medical professionals. Open, targeted discussions with clinicians are essential such that practitioners

from various clinical backgrounds can present their work, the current state of the art and the challenges faced, and that researchers can propose what spectroscopy has to offer in relation to their clinical needs and how it relates to their approaches. There is a need to educate the community better in the technologies of optical/spectroscopic diagnostics and data analysis. This is a current need, but successful translation of the technologies will also create a need for training at medical school level. An accessible handbook on optical diagnostics for various clinical specialities could be valuable as an introduction to the field.

It is equally important to increasingly publish clinically relevant spectroscopic studies in clinical journals and to promote the technologies at clinical conferences and at large medical diagnostics exhibitions. Early adopters from the clinical community can help in this context, and also to develop relationships with key thought leaders in the field and further promote adoption of the technologies within their clinical communities. Where clinical trials have been carried out, patients are powerful advocacy groups!

The research community should also seek publicity for their results using public and social media wherever/whenever possible.

(ii) Ex vivo tissue biopsies, body fluids and cytological samples for diagnostics and disease studies

For the purposes of this discussion, *Ex vivo* applications of Vibrational spectroscopy are interpreted to include analyses of samples taken directly from the body for diagnostic purposes, namely tissue biopsies, cytological samples and body fluids.

From a surgical point of view, an alternative to *in vivo* spectroscopic monitoring would be "near patient" screening of tissue biopsies taken intra-operatively to guide the surgeon in identifying tumour margins. Ideally, analysis should be performed on fresh tissue, and

screening time, including data processing, should be kept to a minimum. As in the case of *in vivo* measurement, time is of the essence, and so a fast, simple spectroscopic modality, which is cheaper, more cost effective but has lower sensitivity and specificity may be a viable solution.

However, there is a lack of concrete understanding of issues such as: What is the competition? How much better can spectroscopy be? Within what reasonable timeframe for measurement? The measurements should take a matter of minutes otherwise their clinical utility will be questionable. Surgeons cannot afford to wait.

Such demands of speed of acquisition and data processing are considerably eased in applications for histological screening, although it is recognised that current mapping/imaging times of large areas of tissue followed by current pre- and post- data processing protocols need to be improved [13], and, although significant progress has been made, there is much to be done in terms of standardising procedures and protocols.

The demands on the ability to rapidly scan large areas of tissue probably currently favour the use of FTIR rather than Raman spectroscopy for such applications.

In terms of sample presentation, fresh frozen sections are recommended as the tissue architecture and biochemistry is kept largely intact and, notably, the lipidic information can be accessed [14]. Furthermore, they are more amenable to combining immunohistochemistry, proteomics, and biospectroscopy. However, clinically, fresh tissue is normally only used for intraoperative work and stained, fixed sections are preferred for histopathology [15]. Therefore, standardised protocols for spectroscopic analysis of Formalin Fixed Paraffin Processed (FFPP) tissue sample are of paramount importance.

Notably, analyses of archived tissue libraries may add much to understanding disease progression and patient prognosis.

It has been demonstrated that it is not necessary to remove the paraffin to obtain usable spectral information, particularly in the case of FTIR spectroscopy [16]. Standard tissue microarray protocols involve paraffin embedded tissue. Leaving the paraffin in place reduces scattering artefacts and effects of further variable removal of aromatic solvent soluble components. However, it may be argued that greater consistency of spectral information is achieved when sections are deparaffinised. Deparaffinising also allows post-staining of the sections, although it has been demonstrated that the efficiency of the deparaffinisation process can depend on the tissue pathology [17]. Nevertheless, even for research purposes, protocols for such tissue processing should be maintained as close as possible to those currently employed in the clinical environment.

In the case of FTIR based spectro-histopathology, there remains much debate on the questions of measurement geometry, and therefore optimum choice of substrates. In terms of cost, low-E, reflective slides appear most attractive, implying the use of a transflection measurement configuration. However, questions have been raised concerning additional spectral artefacts which can result from the so called “Electric Field Standing Wave” effect in such measurements [18], although it has been argued that the effects are diminished by thickness inhomogeneities, the range of sampling angles, and the source incoherence [19]. The alternative, transmission, geometry requires (at least partially) transparent substrates. It has been demonstrated that even glass substrates may provide transmission in a sufficiently broad (high wavenumber) region to provide diagnostic capabilities [20].

However, access to the broader spectrum is only provided by more costly polycrystalline substrates such as CaF_2.

Choice of substrate may ultimately be dictated by cost, and therefore by sample throughput, and therefore by the target application. A full cost analysis is required to assess the relative demand and costs of applications for (i) near patient intra operative diagnostic (ii) postoperative histological and (iii) research purposes.

Cytological screening is commonly employed as a routine preventative measure or for early stage disease detection, notably for cervical and oral disease. In the case of cervical, screening programmes are well established in the “third world” and so sample throughput is very high. This puts increased demands on the cost effectiveness of alternatives to currently employed clinical practice.

Cervical screening is traditionally performed by the Pap smear methods, or more recently by liquid based methods such as Sure-Path or Thin-Prep. In all cases, the samples are stained by a combination of dyes. The Pap Smear test is reported to provide a sensitivity of ~72% and specificity of ~94% [21]. Studies of the accuracy of liquid based monolayer cytology report sensitivity of ~ 63% and specificity ~85% [21, 22]. The aetiology of the disease in the case of cervical is predominantly linked with HPV infection, and so screening for HPV infection has become increasingly popular, although, as it is more costly the procedure is most often used as a further screen of suspicious cytological tests. Studies of the accuracy of HPV testing report a sensitivity of ~90% and a specificity of ~ 80% [22, 23].

The use of vibrational spectroscopy as an adjunct or alternative to currently employed cytological screening methods may be a viable strategic target objective. Sensitivities and specificities of >90% as well as sensitivity to HPV infection have been reported [24, 25].

The use of glass slides for high throughput in all current clinical practices, as well as the smaller spotsize/higher spatial resolution may favour the use of Raman spectroscopy in this case. Raman can potentially selectively target either cell nuclear or cytoplasmic regions, although the denser nuclear region provides greater diagnostic potential. In development of Raman protocols, choice of wavelength is intimately linked with choice of substrate, and it has been demonstrated that although conventional glass microscope slides have a substantial background at 785nm, this is greatly reduced at 532nm [26].

In unstained cytological samples, 532nm causes negligible observable damage to the samples over the measurement period. However, photodamage due to absorption and/or large fluorescent backgrounds due to clinical stains presents a significant problem for the use of Raman spectroscopy as an adjunct to cytological screening. Stained samples cannot be simultaneously optically and spectroscopically screened [27], suggesting that a fully automated procedure for unstained cell recognition, spectral analysis and assessment may be required.

Screening of bodily fluids is a further *ex-vivo* application which is currently attracting increasing attention and may represent a strategic, achievable target [28]. Suspended or dissolved analytes are present in rather low concentrations, however, and many studies to date have been performed on dried samples [29, 30]. The analysis of such samples can suffer from problems associated with the chemical and physical inhomogeneity of the deposit, reducing reproducibility and sensitivities. Bulk ATR FTIR measurements have been shown to reduce such effects [31], however, and multi-well ATR devices have been proposed to potentially offer high throughput screening.

Concentration of samples using centrifugal filtration devices has been shown to offer an alternative which allows measurement of the analytes in the native aqueous environment. Although Raman appears most promising in this context, due to the relatively lower contribution of the water, sufficient concentration of the sample also allows analysis of the fingerprint region by FTIR [32]. Centrifugal filtration also allows fractionation according to molecular weight of the constituent analytes, potentially allowing targeting of molecular biomarkers of disease [33]

As for the case of *in vivo* applications, translation of technologies to clinical practise will be critically dependent on large scale studies with clinically relevant statistics. Diagnostic applications rely heavily on multivariate statistical classification methodologies and each analytical protocol must be "trained" For each potential application, the data can be influenced by instrument, sample presentation and preparation, measurement protocol and data processing. It is critical therefore that consensus be reached on Standard Operating Procedures, to include all of these variables. Inter-laboratory and even inter- instrument consistency and transferability needs to be established. Only then can large data bases be established both for translational and research purposes.

There is also a need for extensive validation and prospective testing of data preprocessing protocols as well as classification and regression models. In this context, using patient data, it is impossible to know what is the "correct" result. Simulated data sets could play a key role in validating data pre- processing methodologies, ensuring that the spectral integrity is preserved [34, 35]. However, while they can play a similar role in validating classification algorithms, correlation with disease pathology and patient prognosis still relies on

consensus clinical standards. The use of archived tissue banks for retrospective studies may play a critical role in establishing such a clear correlation.

There is much scope for instrumental development, in collaboration with the instrument companies. Ideally, instrumentation should be optimised for the specific purpose, ultimately of automated *ex-vivo* screening of histological, cytological or biofluidic samples. However, clinicians may be adverse to automated decision systems, and that they need to be the ultimate arbiters. There may also be a disadvantage in some biophotonic-based diagnostics, as they do not generate a consumable market, and thus it may be more difficult to garner support from some companies. Much progress has, however, been made in the continued development of portable devices, particularly in the case of Raman. This may generate specific applications for example in contamination-restricted environments, and particularly in field clinics.

(iii) *In vitro* cell culture and 3D models for Research and Medical applications

The definition of "*ex-vivo*" to include tissue biopsies, cytological samples and biofluids directly from the patient for diagnostic purposes restricts the discussion of "*in vitro*" to cell culture models for both research and medical applications. As such, direct clinical translation is probably limited, but, nevertheless, such models can prove invaluable for the development and validation new measurement technologies, measurement and data processing and analysis protocols, and ultimately the exploration of the limits of the techniques in identifying and screening biomarkers associated with biological function and dysfunction. The techniques may also provide valuable information on, for example, radiation and chemotherapeutic resistance, and present opportunities in their own right for potential applications in screening for drug delivery mechanisms and efficacy, radiation

damage and toxicology [36-38], given the drive for a reduction in the use of animal models for evaluating toxicity, due to regulatory developments in both the EU and US (EU Directive-2010/63/EU and US Public Law 106-545, 2010, 106th Congress) generally based on the principle of the 3 R's, to replace, reduce and refine the use of animals used for scientific purposes. Therefore, there is currently much promotion of the development of *in vitro* models which can accurately infer *in vivo* results.

In terms of basic research tools, the imperative for standardisation of measurement protocols is not as urgent. Nevertheless, it is important that the spectral data acquired is representative of the biochemical profile of the sample and free of "spectral artefacts" which may arise from the measurement geometry, substrate, or sample. Much has been achieved over the past decade to understand such artefacts [39, 40], but there remain significant issues to be addressed. Ultimately, for successful application for *in vitro* drug or toxicity screening, standard operating procedures will be required.

In the case of Infrared spectroscopy, the debate over the choice of measurement geometry and hence choice of substrate is (at least) as relevant for analysis *in vitro* as it is for diagnostics *ex vivo*. The transflection geometry appears to maximise both multiple beam interference (e.g. EFSW) and scattering effects [18, 40], although neither are completely absent in the transmission geometry. The severity of both is dependent on the homogeneity of the sample, although algorithms for the removal of resonance scattering/reflection are well established [34]. In the research environment, cost is less of a consideration that for high throughput routine clinical screening and so, where possible the use of the more expensive polycrystalline substrates (e.g. CaF_2), in the transmission geometry, are recommended. Notably, UV grade CaF_2 is also an optimum substrate for Raman

measurement [26]. Measurement using ATR minimises (although does not completely eradicate) scattering artefacts and has been gaining increased popularity, although the sampling depth is limited.

In the case of Raman spectroscopy, most instrumentation operates in a back scattering (microscopic) geometry, and consideration of the influence of substrate depends on sample thickness and focal depth of the objective employed, and where substrate contributions are significant, they depend on the source wavelength. Glass substrates have been shown to be acceptable at visible wavelengths. Although they can contribute in the case of thin samples (e.g. cytoplasm) the contribution can be removed by careful preprocessing. In the near infrared, common microscope slides contribute a strong background which can completely obscure the sample response, and normally quartz or ideally UV grade CaF_2 is preferable [26].

In terms of sample preparation/presentation, it is well accepted that measurement of live cells is most desirable. In the case of infrared spectroscopy, the strong absorption of the water bands in the region of ~1600cm^{-1} and 3300cm^{-1} presents a problem, although it should be stressed that this does not prohibit measurement of live cells (or other aqueous based biological samples) [41, 32]. Specifically designed sample compartments can minimise the extracellular pathlength and the use of ultrabright synchrotron sources significantly improve signal to noise [41]. In Raman spectroscopy, contributions from water are less of a consideration and live cell imaging in buffer of complete cell culture medium has been demonstrated [42]. It is important to note that, although the signal is small, water does contribute to the underlying background and careful preprocessing of the data is essential, remembering that water is also a constituent intracellular component.

In both cases, given current technologies, particularly in the case of Raman, whole cell studies at high spatial resolution can be protracted and, to avoid bacterial contamination, cell movement in the liquid environment, etc., it may be more advantageous to fix the cells before measurement. A number of studies have demonstrated that formalin fixation best, although not completely, preserves the biochemical integrity of the cells [43].

Commercial tissue models, notably for skin, are available and can aid in research purposes. These can be measured “live”, or can be processed as normal tissue. They reduce sample variability compared to human or animal samples and can be employed to optimize measurement protocols, although it should be emphasized they are not exact replicas. Notably, in the case of skin models, the basal layer is lacking in melanin, and also the lipidic architecture of the stratum corneum does not well reproduce the barrier function of real skin, limiting the suitability for perfusion studies [44].

Notably, it has been increasingly argued that 2D cell cultures are a poor representation of the cellular environment *in vivo*, and that true cell morphology and cell behaviours, such as drug uptake and response, would be much more closely mimicked in 3-D cell matrices. The use of such constructs may also help to better understand cell/microenvironment interactions and analysis of single cells in such environments seems to partly circumvent the scattering issues which contribute to scattering backgrounds in both FTIR and Raman since cells are no longer isolated.

Independent of use in spectroscopic research, it is important to develop and optimise these models. In doing so, it is important to note that the diffusion and bioaviability of both cytotoxicological assays and test substances in 3D matrices must be considered and adaption of the protocols is necessary for direct comparison with the traditional 2D models

[45]. Nevertheless, such models represent an exciting new development for *in vitro* models which better mimic *in vivo* conditions, and the emergence of IR tomographic image reconstruction using synchrotron sources to image these structures holds great promise [46]. The usefulness of Raman microspectroscopy "optical sectioning" should also be emphasised.

Whereas diagnostic applications rely largely on classification or regression algorithms, *in vitro* applications can potentially exploit the full analytical capabilities of biospectroscopy. In this context, maintaining the integrity of the spectral information during data processing is imperative. As in the case of *ex vivo* measurements, data preprocessing methodologies can, and should, be validated using simulated datasets. Ideally, such data sets should include spectral variability due to all potentially confounding experimental factors, as limits of detection may ultimately be determined by such factors [47].

Postprocessing and analysis protocols can similarly be validated and optimised to ensure that they produce the correct result [33, 35]. Simulated datasets, can be employed to explore and develop the limits of biospectroscopy as an analytical technique, for example to minimise the limits of detection of and maximise the specificity of regression algorithms and feature selection based on spectral biomarkers [47]. With properly validated analytical techniques, biospectroscopy could aspire to realisation of its potential as a truly label free, high content screening technique based on the field of “specral-ohmics”.

Achieving such goals, as well as those of clinical translation, relies much on continued instrumental development. Increased signal throughput and novel sampling techniques, such as those afforded by Quantum Cascade Lasers in IR and Bragg filters in Raman may significantly reduce sampling times over large areas. Emerging technologies such as Surface

Enhanced, Stimulated and Coherent Anti-Stokes Raman Spectroscopy may similarly impact on sensitivity of data collection [48]. Atomic Force Microscopy- IR (AFMIR) and Tip enhanced Raman Spectroscopy open up the realm of nanospectroscopy for both IR and Raman spectroscopy. At present, at least, these developments are very much in the research domain, however, and are most applicable to *in vitro* studies.

Summary

There are clearly many challenges facing the field of diagnostic applications of vibrational spectroscopy. Many of these require a more significant engagement between the broad range of stakeholders, from academic research scientists to clinical practitioners, and including medical and spectroscopic instrument manufacturers.

Raising awareness of the field amongst the medical community can be achieved by academic researchers by targeting medical journals and conferences, and similarly targeted series of conferences can play a pivotal role in bringing the communities together.

In the move towards establishing and promoting SOPs, for measurement and data handling protocols, national and international research networks such as the UK EPSRC Network CLIRSPEC (www.clirspec.org) and the EU COST Action Raman4Clinics (http://www.cost.eu/domains_actions/bmbs/Actions/BM1401) can potentially make significant headway. Such networks can also address the question of what constitutes robust statistics, to take account of population variance rather than simply technical variance, blinded trials etc., and these should be expressed in terms of clinical language to encourage clinical acceptance.

Ultimately, however, more targeted engagement with the medical community must be undertaken to establish strategic target applications and performance levels for Minimal

Viable Solutions. Notably, substantial funding will be required to conduct large scale multi-lab and instrument inter-comparisons and ultimately clinical trials.

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