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Vibrational spectroscopic imaging of pathogens, microorganisms, and their interactions with host systems

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ABSTRACT

Vibrational spectroscopy has recently been increasing in popularity, especially when implemented as a label-free imaging mode. The use of light scattering in Raman imaging, or light absorption in infrared (IR) imaging, where the light wavelengths are selected to probe molecular bonds in the sample, allows a unique view of the molecular makeup of a sample. Generally non-invasive and highly sensitive to spatiotemporal changes in sample composition, vibrational spectroscopic imaging has very large potential in a wide range of fields. Here we review recent work in implementing both Raman and IR imaging, with particular attention to how the imaging itself, meaning the particular spatial distributions of spectra plays a role in generating novel findings. In general, for both Raman and IR imaging, technological developments are rapidly progressing, leading to higher imaging resolutions (especially for IR), images with higher signal-to-noise, substantially faster measurements, and more refined chemometric analysis by way of computational advances. In light of this, the current work highlights emerging applications that are now possible and indicates the potential for future applications of vibrational spectroscopic imaging for biological analysis.

1. Introduction

Infectious and communicable diseases caused by microorganisms and other pathogens represent a significant burden on global human health. In 2015, five of the 20 leading causes of mortality worldwide were linked to such infections: lower respiratory infections were responsible for 4.91% of global deaths, diarrhoeal diseases for 2.35%, tuberculosis for 1.99%, HIV/AIDS (leading to other diseases) for 1.76% and malaria for 1.31%, with the majority of these deaths in low- and middle-income countries (https://vizhub.healthdata.org/gbd-compare/). However, these figures only tell part of the story as they do not include the impact on the quality of life for those individuals who live with these infections. The consequences of co-infection with more than one pathogen are perhaps most clearly demonstrated for HIV/AIDS infection, where disruption of the host immune system often results in increased severity of symptoms from infection with secondary pathogens [1]. However, the health consequences of many infections can be exacerbated by co-infection with other pathogens, such as seen for helminth co-infection affecting the host immune response to tuberculosis [2] or malaria [3]. Early detection of infection is, of course, key to ensuring effective and timely treatment and, in the case where co-infection with additional parasites is suspected, detection and identification of multiple parasite species with a single measurement is particularly advantageous.

Optical techniques can have significant advantages over wet-lab based detection or analysis methods since they are often non-destructive, have low consumable costs, and can provide rapid results. A number of different optical approaches have been employed to analyse pathogens [4], but in particular, label-free techniques do not require any additional chemical labels or changes, and can therefore be an elegant approach to analysis of infected bodily fluids, cells and tissues since no *a priori* knowledge of the nature of the infection is required. Raman and Infrared (IR) spectroscopy are vibrational spectroscopy techniques that probe the molecular signatures of the sample. They are both well-suited to evaluation of complex biological samples, and provide rich spectral information that can be used to discriminate subtle differences between different pathogens allowing detection and identification of the microorganisms responsible for infection, as well as observing local changes in the host cells/tissues surrounding the pathogen. There are a number of ways in which label-free spectroscopic imaging can contribute to solving problems related to pathogenic infection; in diagnosis, understanding the infection process, to predict disease outcome, or to evaluate various treatments or drug effects on the disease. It is also possible to carry out significant and important analyses using only single point measurements, or measurements based on an area of a sample that do not fit the definition of "imaging". Unique to imaging, however,

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is the ability to use spatial information to discriminate the location of pathogens, the spatial extent and heterogeneity of infection, as well as the spatial dependence of the host reaction to the pathogen. This can be on the intracellular level, where the cell reaction includes pathogen-specific spatially dependent responses and/or reorganisation of the cell in response to the pathogen. It also applies at larger scales, such as tissue level responses where, for example, the response of the host to treatment of infection can also be spatially dependent. In line with the theme of this issue, we aim here to focus on work where the spectroscopic imaging itself contributed to the novelty or findings of the paper, although this is somewhat subjective in some cases.

Amongst the wide range of label-free imaging modalities we limit discussion here to spontaneous Raman spectroscopic imaging, as well as Infrared absorption spectroscopic imaging. In general, however, cells and tissues can readily be evaluated by both linear (spontaneous Raman) and nonlinear Raman (see [5] for a review), and a number of different nonlinear methods can be used to generate Raman images. Examples include coherent Anti-Stokes scattering [6], stimulated Raman scattering [7], and hyper Raman scattering [8] which exploit nonlinear interactions between the sample and the irradiation light and can significantly enhance the measured spectral signal quality and/or imaging resolution. Surface-enhancement by a nano-scale rough metal surface can also be exploited to enhance both Raman and Infrared absorption [9], but can make the evaluation of the resulting spectra significantly more difficult. The main advantage of using a linear spectroscopy approach, whether Raman or IR-based, is that the resulting spectra are rich in spectral information, ease of implementation, and the results are relatively straightforward to interpret. Several works also combine different label-free modes in creative ways exploiting the advantages of each (see for example [10]), but here we will focus on Raman and IR spectroscopic imaging.

For some studies, in order to simplify experimental protocols, non-pathogenic versions of otherwise pathogenic organisms have been studied. This can be for improved experimental safety, but with results designed to be used as a model for the pathogenic strain. For this reason, we also include here microorganisms that are not strictly pathogenic, but illustrate the capabilities of the spectral imaging techniques, and with a view towards their similarity with pathogenic strains.

2. Instrumentation

The features of both IR and Raman microscopes and spectrometers have been covered in detail in many previous publications (such as [11–13]). Therefore, here we will only briefly describe some of the instrumentation considerations that are specific for imaging and not for vibrational spectroscopic analysis in general.

For IR spectroscopy, two aspects of the instrumentation are worth considering in terms of imaging systems: the light source and the detector. Although images can be collected using systems equipped with a single element detector, by mapping the sample and recording a spectrum at each point. However, this process can be extremely time consuming. Consequently, IR imaging only really became a broadly applicable analysis technique after the introduction of array detectors, with early studies employing an Indium antimonide (InSb) focal plane array (FPA) detector [14]. Detectors can be made from various materials, each with differing performances in the IR region (for a summary see [15]) and today many commercial IR imaging systems contain a Mercury Cadmium Telluride (HgCdTe, often abbreviated to MCT) linear array or FPA detector due to their wide spectral range and high quantum efficiency in the IR region [16].

Standard IR spectroscopy systems (both single element and array detectors) employ a globar light source. These are simple, low cost and robust sources with broadband emission which produce the necessary light levels for IR spectroscopy [17]. However, it should be noted when using array detectors that the light intensity per element is lower, along with increased noise inherent in the FPA detectors, which can result in longer exposure times needed to record sufficient signal-to-noise spectra for each pixel in an image [17,18]. As a result, some researchers have utilized synchrotron radiation which is on the order of 100–1000 times brighter and produces less noise than the globar sources [19], while the high level of collimation improves both spectral and spatial resolution [17,19]. Although synchrotron radiation provides a number of benefits for IR imaging, this approach is limited to a small number of facilities worldwide. Several alternative light sources that can provide greater flux for standard laboratory-based systems have been investigated, but perhaps the technology with the greatest potential is that of Quantum Cascade Lasers (QCLs). QCLs provide mid- to near-IR, narrow band, high intensity light [17] and are small enough for benchtop operation. Single wavelength QCLs can provide fast imaging at a single wavenumber, while tuneable QCLs can produce multispectral/hyperspectral images, albeit with an increase in time needed to step the emission of the laser [18]. In recent years the use of single wavelength and tuneable wavelength QCLs for imaging selected regions of the IR spectrum for biological samples has been demonstrated [e.g. [20–23]] and while these studies have not included applications to microorganisms, they demonstrate the potential for higher resolution imaging capabilities.

Attenuated total reflection (ATR) can also be employed in conjunction with FTIR imaging instrumentation. In an ATR experiment, in the form of an evanescent light wave, penetrates a certain depth into the sample, typically 0.2–5 μm. This means that the ATR-FTIR spectra generated are independent of sample thickness in that they originate from a small layer on top of the sample. The same holds for when ATR elements are used for imaging, but it should be noted that while conventional (single-point) ATR experiments may employ multiple reflections within the ATR element, imaging requires that this be limited to a single reflection [24]. To date, only a few studies have used ATR-based infrared spectroscopy for the study of microorganisms (discussed in the IR applications section of this paper) but detailed information on the use of ATR in imaging, and examples of other biological applications can be found in [24].

For Raman imaging instrumentation, in this case limiting the definition to building an image using the contrast mechanism of spontaneous Raman scattering, there has been a steady progression in capabilities. From first single point measurements, to a series of point measurements mapped over spatial regions with increasingly fine spacing, Raman measurements have evolved into a full imaging technique over several decades. Early attempts at Raman imaging started around the 1970s [25], with some significant advances in the 1990s [26]. With the increased availability of 2D detectors with relatively large area, high pixel count, low noise and quantum efficiencies near 90%, the parallel acquisition of large numbers of spectra became possible, which could then reduce imaging time from hours to minutes [27].

The choice of excitation wavelength is critical for Raman imaging, and was originally limited by the availability of lasers. Currently, with an expanded choice of laser sources, the excitation wavelength is usually between ~244 nm and ~1064 nm. Due to the low signals inherent in Raman measurements, the wavelength is often chosen to optimize measurement of a particular sample of interest. Shorter wavelengths, especially 488 and 532 nm, can produce stronger Raman signals and can generate resonant Raman scattering (depending on the sample), but often produce fluorescence which overlaps with the detection of the Raman signals. The potential for sample damage also needs to be managed carefully with shorter wavelengths. As the wavelength increases, the fluorescence is usually reduced, and sample damage can be reduced, but the Raman scattering probability itself reduces with the 4th power of the wavelength, so that higher powers and longer exposure times are often needed to compensate for the lower efficiency of Raman scattering. Even with these issues, samples that produce strong Raman signals can be measured with a range of different excitation wavelengths and generating a library of spectra where different molecular features of the sample dominate individual spectra, so the ability to measure the same sample