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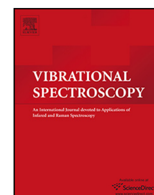

Multimodal vibrational imaging of cells

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ABSTRACT

Fourier Transform Infrared (FTIR) and Raman imaging offer complementary information about the spatial location of molecules within cells. In this paper we investigate the integration of both imaging modalities in an extended image containing unique FTIR and Raman spectra for each pixel. Two types of cells were investigated: red blood cells infected with the *Plasmodium falciparum* parasite and *Micrasterias*, a desmid microalgal species. The microscope configuration and pixel size were selected specifically for acquiring images with the same pixel size, and samples were fixed to a sample holder in order to measure the same cells with the same orientation. Images were first analysed individually and then registered in an extended matrix containing an FTIR and a Raman spectra for each pixel. The results indicated that the combination of both techniques provide complementary information not evident in the analysis of individual images. The assignment of haemozoin FTIR bands from malaria trophozoites was only possible after correlating the FTIR spectrum with the Raman spectrum. The correlation of the Raman and FTIR spectral variables using statistical heterospectroscopy (SHY) enabled the assignment of overlapping lipid and carbohydrate bands. In summary, although the approach can be computing intensive the complementarity of the two techniques in terms of pixel resolution, signal to noise ratio and the assignment of vibrational modes makes this a powerful approach to diagnostic imaging.
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1. Introduction

Fourier Transform Infrared (FTIR) and Raman spectroscopy (RS) are powerful techniques for investigating the composition and molecular structure of biological materials [1]. Both techniques rely on the detection of molecular vibrations providing a phenotypic fingerprint of the general metabolome [2,3], which includes changes in the structure and/or oxidation state of lipids [4], changes in the secondary structure of the proteins [5] and DNA conformation [6]. In recent years, the development of FTIR and Raman based methods in biological analysis has become an expanding field of research [7], driven by recent improvements in instrumentation and advanced multivariate data analysis strategies [8,9]. Both Raman and FTIR spectrometers can be integrated into optical microscopes enabling imaging of single cells [10].

Infrared spectroscopy is based on the absorption of the FTIR light from oscillating nuclei when the wavenumber value of the incident radiation is the same as the oscillating nuclei or group transitional energy [11]. The Raman effect, on the other hand, is observed after illuminating the sample with an ultra violet (UV), visible (VIS) or near-IR (NIR) and monitoring the inelastic scattered light component [12].

The fundamental differences in the mechanism of the two techniques results in dissimilarities in the properties and features of each technique. For instance: i) IR absorption results from changes in the dipole moment as the nuclei oscillate, and therefore asymmetric and polar molecules show strong IR activity compared to more symmetric non-polar molecules. In contrast, Raman activity is related to changes in the polarizability tensor, of the functional group or molecule and in general only symmetric modes and non-polar oscillating nuclei are strong Raman scatterers. ii) The Raman efficiency is quite low compared with FTIR unless resonance or surface enhanced Raman scattering mechanisms are involved. In IR, almost all organic molecules show considerable absorbance, and the spectra are normally composed of many

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overlapping bands especially when recording FTIR spectra of cells or tissues. iii) Raman can utilise visible laser light, which enables a lateral resolution of under half a micron to be achieved compared with the low resolution (around 2–10 μm depending on the wavelength) provided by the IR light. iv) Liquid water is a strong absorber of IR light and generating a spectrum of hydrated cells without a bright IR source is indeed challenging [13]. In contrast, water shows negligible Raman scattering and consequently the technique is eminently suitable for the analysis of living cells providing the laser light is of minimal power to avoid cell damage. v) Focal plane array (FPA) FTIR imaging enables the study of larger and more representative areas of cells or tissues in reasonable time, whereas with RS only small areas can only be analysed $\sim 20 \times 20$ μm unless under-sampling is employed. This can be a problem when the area under investigation is not necessarily representative of the sample, *e.g.* for a small area in a whole tissue section, but they do allow a more detailed insight into regions of interest. Due to the small number of inelastically scattered photons in a Raman experiment only a poor S/N ratio is usually achieved especially for non-chomophoric asymmetric type molecules. This necessitates longer collection times, especially in the case of thin (<1 μm) biological materials. vi) Although both, FTIR and Raman, are considered to be non-destructive, in reality imaging *via* RS is achieved using intense VIS, UV and NIR laser excitation sources The use of such intense light for excitation of the sample can result in localised thermal heating and photo-decomposition. The level of damage depends on the type of sample being interrogated, the laser power and the acquisition time per spectrum [14]. By decreasing the laser power or reducing the integration time will reduce the signal-to-noise ratio and therefore care must be taken to ensure adequate S/N without photodecomposition or burning.

The complementarity of the information obtained from both techniques makes their combination appealing. Several studies have compared the results of FTIR and RS for the analysis of tissues and cells. Nallala et al. [15] characterized colon tissue with both techniques and concluded that ATR-microscopy produced the best combination of imaging contrast and speed of analysis. Brain tumours have been investigated by Meyer et al. [16] employing non-linear techniques which were validated with classical FTIR and RS. Other examples includes the combination of Raman and FTIR for studying bone tissue [17], atherosclerotic plaque [18], breast cancer tissue [19], liver rate tissue [20], pollen [21] and oocytes [22]. In general, those studies combine images from different samples, different sections of the samples, or the same section but using different resolutions. The most logical approach according to the lateral resolution of each sample is normally to take a large area with the FTIR image and studying specific small areas with Raman microscopy [23].

Here we propose a novel approach that enables the co-registration of FTIR and Raman images obtained with the same pixel size over the same section in order to create an extended hyperspectral image containing both, a FTIR and Raman spectrum for each pixel. The enormous differences in features and acquisition modes makes multimodal imaging based on a pixel to pixel registration very challenging. This necessitates measuring exactly the same region of the sample with both techniques using the same pixel size and overcoming the dissimilarities in spatial resolution by over- or under- sampling. This approach of combining image data is known as multimodal imaging and can be applied to study spatial and spectral correlation using data fusion methods.

Extracting information from the large amount of spectral data contained in a concatenated Raman and FTIR image is another challenging issue. Vibrational spectra are already complex *per se*. They contain the complexities of the composition of biological materials and the intricate optical interactions between light and matter. Combining the two different data sources adds greater complexity to the system, but selected data analysis methodologies can provide biologically meaningful interpretations from the data amalgam.

In this study of complex biological materials, data from complementary sources can be jointly analysed to obtain a comprehensive understanding of the system [24] and can help in the study or diagnosis of various illnesses [25]. Correlation studies among data from different sources range from univariate to multivariate methods [26]. Some examples of data fusion methodologies are hierarchical Principal Component Analysis (PCA) [27], Multiblock Partial Least Squares (MBPLS) [28] or Joint and Individual Variation Explained (JIVE) [29]. In this paper we used statistical heterospectroscopy, (SHY) which analyses the covariance between signals measured across the same set of samples, facilitating the identification of associations between different sets of variables and thus improving the efficiency of biomarker identification [30].

We tested the multimodal imaging over two common cell types; malaria infected red blood cells and algal cells. Red blood cells (RBCs) infected with the malaria-causing parasite *Plasmodium falciparum* have been widely studied with Raman imaging [31,32] but to a lesser extent with FTIR [33,34] due to limitations in spatial resolution (~6–8 μm diameter). To complement these studies, we also assessed the utility of these two methods using *Micrasterias,* which is a genus of large microalgae (~200 μm diameter). This size makes them ideal for study by FTIR spectroscopy [35,36] but there is no literature on the use of RS.

The aim of this study is to investigate the benefits and challenges of multimodal vibrational imaging of cells, defining multimodal images as hyperspectral images where each pixel is characterized by generating FTIR and Raman spectra. The differences between the analysis of the individual images and the analysis of the merged image will be considered, and the question addressed as to whether the fusion of vibrational modes is more informative than the analysis of the individual bands from the one technique.

2. Experimental and methods

2.1. Sample preparation

Blood was collected into lithium heparin vacutainers (BD) from donors at Burnet Institute, then centrifuged at 1500 r.p.m. for 5 min and the plasma removed. These RBCs were then used for *in-vitro* culture of *Plasmodium falciparum* parasites (laboratory strain D10) at 3% haematocrit in RPMI media supplemented with 10% AlbuMAX II (Gibco). Once a synchronised population of trophozoite-stage parasites was obtained at a parasitemia of 5%, these infected RBCs were centrifuged to remove culture media and reconstituted to a physiological haematocrit using human plasma. Thin smears of infected RBCs were prepared on CaF_2 slides, air-dried, fixed in methanol for 5 min and then allowed to air dry again. After FTIR and Raman spectral acquisition, the cells were then stained with 10% Giemsa stain for 5 min and rinsed with distilled water and allowed to air-dry. Giemsa stain is typically used in the staining of marrow and blood smears, differentially colouring biological components. Giemsa stains DNA varying shades of purple, while the rest of the cell is stained light pink to blue which clearly highlights the presence of the plasmodium parasite in RBCs [37]. Visual images were taken on a Leica DM4000 UV–vis microscope using the Visible/NIR light source in transmission mode through a quartz fibre optic with an 80 × objective lens.

An axenic culture of *Micrasterias hardyi* (strain CCAP649/15) was obtained from Sam Research Services Ltd., Dustaffnage Marine Laboratory, Oban, Scotland. Cells were inoculated into complete JM

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