Vibrational Spectroscopy 60 (2012) 34–42

Contents lists available at SciVerse ScienceDirect

Vibrational Spectroscopy

journal homepage: www.elsevier.com/locate/vibspec

Label-free non-destructive *in situ* biochemical analysis of nematode *Steinernema kraussei* using FPA-FTIR and Raman spectroscopic imaging

Katherine Lau[a,*,1], Alison Hobro[b,1], Tim Smith[a], Thomas Thurston[a], Bernard Lendl[b]

[a] *Spectroscopy Product Division, Renishaw plc, Old Town, Wotton-Under-Edge, Gloucestershire GL12 7DW, England, United Kingdom*
[b] *Institute of Chemical Technologies and Analytics, Vienna University of Technology, Getreidemarkt 9/164-AC, A-1060 Vienna, Austria*

ARTICLE INFO

Article history:
Received 9 September 2011
Accepted 16 January 2012
Available online 25 January 2012

Keywords:
FTIR imaging
FPA
Raman imaging
Nematode
Model organism
Steinernema kraussei

ABSTRACT

Here we present Fourier transform infrared (FTIR) and Raman images of *Steinernema kraussei* nematode worms and, in conjunction with chemometric analysis. We also distinguished the biochemical differences associated with different regions of these images. The nematodes are complex, multicellular organisms that have a simple, but defined body plan with distinct digestive, muscular and reproductive systems. As such, nematodes have often been used as model organisms for the study of biological processes in higher organisms. We show that FTIR spectra, collected in transmission mode, contain information from the entire thickness of the nematode, but still exhibit biochemical differences reflecting different tissues or anatomical structures, such as the digestive tract. Raman spectroscopy, in contrast, can be used to investigate the distribution of biological molecules on one plane within a constrained depth of the nematode, allowing imaging of fine details within the body of the worm, including the distribution of lipid, protein and collagen. Together these vibrational spectroscopic techniques provide complementary, non-invasive and label-free information on the spatial distribution of biomolecules within multicellular organisms and, therefore, have potential future applications in developmental studies of such model organisms.

1. Introduction

Genetically amenable multicellular organisms have been instrumental in elucidating gene function and regulation, cellular organisation and the developmental stages from embryo to adult. Multicellular organisms are evolutionarily highly conserved. Simple multi-cellular organisms contain direct or similar orthologues to developmental processes in humans. They also have faster life cycles and reproduce more rapidly compared to higher organisms, allowing studies of development and metabolism on realistic timescales. According to Nobel Laureate Sydney Brenner, the nematode *Caenorhabditis elegans* is, "the simplest differentiated organism". Since Brenner proposed its use as a model organism in the 1960s, *C. elegans* has been widely adopted as a model for understanding gene functions [1] and gene–protein interaction, as well as analogues of human diseases [2]. In this study on the nematode species *Steinernema kraussei*, we explore the use of FTIR and Raman spectroscopies as non-invasive and label-free techniques to provide *in situ* biochemical information within the nematode.

Genetic manipulation can result in phenotypic changes, which are currently studied by techniques that are either invasive or involve labelling [3]. Although invasive techniques such as quantitative polymerase chain reaction [1], Western blotting [1] and mass spectrometry, can provide very specific information, spatial information of the bio-molecules in the organism is lost. Labelling can retain spatial information, but requires *a priori* knowledge. Through the use of confocal laser scanning microscopy, 3D non-invasive examination can be achieved [3]. Fluorescent labels, in this case, can be applied to visualise the presence and locality of target molecules [3]. However, the number of labels applicable to an organism is limited, since fluorescent bands are broad and overlap in the electromagnetic spectrum.

FTIR and Raman spectroscopies have been applied to a wide range of cellular and tissue studies [4–8], as well as microbes [9]. Vibrational spectroscopy gathers information on the biomolecules present in biological samples based on their vibrational characteristics. FTIR spectroscopy measures the infrared (IR) radiation of the mid-IR range absorbed by the sample. Raman spectroscopy measures the Raman scattering of the volume illuminated by the laser. Through measuring the vibrational modes associated with the molecules present in defined regions of a sample, and reorganising the data into a hyper spectral data cube, biochemical contents can be presented in a spatial manner. Vibrational spectroscopic techniques are label-free, and prior knowledge of the sample is

* Corresponding author. Tel.: +44 1453 532935; fax: +44 1453 523901.
E-mail address: katherine.lau@renishaw.com (K. Lau).
[1] Equal contribution to this work.

0924-2031/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
doi:10.1016/j.vibspec.2012.01.009

not necessary as the full range of chemical information is recorded simultaneously.

Hitherto, FTIR has been used to differentiate nematode species based on single point spectra [10,11] and, recently, FTIR imaging has been used to study the distribution of biomolecules within nematodes [12]. Previous spontaneous Raman scattering studies, however, have generally relied on the presence of dye molecules, such as nile red, incorporated into the nematode, localising within particular tissues [13], or have concentrated on specific molecules within the nematode, which are strong Raman scatterers, such as S8 sulfur [14].

FTIR and Raman imaging are often used together as they provide complementary and insightful information. This study shows that FTIR and Raman spectroscopies can be applied to nematode species *S. kraussei* to reveal chemical structural contents over the entire organism length, in a non-invasive and label-free manner.

2. Materials and methods

2.1. Material and sample preparation for spectroscopic imaging

Living *S. kraussei* was purchased from a garden centre and came in a water soluble gelatinous substance. The nematodes are typically approximately 20 μm in diameter and 80–100 μm in length.

For FTIR imaging, a small spatula of nematodes was rehydrated in 1 ml of distilled water in an Eppendorf tube. The suspension was mixed thoroughly, centrifuged and the supernatant removed. The resultant pellet was re-suspended in water and the process repeated twice to ensure any media from the dried nematode preparation was removed from the rehydrated nematodes prior to analysis. After the final centrifugation the pellet was re-suspended in water and allowed to stand for approximately 20 min before opening the tube carefully (to prevent disturbing any debris that may have settled to the bottom). Finally, a small volume of the supernatant containing the nematodes was spotted onto a mid-infrared (IR) transparent ZnSe plate. As the water evaporated the nematodes adhered to the ZnSe slide and were therefore immobilised prior to the IR measurements.

For Raman imaging, a small spatula of *S. kraussei* nematodes was rehydrated using distilled water in a 10 ml beaker and washed by increasing the dilution. A small volume of single nematodes in water was pipetted onto a quartz slide and separated by a small brush. The nematodes were dehydrated and immobilised on the quartz surface. The sample was immersed in 0.9% NaCl solution for Raman analysis.

2.2. Methods

2.2.1. FPA-FTIR imaging

IR images of the nematode were collected using a Bruker Hyperion 3000 IR microscope (Bruker, USA), attached to a Tensor 37 FT-IR spectrometer, operating in focal plane array (FPA) imaging mode. A 15× objective was used. The image spectra were collected over the spectral range 900–3400 cm^{-1}, at 4 cm^{-1} resolution. Each spectrum was a result of 128 accumulated scans. The spectra were recorded using an FPA detector with 64 × 64 pixels covering an area of 170 μm × 170 μm (approx 2.7 μm × 2.37 μm per pixel). For an area covering a whole nematode, the image presented here is a composite of 2 × 3 FPA images. The spatial resolution in FTIR spectroscopy is wavelength dependent. For the mid-IR range it ranges from approximately 3 μm to 10 μm. All image spectra were collected as absorbance spectra in transmission mode. A background spectrum was collected from the ZnSe plate outside of the areas with the nematode suspension, prior to recording the nematode images.

2.2.2. FPA-FTIR data processing and analysis

Spectra were corrected for Mie scattering effects using a correction algorithm [15] operating in Matlab R2010b (MathWorks, USA), prior to spectral analysis. The non-resonant Mie scattering correction option, based on Ref. [16] was applied using a matrigel reference spectrum, and 8 principal components (PCs). The size of the scattering particle was assumed to be 2–18 μm and the refractive index was assumed to be 1.1–1.5. These settings have been shown to provide effective scattering correction for FTIR spectra of nematodes [12]. Once corrected, the image was cropped in order to remove pixels recorded from outside the worm, using the MIA toolbox (Eigenvector Research Inc., WA, USA) operating in Matlab.

All IR image reconstruction, display and cluster analyses were performed in CytoSpec© software (http://www.cytospec.com/). Two data processing steps were performed on the non-resonant Mie scattering corrected data prior to cluster analysis. Initially, the nematode spectra were selected as the 'region of interest' and spectra originating from the ZnSe slide or cellular debris were therefore omitted from further analysis. Subsequently, the IR spectra in the 'region of interest' were transformed into second derivative spectra (using an 11 point smoothing function). Hierarchical cluster analysis (HCA) was limited to the spectral ranges between 1000–1770 cm^{-1} and 2800–3000 cm^{-1}. HCA was performed using the *D*-value distance method and the Ward's algorithm cluster method. This combination has been shown to provide a good correlation between spectroscopic and histological data [7]. In this paper the appropriate number of clusters was defined according to the dendrogram, using the minimum number of clusters required to explain the biological condition, where the standard deviation of each cluster was low.

2.2.3. Raman microspectroscopic mapping

Raman map data of the entire worm, the middle section, and the tail section of the same organism, were collected using an inVia Reflex Raman Microscope (Renishaw plc, Wotton-Under-Edge, Gloucestershire, England). A 532 nm laser source and 2400 l/mm grating were used, giving better than 1 cm^{-1} spectral resolution. A 50× water immersion objective NA 0.75 (Nikon) was used resulting in sub-micrometre lateral resolution. The spectral range was approximately 750–1700 cm^{-1}. The map collected over the entire length of the nematode was obtained using StreamLine Plus™ Raman imaging, which uses a line focus. Using a line focus geometry enabled multiple spectra to be collected simultaneously and power density was thus minimised, preventing photothermal degradation. For the full organism map the step size was 1.2 μm in both *x* and *y* directions. The mapping completed in just over 60 min.

The maps collected from the middle and tail sections were obtained using StreamLineHR™ imaging, which is a high spatial resolution mode using a spot focus with spatial over-sampling. The step size was 0.5 μm in both *x* and *y* directions. The tail section map took approximately 45 min to complete. The middle section map took under 6 h to complete.

2.2.4. Raman data analysis

The Raman data were analysed using principal component analysis (PCA). PCA looks for the most significant trends in the data set. The data volume is rotated in such a way that the first PC explains the largest possible proportion of the variance in the data matrix. The second PC explains the largest possible proportion of the residual variance in the data matrix that is not explained by the first PC. Subsequent PCs continue to explain the largest possible proportion of the residual variance not accounted for by earlier components. The full length nematode Raman data set was imported to Matlab R2010b (MathWorks, USA) and analysed using an in-house script. The mid and tail section maps were analysed using WiRE 3 software (Renishaw plc, Wotton-Under-Edge, Gloucestershire, UK). In