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Label-free Raman characterization of bacteria calls for standardized procedures

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ABSTRACT

Raman spectroscopy has gained relevance in single-cell microbiology for its ability to detect bacterial (sub) populations in a non-destructive and label-free way. However, the Raman spectrum of a bacterium can be heavily affected by abiotic factors, which may influence the interpretation of experimental results. Additionally, there is no publicly available standard for the annotation of metadata describing sample preparation and acquisition of Raman spectra. This article explores the importance of sample manipulations when measuring bacterial subpopulations using Raman spectroscopy. Based on the results of this study and previous findings in literature we propose a Raman metadata standard that incorporates the minimum information that is required to be reported in order to correctly interpret data from Raman spectroscopy experiments. Its aim is twofold: 1) mitigate technical noise due to sample preparation and manipulation and 2) improve reproducibility in Raman spectroscopy experiments studying microbial communities.

1. Introduction

Single-cell technologies have been proposed to observe and characterize phenotypic heterogeneity (Davis and Isberg, 2016). For example, flow cytometry offers high throughput measurements and the possibility to employ numerous dyes that can be used to characterize bacteria (Ambriz-Avina et al., 2014). Imaging techniques can be used to detect gene expression (Ceuppens et al., 2013; Li et al., 2008). Spectroscopy methods, such as Fourier-transform infrared spectroscopy (FT-IR) or Raman spectroscopy are also used to identify bacteria subpopulations (Athamneh et al., 2014; Wehrli et al., 2014).

Raman spectroscopy is an advantageous technology as it can be used without labelling the sample, is rapid and non-destructive, allowing to keep the bacteria alive after the analysis. It detects the inelastic scattering of the molecules present in the sample, resulting in a molecular fingerprint that gives information about lipids, carbohydrates, proteins and nucleic acid content of the bacteria (Huang et al., 2010). With this information, both the structure and metabolic state of individual cells, bacterial species, subspecies and phenotypes can be identified (Davis and Isberg, 2016; Lorenz et al., 2017). The potential of Raman spectroscopy to identify bacteria has aroused interest of the medical, pharmaceutical and defense field (Hakonen et al., 2015; Neugebauer et al., 2015).

The Raman signal is weak – it is estimated that only 1 in 10^8 incident photons are Raman scattered (Jarvis and Goodacre, 2004). To enhance the signal, bacteria can be labelled (i.e. deuterium or isotope probing) and techniques such as Surface Enhanced Raman Spectroscopy (SERS) can be used (Berry et al., 2015; Taylor et al., 2017). However, the signal-to-noise ratio might be too low in unlabelled samples to detect biologically relevant information. Especially when measuring phenotypes in unlabelled samples, this noise could mislead in the result interpretation. It is known from literature that parameters such as laser power, acquisition time and fixation can affect the Raman spectra. While progress has been made towards standardization (Butler et al., 2016, Chen et al., 2014, Guo et al., 2017, Hutsebaut et al., 2005, Rodriguez et al., 2011), there is currently no general protocol available on how to optimally handle bacterial cells for the purpose of identification of subpopulations using a label-free Raman approach. Neither is there a publicly available standard for the annotation of metadata describing the acquisition of Raman spectra.

This study outlines standardization of label-free bacterial phenotypic identification and investigates the impact of sample

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manipulations on the analysis of Raman spectra. The impact of technical manipulations in the spectra (i.e., the effect of storage time, the time on the slide or the influence of different centrifugation and resuspension steps) was evaluated using different multivariate statistical techniques, with and without prior knowledge of sample manipulations (i.e., using supervised or unsupervised methods). We show that these manipulations induced 'phenotypes' that had no biological relevance, but were identified as separate groups in both the supervised and unsupervised setting. To assist researchers with the annotation of metadata, we combined our results with existing literature on Raman standardization and created a Raman metadata recording tool.

2. Materials and methods

2.1. Inducing phenotypes with different media

Escherichia coli DSM 2092 was grown in Nutrient Broth (NB, Oxoid) or in Luria Bertani broth (LB, Oxoid) in a shaking incubator at 120 rpm at 28 °C. Cells were harvested in the stationary phase. To determine the stationary phase, 10^6 cells/mL were inoculated in the media and samples were incubated in the dark for 30 h at 28 °C, during which optical density measurements were automatically collected each hour using a microtiter plate reader (OD, $\lambda = 620$ nm, Tecan Infinite M200 Pro; Tecan UK, Reading, United Kingdom). The growth phases were visually determined after plotting OD over time. The stationary phase was reached in both cultures after 24 h, with a final concentration of approximately 10^8 cells/mL. Three replicates of the cell culture were analyzed for each condition (LB or NB media).

2.2. General fixation procedure

After the cultures reached the stationary phase (24 h), bacteria were fixed in 4% formaldehyde (Sigma- Aldrich) dissolved in PBS (protocol from Bio-Techno Ltd. Belgium). Formaldehyde was chosen as fixation method to preserve the physical characteristics of the cell (Read and Whiteley, 2015). First, 1 mL of the cell suspension was centrifuged for 5 min at room temperature and 1957 ×*g*. The supernatant was discarded and cells were suspended in filtered and cold (4 °C) phosphate-buffered saline (PBS, Thermo-Fisher). The samples were again centrifuged at 1957 ×*g* for 5 min at room temperature. The supernatant was discarded and the pellet was resuspended in 0.2 μm filtered formaldehyde 4% (RC Minisart filter, Sigma-Aldrich). The cells were fixed for 1 h at room temperature. Subsequently, the samples were centrifuged at 1957 ×*g* for 5 min at room temperature and washed twice with equal volumes of cold PBS. Then, samples were resuspended in Milli-Q water (Merck-Millipore) and four 5 μL drops were put on the CaF_2 slide (grade 13 mm diameter by 0.5 mm polished disc, Crystran Ltd.) and allowed to dry until complete evaporation at room temperature. Samples were resuspended in 1 mL of PBS and stored at 4 °C.

2.3. The effect of storage time

To assess how many days bacteria can be stored without inducing changes in their Raman spectra, a sample grown in Luria Bertani (LB) and another sample grown in Nutrient Broth (NB) were harvested and fixed immediately (time 0 h) and measured on that day, after 5 days and after 12 days. They were resuspended in 100 μL of Milli-Q water and four 5 μL drops were put on the CaF_2 slide and allowed to dry until complete evaporation. After sampling, bacteria were resuspended in 1 mL of PBS and stored at 4 °C.

2.4. Time on the slide and centrifugation

To investigate the effect of the drying time on the slide of the sample, four 5 μL drops were dried on a CaF_2 slide for 15 min. The slide was kept at room temperature and measured again after 3 h and 6 h. One sample from this batch was centrifuged at 1957 ×*g* for 5 min and resuspended in 1 mL of PBS 6 six additional times.

An overview of the different technical manipulations is given in Table 1.

Table 1
Sample description.

Growth medium	Replicate number	Days stored	Time on slide	Cells analyzed	Centrifugations
LB*	1	0 days	0 h	45	Standard
		5 days	0 h	38	Standard
		12 days	0 h	39	Standard
NB*	1	0 days	0 h	45	Standard
		5 days	0 h	38	Standard
		12 days	0 h	39	Standard
LB	2	0 days	0 h	45	Standard
LB	3	0 days	0 h	44	Standard
NB	2	0 days	0 h	44	Standard
NB	3	0 days	0 h	45	Standard
LB*	4	0 days	0 h	40	Standard
			3 h	39	
			6 h	40	
		0 days	0 h	40	Extra centrifugations

Description of the samples produced for every condition. Replicates of the cell culture were made for bacteria grown in Luria Bertani (LB) and nutrient broth (NB). Different storage days, time on the slide and centrifugations were tested.

2.5. Raman spectroscopy

The spectra were measured with a WITec Alpha300R+ spectroscope using a 785 nm laser (Toptica). As a control for the instrument performance, a silica gel was measured with a grating of 600 –mm/g, with a 1 s time exposure and 10 accumulations. Laser power was also monitored to detect possible variations. Bacteria were measured with a grating of 300–mm/g, with a 40 s exposure time and 1 accumulation. More information on the Raman spectroscope and data collection is included in the Supplementary Information (see Table S1).

Three replicates of the cell culture were made for cells grown in in Luria Bertani (LB) or nutrient broth (NB). They are labelled as replicate 1, 2 and 3 respectively. The samples 'LB replicate 1' and 'NB replicate 3' were stored at 4 °C and analyzed after 5 and 12 days. The sample 'LB replicate 4' was spotted on a slide an measured after 3 h and 6 h. From the sample 'LB replicate 4' two aliquots were made: one was treated following our standard protocol (see 'General fixation procedure'), the second followed extra centrifugation steps.

2.6. Data preprocessing

The obtained spectra were imported as SPC files in R (R Foundation for Statistical Computing, version 3.4.4) (Team, 2015) for pre-processing and analysis. The GitHub package 'MicroRaman' was used (Kerckhof et al., 2018). First, the region between 600 and 1800 cm^{-1} that has most biological significance was selected using the *Hyperspec* package v0.98.20161118 (Beleites and Valter, 2017). Next, the baseline was estimated using the SNIP algorithm with ten iterations and corrected by subtraction. The data was also normalized using the area under the curve (AUC) algorithm. Both functions are implemented in the *MALDIquant* package v1.16.2 (Gibb and Korbinian, 2012).

Raw data can be found in the GitHub repository 'MicroRaman' (Kerckhof et al., 2018).

2.7. Multivariate analyses

To investigate the impact of technical manipulations in the Raman spectra, two analyses were performed. The first one in a supervised