**Spectral artefacts induced by moving targets in live hyperspectral stimulated Raman spectroscopy: the case of lipid droplets in yeast cells**

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**Abstract:**. In this study, we used stimulated Raman spectroscopy (SRS) microscopy to collect Raman signatures from live *Saccharomyces cerevisiae* cells in the spectral range 2804-3060cm-1 and 830-2000 cm-1 with a spectral resolution of 8 cm-1. To effect this, we tuned the pump beam to several distinct wavelengths and thus acquired a series of chemical maps in order to reconstruct SRS spectra based on the intensity of the pixels, an approach also referred as hyperspectral SRS (hsSRS). One of the advantages of hsSRS over spontaneous Raman is that it is not overtly plagued by fluorescence and so fluorescent samples like yeast can be analysed. We show however that Raman signatures acquired by this approach may be subject to spectral artefacts that manifest as drops in intensity of Raman signal due to the movement of lipid droplets (LDs) within the yeast cells. To overcome this issue, yeast cells were chemically fixed with 4% formaldehyde and no artefacts were observed in the Raman signatures acquired from ‘stationary’ samples. Our findings indicate that caution must be applied when analysing SRS signatures obtained through hsSRS from mobile LDs and/or any other moving target within a system, whether biological or not.

Keywords: Yeast, hyperspectral SRS, fixatives, live cell imaging, Raman

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**Highlights**

* The motion of lipid droplets (LDs) may introduce spectral artefacts into Raman signatures acquired from yeast cells via hyperspectral SRS in live cell imaging;
* Selecting a region of interest (ROI) that includes the path of any mobile element in the sample may decrease the spectral artefacts induced by the motion of LDs;
* Fixing the cells with formaldehyde leads to stationary LDs and therefore, no spectral artefacts;
* Spectral artefacts reported in this study may be expected to any other system with a moving target whether biological or not;

**Graphical abstract**



1. **Introduction**

In spite of the use of microorganisms by humans throughout the different eras, it is only in the last few decades that modern biotechnology has provided the tools and strategies required to modify these microorganisms specifically for producing products of interest. Yeasts are a prime example of such microorganisms employed in ancient biotechnologies [1, 2]. They are unicellular fungi that are well known for their remarkable biodiversity, novel catabolic and anabolic capabilities, and broad range of industrial applications [3, 4]. *Saccharomyces cerevisiae* is perhaps one of the most extensively studied yeast species, with a variety of industrial applications including: production of enzymes and proteins [4, 5], fermented food and beverages [6], and other metabolites and chemicals with significant and wide-ranging health benefits [7-9]. Microbial conversion of renewable resources into biofuel and other chemicals represents another attractive application for such organisms, and an alternative to conventional petroleum refining processes, which are deemed as a major contributor to one of the main concerns of this century – global climate change [10]. Biodiesel, produced from triacylglycerols (TAGs) obtained mainly from plants, animals, and microorganisms, has attracted a lot of interest as a renewable and biodegradable energy source. Microbial cells are in fact considered the most desirable source of TAG due to their short life cycle, low production costs, and the fact that they are minimally affected by changes in the climate and seasons [11, 12]. Such demand and interest have resulted in the application of various genetic engineering strategies to enhance the production and accumulation of TAG in microorganisms [3]. Despite the fact that genomics and synthetic biology tools have successfully been applied for redesigning and engineering of these model organisms, nevertheless a much better understanding of the complex and fundamental biological processes at the cellular level is required to achieve a system that is widely applicable for industrial purposes [13, 14]. There are various analytical techniques available to study the lipid composition of these cells, including gas chromatography (GC) and liquid chromatography (LC), most commonly combined with mass spectrometry (MS) [15, 16], and of course a variety of well-established fluorescence staining techniques[17]. Although techniques such as GC-MS and LC-MS can provide detailed information on the lipid composition (lipidome) of the cells, they cannot provide evidence on the spatial distribution of these lipids inside the cell, and while fluorescence imaging can provide the latter, it lacks the required chemical information. These limitations may hamper the holistic understanding of a biological system. This is perhaps where Raman microspectroscopy shines best, as an alternative technique with high chemical specificity, providing spatially resolved information on the structure, abundance, and distribution of multiple biomolecules simultaneously, while being non-destructive and label free [18]. In a study by Huang *et al.* [19], spontaneous Raman imaging and coherent anti-Stokes Raman scattering (CARS) microscopy were employed in a time-lapse manner (~ 18 min resolution) to track cell division in *Schizosaccharomyces pombe* by monitoring changes in the spectral features of the cells, corresponding to various biomolecules in single cells. The authors provided evidence that the concentration and distribution of phospholipids and proteins in *S. pombe* are in fact linked to the cell cycle stage [19]. A recent study by Kochan and colleagues also used confocal Raman imaging to monitor cyclopropane fatty acids (CFA) production in various engineered *S. cerevisiae* strains and reported a significant reduction in the unsaturation of the detected lipid bodies in these modified strains [12]. Other examples include the identification of single yeast cells [20], accumulation of ethanol in yeast cells during aerobic fermentation [21], imaging the cell wall of living single yeast cells [22], and observation of the fungal infection of neutrophils [23]. These studies clearly demonstrated the potential applications of Raman spectroscopy for monitoring metabolic activity and concentration and distribution of various biomolecules at the single cell level. Although spontaneous Raman spectroscopy has been the most used Raman-based technique to analyse yeast samples, stimulated Raman spectroscopy (SRS) holds a number of advantages to study these microorganisms. These include higher sensitivity (SRS is a resonantly enhanced process), less susceptibility to fluorescence, and the fact that it is suitable for live cell imaging due to the video-rate acquisition speed, unlike spontaneous Raman that requires long acquisition time for imaging. The Raman signatures can be obtained from live cell imaging through SRS microscopy by tuning the pump beam to several distinct wavelengths, and thus acquiring a series of chemical maps in order to reconstruct the SRS spectra based on pixel intensities, also referred as hyperspectral SRS (hsSRS). Despite the advantages of this approach, acquiring SRS spectra from moving targets may be complicated due to the motion of the targets during the image collection. This is because in hsSRS the whole image is not generated immediately but rather ‘slices’ at specific pump-probe combinations (wavenumber shifts) are generated sequentially. Farkas *et al.* [24] used SRS microscopy to evaluate the fatty acid content in yeast by monitoring lipid droplets (LDs) within the cells through hsSRS. According to the authors, the image acquisition was performed in < 10 min per spectrum in order to avoid the motion of LDs, which was tracked throughout the course of the experiment by white-light transmitted images. Spectral artefacts induced by moving targets in live hsSRS may necessarily be observed in any system with a moving target whether biological or not. Thus, in order to obtain a better understanding of the effects of moving targets on the SRS spectra obtained by hsSRS, the present study aimed to investigate the spectral artefacts induced by the motion of lipid droplets (LDs) in yeast cells during 50 min scans, which is usually the time required to obtain information over the 700-3200 cm-1 spectral range.

1. **Materials and Methods**
   1. *Yeast strains and growth conditions*

*Saccharomyces cerevisiae* strain BY4741 was cultivated in YPG broth (1% w/v yeast extract, 2% w/v peptone and 2% w/v galactose) at 30°C with shaking for 48 h. Untreated/fresh cells were harvested by centrifugation at 5000 *g* for 5 min and resuspended in 0.9% w/v sodium chloride solution (physiological saline solution). Treated/chemically-fixed cells were treated with 4% w/v formaldehyde for 1 h at 30°C with shaking before being harvested and washed with 0.9% w/v sodium chloride solution.

* 1. *Sample preparation*

Yeast cells were transferred to a 2 mL microcentrifuge tube and centrifuged at 4°C for 4 min at 4000 *g* using a benchtop Eppendorf microcentrifuge 5424R (Eppendorf Ltd., Cambridge, U.K.). The supernatant was discarded and the biomass was washed by resuspending it in 2 mL of deionized water. After repeating this step three times to remove any residues from the medium, the biomass was resuspended in deionized water and 1 mL (OD600 = 15) was transferred to 35 mm glass-bottom culture dishes (Greiner Bio-one, U.K) coated with poly-L-lysine and allowed to settle for 15 min to attach the cells to the surface and thus to keep the cells stationary during SRS microscopy. Unattached cells were removed by gently washing the dish with deionized water. In the following, 1 mL of deionized water was added to keep the cells hydrated during data collection.

* 1. *SRS microscopy*

A picosecond one-box laser system (picoEMERALD™, APE GmbH) coupled to an inverted laser-scanning microscope (Leica TCS SP8, Leica Microsystems) was used to generate spatially and temporally overlapped (2 ps pulses with 10 cm-1 spectral width) Stokes (1031.4 nm) and tunable pump (700–990 nm) beams. Hyperspectral images over the high wavenumber region (3060-2804 cm-1) were recorded by tuning the pump laser frequency from 784-800 nm and from 855-950 nm to cover the fingerprint region (2000–830 cm-1) with step size of 0.5 nm and scan speed of 400 Hz. The pump and Stokes laser power were set to 15 and 30 mW, respectively, and a water immersion objective (25×, N.A. 0.95) was used to collect images from a field of view of 93.56 × 93.56 μmwith 512 × 512 pixels using a digital zoom of ×5, resulting in a pixel size of 0.183 × 0.183 µm. For time-series scan (See Supplemental Videos S1 and S2), the pump beam was tuned to 797 nm (2850 cm-1) and 2311 images were collected every 1.295 s. The 50 min scans were speeded up ~ 80 × to facilitate the visualisation of the movement of LDs within the cells, resulting in 38 s videos. The system was operated by Leica Application Suite X (LAS X).

* 1. *Data analysis*

Spectral data subjected to Principal Components Analysis (PCA) were pre-processed using MATLAB software version 2020a (Mathworks Inc., Natwick, USA). Spectra were submitted to baseline correction using Asymmetric Least Square algorithm, smoothed via Savitzky−Golay filter using a polynomial of second order in a 5-point window, vector normalized and mean-centered prior to PCA.

1. **Results and Discussion**

Fig. 1A illustrates the high wavenumber region (2800-3020 cm-1) of an SRS spectrum acquired from a single untreated yeast cell. This region is dominated by Raman signatures attributed to C−H stretching vibrations from lipids (2850 cm-1), proteins (2937 cm-1), and nucleic acids (2970 cm-1) [25]. Fig. 1B shows a single-frequency image obtained by tuning the OPO to the band associated to C−H stretching in CH3 groups from lipids and proteins (2936 cm-1) [26]. The whole cell is seen as these molecules are distributed throughout the cell. Fig. 1C was obtained by tuning the OPO to the band peaking at 2850 cm-1, which is attributed to C−H symmetric stretching vibrations of CH2 in lipids [26]. The organelles lighting up in the cytoplasm of the cells are LDs, which consist mainly of a core of neutral lipids (triacylglycerols (TAGs) and sterol esters (SEs)) enclosed in a monolayer of phospholipids as well as a series of trafficking proteins [27]. LDs play important roles in the dynamic of the cell including in regulation of metabolism, cell signalling, and can also be found in all eukaryotes and even some bacteria [28].



**Fig. 1.** Hyperspectral SRS imaging of yeast cells. (A) High wavenumber region (2800-3020 cm-1) of SRS spectrum acquired from a single yeast cell. (B,C) Single-frequency images obtained by tuning the OPO to the bands peaking at 2936 and 2850 cm-1, respectively. (Scale bar = 4 μm).

Panels A-F in Fig. 2 show time-lapse images of a yeast cell collected at 0, 10, 20, 30, 40, and 50 min. The images were obtained by tuning the OPO to the wavelength corresponding to the vibration peaking at 2850 cm-1 and single-frequency images were acquired every 1.295 s during 50 min in a time series scan (See Supplemental Video S1). The images clearly show LDs within the cell as well as the movement of these organelles during the scan. LDs can move freely within the cell in any direction including in and out of the image focus plane of the SRS. The apparent ‘random’ movement of the LDs causes fluctuations in the intensity of pixels but with no image artefacts due to the video-rate acquisition speed that each image is collected. However, moving LDs may induce spectral artefacts on the SRS signatures obtained by hsSRS, since it takes a few seconds to tune the pump beam to multiple wavelengths in order to collect the chemical maps that will ultimately provide information to reconstruct the SRS spectra based on the intensity of pixels.



**Fig. 2.** hsSRS chemical mapping of a single yeast cell highlighting the movement of LDs within the yeast cell. (A-F) SRS images showing LDs in live yeast cells collected at 2850 cm-1 every 10 minutes in a 50 min time series scan. (G) Variation in averaged pixel intensities within 7 regions of interest (ROIs) in a yeast cell (6 ROIs within the cell and 1 ROI containing the whole cell, highlighted in F). (Scale bar = 2 μm).

Fig. 2G illustrates the variation in the averaged pixel intensities within 6 regions of interest (ROIs) in a yeast cell as well as in a larger ROI containing the whole cell (Fig. 2F). The pixel intensities in ROIs within the cell fluctuate considerably during scanning due to the movement of LDs in and out of each ROI. On the other hand, relatively lower variations were observed in the ROI containing the whole cell, since the LDs do not move outside the cell as they are confined by the cell wall. Representative SRS spectra obtained from the 6 ROIs within the yeast cell (Fig. 3C-H) and ROI containing the whole cell (Fig. 3B) are displayed in Fig. 3.



**Fig. 3.** Image of a single yeast cell (A), highlighting 7 ROIs where SRS spectra were generated from the whole cell (B) and 6 sub-cellular locations (C-H). (Scale bar = 2 μm).

Spectral artefacts caused by the movement of LDs are seen as drops on Raman signal intensity, while shifts on peak position were not observed. The SRS spectrum acquired from ROI 3 (Fig. 3E) resembles the signatures obvserved in the ROI containing the whole cell (Fig. 3A), while the signatures acquired from other ROIs varied considerably. Spectral artefacts like these can easily lead to misinterpretation of the data, especially in situations where multivariate statistical methods are employed, since such techniques are able to identify small variations in the spectral signatures used as input to these algorithms. In general, caution must be taken when analyzing SRS signatures obtained through hsSRS from moving LDs and indeed any other moving target, since there is a risk that the spectral changes might be due to the motion of the targets rather than to changes in the biochemistry of the sample. Our findings show the importance of selecting the appropriate ROI to reconstruct SRS spectra on situations like these. Selecting a ROI that includes the whole moving path may decrease the spectral artefacts created by objects moving on XY axis; however, this does not apply if the target moves out of the image focus (Z axis).

The spectral artifacts shown in Fig. 3 arise mainly due to the long time required to scan the given spectral region while the LDs move within the cells. The SRS system used in the present study operates by sequential wavelength tuning, which features the slowest spectral-scanning speed compared to other approaches [29]. Typically, SRS systems with this configuration use two synchronized picosecond lasers to excite the sample, thereby acquiring Raman information on a specific Raman band. In order to acquire the following Raman peak, the pump beam is tuned to a different wavelenght. This process involves changing the nonlinear crystal temperature, electro-optical Lyot filter or resonator length of the laser, which is usually slow and susceptible to drifts on optical power and/or wavelength [29]. Over the last years, other SRS setups have been proposed with the aim of enhancing the sampling time and, consequently, improve the observation of dynamic events in a living system as well as to avoid spectral artefacts induced by the movement of subcellular structures. Another SRS configurations used to scan Raman frequency shifts in sequence includes spectral focusing and parallel multiplex excitation and detection (multiplex SRS) [29]. In spectral focusing, pulses from the two lasers are positively chirped by dispersion materials in order to obtain a linear distribution of spectral frequency on the time axis [29-31]. In this way, each temporal delay between the chirped pulses corresponds to a Raman shift and the Raman spectrum is retrieved by scanning the time delay between the two lasers by adjusting the two chirps [29, 32]. In multiplex SRS, a picosecond and femtosecond lasers are used to excite multiple Raman bands simultaneously as well as to apply a parallel-detection and multi-modulation strategy to retrieve the Raman shifts [29, 33, 34]. Despite the great advances in SRS technology and instrumentation, the acquisition speed for SRS image stack is still a challenge [29].

Fixing and thus imobilising the moving target may be a good alternative to avoid these issues. Therefore, yeast cells were chemically fixed with 4% formaldehyde in order to prevent the movement of LDs during hsSRS scan. Chemical fixation is a well known method used to preserve the structure of cellular components including their location within the cell and thus, it may prevent the spectral artefacts induced by moving targets. Formaldehyde induces denaturation of intracellular proteins through the formation of cross-links between the amide residues, resulting in changes in the conformational structure of proteins as well as a reduction in the total cell volume [35, 36]. Of course, once cells are fixed then one can not claim live cell imaging as the fixative kills the cell.

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**Fig. 4.** SRS chemical maps collected from a single formaldehyde-fixed yeast cell. (A-F) SRS images acquired at 2850 cm-1 in a 50 min time series scan. (G) Variation in averaged pixel intensities within 7 regions of interest (ROIs) in a yeast cell (6 ROIs within the cell and 1 ROI containing the whole cell). (H) SRS signatures obtained from each ROI. (Scale bar = 2 μm).

Panels A-F in Fig. 4 show SRS images acquired at 2850 cm-1, indicating stationary LDs within a yeast cell, even where data were acquired over a 50 min period after formaldehyde fixation. The averaged pixel intensities in all 7 ROIs (Fig. 4G) within the cell and containing the whole cell varies only slightly during these scans, unlike the results obtained for unfixed cells (Fig. 2G). This results in SRS spectra with no spectral artefacts due to the lack of moving targets within the yeast cell (Fig. 4H). These findings indicate that chemical fixation is a good solution to overcome the spectral artefacts induced by movment of LDs and indeed other subcellular targets in live imaging hsSRS. A video of fixed cells can be seen in Supplemental Video S2 where it is clear that the lipid droplets are now stationary and not moving within the yeast.



**Fig. 5.** (A)PCA scores plot of preprocessed SRS spectra acquired from 15 fixed and unfixed single yeast cells; values in parentheses represent total explained variance (TEV) percentage on each principal component (PC). (B) Raman signatures acquired from 4% formaldehyde solution (green), unfixed and formaldehyde-fixed yeast cells using SRS (blue and red) and spontaneous Raman spectroscopy (orange and purple); dashed line displays the loadings related to PC-1 in (A).

To the best of our knowledge, there is no study evaluating the effects of chemical fixatives to the Raman signatures of yeast cells. Therefore, SRS signatures acquired from 15 fixed and unfixed cells were subjected to PCA (Fig. 5). Satisfactory discrimination was observed along PC-1 axis which accounted for 70.5% of the variance with negative loadings to molecular vibrations associated to proteins (1666 and 2936 cm-1) from chemically-fixed yeast cells. These findings are expected due to the well known effects of formaldehyde on cellular proteins which have been documented by other studies evaluating the effects of chemical fixatives in mammalian cells [35]. Although formaldehyde has strong peaks in the Raman spectrum (as observed through spontaneous Raman spectroscopy (green line, Fig. 5B)), no signatures from formaldehyde were observed in the Raman spectra collected from fixed cells, indicating that there was minimal influence of fixative residuals directly.

1. **Conclusions**

In this study, SRS microscopy was used to collect Raman signatures from yeast cells via hsSRS. This approach has higher sensitivity and it is less affected by fluorescence compared to spontaneous Raman spectroscopy. We have shown that Raman signatures acquired through hsSRS are susceptible to spectral artefacts induced by the movement of lipid droplets within yeast cells. These are seen as variations in pixel intensities within series of chemical maps that are collected in order to reconstruct SRS signatures. We found that selecting a ROI that include the whole cell may decrease the spectral artefacts created by moving LDs; however, this means that highly accurate sub-cellular Raman chemical mapping is no longer possible. By contrast we show that collecting data from stationary samples avoids these issues and we recommend this as an alternative means of generating sub-cellular images within eukaryotic cells. Our findings indicate that formaldehyde fixation is a good solution for the case of LDs within *S. cerevisiae* cells. Although the spectral artefacts reported in this study were observed in yeast cells due to the motion of LDs, similar issues may be expected in any other system where there may be movement of biochemical or chemical targets.

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