

Chapter

Detecting phenotypically resistant *Mycobacterium tuberculosis* using wavelength modulated Raman spectroscopy.

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Summary

Raman spectroscopy is a non-destructive and label-free technique. Wavelength modulated Raman (WMR) spectroscopy was applied to investigate *Mycobacterium tuberculosis* cell state, lipid rich (LR) and lipid poor (LP). Compared to LP cells, LR cells can be up to 40 times more resistant to key antibiotic regimens. Using this methodology single lipid rich (LR) from lipid poor (LP) bacteria can be differentiated with both high sensitivity and specificity. It can also be used to investigate experimentally infected frozen tissue sections where both cell types can be differentiated. This methodology could be utilised to study the phenotype of mycobacterial cells in other tissues.

Key words: Raman spectroscopy, mycobacteria, phenotypic resistance, lipids.

1. Introduction

Tuberculosis is a major health issue worldwide and a major cause of death due to infectious disease. Treatment of tuberculosis has not improved in the past fifty years. Shortening therapy would make an important step forward to reducing the global burden of tuberculosis. Recent clinical trials using more bactericidal regimens to shorten TB therapy to 4 months failed to do so due to higher relapse rate after successful treatment (1-3). Those observations confirmed that relapse is the main barrier to shorter tuberculosis treatment. The bacteriology of relapse remains largely unknown and due to its importance represents a key research area in tuberculosis. Patients that clear *Mycobacterium tuberculosis* from their sputum rapidly during treatment can still undergo relapse (4). In order to improve our knowledge of relapse and its bacteriology, we need non-destructive methods to study bacteria directly at the site of the disease. Relapse could be linked to bacteria that survive therapy, and several studies have shown that mycobacteria accumulate lipid in intracellular bodies and these cells exhibit a lower metabolic rate (5-8). Recently lipid body positive mycobacteria were shown to be much more resistant to key components of the tuberculosis therapy, up to 40 times more resistant to rifampicin (9). Both phenotypes, lipid rich (LR) and lipid poor (LP), can be observed in any mycobacterial population in a range of species. To study the lipid content of *Mycobacterium tuberculosis*, we describe a novel method consisting of an all-optical label-free Raman spectroscopy based system that can be applied to bacteria directly in tissue.

Raman spectroscopy has been applied previously to discriminate cultured bacteria and mycobacteria species but never discriminate between two phenotypes or target mycobacteria in tissue (10-12). We use wavelength modulated Raman (WMR) spectroscopy to improve both sensitivity and specificity. Rather than using a single excitation wavelength, WMR spectroscopy scans over a small range of the laser wavelengths. Combined with subsequent multivariate statistical analysis, all background fluorescence from biological samples can be removed. Importantly, WMR spectroscopy is a label-free technology and can be therefore combined with other techniques such as immunostaining.

2. Materials

2.1. *In-vitro* investigation

- 2.1.1. Test organism in this case: *Mycobacterium tuberculosis* (NCTC7416).
- 2.1.2. Growth medium: Middlebrook 7H9 medium (Sigma-Aldrich, UK).
- 2.1.3. Glycerol (Sigma-Aldrich, UK).
- 2.1.4. Tween 80 (Fisher BioReagents, UK).
- 2.1.5. Middlebrook ADC enrichment (Sigma-Aldrich, UK).
- 2.1.6. Bacterial culture tube.
- 2.1.7. Incubator set at 37°C.
- 2.1.8. PBS.
- 2.1.9. Quartz coverslip (01015T-AB, SPI Supplies, PA, USA).
- 2.1.10. Quartz slide (01016-AB, SPI Supplies, PA, USA).

2.1.11. Transparent Nail polish.

2.2. Tissue investigation

2.2.1. Frozen infected tissue to investigate: in this example infected guinea pig (Specific pathogen-free Dunkin Hartley strain guinea pigs) lung sections.

2.2.2. Cryostat to produce 3 to 5 μ m thick frozen tissue sections (see **Note 1**).

2.2.3. Formalin solution.

2.2.4. Sucrose.

2.2.5. PBS.

2.2.6. Quartz slides both coverslip (01015T-AB, SPI Supplies, PA, USA).

2.2.7. Quartz slide (01016-AB, SPI Supplies, PA, USA).

2.2.8. Transparent Nail polish.

2.3. Raman system

An example of the Raman system design is presented in figure 1.

2.3.1. Tunable laser that can generate light at 785 nm (see **Note 2**).

2.3.2. Microscope objective (Nikon, 50x, oil immersion)

2.3.3. Spectrometer: formed with a monochromator (Andor Shamrock SR303i, 400 lines/mm grating @850nm) and a cooled CCD camera (Newton 920, Andor Technology, UK).

2.3.4. A computer linked to the Raman system and connected to the Internet.

2.3.5. Software list: Matlab, Andor Solis.

List of optics:

- 2.3.6. **LF**: Laser line filter (LL01-785, Semrock, USA).
- 2.3.7. **EF**: Edge filter (LPD02-785RU, Semrock, USA).
- 2.3.8. **NF**: Notch filter (NF03-785E, Semrock, USA)
- 2.3.9. **FM**: flip mirror.
- 2.3.10. **CCD₁**: Imaging Source USB camera (DFK 42AUC03, Imaging Source, Germany).
- 2.3.11. **CCD₂**: Andor Newton Camera (cooled at -70°C).
- 2.3.12. **L1~L6**: lenses; **F/#**: F number matcher.

[Fig 1 near here]

3. Methods

3.1. Bacteria from culture

3.1.1. Culture

Perform all steps before the heat killing of bacilli in a level 3 laboratory facility (or equivalent). Grow *M. tuberculosis* (NCTC7416) at 37°C in Middlebrook 7H9 medium supplemented with 0.05% tween 80 and 4mL of 50% glycerol (for 450mL). Add one vial of Middlebrook ADC supplement (M0678, FLUKA) to the 450mL.

3.1.2. Heat killing of bacilli

Harvest one millilitre of *M. tuberculosis* culture and place it at 80°C for 20 minutes to heat inactivate the bacteria. Take the inactivated bacteria out of the Cat3 facility (see **Note. 3**).

3.1.3. Wash

Take 100µL of bacterial suspension and spin it at 20,000 g for 3 minutes at room temperature. Discard the supernatant. Wash the cells twice with 100µL of PBS.

3.1.4. Raman slide preparation from *in-vitro* cell.

Resuspend the bacteria in 20µL of PBS, take 10µL out and heat fix onto a quartz coverslip (SPI Supplies). Mount the fixed bacteria that are on the coverslip, on top of a quartz slide (SPI Supplies). The cells end up between the quartz slides and coverslip. Seal mount using a transparent nail polish (leave to air dry for an hour before use). Interrogate the sample with WMR spectroscopy.

3.2. Tissue

3.2.1. Tissue

Treat the tissue to investigate with 10% neutral buffered formalin for an appropriate amount of time if it is infected with *M. tuberculosis* (or another pathogen). Remove the tissue from the formalin solution and freeze the tissue sample on a bed of dry ice

in OCT (optimal cutting temperature) solution (30% sucrose in PBS) for future Raman investigation. From the OCT block cut 3 to 5 μ m sections using a cryostat. Place the tissue section on the quartz coverslip.

3.2.2. Tissue section mounting

Mount the frozen tissue section that is on the quartz coverslip (SPI Supplies, 01015T-AB), directly on top of a quartz slide (SPI Supplies, 01016-AB). Seal the mount with transparent nail polish (leave to air dry for an hour before use). Interrogate the tissue sample with WMR spectroscopy. An example of single *M. tuberculosis* bacillus in guinea pig lung tissue section observed under the Raman system is shown in **Fig. 2** (see **Note 4**).

[Fig 2 near here]

3.3. Raman microscopy methods

3.3.1. Raman system

Use your Raman system to acquire the spectra, an example of a confocal Raman system shown in **Fig. 1**. This system uses a green laser (Verdi V6, 532nm, 5W) to pump a tunable Ti:Sapphire laser (Spectra-Physics 3900s, 785nm, 1W). To focus the laser on the bacteria use an oil immersion objective a Nikon, 50x, oil for example. A spectrometer with a monochromator (Andor Shamrock SR303i, 400 lines/mm grating @850nm) with a cooled CCD camera (Andor Newton) is used to collect the

Raman photons. Determine the laser power to use according to the sample type being investigated (see **Note 5**).

3.3.2. Acquisition

3.3.2.1. Standard Raman spectra

Use for each single bacteria a 30 seconds integration time with a stable excitation laser wavelength at 784.6nm. Record a separate background Raman spectrum with the same condition from a (bacteria-free position) position near the bacteria. Use it to subtract the background signals afterwards. Determine the integration time you need to obtain an optimal signal to noise ratio; this could vary depending on your system, the organism under investigation and the power on the sample plane (see **Note 6**).

3.3.2.2. WMR spectra

Record five spectra continuously, with 30 seconds of integration each, while the laser is tuning over a small range of 1nm. From these five original spectra calculate a single WMR spectrum with the auto-fluorescence background removed. Compare to the standard Raman spectrum; all Raman peaks will locate at the zero crossings while their peak intensity corresponds to the peak-to-valley value (see **Note 7**).

3.3.3. Raman Calibration and spectra processing

Use control Raman spectra from polystyrene beads (1 μ m in diameter) to monitor any possible drift in the laser wavelength or the optical system. Acquire the Raman spectra of the polystyrene beads with the same integration time as the experimental conditions.

The laser wavelength may vary during the experiments. We use a standard chemical, polystyrene, to monitor this variation. As the known largest peak position of polystyrene is at 1001.4 cm^{-1} , the actual laser wavelength can be calculated (**Note 8**).

If the drift in laser wavelength is very small (typically <0.2nm over a day) and slow, the actual laser line used to acquire each Raman spectrum can be calculated using an interpolation.

To avoid any influence from laser power fluctuation during wavelength tuning, normalise each Raman spectrum by its total intensity (i.e. the integration over the area covered by the spectrum). To compare your data sets and do the data processing use mainly the fingerprint region from 1000 cm^{-1} to 1800 cm^{-1} (see **Note 9**).

After Raman investigation, the tissue sample can be used for another method (see **Note 10**).

3.3.4. Principal component analysis

To distinguish between two different cell phenotypes or species, apply principal component analysis (PCA) to each training dataset containing standard Raman spectra or WMR spectra. Use approximately 60~80 cells for each phenotype or specie.

Use a number of principal components (PCs) that corresponds to more than 70% of variances in the training dataset. In this example, the first seven principal components (PCs) have been used (see **Note 11**).

3.3.5. Leave-one-out cross validation

Use the method of leave-one-out cross validation (LOOCV) to estimate the ability of classification for the different cell phenotypes or species. Without considering one Raman spectrum, a multiple-dimensional space is defined by all the PCs in the training data set. This leave-out spectrum is then classified to be a spectrum taken from either of your cell types based on its position in the multiple-dimensional space. With this LOOCV for each spectrum in the data set, the specificity and sensitivity for a data set containing two cell types are calculated (see **Note 11**).

4. Notes

1. Frozen tissue slicing

We have used 5 μ m thick formalin fixed, frozen tissue sections successfully. However, the tissue thickness can be adjusted according to the experiment. Commercial

companies can slice fixed, frozen tissue and mount onto quartz slides if cryostat equipment is not available.

2. Laser

A laser that can generate light at 785nm and its wavelength can be tuned over 1nm. For example, a tunable Ti:Sapphire laser (Spectra-Physics 3900s, 1W@785nm) pumped by a green laser (Coherent Verdi V6, 532nm, 5W) or a tunable Ti:Sa laser system (SolsTis M Squared lasers, 1W@785nm).

3. Heat killing

To heat kill mycobacteria prior to the transfer of material from level 3 containment to a lower level for analysis, one mL of cell culture (up to 10^8 cfu.mL⁻¹) is placed in a heat block for 20 minutes at 80°C. This protocol was validated as follows:

After heat killing, *M. tuberculosis* was plated onto 7H10 (Sigma-Aldrich, UK) supplemented with 0.05% glycerol (Sigma-Aldrich, UK) and 10% OADC (Sigma-Aldrich, UK) and no growth was observed after four weeks. Completion of this protocol allows transfer of fixed *M. tuberculosis* out of the containment level 3 (CL3) laboratory to be analysed at a lower containment level.

4. Preparation storage

Store tissue sections at -80°C before use. Once sliced and mounted onto quartz slides store the frozen tissue section preparation at -20°C between two experimental days.

5. Laser power adjustment

Adjust the power on the sample plane to obtain an optimal signal to noise ratio. The power used will depend on the sample being targeted. In the case of single mycobacterial cells, you can use 150mW on the sample plane. This condition does not produce any damage to the cells during the acquisition of the spectra. However, the integration time and laser power can be adjusted depending on the cell size, specie or if a group of bacteria is targeted instead of a single cell. It is important to optimise these parameters on the different organisms in advance.

6. Standard Raman acquisition

The acquisition time set to obtain a standard Raman spectrum is 30 seconds, six seconds five times accumulated. The laser wavelength is constant through the measure and set at 784.6nm. To produce a final spectrum, a background Raman spectrum is first acquired next to a single bacterium. Once the background is taken the signal from the single cell is recorded, and the background signal is subtracted afterwards. It takes 30 seconds to have the background signal and 30 seconds of acquisition to obtain the bacterial spectrum. So, in this case, it takes around one minute in total per single bacterium standard Raman spectrum.

7. WMR spectroscopy acquisition

During acquiring WMR spectra, each spectrum was taken at an integration time of 30 seconds that accumulates six seconds for five times. Five spectra were acquired continuously over 150 seconds when the laser was tuning over a range of 1nm around 785nm. So, in this case, it takes around two and half minutes in total per single bacterium to acquire a WMR spectrum.

8. Laser wavelength calibration

To calculate the laser wavelength using the known position of the main polystyrene peak ($\Delta\omega = 1001.4 \text{ cm}^{-1}$) use the following equation:

$$\Delta\omega(\text{cm}^{-1}) = \left(\frac{1}{\lambda_0(\text{nm})} - \frac{1}{\lambda_1(\text{nm})} \right) \times \frac{(10^7 \text{ nm})}{(\text{cm})}$$

Where $\Delta\omega$ corresponds to the Raman shift, λ_0 (nm) is the laser wavelength to calculate and λ_1 (nm) the measured wavelength of the main polystyrene peak in the Raman spectrum.

9. Raman spectra analysis

We observed that both standard and WMR spectroscopy could discriminate LR and LP cells. However, WMR spectroscopy brings both higher sensitivity and specificity

(13). The auto-fluorescence background is completely removed by the use of the modulation method showing the spectrum with only the Raman peaks and with the accurate ratio between peaks (14,15). LR mycobacteria display higher intensity in the Raman bands associated with lipids mainly at 1300 cm^{-1} and around 1440 to 1450 cm^{-1} . (Paper title: "Label-free optical vibrational spectroscopy to detect the metabolic state of *M. tuberculosis* cells at the site of disease" submitted)

10. Raman spectroscopy a label-free and non-destructive methodology

Raman spectroscopy is label-free and non-destructive; therefore the tissue sample investigated can be used again to perform another test such as immunostaining (cite our paper here). If the tissue is studied with a staining method, the sample can be damaged meaning that it cannot be re-used. However, Raman spectroscopy is an interesting method to study single bacteria both *in vitro* and in tissue especially if the sample is to be analysed further using another method.

11. PCA LOOCV and matlab

Additional information on PCA can be found in this article (16). We used Matlab to run the PCA and the LOOCV analysis (MathWorks, UK, version R2014b).

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Figure Caption:

Figure 1. Schematic diagram of experimental setup. **SF:** single-mode fibre; **MF:** multi-mode fibre (200µm core diameter); **LF:** Laser line filter (Semrock LL01-785); **EF:** Edge filter (Semrock LPD02-785RU); **NF:** Notch filter (Semrock NF03-785E); **TRITC/FITC:** Fluorescence Filter cubes (FITC: excitation 475-490nm/emission 500-540nm, TRITC: excitation 545-565nm/emission 580-620nm); **FM:** flip mirror; **CCD₁:** Hamamatsu ORCA_ER; **CCD₂:** Imaging Source USB camera (DFK 42AUC03); **CCD₃:** Andor Newton Camera (cooled at -70 °C); **L1~L6:** lenses; **F/#:** F number matcher. See methods section for more details.

Figure 2. *M. tuberculosis* in infected guinea pig lung tissue section observed under the Raman system (bright field). A single bacillus can be observed (black arrow) in alveoli. The scale bar represents 5 μm .

Running title: "Detecting phenotypically resistant *M. tb*"