

## Raman-based evaluation of cells and tissues: Sample preparation and hyperspectral data analysis

Version: 1.0

Version Date: April 2024

### 1. RATIONALE/AIM

Raman spectroscopy relies on the detection of photons scattered inelastically upon interaction with a sample, providing insights into the molecular bonds present within the analyte. This non-invasive technique shows significant potential for in-depth chemical analysis of cells and tissues, as well as for quantitative evaluation of (patho)physiological changes and effects of therapeutic molecules. This protocol outlines the necessary steps for sample preparation of liquids (e.g. supernatants, lysates), cells, and whole tissues, along with the analysis of acquired hyperspectral Raman datasets. We present an optimized workflow for spectral processing, based on a detailed assessment of various approaches described in Haessler et al., 2023, doi: [10.1021/acs.analchem.3c03368](https://doi.org/10.1021/acs.analchem.3c03368).

### 2. EXPERIMENTAL CONDITIONS

#### 2.1. Key Requirement:

##### 2.1.1. Raman-compatibility of sample

For Raman spectroscopic analyses, samples must be mounted on a Raman-compatible surface (e.g. CaF<sub>2</sub>), be free of spectral contaminants (e.g. fixatives, fluorophores, complex cell culture media) and contain concentrations of the desired sample above the detection limit.

##### 2.1.2. Quality of Raman data sets

Acquired hyperspectral data sets should display high signal to noise ratio to enable optimal data analysis. For confocal Raman imaging of cells and tissues, scans should be acquired in high resolution.

##### 2.1.3. Analysis using MATLAB

Although analysis of hyperspectral data sets is explained in a step-by-step manner and code is provided in the protocol below, basic knowledge of the software MATLAB is necessary to perform the evaluation.

#### 2.2. Key resources table:

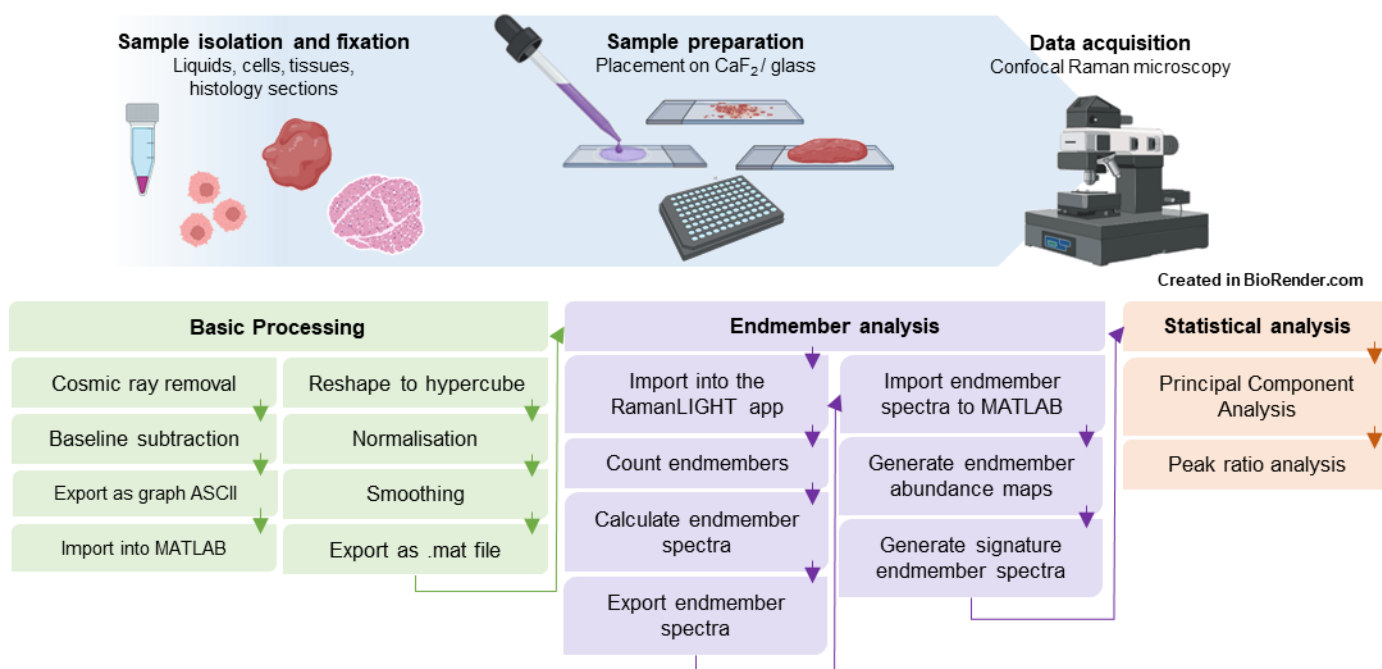
<b>Reagent</b>	<b>Supplier</b>	<b>Code</b>
DBPS	Gibco, Life Technologies	14190-094
Formaldehyde 3.7%	Sigma Aldrich/Merck	11-0705
Glass slides	Avantor/VWR	631-1553
High precision cover slips	Carl Roth	LH26.2
CaF <sub>2</sub> windows	Korth Kristalle	

<b>Equipment</b>	<b>Supplier</b>	<b>Model</b>
Confocal Raman microscope	WITec/Oxford Instruments	Alpha 300R <sup>+</sup>

<b>Software</b>	<b>Supplier</b>	<b>Version</b>
ProjectFOUR	WITec/Oxford Instruments	FOUR (or higher)
MATLAB	MathWorks	R2023b (or higher)
Optional: ImageJ	ImageJ, open source	1.54h (or higher)
Optional: Origin	OriginLab	Origin 2022 (or higher)

### 3. PROTOCOL

#### 3.1. Workflow



#### 3.2. Protocol

This protocol describes general considerations for preparation of biological samples and acquisition of Raman spectral data sets as well as an improved workflow for hyperspectral data analysis. The optimisation of the data analysis workflow including assessment of different algorithms and an extensive background can be found in the publication by Heassler et al., 2023 doi: [10.1021/acs.analchem.3c03368](https://doi.org/10.1021/acs.analchem.3c03368).

### 3.2.1. Preparation of biological samples

In general, all samples should be mounted on a Raman-compatible sample holder, meaning a substrate that does not provide significant spectral background, to allow for best measurement results. CaF<sub>2</sub> windows are the material of choice, but samples can also be placed on glass, if no CaF<sub>2</sub> windows are available. Samples can also be placed in 96 well glass bottom plates to achieve higher throughput. Be sure to record reference spectra for comparison during analysis if you use alternative substrates.

Liquids: Liquids (e.g. blood, isolated extracellular vesicles, tissue lysates) can be analysed via Raman spectroscopy in their liquid state, but depending on the concentration of the analyte, Raman signals might be too weak. It is therefore suggested to place a droplet (10-30 µL) of the sample on a CaF<sub>2</sub> slide and let the sample dry completely. Due to the coffee ring effect, the analyte will concentrate in the border region of the dried droplet.

Cells: Ideally, adherent cells are directly seed and cultured on CaF<sub>2</sub> windows. If cells do not adhere to CaF<sub>2</sub>, the windows can be coated using e.g. poly L-lysine or other appropriate coatings. If cells are imaged in native state (alive), be sure to choose an imaging solution with minimal spectral background (avoid glucose, phenol red etc.). Cells can be fixed to avoid artefacts from imaging solutions or cells dying during the imaging process by using different fixatives. Formalin has been shown to preserve spectral features of cells better than other fixatives (Meade et al., 2010 doi: [10.1007/s00216-009-3411-7](https://doi.org/10.1007/s00216-009-3411-7)). For fixation, wash cells twice with DPBS or H<sub>2</sub>O and incubate for 15 min with a 3.7 % formalin solution. Wash cells twice and cover with DPBS for imaging.

Tissues: If samples are thick enough (e.g. whole tissues, > 0.5 mm), they can be placed on any substrate for imaging. If desired, fix tissue using formalin solution and wash thoroughly. Cover tissue with DPBS or H<sub>2</sub>O for imaging.

Histological sections: Prepare sections of native or fixed tissues via cryo-sectioning and place them on a CaF<sub>2</sub> window or glass slide. If tissues were embedded in paraffin, remove paraffin, rehydrate, and wash thoroughly before Raman imaging. Histological sections can be imaged in dry state, however, if fluorescent background arises due tissue properties (e.g. high collagen content), cover the sample with DPBS for imaging.

### 3.2.2. Raman imaging of biological samples

In general, biological samples can be analysed using e.g. 532 nm, 633 nm, or 785 nm lasers. 532 nm lasers offer the highest signal intensity but may be experiencing higher fluorescent background compared to e.g. 785 nm lasers. For most biological samples the following parameters produce good results using objectives with short working distances:

Laser	532 nm
Laser power	20 – 40 mW
Accumulation time	0.2 – 0.5 sec

However, samples should always be checked for thermal damages and parameters adjusted accordingly. For samples covered in DPBS, a water-dipping objective is recommended. In general, samples should be imaged at the highest possible resolution to allow differentiation between sub-cellular structures. If high autofluorescence of the sample is detected, a line scan spanning approx. 50 – 70  $\mu\text{m}$  above the designated imaging area can be performed to bleach neighbouring tissue sections.

### 3.2.3. Basic data processing

The here outlined steps describe the basic processing of hyperspectral Raman data sets that should routinely be performed to ensure consistent spectral data quality before further analysis of the data. Although these processes have been well tested on liquids, cells, and tissues, it is highly recommended to check if the here chosen methods yield the best results for your specific data set.

The outlined steps for data processing are described using the software ProjectFOUR (WITec, Oxford Instruments) and MATLAB, but can be done using other software for spectral processing. MATLAB scripts can be provided upon request.

1. **Background subtraction:** Minimisation or elimination of background signals in acquired Raman data sets can be achieved in most acquisition software using polynomial fits or more customisable functions, e.g. WITec ProjectFOUR's 'shape' function, which operates based on a rolling circle background subtraction.
2. **Cosmic ray removal:** apply appropriate settings to detect and eliminate Raman peaks originating from cosmic rays, e.g. by identifying and eliminating peak with tenfold higher intensities as the mean at a given wavelength.
3. **Export/Import:** Use the Graph ASCII file format to export the data file from the acquisition software and import into MATLAB.
4. **Reshaping:** The dataset must be reshaped to list all spectra in a matrix. Create a 2-dimensional matrix with all recorded spectra listed in the rows and each column representing one wavenumber of the respective spectra. For analysing Raman image scans, the data must be reshaped into a hypercube-formatted matrix. This is a 3-dimensional matrix with each x representing the x coordinate of the pixel, each y representing the y coordinate of the pixel and the z values being the spectral data.
5. **Trimming:** By cropping the data set, spectral areas that contain no information (before 400  $\text{cm}^{-1}$ , between 1800 and 2800  $\text{cm}^{-1}$ , and after 3140  $\text{cm}^{-1}$ ) are eliminated to reduce the total data size and enable faster processing.
6. **Normalisation:** To enable the comparison of different data sets, normalisation is performed by e.g. z-scoring, setting the mean of the data to 0 and the standard deviation to 1. Depending on the type of data, other normalisation types might be better suitable such as Pareto scaling.
7. **Smoothing:** Optionally, data is smoothed using e.g. the Savitzky-Golay filter (adjust polynomial filter and frame length depending on your data).

### 3.2.4. Endmember analysis

Endmember analysis constitutes a reliable and reproducible approach to create Raman images from hyperspectral data sets. Endmembers are pure spectral signatures of distinct entities in your sample (e.g. nucleic acids or lipids) that can be identified, extracted, and their abundance is subsequently visualised in

2D maps. MATLAB offers different options to perform the analysis, this protocol requires the installation of the 'Image Processing Toolbox™ Hyperspectral Imaging Library' and the 'RamanLIGHT' add-ons.

8. Optional pre-processing: The RamanLIGHT app provides the option to pre-process spectra, but we suggest performing these in a separate script in MATLAB (see above) for more control about the parameters.
9. Calculate endmembers: Switch between the different tabs of the app to choose options for endmember calculations. In tab 'Endmember 2', you can perform vertex component analysis, which usually calculates endmembers that give very good abundance maps for biological samples. However, it is suggested to generate abundance maps with different endmember analysis methods to identify the method that results in the best images for your specific data set. To evaluate the quality of endmember analysis, check if the respective algorithm can distinguish e.g. sub-cellular structures with clear contrasts.
10. Extract endmember spectra: For further analysis in MATLAB, extract the mean endmember spectra from the 'Mean Spectra' tab.
11. Create abundance maps: Calculating the abundance maps in MATLAB instead of the Raman LIGHT app enables direct .jpg image exportation. Further image processing is then performed in ImageJ.

### 3.2.5. Statistical analysis

Beyond the visualisation of the distinct biological entities within the scan region, endmember spectra can further be used for statistical evaluation.

12. Calculate signature spectra: From each abundance map, a percentage or fixed number of the highest correlated spectra per endmember can be used to calculate a mean, which represents the signature spectrum of the respective endmember from your data set. At this point the spectra might be further analysed in MATLAB or exported to any other software (e.g. Origin).
13. Principal component analysis (PCA) can be applied to compare the signature spectrum of a specific endmember (e.g. lipids, nucleic acids, cytochrome c, etc.) between samples. The resulting plots represent a visual indication of the similarity of the test groups. Closer observation of the PC loadings of each plot indicates positively and negatively correlated Raman peaks, guiding further qualitative investigations in e.g. peak ratio analysis.
14. Peak ratio analysis is implemented to semi-quantitatively assess the relative change of a specific Raman peak. By correlating a peak of interest to a fixed constant peak, a value is created that is then normalised to the value of the control group. After statistical analysis, the significance of the observed change can be determined.  
It is highly recommended to investigate the consistency of the chosen reference peak(s) and employ multiple peak ratios to evaluate a specific biological event.

The following table lists Raman peaks of general interest, their assignment as well as possible biological contexts.

Raman shift [cm <sup>-1</sup> ]	Assignment	Biological context	Reference
702	Cholesterol	Lipid metabolism, inflammation	1, 2
750	Cytochrome c	Cell stress, apoptosis	1
785	DNA, RNA	Chromatin condensation	1
826	Nitrated tyrosine	Oxidative stress	3
1002-1008	Phenylalanine	Popular reference peak	1, 2
1030-1034	Phenylalanine	Popular reference peak	1, 2
1050	deoxyribose	DNA integrity	4
1070	Fatty acids, lipids	Lipid metabolism, inflammation	1
1090	Phosphate	Phosphorylation, DNA integrity	1
1129	Cytochrome c	Cell stress, apoptosis	5, 6
1224-1242	$\beta$ -sheet	Protein conformation	1, 2
1268	Unsaturated lipids (=CH)	Lipid metabolism, inflammation	1
1307	$\alpha$ -helix	Protein conformation	1, 2
1310-1315	Cytochrome c	Cell stress, apoptosis	5, 7
1338-1342	DNA, nucleic acids	Chromatin condensation	1
1369-1375	DNA, nucleic acids	Chromatin condensation	1, 8
1421-1425	DNA, nucleic acids	Chromatin condensation	1, 7, 9
1442-1452	Lipids, proteins (-CH <sub>2</sub> , -CH <sub>3</sub> )	Lipid metabolism, inflammation	1
1485	DNA, nucleic acids	Chromatin condensation	1
1584	Cytochrome c	Cell stress, apoptosis	5
1650-1655	Unsaturated lipids, proteins (C=C, -CH <sub>2</sub> )	Lipid metabolism, inflammation	1
1665-1670	$\beta$ -sheet	Protein conformation	1, 2, 10
1740	Lipid esters	Lipid metabolism, inflammation	1
2850	Lipids	Lipid metabolism, inflammation	1, 2
2883	Lipids	Lipid metabolism, inflammation	1
2930-2935	Lipids, proteins (-CH <sub>3</sub> )	Lipid metabolism, inflammation, popular reference peak	1
3010-3015	Unsaturated lipids (=CH)	Lipid metabolism, inflammation	1

#### References

1. Movasaghi, Z., Rehman, S. & Rehman, I. U. Raman Spectroscopy of Biological Tissues. *Appl. Spectrosc. Rev.* **42**, 493–541 (2007).
2. Niederhafner, P. et al. Monitoring peptide tyrosine nitration by spectroscopic methods. *Amino Acids* **53**, 517–532 (2021).
3. Fonseca, E. A. et al. Micro-Raman spectroscopy of lipid halo and dense-core amyloid plaques: aging process characterization in the Alzheimer's disease APPswePS1 $\Delta$ E9 mouse model. *Analyst* **146**, 6014–6025 (2021).
4. Chen Y, Dai J, Zhou X, Liu Y, Zhang W, Peng G. Raman spectroscopy analysis of the biochemical characteristics of molecules associated with the malignant transformation of gastric mucosa. *PLoS One*. 2014 Apr 7;9(4):e93906. doi: 10.1371/journal.pone.0093906.

5. Russo, V. et al. Key Role of Cytochrome C for Apoptosis Detection Using Raman Microimaging in an Animal Model of Brain Ischemia with Insulin Treatment. *Appl. Spectrosc.* **73**, 1208–1217 (2019).
6. Abramczyk, H. et al. Revision of Commonly Accepted Warburg Mechanism of Cancer Development: Redox-Sensitive Mitochondrial Cytochromes in Breast and Brain Cancers by Raman Imaging. *Cancers* **13**, 2599 (2021).
7. Kunapareddy, N., Freyer, J. P. & Mourant, J. R. Raman spectroscopic characterization of necrotic cell death. *JBO* **13**, 54002 (2008).
8. Ichimura, T. et al. Visualizing the appearance and disappearance of the attractor of differentiation using Raman spectral imaging. *Sci Rep* **5**, 11358 (2015).
9. Maiti, N. C., Apetri, M. M., Zagorski, M. G., Carey, P. R. & Anderson, V. E. Raman spectroscopic characterization of secondary structure in natively unfolded proteins: alpha-synuclein. *JACS* **126**, 2399–2408 (2004).
10. A.L.M. Batista de Carvalho, M. Pilling, P. Gardner, J. Doherty & MPM Marques. Chemotherapeutic Response to Cisplatin-like Drugs in Human Breast Cancer Cells Probed by Vibrational Microspectroscopy. In *Advanced vibrational spectroscopy for biomedical applications. Faraday Discuss.* **187**, 273–298 (2016).