Identification of Subcellular Structures with Raman Microspectroscopy

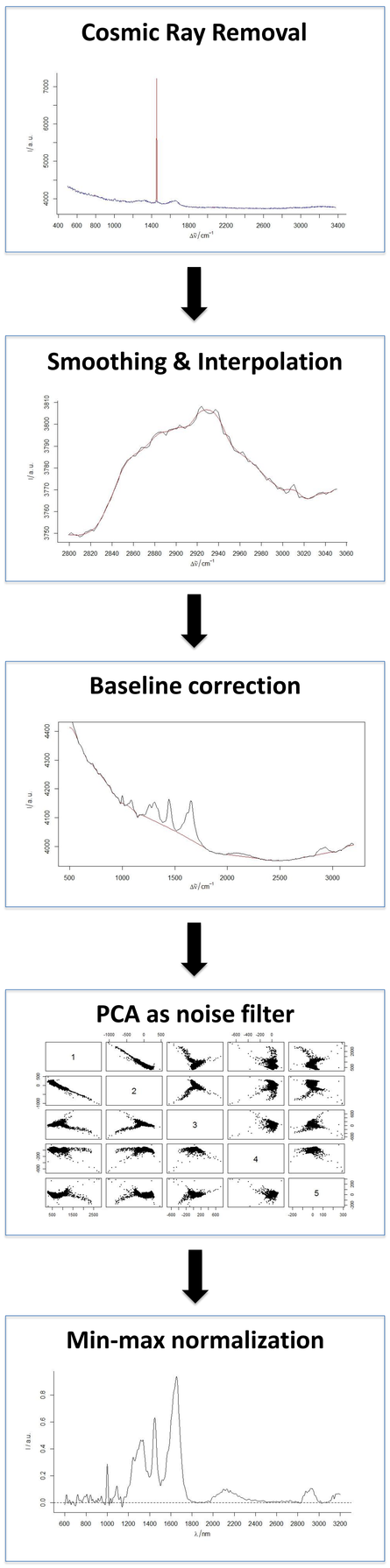
Tobias Corne[1,2], Pieter Wuytens[1,2,3], Claudia Beleites[4], Andre Skirtach[1,2], Winnok De Vos[1,2,5]

Introduction

Raman microspectroscopy (RMS) is a technique which enables noninvasive, label-free imaging of cells. RMS probes molecular vibrations and generates a molecular fingerprint which can be used to discriminate different subcellular structures within a cell. Here we collected Raman spectra by raster-scanning fixed Hela cells under a confocal Raman microscope (alpha300 R, WITec, GmbH, Ulm, Germany) at 785 nm excitation with 0.5 µm step size and 1 s integration time per spectrum. Preprocessing and data analysis was performed in R using the hyperSpec package (Beleites & Sergo, http://hyperspec.r-forge.r-project.org, 2014). Cluster analysis allowed us to segment several subcellular structures, such as the nucleus and lipid droplets (LDs). The number of LDs was increased in Hela cells by cycloheximide treatment, Hoechst (DNA stain) and bodipy 493/503 staining (neutral lipid stain) correlated with the segmented nucleus and LDs, respectively. The average Raman spectrum of the nucleus contained a DNA specific peak (788 cm^{-1}) and the average spectrum of LDs showed great similarities with the reference spectrum of triolein, the symmetrical triglyceride of oleic acid.

Data preprocessing

After data acquisition the hyperspectral datasets were preprocessed. In a first step pixels containing cosmic rays were removed and replaced by the mean spectrum of four adjacent pixels. Next, signal-to-noise ratio (SNR) was increased by smoothing and interpolation. The spectra were smoothed by local polynomial regression fitting and in parallel interpolated on a new wavelength axis with equidistant spacing. The background was removed by baseline correction using the baselineWavelet package (Zhang et al., http://code.google.com/p/baseline wavelet, 2009). To estimate the baseline, peak position was accurately detected by continuous wavelet transformation (CWT), peak-width was estimated by SNR enhancing derivative calculation based on CWT, and finally the baseline was fitted using penalized least squares with binary masks. After baseline correction the data distribution was studied with principal component analysis (PCA) and outliers were removed and replaced by the average spectrum of eight adjacent pixels. Finally the spectra were min-max normalized to minimize the influence of the focal volume on the signal. The minimum of each spectrum was scaled to 0 and the maximum to 1. Following preprocessing the data was analyzed using different clustering and spectral unmixing methods.



Identification of subcellular structures by crisp and soft clustering

Subcellular structures were identified by cluster analysis. In first instance crisp cluster analysis methods were used, such as *hierarchical cluster analysis* (HCA) and *k-means cluster analysis* (KMCA). Next soft cluster analysis was tested using *fuzzy C-means* (FCM) and *vortex component analysis* (VCA). Crisp clustering implies that a spectrum in a Raman image is assigned to one cluster only, on the other hand soft clustering methods can assign each spectrum to more than one cluster with probability values between 0 and 1. In fig. 1 false color images are shown, which were generated by crisp and soft clustering methods.

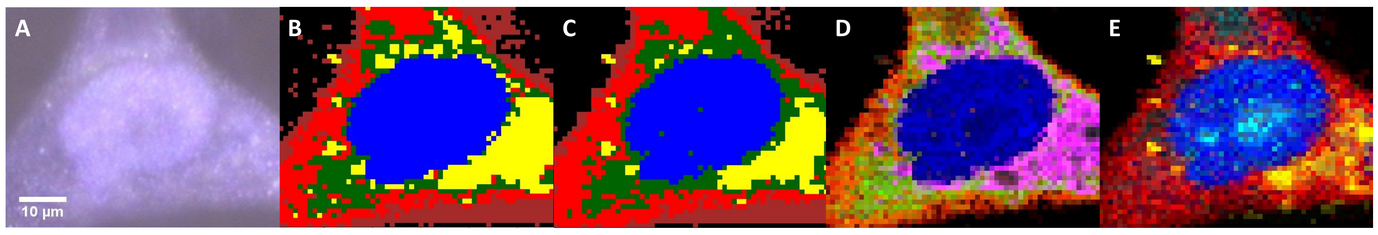


Fig. 1: *A) Reflection image of a fixed Hela cell. B) HCA image using 6 clusters. C) KMCA image using 6 clusters. D) FCM image, overlay of abundance maps of 6 clusters. E) VCA image, overlay of abundance maps of endmember 1 to 6.*

All cluster analysis methods were able to discriminate the nucleus and cytoplasm, inside the cytoplasm globular structures were detected which resembled lipid droplets (LDs). LDs provide reservoirs for lipids for membrane synthesis, lipoprotein synthesis, and as energy storage (Thiam et al. 2013). These organelles are found in nearly all cell types and an imbalance leads to many different human diseases, including obesity, diabetes and atherosclerosis (Krahmer et al. 2013). To ensure that these structures were LDs, their number was increased by cycloheximide (CHX) treatment (10 µg/ml for 24 hours) (Suzuki et al., 2012). After data acquisition the cells were stained with Hoechst (DNA stain) and bodipy 493/503 (neutral lipid stain). The Hoechst and bodipy stain clearly colocalized with the cluster corresponding to the nucleus and LDs, respectively (fig. 2). The average spectrum of LDs showed great similarity with the reference spectrum of triolein, the symmetric triglyceride of oleic acid (fig. 3). The average spectrum of the nucleus contained a DNA specific peak at 788 cm^{-1} and predominant CH_3 stretching vibration (fig. 4).

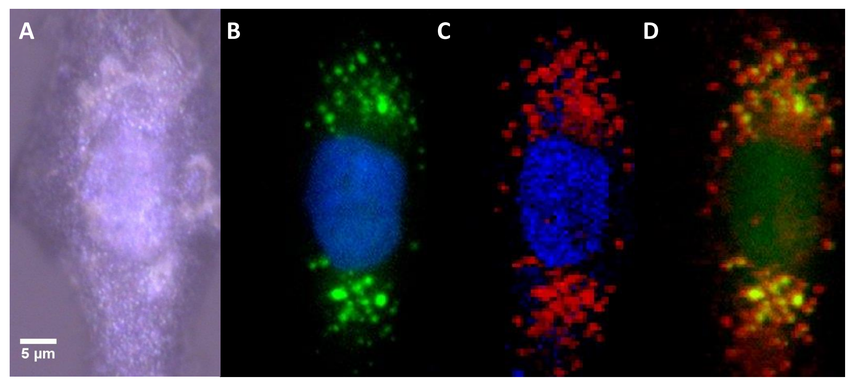


Fig. 2: *A) Reflection image of a fixed cycloheximide treated Hela cell. B) Fluorescence image after lipid droplet staining with bodipy 493/503 (green) and nuclear staining with Hoechst (blue). C) VCA image, overlay of abundance maps of endmember 3 (blue) and 5 (red). D) Merged image of bodipy stain (green) and VCA endmember 5 (red).*

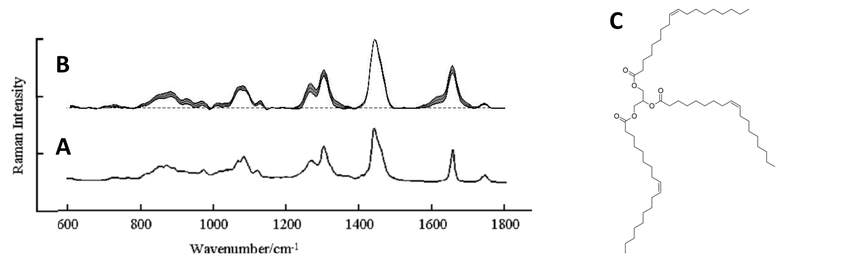


Fig. 3: *A) Reference spectrum of triolein (Gelder et al. 2007). B) Average spectrum of LDs. C) Chemical structure of triolein.*

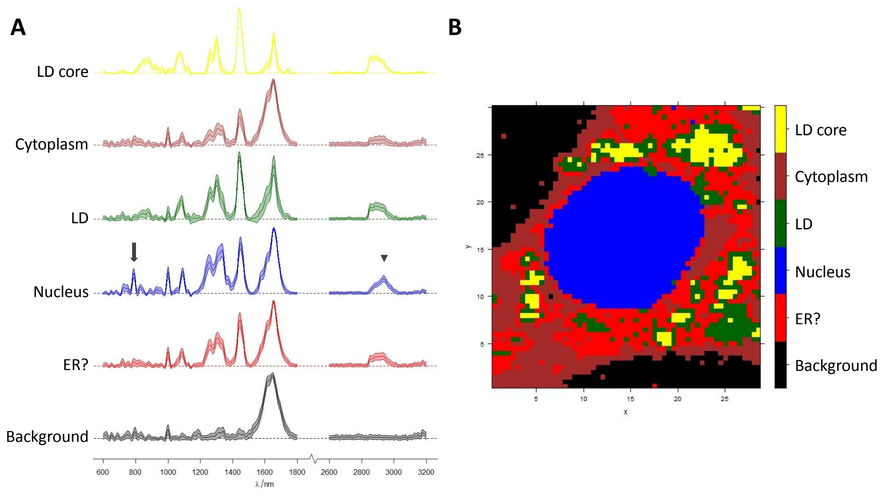


Fig. 4: *A) Average spectra of 6 different HCA clusters of a CHX treated Hela cell, the arrow indicates a DNA specific peak around 788 cm^{-1}, and the arrowhead highlights the predominant CH_3 stretching vibration. B) HCA image of the cell with labeled subcellular regions.*

Conclusion and future perspectives

We have shown that RMS can be used to identify subcellular structures in a label-free manner. By using clustering methods we were able to identify the nucleus and LDs. In future more subcellular regions should be correlated with a fluorescent stain, i.e. ER-Tracker and MitoTracker staining. In addition, the effect of altered lipid metabolism can be studied by searching for changes in the average spectrum of LDs.

[1] Department of Molecular Biotechnology, Faculty of Bioscience Engineering, Ghent University, Ghent, Belgium;
[2] Center of Nano- and Biophotonics, Ghent University, Ghent, Belgium;
[3] Photonics Research Group, Department of Information Technology, Ghent University, Ghent, Belgium;
[4] Institute of Photonic Technology, Jena, Germany;
[5] Department of Cell Biology and Histology, Faculty of Veterinary Sciences, Antwerp University, Antwerp, Belgium;

CONTACT
Tobias Corne
Tel. +32-9-2645971
Email Tobias.Corne@UGent.be