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Investigation of lipid bodies in a colon carcinoma cell line by confocal Raman microscopy

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Summary
Objective: The investigation of living cells under physiological conditions requires sensitive, sophisticated and in particular, non-invasive methods. Raman spectroscopy provides vibrational information about the sample. Combined with high-resolution confocal microscopy, it allows a complete Raman spectrum to be recorded at every confocal image point. This technique was applied here for the investigation of lipid bodies in a colon carcinoma cell line.
Materials and methods: The colorectal adenocarcinoma cell line Caco-2 and the rat intestine epithelial cell line IEC-6 were analysed with the confocal Raman microscope alpha300 R (WITec GmbH, Germany), using a frequency-doubled Nd:YAG laser at 532 nm and 10 mW for excitation. The use of a water immersion lens (63×, NA 1.0) allowed a lateral resolution in the sub-micrometer range. Raman images of cells were generated from the data sets by integrating over specific Raman bands. Mapping of the C—H stretching band (2800—3030 cm^{-1}) allowed for the visualisation of the whole cell, whereas the automated statistical evaluation of all spectra by *k*-means cluster analysis resulted in spectral unmixed images which provided an insight into the chemical composition of the sample.
Results: With the described method, it was possible to visualise the distribution of different cellular biomarkers. Lipid bodies, in particular, which are reported to be present in increased numbers in colorectal cancer cells as compared to normal tissue, could be characterised and localised. A quantitative approach was developed to assess the fraction of lipid bodies in the total cell area. This method was applied for comparison between malignant and non-malignant cell lines. The fraction of lipid bodies turned out to be significantly higher in malignant (13.8%, $n = 21$) than in non-malignant cells (1.8%, $n = 16$).
Conclusion: Confocal Raman microscopy is shown to be a powerful method for the investigation of lipid bodies.

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Introduction

Confocal Raman microscopy combines the advantages of Raman spectroscopy, a non-invasive technique which allows detailed chemical information of a sample to be obtained, without a need for preparation or labelling, with those of confocal microscopy. It gives highly and spatially resolved, diffraction-limited images with signals collected only from the sample focal plane [1,2]. This method has developed into a powerful tool for the investigation of biological samples and of living cells [3—5]. Sophisticated fitting procedures [6], such as basic spectral analysis, principal component analysis and cluster analysis, can be applied to Raman microspectroscopy [7] to improve the signal-to-noise ratio and allow a faster data interpretation. Several works have reported the discrimination of normal and malignant tissues by Raman spectroscopy [8,9]. A method using Raman microspectroscopic detection for skin cancer cells is described in [10].

Colorectal neoplasm is an epidemiologically relevant disease which profits from advanced optical techniques for the early detection of neoplastic lesions [11,12]. Colorectal cancer is the fourth most common cancer in men and women in the United States [13]. Treatment for colorectal cancer is more likely to be effective when the disease is diagnosed early which has led to screening using fecal occult blood tests and colonoscopy being recommended for those over 50 years old [14]. As these methods still fail to detect small and flat adenomas, methods for early detection of the disease are required that are based on biochemical changes rather than morphological ones. Raman microspectroscopy would seem to be a promising method for the definition of biomarkers for colorectal cancer. Recently researchers' interest has been drawn to lipid bodies as dynamic cell organelles [15] and their incidence has been correlated to colon adenocarcinoma [16]. The composition of lipid bodies was studied by Raman microscopy by van Manen et al. [3] in neutrophilic granulocytes.

In this study we aim to investigate the role played as well as the incidence and composition of lipid bodies as biomarkers in a colon carcinoma cell line using Raman microscopy.

Materials and methods

Materials

The cell growth media Dulbecco's modified Eagle's medium (DMEM; PAA Cat. No. E15-011), Eagle's minimal essential medium (EMEM; PAA Cat. No. E15-024) and non-essential amino acids supplement (PAA Cat. No. M11-003) were purchased from PAA, Pasching, Austria. Fetal calf serum was from Biochrom (Berlin, Germany). Glutamax and sodium pyruvate were from Invitrogen (Darmstadt, Germany) while bovine insulin and arachidonic acid were from Sigma (Steinheim, Germany). A ready-to-use sodium chloride solution (0.9%) was purchased from B. Braun (Melsungen, Germany).

Cell culture

Human colorectal adenocarcinoma cells from the Caco-2 cell line (ATCC® Cat. No. HTB-37™) were cultured as mono-layers in EMEM supplemented with 1× non-essential amino acids, 2 mM Glutamax, 1 mM Na-pyruvate and 20% fetal calf serum at 37 °C in a humidified atmosphere containing 5% CO_2. Rat intestine epithelial cells IEC-6 (ATCC® Cat. No. CRL-1592™) were grown in DMEM containing 4 mM L-glutamine, 0.1 U/ml bovine insulin and 10% fetal calf serum. Cells were seeded at 10—30 cells/mm^2 and sieved every 3—4 days.

For microscopy, cells were sub-cultured in 35 mm diameter glass bottom Petri dishes (MatTeK, Ashland, MA, USA) over 4 days before investigation.

Experimental setup

Raman spectra and images were recorded with a WITec Confocal Raman Microscope (WITec alpha300 R, Ulm, Germany). This instrument has already been described in detail [1]. In short, it consists of a confocal microscope which is combined with a Raman spectrometer. For excitation, the frequency-doubled Nd:YAG laser (532 nm), which is part of the instrument, is used with an output of 10 mW on the sample. The laser source is coupled into the microscope via a single mode fibre for 532 nm having a core diameter of 3.5 μm. For reference compounds, a drop or a crystal of undiluted substance was spotted on a glass slide and single spectra were recorded with a Nikon 20× lens. For living cells, the culture medium was removed; cells were washed with 0.9% sodium chloride solution, and then observed with a Zeiss 63×, NA 1.0 water immersion objective. Sample scanning is achieved by a piezoelectric scan stage. Reflected laser and Rayleigh-scattered light are suppressed by an edge filter while Raman scattering is focused into a multimode optical fibre with a core diameter of 50 μm, which acts as a pinhole for confocality and serves as an entrance slit for the WITec UHTS 300 spectrometer.

Data acquisition

Data acquisition was controlled by the WITec Control software. Images of 150 × 150 pixels and 50—80 μm edge length were recorded, depending on cell form and size. The integration time per pixel was at least 10 ms, i.e. the acquisition time per image was approx. 8 min. A complete Raman spectrum was recorded at each pixel covering the region from −131 to 3750 cm^{-1}. Signals were detected by a 1024 × 127 pixel Peltier-cooled CCD camera to yield a spectral resolution of 4 cm^{-1}. Raman images were generated during data acquisition by integrating over the C—H stretching band (2800—3030 cm^{-1}).

Data analysis

Data matrices were evaluated using the WITec Project software. Data sets were submitted to *k*-means cluster analysis using a multi-clustering procedure. For each image data set, 4 clusters were generated. This algorithm calculates the number and distribution of the pixels showing common spectral features and the average common spectrum. The fraction of a single component was calculated by relating