Available online at www.sciencedirect.com
SCIENCE DIRECT®
Vibrational Spectroscopy 38 (2005) 85–93
VIBRATIONAL SPECTROSCOPY
www.elsevier.com/locate/vibspec

Identification of organelles and vesicles in single cells by Raman microspectroscopic mapping

Christoph Krafft [a,*], Thomas Knetschke [b], Richard H.W. Funk [b], Reiner Salzer [a]

[a] *Institute for Analytical Chemistry, Dresden University of Technology, D-01062 Dresden, Germany*
[b] *Institute for Anatomy, Medical Faculty, Dresden University of Technology, Fetscherstr. 74, D-01307 Dresden, Germany*

Available online 14 March 2005

This paper is dedicated to Henry H. Mantsch for his outstanding contributions to spectroscopy and his valuable support to spectroscopists around the world.

Abstract

Scope of the present paper is to develop non-resonant Raman microspectroscopic mapping techniques for single cells studies in order to identify subcellular structures by their spectral signatures. Human lung fibroblast cells were fixed in formalin and stored in buffer in order to prevent morphological and chemical changes during data acquisition. Raman maps were recorded with 1 μm step size at 785 nm excitation and with 0.3 μm step size at 532 nm excitation. Spectral details could be resolved such as the distinction of RNA and DNA, proteins, cholesterol and phospholipids like phosphatidylcholine (PC) and phosphatidylethanolamine (PE). Fitting of cluster averaged spectra by linear combinations of reference spectra was used to approximate the subcellular composition. Images could be reconstructed revealing the protein, nucleic acid and lipid concentration. Based on these compositional information, color coded cluster memberships were correlated with nucleus, cytoplasm, endoplasmic reticulum, vesicles and peripheral membrane.

Keywords: Single cell spectroscopy; Biomedical spectroscopy; Raman spectroscopy; Human cells

1. Introduction

Fluorescence microscopy, the coupling of fluorescence spectroscopy with microscopy, is the most highly developed and widely used means of imaging single cells. But this approach is sometimes limited in application. External labeling of the molecules of interest by chemical or recombinant methods is so far necessary, as most molecules naturally occurring in cells cannot directly be identified. However, the accessibility of certain structures to external fluorescent dyes is restricted, e.g. labeling of DNA in the nucleus depends on the dye, the metabolic state of the cell as well as the phase of cell differentiation [1]. Other problems result from the limited stability and bleaching of fluorophores. Raman microspectroscopy, the coupling of Raman spectroscopy with microscopy, has the potential to complement fluorescence microscopy for studies of single cells because it combines molecular specificity with diffraction limited resolution in the submicrometer scale, so it can image directly the chemical composition and the distribution of molecules without prior labeling. Difficulties of Raman spectroscopy result from low scattering cross sections of naturally occurring compounds such as DNA, RNA, proteins and lipids which make up the majority of biological cell's content.

To compensate low scattering cross sections, resonance enhancement, surface enhancement and coherent anti-Stokes Raman scattering (CARS) have been applied for cellular imaging. However, resonance Raman spectroscopy (RRS) only probes selected molecules in single cells like carotenoids [2] or hemoglobin [3] which show absorption

* Corresponding author. Fax: 49 351 4633 7188.
E-mail address: christoph.krafft@chemie.tu-dresden.de (C. Krafft).

0924-2031/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.vibspec.2005.02.008

near the excitation wavelength. As a disadvantage, these light absorbing molecules tend to photodegradation which is induced by the excitation laser. Surface enhanced Raman spectroscopy (SERS) only probes molecules in the close vicinity (several nanometers) of metal surfaces which results in different Raman spectra at almost all places in cells [4]. In CARS microscopy the low non-resonant scattering cross sections are overcome by high peak powers in ultra-short laser pulses used to generate the coherent signal [5]. Furthermore, the CARS technique can suppress non-resonant background which was utilized for imaging lipids in cells [6]. However, this complicated technique using 2 ps pulsed lasers is difficult to implement as a routine method.

In contrast to RRS, SERS or CARS, non-resonant Raman spectroscopy can be generally applied to all molecules using commercially available spectrometers and minimal sample preparation. The requirement of high sample concentrations is automatically fulfilled in the extremely condensed volume within a biological cell. The protein concentration may be as high as 250 μg/μl, the DNA concentration 100 μg/μl, and the RNA concentration 100 μg/μl depending on the cell type, the phase of the cell cycle and the location inside the cell [7]. The spectral contributions of all molecules overlap, which results in complicated but highly informative Raman spectra. To image single cells, sensitive Raman systems using high power excitation laser at 647 nm [8,9] and 785 nm [10], confocal microscopes, high throughput optical components and CCD detectors have recently been employed. Signal intensities were sufficiently high and fluorescence background was sufficiently low allowing to acquire spectra within seconds. Acquisition of Raman images in point detection, mapping mode takes 10 min and more because hundreds or even thousands of spectra have to be subsequently recorded.

In this study Raman images were recorded in multiplex mode in which the whole spectrum ($\sim$3500 cm^{-1}) including the fingerprint area and the valence vibrations area is simultaneously obtained. Fixed lung fibroblasts were studied because (i) cell cultivation can easily be controlled, (ii) cell geometry and morphology are very suitable for depicting and for discrimination of visible compartments, (iii) they grow adherent to the quartz slides, (iv) and they keep their shape over time after fixation. Cells were fixed in order to prevent morphological and chemical changes during data acquisition. The broad spectral response with numerous Raman bands is analyzed by multivariate algorithms like cluster algorithms and fitting procedures. Averaging spectra in each cluster of the Raman image improved the signal to noise ratio and, consequently, the data interpretation. The analyses identify spectral contributions of DNA, RNA, phospolipids, cholesterol, proteins and water, approximate the composition of subcellular structures and permit their assignment to nucleus, cytoplasm, endoplasmic reticulum, vesicles and peripheral membrane. The Raman images of two commercial systems are compared. Both systems differ in laser excitation wavelengths, microscopes, spectrographs and detectors.

2. Experimental

2.1. Preparation of cells

Human embryonic lung fibroblast cells (strain L132) were directly grown on quartz slides. The cells were fixed by formalin and stored in isotonic, 10 mM phosphate buffer, pH 7, with 1 mM sodium azide at 4 °C until data acquisition.

2.2. Raman microspectroscopy

In contrast to our former studies using a Raman spectrometer from Kaiser Optical Systems (Ann Arbor, MI) with a diode laser from SDL (San Jose, CA) [10], a high power, single-mode, 785 nm diode laser from Toptica (model Xtra, Germany) was coupled via a single-mode fiber to the Raman microscope. The microscope was equipped with a water immersion objective (Nikon, 60x/1.0) and a motorized stage (Prior Scientific, United Kingdom). Single cells were excited with 100 mW intensity. The backscattered light was guided via a multi-mode fiber to the spectrograph HoloSpec f/1.8 (Kaiser) equipped with a transmissive holographic grating and registered by a liquid nitrogen cooled, back illuminated deep depletion CCD detector (Princeton Instruments, Trenton, NJ). The spectrograph covered the region from 125 to 3555 cm^{-1} with 4 cm^{-1} spectral resolution.

The confocal Raman microscope system CRM200 was developed by Witec (Germany). The general setup was similar as described above. However, the components were different. A 532 nm diode laser (model Compass 315 M, Coherent, USA) was coupled via a single mode fiber to the confocal microscope, which was equipped with a high precision xyz motorized stage and a water immersion objective (Nikon, 60x/1.0). Single cells were excited with 20 mW intensity. The backscattered light was analyzed by a reflective grating spectrograph (300 mm, f/4) and detected by a Peltier cooled, back illuminated CCD camera (Marconi Applied Technologies, USA). The spectrograph covered the region from -162 to 3666 cm^{-1} with 8 cm^{-1} spectral resolution.

2.3. Data acquisition

Raman spectroscopic maps at 785 nm were obtained by collecting spectra with 10 s exposure time and subsequently moving the sample in a raster pattern in increments of 1 μm. Data acquisition was controlled by the HoloMap module of the HoloGrams software package (Kaiser). The software automatically performs a cosmic ray correction, intensity normalization and wavelength calibration.