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IR microspectroscopy of live cells

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Abstract

The objective of the present study was to establish the conditions for infrared spectroscopy of single living cells in cell culture under aqueous media, using a high brilliance synchrotron light source to achieve acceptable S/N at the necessary high spatial resolution. This is experimentally challenging, but brings a number of significant advantages. The data presented show that the infrared spectra of single living cells can be monitored over several hours with high reproducibility, as well as confirming that acquisition of spectral data from single cells can contribute new insights that are not available from measurements of cell populations.

Keywords: FT-IR spectroscopy; Synchrotron; Cell culture; Cancer diagnosis

1. Introduction

Following the successful establishment of infrared spectroscopy as a method for the structural investigations of biological molecules, over the last decade the biospectroscopy community has turned its attention to cells and tissues [1,2]. Although the extraction of specific molecular information from the infrared spectra such complex systems has not really been achieved, assessment of the spectra in the sense of 'pattern recognition', often with sophisticated mathematical algorithms, has produced promising results in such fields as tissue identification, disease diagnosis and classification of bacterial strains [1,2].

As noted by Jackson and Mantsch [3], Raman spectroscopy has so far been more useful than infrared spectroscopy in studies of single cells, due to the inherently better spatial resolution achievable with the shorter measuring beam wavelength [4–6]. Infrared studies have largely concentrated on cell populations suspended in aqueous media or, more usually, dried down to a film to minimize the background absorbance. This means that the spectrum obtained is the average over the cell population, raising issues of sample heterogeneity [3] for example due to the cells being at different stages of the cell cycle [7].

Synchrotron infrared radiation can in principle provide the spatial resolution needed for recording the infrared spectra of single cells. The Abbe diffraction limit of 0.61 λ/NA obviously still applies regardless of the light source, however, this is not inherently a problem since the resulting spatial resolution limit of 2.5–10 μm in the mid-infrared range is still below the size of many single cells. The synchrotron advantage results rather from their much higher brilliance (photon flux per unit source area into unit solid angle), resulting in up to 1000× higher flux through a diffraction-limited aperture at the sample plane [8]. With this method, Holman et al. recorded the spectra of cells after various times of exposure to environmental toxins [9], and Miller et al. studied the spectral responses of cells to apoptosis-inducing agents [10]. However, these studies involved removal of the cells from the culture medium after treatment and transfer to a suitable IR-transparent substrate, i.e. the cells were not living when the spectra were recorded. Also, we consider it would be fair to say that these were essentially feasibility demonstrations performed by spectroscopists, and have yet to yield any new insights of value to the biologist.

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The objective of the present study was to establish the conditions for infrared spectroscopy of single living cells in cell culture under aqueous media, using a high brilliance synchrotron light source to achieve optimal S/N at high spatial resolution. This is experimentally challenging, but brings a number of advantages: in addition to the obvious issues of avoiding possible artefacts for example due to drying or fixation, the use of living cells raises the possibility of monitoring the spectral changes resulting from perturbation of the homeostasis of a single cell in real time. In analogy to the use of difference spectroscopy for isolated biomolecules [11–13], this can be expected to yield detailed spectral data due to the precise subtraction of background signals, and is thus more likely to deliver insights at the level of molecular interaction.

2. Experimental

The experiments were carried out at the IR beamline of the ANKA synchrotron light source in Karlsruhe, Germany, in two 5-day beamtime assignments in August 2003 and January 2004, respectively. This beamline exploits synchrotron edge radiation, a new approach to the extraction of radiation from a relativistic electron beam that yields even higher brilliance than conventional synchrotron sources [14]. The beamline is equipped with two Bruker IFS-66vs spectrophotometers, one of which is coupled to a Bruker IRScope II infrared microscope. The visible image obtainable through the IR objective was found to be quite poor, sometimes causing difficulties for the location and identification of cells to be measured, particularly in cultures where the cells had attached especially well to the substrate and minimized their vertical thickness. This problem could be overcome by the addition of a 20× visible microscope objective to the IR microscope for viewing.

Various human colorectal cancer cell lines were studied (HT29, SW-480, WIDR, $CaCO_2$), together with human fibroblasts (primary culture) and human umbilical vein endothelial cells as healthy controls, in both confluent and non-confluent cultures. Primary fibroblasts isolated from skin biopsies were obtained from the Dermatology Department of the Faculty of Clinical Medicine Mannheim [15]. Cells were cultured at 37 °C, 5% CO_2 in RPMI-medium containing 10% FCS and 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco) until confluency and could be passaged up to 20 times. HUVEC cells were isolated as described [16] and cultured in Endothelial Cell Culture™ medium (Promocell) supplemented with 10% fetal bovine serum (FCS), 1.0 ng/ml bFGF, 0.1 ng/ml EGF, 1.0 μg/ml hydrocortisone, 100 U/ml penicillin, 100 μg/ml streptomycin and 1% endothelial cell growth factor (ECGF) (all from Sigma, Deisenhofen, Germany) in gelatin-coated culture flasks (Falcon; Becton Dickinson GmbH, Heidelberg, Germany) at 37 °C, 95% relative humidity and 5% CO_2. Confluent monolayers were passaged by trypsin 0.025%/EDTA 0.01% (CellSystems, Remagen, Germany). Tumor cell lines were all derived from ATCC™ and cells were cultured at 37 °C, 5% CO_2 in McCoy's™ (HT29, WIDR and $CaCO_2$) or RPMI™ (SW-480) medium (Gibco) supplemented with 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin.

For the spectroscopic studies, cell cultures were established on 32 mm × 3 mm CaF_2 windows (Korth Kristalle, Kiel), generally 24 h before the experiments. The windows were heat-sterilized directly prior to use. Pretreatment of the windows through coating with polypeptides or proteins was explored, but so far no clear benefits were found, i.e., adherence of the cells to the CaF_2 window was found to be adequate without such pre-treatments. Immediately before the measurements, the windows were assembled into a standard demountable liquid cuvette (SpectraTech EZ-Fill with Bolt-Down Holder) with a 15 μm Teflon spacer, giving a measured pathlength of 11–12 μm. The cuvette was placed on the microscope stage, thermostatted at approximately 35 °C and provided with a flow of the cell culture medium described above at 230 μl/h, prewarmed to the same temperature and transported through the cuvette with a peristaltic pump, model Reglo MS-2/12 (Ismatec, Zurich).

It should be noted that synchrotron infrared radiation is neither toxic nor mutagenic for living cells and does not cause heat damage, as clearly demonstrated by experiments of Holman et al. [17].

Single-sided interferograms were recorded from 0 to 10,000 cm^{-1} at 4 cm^{-1} resolution in single sided/fast return mode at a scanner velocity 80 kHz, using a liquid N_2 cooled mid-range MCT detector with a 250 μm element. Each interferogram acquired was the mean of 256 scans. The Fourier transformation was carried out with Mertz phase correction and Blackman-Harris-3 term apodization. Aperture sizes of 20–30 μm were found to be sufficient to restrict the measuring beam to a single cell.

No baseline corrections, smoothing, atmosphere corrections or similar mathematical manipulations of the data were applied, since we considered these to be a potential source of error and therefore to be avoided whenever possible.

3. Results and discussion

In initial experiments, basic questions concerning how best to monitor the infrared spectra of single living cells were explored. The approach most compatible with the philosophy of reaction-induced difference spectroscopy [11] would be to position a single cell statically in the measuring beam and to record single-beam spectra before and after a defined stimulation of the cell (e.g. with a chemical agent), then to use the log ratio of these two signals to calculate directly the absorbance changes triggered by this stimulus. However, this approach presupposes sufficiently constant source intensity over the period of the experiment. Due to