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Development of Raman Imaging System for time-course imaging of single living cells

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Abstract. Development of novel inverted Raman micro-spectrometer with the ability to perform multi-hours spectral measurements on living cells is presented. Our system combines a Confocal Raman Micro-Spectrometer and Fluorescence Microscope with cell incubator enclosure allowing measurement of cells in extended time period. To illustrate the feasibility of this Raman micro-spectroscopy system for *in vitro* time-course studies of cells we performed an experiment where the same group of cells were scanned with the laser at 2 hours intervals between the scans over 8 hours to build Raman spectral images and ensure that no changes occur due to laser damage or environmental conditions. Cell viability test was performed with fluorescence microscopy on exactly the same cells at the end of the time-course Raman measurements.

Keywords: Raman spectroscopy, live cells, imaging

1. Introduction

Raman micro-spectroscopy is based on the inelastic scattering of laser photons. The technique has high chemical specificity and its attractiveness comes from the ability to provide quantitative information about the biochemical and morphological states of cells. It provides a fingerprint of the intrinsic chemical composition of the measured sample, in a minimally invasive or non-invasive manner and with no sample preparation. Raman micro-spectroscopy is well suited for studying live cells as water solutions (buffers and culture media) do not interfere with experiments and there is no need for cell labeling or other cell modification. In Raman micro-spectroscopy a spectrometer is combined together with a microscope to allow micrometric resolution. This kind of system usually allows point measurement as well as spectral mapping with diffraction limited pixel sizes.

Despite its potential on live cell work, Raman spectral imaging focused on fixed cells because employs up-right microscopes in conjunction with water immersion objectives dipped in the fluid (PBS or culture medium) [2,4,7]. This approach provides a higher Raman signal strength than inverted microscopes where optical losses occur due to reflections at the cell-chamber window and coverslips on which cells are cultured. However, the use of the inverted Raman micro-spectrometer offers more flexibility for performing time-course Raman spectral imaging on live cells since cells are not disturbed during experiments and issues related to bacterial contamination can be avoided.

The correct selection of the laser wavelength and power is an important consideration for Raman spectroscopy of live cells. Biomolecules are sensitive to light and it makes the choice of laser wave-

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length crucial for studying living cells. Lasers with shorter wavelengths give stronger Raman signal, but using low wavelengths can be destructive for some samples due to absorption and the associated rise in temperature. Additionally, most samples of a biological nature, are fluorescent. Although it was recently reported that certain cell types, e.g. HeLa cells, do not produce fluorescence background. Raman mapping of this cell type allowed obtaining high-quality spectral images of fixed cells, which were acquired with short integration time (0.5 s/pixel) and with 488 nm excitation wavelength [3,4]. More recent studies showed Raman imaging of living HeLa cells under mitosis when 532 nm laser line is used and Raman resonance effect observed [1]. Unfortunately, most cell types undergo autofluorescence when excited with visible light. Furthermore cell culture media, which is the environment required for live cells experiments, may also experience fluorescence; therefore it is not possible to perform similar experiments with the above mentioned laser wavelengths on most cell lines.

Since the development of high-power near-infrared lasers, 785 nm lasers have become an attractive choice for Raman spectroscopy of cells and tissue. Firstly, the use of this wavelength avoids the excitation of the strong fluorescence background typical of biological materials when visible lasers are used. However, fluorescence is not the only concern when studying live cells; laser-induced cell damage is another important factor. While visible lasers induce cell death even at low laser powers and short exposure time, there is increasing evidence that 785 nm laser light does not induce cell damage in the power range and exposure times typical to Raman spectral imaging [5,6].

Another significant issue when performing Raman imaging experiments of living cells is overall measurement time. Raman maps on fixed cells may be acquired with longer acquisition times and smaller stepsizes (therefore more pixels in the image), since fixed cells cannot move and internal changes does not occur during measurement. When acquiring Raman image of living cells, cell motility (which refers to both cell migration as well as internal movement of molecules) could be a substantial problem, therefore short measurement times guarantee a more reliable picture of the state of the observed cell in a given time-point.

Additional concern in living cells studies is environmental issues. Cell cultures require a very controlled environment for growth and maintenance of healthy conditions. Therefore during long measurements, like Raman spectroscopic cell imaging, a cell incubator could provide right settings to preserve cell culture. The incubator keeps cultures at an optimal temperature and humidity, and carbon dioxide levels are regulated. Without a cell incubator in place, extended time Raman experiments on living cells are not possible, as studied changes could be due to environmental adjustments.

2. Experimental procedure

2.1. Instrumentation

Raman spectra were recorded using a home-built Raman micro-spectrometer optimized for live-cell studies (Fig. 1A). An inverted microscope (IX71 Olympus) was chosen for the setup as it allows live cells measurements without additional disturbance of objective dipping in culture media or PBS as opposed to up-right microscopes. Due to optical losses occurring caused by the light scattering from the sample holder surface, the optimization of the optical throughput for the inverted Raman spectrometer was very important to reduce the integration time of acquired spectra to 1 second.

A near-infrared 785 nm laser was chosen to avoid cell damage and autofluorescence. The Raman photons are focused and collected by the same microscope objective (water-immersion 60× objective, Olympus) and collimated through carefully chosen optical fiber to a spectrometer equipped with a

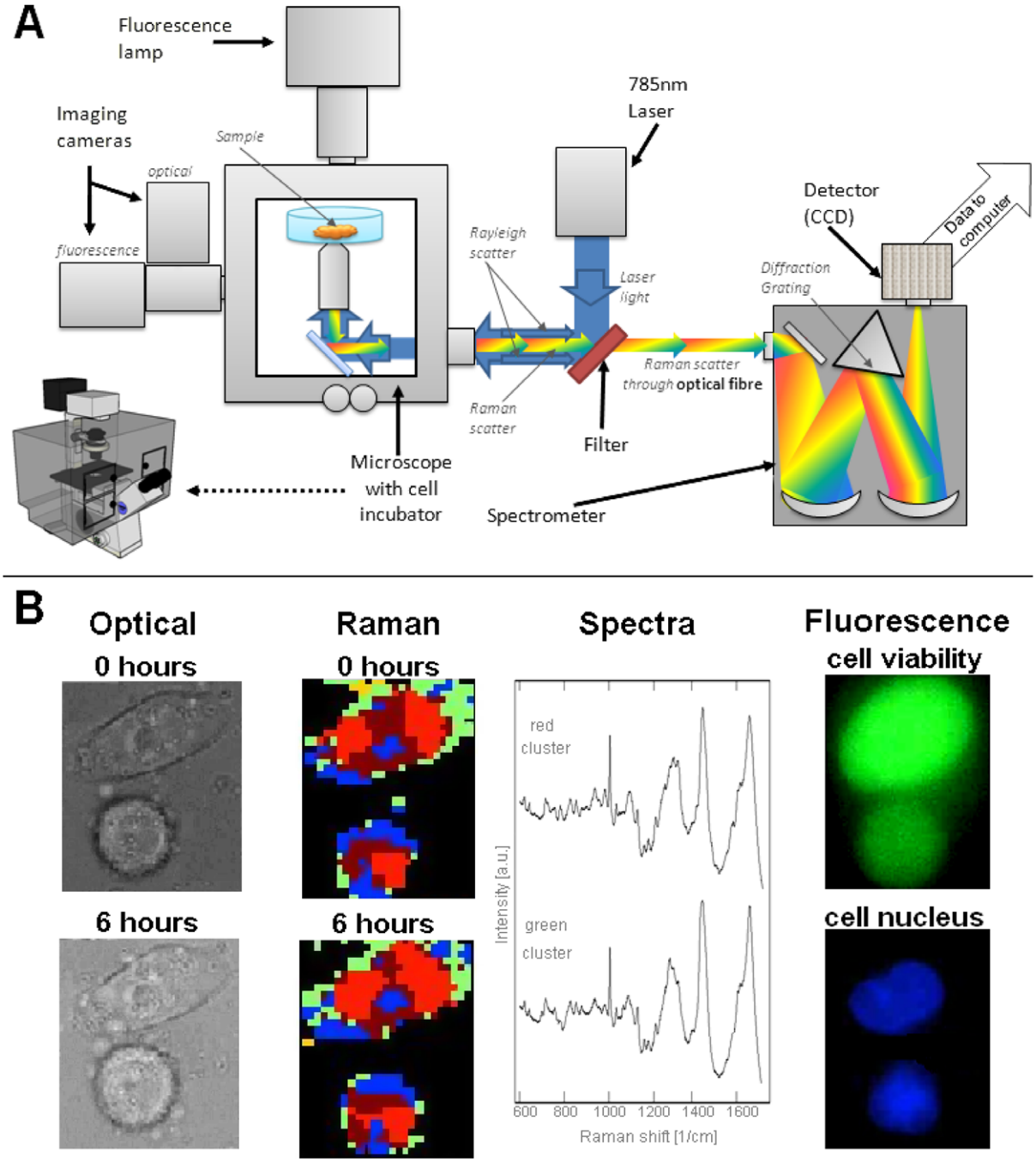

Fig. 1. (A) Simplified schematics of the system. (B) Optical and color-coded Raman images of two cells obtained in time 0 and 6 hours later, along with spectra corresponding to red and green clusters (DNA rich and cytoplasm regions respectively) and fluorescence images of the same 2 cells obtained with two different dyes (6-CFDA – viability test and DAPI – nuclei stain).

830 lines/mm grating and cooled deep-depletion back-illuminated CCD detector (Andor Technologies, UK).

The system is in a confocal setup to reduce background signal and enhance resolution. Confocal alignment usually uses an additional aperture to block the out-of-focus light. In our system an optical fibre is acting as a pinhole and ensuring high stability of the setup. Optical fibers with different core sizes may be chosen and coupled to the system according to resolution and signal strength needed. Moreover, to improve the laser focused spot size in the XY plane a beam expander was aligned in the laser path. A beam expander ensures that the objective entrance is completely filled with laser light that can then be focused to a tighter spot size by the objective.

A high-precision step-motor stage (Prior, UK) is attached to the microscope to enable Raman spectral imaging. Dedicated software was written in Labview language (Labview 7.1, National Instruments) for automatization of the system and data collection. Raman mapping is performed by automated movement of the sample over the laser focus in a raster pattern. The spatial resolution of the spectrometer was measured using 1 μm polystyrene beads; it is $\sim$1 μm in the sample plane and $\sim$4 μm along optical axis when an optical fiber with a 50 μm core.

Wide-field fluorescence images of the same cells could be completed after collecting Raman spectra. The system has the ability to perform fluorescence imaging of cells in several filter setups, which allows flexibility in choosing fluorescence staining according to the cell type or experimental interest.

The sample can be removed from the microscope for the fluorescence staining and then carefully repositioned back in order to find exactly the same cells. This is possible due to specially designed sample holders on which the cells are grown. An accuracy of $\sim$5 μm is achieved.

To ensure proper environmental conditions for living cells the cell incubator (Solent, UK) is placed over the microscope with temperature controller and especially design gas chamber with CO_2 concentration control.

2.2. Materials and methods

2.2.1. Cell culture

Human breast cancer cells (MDA-MB-231 cell line) were grown to confluence (24 hours) on the sample holders with MgF_2 window, incubated in MEM culture medium supplemented with 10% DMSO, 30% fetal calf serum (Gibco) and 1% L-glutamine at 37°C 5% CO_2.

2.2.2. Raman measurements and analysis

Prior to experiment the spectrometer wavenumber axis was calibrated using a Tylenol (Sigma-Aldrich) sample (10 points) indicating an accuracy of 0.5 cm^{-1}. For measurements, the sample holder with cells was placed inside the microscopes' incubator and an area containing 2 cells was selected. Raman spectra were collected by scanning the cells in raster pattern with 1 μm step size, with an integration time of 1 second at each position. This was repeated 4 times with 2 hours intervals between the scans. The laser power incident on cells was $\sim$200 mW.

All spectral analysis was performed using home-built software in Matlab (Mathworks, UK). Raman spectral images were produced using the k-means clustering analysis (5 clusters). Prior to clustering analysis the spectra was pre-processed, meaning that the background spectra was identified and removed (color black in k-means clustering images) and standard correction of the spectra performed (smoothing, baseline correction and normalization). K-means cluster analysis is a well-established spectral analysis technique which has been successfully used for Raman spectral imaging of cells [2]. This technique

identifies regions of the sample with similar Raman spectra by minimising intra-cluster spectral differences and maximising intercluster differences.

2.2.3. Fluorescence staining and cell fixation, retro-positioning

After completing the Raman experiment the sample was removed from the microscope and incubator and stained with 6-carboxyfluorescein diacetate (6-CFDA, Sigma-Aldrich) for 10 minutes for cell viability test, then fixation of the cell was performed by 4% formal saline for 10 minutes. Following, the cells nucleuses were staining with 4′,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) for 5 minutes. Thereafter the sample was put onto the microscope, re-positioned back and fluorescence imaging of the same cells was performed with appropriate fluorescence filters. Fluorescence imaging could help in interpretation of Raman spectroscopic data.

3. Results

Raman scans were performed with 2 hours intervals for 4 times, therefore images at 0, 2, 4 and 6 hours time points were obtained. In Fig. 1B time-course bright field white light images and images using k-means clustering of Raman spectra of MDA-MB-231 cells at 0 and 6 hour time-points are presented. The white light images of the cells show no morphological changes over the studied time. These images suggest that the conditions under which experiments were performed were suitable for maintaining cell viability over the time period of the study and it was confirmed with fluorescence staining afterwards.

The color-coded images built using k-means clustering analysis of Raman spectra of cells show good correspondence with the bright field white light images. The Raman spectral images provide additional information regarding the spatial distribution of cellular molecules. For instance, red and brown clusters in Raman spectral images of the cells show the shape and location of cell nucleuses, which are confirmed with DAPI staining. Spectra of red and green clusters are shown in Fig. 1B for comparison of DNA rich region and cytoplasmic regions respectively.

4. Conclusions

The experiment shows the capability of novel Raman spectroscopy system combined with fluorescence microscopy and incubator to provide extended in time experiments on living cells without prior sample preparation and possibility of fluorescence imaging of the cells under investigation after Raman measurements. Exactly the same cells were Raman mapped a few times with 2 hours intervals and cell viability test and nucleus staining was performed afterwards.

Time-course Raman spectral images of live cells may be used to detect time-dependent spatially-resolved biochemical changes in cells. This technique has a potential in drug delivery studies, tissue engineering or biosensing. Similar experiments cannot be completed by other techniques used in cell biology as they need sample preparations which interfere with living cell and experiments cannot be completed on exactly the same cells.

References

[1] K. Hamada, K. Fujita, N.I. Smith, M. Kobayashi, Y. Inouye and S. Kawata, Raman microscopy for dynamic molecular imaging of living cells, *J. Biomed. Opt.* **13** (2008), 044027.

[2] C. Krafft, T. Knetschke, R.H.W. Funk and R. Salzer, Mapping of single cells by near infrared Raman microspectroscopy, *Anal. Chem.* **78** (2006), 4424–4429.
[3] C. Matthaus, S. Boydston-White, M. Miljkovic, M. Romeo and M. Diem, Raman and infrared microspectral imaging of mitotic cells, *Appl. Spectrosc.* **60** (2006), 1–8.
[4] C. Matthaus, T. Chernenko, J.A. Newmark, C.M. Warner and M. Diem, Label-free detection of mitochondrial distribution in cells by nonresonant Raman microspectroscopy, *Biophys. J.* **93** (2007), 668–673.
[5] I. Notingher, S. Verrier, H. Romanska, A.E. Bishop, J.M. Polak and L.L. Hench, *In situ* characterization of living cells by Raman spectroscopy, *Spectrosc. Int. J.* **16** (2002), 43–51.
[6] G.J. Puppels, J.H.F. Olminkhof, G.M.J. Segers-Nolten, C. Otto, F.F.M. de Mul and J. Greve, Laser irradiation and Raman spectroscopy of single living cells and chromosomes: Sample degradation occurs with 514.5 nm but not with 660 nm laser light, *Exp. Cell Res.* **195** (1991), 361–367.
[7] N. Uzunbajakava, A. Lenferink, Y. Kraan, B. Willekens, G. Vrensen, J. Greve and C. Otto, Nonresonant Raman imaging of protein distribution in single human cells, *Biopolymers* **72** (2003), 1–9.