Development and Application of Raman microspectroscopic and Raman imaging techniques for cell biological studies

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Raman spectroscopy is being used to study biological molecules for some three decades now. Thanks to continuing advances in instrumentation more and more applications have become feasible in which molecules are studied in situ, and this has enabled Raman spectroscopy to enter the realms of biomedicine and cell biology [1–5].

Here we will describe some of the recent work carried out in our laboratory, concerning studies of human white blood cells and further instrumental developments.

1. Carotenoids in lymphocytes

Lymphocytes are white blood cells, responsible for the body's defense against virus infected cells and tumor cells. Epidemiological and experimental investigations have indicated that there may exist an inverse relationship between blood plasma carotenoid levels, and the risk of developing cancer, especially lung cancer [6,7]. Recently we studied, combining fluorescence activated cell sorting and Raman microspectroscopy, the presence and subcellular location of carotenoids in lymphocyte subtypes, specifically in $T_{\text{helper/inducer}}$ -lymphocytes (CD4+), $T_{\text{cytotoxic/suppressor}}$ -lymphocytes (CD8+), $T_{\gamma/\delta}$ -lymphocytes (TCR γ/δ +), Natural Killer cells (CD16+) and B-lymphocytes (CD19+) [8]. The carotenoids appeared to be very specifically distributed among the different lymphocyte subtypes, which all have their own specific function in the immune system. The highest concentration of carotenoids ($\sim 10^{-3}\text{M}$) was found in the Gall body of $T_{\text{helper/inducer}}$ -lymphocytes and $T_{\text{cytotoxic/suppressor}}$ -lymphocytes. For comparison, the carotenoid concentration in blood plasma is of the order of 10^{-6}M . In $T_{\gamma/\delta}$ -lymphocytes, Natural Killer cells and $T_{\text{cytotoxic/suppressor}}$ lymphocytes the Golgi-complex appeared to be rich in carotenoids ($\sim 10^{-4}\text{M}$). B-lymphocytes appeared to contain little or no carotenoids (i.e. $< 10^{-5}\text{M}$), although they also possess a well-developed Golgi-complex. This very cell type-specific subcellular accumulation of carotenoids, which suggests a specific role for carotenoids in the immunesystem, has prompted further studies, in which lymphocyte carotenoid levels in lung cancer patients are compared with those of healthy individuals. Figure 1 shows a typical result. In comparison with the healthy donor the Gall bodies in the lymphocytes of the small cell lung carcinoma patient contain very little carotenoids. Comparison of blood plasma (measured by HPLC) and lymphocyte total carotenoid levels indicates that there does not exist

a clear correlation between these parameters. First results indicate that there may exist a correlation between blood plasma α -carotene levels and the intensity of the Raman signal obtained from lymphocytes. This is the subject of further investigation. Measurement of lymphocyte carotenoid levels could possibly be used to identify individuals at risk.

figure 1: Raman spectra, illustrating the difference in carotenoid levels (A) of a Gall body in a lymphocyte from a patient suffering from small cell lung carcinoma, and (B) of a Gall body in a lymphocyte from a healthy donor .

Experimental conditions: A laboratory made Raman microspectrometer was used, consisting of a Coherent Innova 90K Krypton+ laser, a Nikon Optiphot microscope frame optically coupled to a single grating 0.6 m spectrometer, a Kaiser Optical Systems, Inc. holographic notch filter and a Princeton liquid nitrogen-cooled, slow-scan CCD camera fitted with a thinned back-illuminated Tektronix 512TKB CCD-chip. Laser power: 3 mW (647 nm), signal integration time: 15 s, microsc. obj.: X63 Zeiss Plan Neofluar water imm. (NA 1.2). Cells on fused silica slides in phosphate buffered saline.

Measurements are carried out, with laser excitation away from the ~ 480 nm absorption band of carotenoids. This is done because the rate of bleaching of carotenoid Raman signal increases much faster than the resonance signal enhancement when approaching the carotenoid absorption band. In other words: working off-resonance many more Raman-photons can be obtained per carotenoid molecule, than working in resonance. Figure 2 clearly illustrates this, whereas virtually no carotenoid signal is obtained from the Gall bodies of lymphocytes (of healthy donors) using 514.5 nm excitation, a signal can be recorded even by means of excitation by a not optimally focused 680 nm diode laser, using only ~ 1 mW of laser power on the sample.

figure 2: Raman spectra obtained from the Gall body of lymphocytes of a healthy individual, using resonance and off-resonance excitation.

A) Spectrum obtained using 514.5 nm excitation. Virtually no carotenoid signal is detected (3 mW on sample, 10 s signal acquisition).

B) Spectrum obtained using 680 nm excitation from a Toshiba TOLD 9140 diode laser (~1 mW on sample, 30 s signal acquisition).

The set up described under figure 1 was used. Cells were in phosphate buffered saline on fused silica slides.

2. EPO in eosinophilic granulocytes

Eosinophilic granulocytes form a small fraction (~2%) of the circulating white blood cells. Not all their functions are known at this moment, but one is to defend the body against invading parasites. They do this by attaching to or by phagocytosis of the parasite after which a range of bactericidal substances, contained in cytoplasmic granules take care of killing and digesting the invading organism. One of these substances is the enzyme eosinophil peroxidase (EPO), which in the presence of hydrogen peroxide and halides, produces bleach-like hypohalous acids. EPO is present in very high concentrations in eosinophilic granulocytes (~15 pg/cell). It is a protein, which possesses an active-site heme. In a recent study we showed that EPO heme structure in situ (i.e. in the cell) and its interactions with the surrounding protein, can be investigated and characterized by Raman microspectroscopy [9]. Using excitation in the Soret band (413.1 nm), in the Q_V -absorption band (514.5 nm) and a charge transfer band (623-676 nm), and methods to determine depolarization ratios and excitation profiles of Raman lines of the enzyme in situ, it was found that EPO contains a ferric high spin protoporphyrin IX heme, with a core size of 2.04 Å, histidine as the proximal ligand and a weakly bound water molecule as the distal ligand. Its unusual low wavenumber spectrum, in which in-phase and out-of-phase vinyl bending modes are very prominently present but in which a propionate bending vibration, which is usually found at ~380 cm⁻¹ is absent (Hu & Spiro: personal communication), appear to indicate heme-protein interactions specific for mammalian peroxidases. The fact that heme-molecules can be characterized in situ at the single cell level means that the road to studies in which changes occur in heme structure and/or heme-protein interactions as a result of enzyme activation, lies wide open, although it will undoubtedly be full of pitfalls.

figure 3: Raman spectra obtained in the cytoplasm of human eosinophilic granulocytes.

- A) Spectrum of a resting eosinophil
- B) Spectrum of an eosinophil after a 15 minute stimulation with 5×10^{-10} M interleukine-5 [10]
- C) Spectrum of an eosinophil that has attached to a $2 \mu\text{m}$ opsonized polystyrene sphere. (opsonization procedure described in Bloom, et al. ,1992 [11])

Experimental conditions: The set up described in the caption of figure 1 was used. Laser power: 0.5 mW (413.1 nm), signal integration time:10 s. Cells in Hank's Buffered Salt Solution on fused silica slides.

Figure 3 shows the first results of such a study with eosinophils. It compares spectra of a resting (not-activated) eosinophil, an eosinophil that was stimulated with interleukine-5, and an eosinophil that had attached to an opsonized polystyrene bead. Clear differences are seen. From spectrum B it is clear that stimulation with interleukine-5, leads to exocytosis of the granule contents, so that little or no EPO remains in the cell. Spectrum C shows a spectacular change in the eosinophil spectrum when compared with spectrum A. Judging from the ν_4 vibration at 1352 cm^{-1} , it appears that the iron oxidation state of EPO has changed from $3+$ to $2+$. This promising result shows that further investigation along these lines is warranted.

3. Instrumentational developments

3.1 Raman imaging

New instrumentational development will further enhance the applicability of Raman spectroscopy in cell biological studies. Where Raman microspectroscopy can be applied to determine with high spatial resolution, which molecules are located at a certain position in a cell or chromosome, often the distribution of molecules in a cell or chromosome is an important parameter. Such questions are more easily addressed by Raman imaging techniques. Although Raman imaging was already pioneered by Delhaye & Dhamelincourt in 1975 [12], the technique became really feasible as a chemical imaging method after cooled slow-scan CCD-cameras became available. Different methods have been introduced, which can roughly be divided in image reconstruction methods and direct imaging methods. The first have the advantage that full spectral information is obtained for each point of the sample but suffer from long signal acquisition times, which make them less suitable for cell biological applications. In direct imaging methods the sample is globally illuminated and the sample is imaged on the CCD-chip. However, only light that is scattered in a very narrow wavelength interval (~ 0.5 nm) is used for image formation. A Raman image showing the distribution of a compound in the sample is obtained, when the wavelength at which one of the Raman lines of this compound is scattered coincides with the wavelength interval transmitted by the instrument.

figure 4: Direct imaging Raman set up.

The sample is globally illuminated by rapidly scanning a focused laser beam over the sample, by means of two scanning mirrors. The dichroic mirror transmits light with a wavelength below 700 nm, and reflects above

700 nm. The sample is imaged onto the CCD-chip. A holographic notch filter (Kaiser Optical Systems, Inc.) is used to suppress the intensity of scattered laser light and an ultranarrowband transmission filter (FWHM: 0.5 nm, Omega Optical, Inc.) is used to select a small wavelength interval from the Raman spectrum. Selection of the wavenumber interval at which an image will be recorded, is achieved by appropriately tuning the wavelength of the laser light, coming from a Spectra Physics 375B dye laser, operated with DCM and pumped by a Coherent Innova 70 Argon+ laser. The instrument is equipped with 2 ultra-narrowband transmission filters (centered at 711 nm and 735 nm) and a range of holographic notch filters, so that the whole DCM laser region can be used and wavenumber selection is possible between ~ 500 cm^{-1} and 2000 cm^{-1} . For a detailed description see Puppels et al. [13].

In our own direct imaging instrument [13] this principle takes on a particularly simple form, as is shown in figure 4. Light is detected at a fixed wavelength and Raman line selection takes place by adjusting the wavelength of the exciting laser light. In practice the instrument can make bright field, fluorescence and Raman images, and it can easily switch between imaging and spectroscopic mode.

Below, we give an example in which this instrument was used to recognize polystyrene beads, which were employed to label a cell. The present instrument suffers from the fact that detection is not confocal. Much signal from the surroundings of the object under investigation is detected, resulting in a poor signal-to-noise ratio unless very strong scatterers (such as polystyrene or carotenoids) are being detected. However, in an improved version of the instrument described above, which is presently being developed, the direct imaging principle will be combined with confocal detection. It is expected that with this instrument it will be possible to image DNA-distribution in cells and chromosomes.

3.2 Raman labelling

Raman spectroscopy is a direct analysis technique, i.e. no labels or probes are needed to obtain a signal. Nevertheless in some cases the use of labels may be advantageous, namely when the signal of the molecules one wants to detect is too low, either due to a low Raman scattering cross section or simply due to the fact that only very few of these molecules are present. Practical advantages of Raman labels over fluorescence labels would be that many more Raman labels than fluorescence labels could be used simultaneously, without spectral overlap and that no bleaching of the labels occurs.

figure 5: Combined bright field, fluorescence and Raman microscopic investigation of human lymphocytes after incubation with CD4 antibodies (specific for *T_{helper}/inducer*-lymphocytes) labelled with a fluorescent label (FITC) or small ($0.96\mu\text{m}$) polystyrene spheres. The set up described in figure 4 was used. Cells in phosphate buffered saline on fused silica slide. (Nederlands Tijdschrift voor Fotonica: in press, copyright Dutch Society for Optics and Photonics)

- A) Bright field image
- B) Fluorescence image
- C) Raman image (laser power: 100 mW, signal integration time: 600 s, microsc. obj.: X63 Zeiss Plan Neofluar, water immersion, N.A. 1.2) Set up tuned to the 998 cm^{-1} ring-breathing vibration of polystyrene. A background image recorded at 970 cm^{-1} was subtracted.

Figure 5 shows the result of an experiment in which human lymphocytes were incubated with CD4-antibodies ($T_{\text{helper/inducer}}$ -lymphocytes), labelled with either a fluorescent molecule (FITC) or a small polystyrene sphere. Bright field, Raman and fluorescence images were made of the cells, using the set-up of figure 4. For the Raman image the set-up was tuned to the 998 cm^{-1} Raman line of polystyrene. It can be seen that both the fluorescently labelled antibodies and the Raman labelled antibodies recognize the cell on the right as CD4⁺, and the cell on the left as CD4⁻. A detailed report on the principles and potentials of Raman labels will be published shortly [15].

4. Concluding remarks

Raman microspectroscopy is establishing itself as a valuable research technique for cell biology, which readily yields information on local molecular composition and conformation in situ & in vivo. Characterization of cellular processes, that lead to changes in these by means of Raman spectroscopy appears to be within reach. It can easily be combined with other techniques, such as fluorescence microscopy and atomic force microscopy [14], due to the fact that no pretreatment of the sample or any special conditions are necessary. Further advances may be expected in the fields of Raman imaging and low wavenumber Raman spectroscopy of cells and chromosomes [16]

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