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RAMAN IMAGING OF DENTAL FOLLICLE MESENCHYMAL STEM CELLS

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ABSTRACT. Raman spectroscopy is slowly but surely becoming an important investigative method in cellular samples. In this study, the potential of Raman mapping was assessed on dental follicle mesenchymal stem cells (MSCs). Raman spectra were recorded with the 532 nm laser line at ~1 μm spatial resolution. Principal Component Analysis (PCA) of the recorded Raman spectra provided false color Raman maps, evidencing cellular components such as the nucleus, nucleolus, endoplasmic reticulum and cytoplasmic RNA. A high concentration of this last component can be considered as a universal marker for stem cells. Given that stem cells are in a state where they are always ready to undergo transcription, a large concentration of RNA is found in the cytoplasm of these cells, as they rapidly need to produce proteins that specialize the cell during differentiation.

Keywords: *Raman mapping, principal component analysis, dental follicle mesenchymal stem cells, DNA, RNA*

INTRODUCTION

Raman mapping (or Raman scanning) is a function of Raman spectroscopy in which the laser spot travels through the investigated sample and acquires a

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spectrum at set intervals. The end result of Raman mapping is an image of the scanned object that has highly precise structural information. The difference between Raman spectroscopy and Raman mapping is that singular Raman spectra only provides concrete chemical information at distinct positions within the sample while Raman imaging provides chemical information coupled with spatial information [1].

There are already many studies that focus on the acquisition of standard Raman point spectra and they provide an important basis on future imaging studies. These existing spectroscopic studies have already assigned the important spectral components for a great number of cells types and they provide a background of knowledge that Raman imaging can take advantage of. The dimensionality gained from Raman imaging takes Raman spectroscopy to new heights as a spectroscopic method, as it offers it the ability to investigate dynamic changes within the cell with a high spatial accuracy and a remarkable specificity [1-3].

Several Raman studies focused on different aspects of stem cell development have been already published. Notingher et al. have investigated the differentiation of both murine embryotic stem cells, as well as mouse neural stem cells. The translation of mRNA was monitored by quantifying both, the peak corresponding to the RNA backbone at 813 cm^{-1} and the phenylalanine peak at 1005 cm^{-1}, which is an indicator of proteins [4]. These studies have shown an easily distinguishable method of detecting cytoplasmic RNA, without interference from DNA, based solely on the peak at 813 cm^{-1}.

Ghita et al. have worked extensively on imaging stem cells through Raman spectroscopy [5]. The Raman band at 813 cm^{-1}, which corresponds to the O-P-O vibration of the RNA phosphate backbone, has been shown to be a good indicator of the differentiation state of the cells, and as such has allowed the fast detection of stem cell derived glial cells from stem cells. Neural stem cells have a high concentration of tRNA in the cytoplasm, unlike glial cells, and this has been shown to be a good distinguishing factor between the two types of cells.

Konorov et al used the DNA band at 783 cm^{-1} from a large number of nuclei in order to acquire information about the cell cycle of human embryotic stem cells [6]. Through this method, they were able to determine the state of division of human embryonic stem cells by quantifying the corresponding DNA and RNA peaks from the Raman spectra. As such, the intensity of the band at 783 cm^{-1} proved to be a good identifier of cell phase.

Another purely spectroscopic study on stem cells was performed by Tan et al., showing that spectra of human induced pluripotent stem cells are very similar to spectra of human embryotic stem cells, whilst at the same time being distinguishably different from spectra of differentiated human embryonic stem

cells [7]. The PCA discrimination method that they employed showed a clear divide between the differentiated and undifferentiated cells, which could be mainly attributed to the reduced protein to nucleic acid ratio within the differentiated cells. The authors have stated that proper Raman imaging has to be employed in order to obtain a more complete overview of the cells.

These preliminary studies show that Raman spectroscopy has the potential to become a leading method in the investigation of stem cells as it provides accurate, highly specific results, a great resolution and, more importantly, it is non-invasive to the cell. Therefore, Raman investigated cells can still be used in biomedical applications afterwards and, as such, Raman might be the ideal discriminating method between usable stem cells and unusable differentiated cells, on a per application basis.

Even with these important advantages, Raman imaging studies on stem cells are still not widespread and only a few groups are focusing on this function of Raman spectroscopy when regarding stem cell research.

Some of the most studied stem cells are hematopoietic and mesenchymal lineages as they are commonly used for bone marrow transplantation. Mesenchymal stem cells (MSCs), are pluripotent stromal cells that can differentiate into a variety of cell types, including osteoblasts (bone cells), chondrocytes (cartilage cells), myocytes (muscle cells) and adipocytes (fat cells). Recently, the presence of mesenchymal stem cells was detected in dental tissues (dental pulp, periodontal ligament, and dental follicle) [8]. The dental follicle is a loose connective tissue surrounding the developing tooth. Dental follicle stem cells could therefore become a safe and cheap source of MSCs. As dental follicle is present in impacted, extracted and disposed of teeth, which are regarded as medical waste in dental practice, dental follicle stem cells can be isolated and grown under defined tissue culture conditions.

In this study we assess the potential of Raman mapping on dental follicle MSCs. The obtained Raman maps show different cellular components, and most importantly, a high concentration of cytoplasmic RNA, an indicator for undifferentiated stem cells.

EXPERIMENTAL

Cell culture. Briefly, dental follicle mesenchymal stem cells were cultured in standard stem cell medium DMEM, 4.5 g/l glucose/F12-HAM, 15% FBS (Fetal Bovine Serum), 100 U/ml penicilin-100 μg/ml streptomicin, 2 mM L-glutamin, 1% NEA (Non-Essential Aminoacids), 1 mM sodium piruvate, 55 μM β-mercaptoetanol (Sigma, UK). Prior to Raman measurements, the cells were fixed on 15 mm diameter, 1 mm thick MgF_2 plates with 4% ice-cold paraformaldehyde and stored at 4°C until analysis.

Raman microspectroscopy measurements. For Raman measurements, a Raman microspectrometer (InVia, Renishaw) was used. The instrument was based on a upright microscope (Leica) with a 60×/NA 0.90 water-immersion objective (Olympus), a 532 nm, 150 mW diode laser, a spectrometer equipped with a 1800 lines/mm grating, CCD detector and an automated step-motor stage. The instrument was calibrated prior to each experiment using an internal standard silicon sample, and the spectral resolution in the 220−1970 cm^{-1} region was ∼4 cm^{-1}. The Raman map was obtained by raster-scanning an area comprising 21x31 points on the cell surface, through the laser focus in ∼1 μm steps. The exposure time for each spectrum was 5 s. A total number of 651 spectra were recorded. Spectra preprocessing consisted of cosmic ray removal.

RESULTS AND DISCUSSION

The optical microscopy image of a typical dental follicle mesenchymal stem cell (MSC), obtained with the 60x objective is displayed in Fig. 1. Optical images were recorded before and after the Raman mapping process, which provided us with the certitude that the cell was not damaged after laser illumination during mapping.

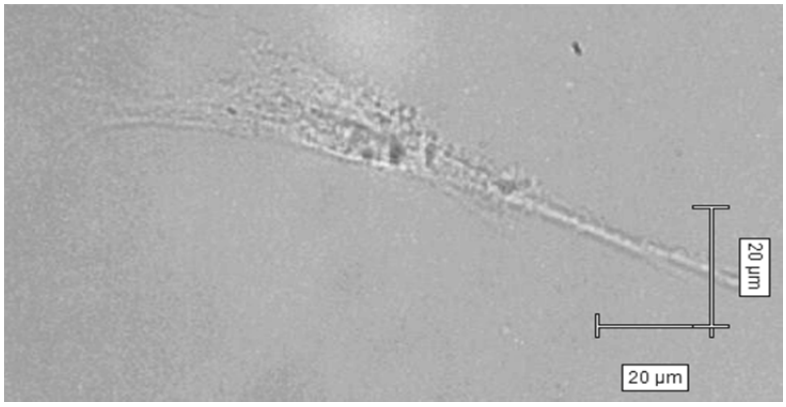

Fig. 1. Microscopic image of a dental follicle mesenchymal stem cell.

Raman mapping at a ~1 μm spatial resolution was performed by using a 532 nm laser line. The literature mentions the preponderant use of the 785 and 532 nm laser lines for cellular mapping [1-3]. Both laser lines show certain advantages and disadvantages, thus choosing the proper laser wavelength remains an option of the operator, depending on the type of cellular probe. If

excitation at 532 nm causes strong fluorescence for a sample, the 785 nm line is a good alternative, as its lower energy photons should overcome this inconvenience. As the intensity of Raman scattering increases with the fourth power of the frequency of the exciting radiation, the intensity of the Raman inelastic scattered photons will be higher with a 532 nm excitation than with a 785 nm excitation. Moreover, regular CCD detectors have a maximum efficiency of detection within the 500-600 nm spectral region, which also coincides with the maximum efficiency of transmittance for most objectives.

In our study, we used the 532 nm laser excitation line in order to maximize the above mentioned parameters especially due to the fact that no fluorescence was observed when exciting with this laser line.

Analyzing the recorded Raman raw spectra, some bands can be assigned by visual inspection, but the main information can be accessed only after noise and redundant information reduction and enhancement of the information rich signal by PCA. PCA provides a certain number of false color score plots and the corresponding loadings plot. In order to decide which of the score plots provide relevant information, the loadings plot has to be evaluated in order to find positive or negative peaks which can be assigned to cellular components.

Fig. 2 shows the scores and loadings plot of the first principal component (PC1).

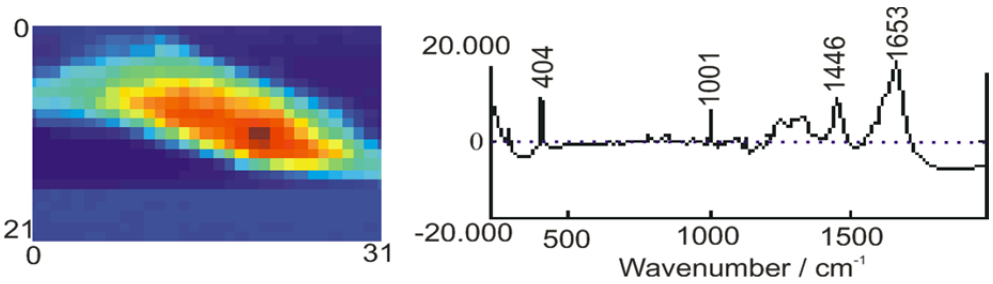

Fig. 2. Scores plot and loadings plot of PC1.

The positive bands from the loadings plot of PC1 can be assigned to bands that are present in all analyzed points during the Raman mapping: 404 cm^{-1} from the MgF_2 substrate, 1001 cm^{-1} due to ring stretching of phenylalanine aromatic amino acid, 1446 cm^{-1} due to C-H bending vibration and 1653 cm^{-1} due to water and amide I band. Given that PC1 contains contributions from both phenylalanine stretching and C-H bending, it is fair to assume that it may provide a good indication of cellular volume, as recently shown by Boitor et al. in an AFM-Raman study [9]. However, no distinct structural information can be extracted from PC1.

Cellular structural information can be first extracted from PC4. Fig. 3 shows the scores plot of PC4, depicting the nucleus (blue) and endoplasmic reticulum (red), as can be deduced from the loadings plot of PC4.

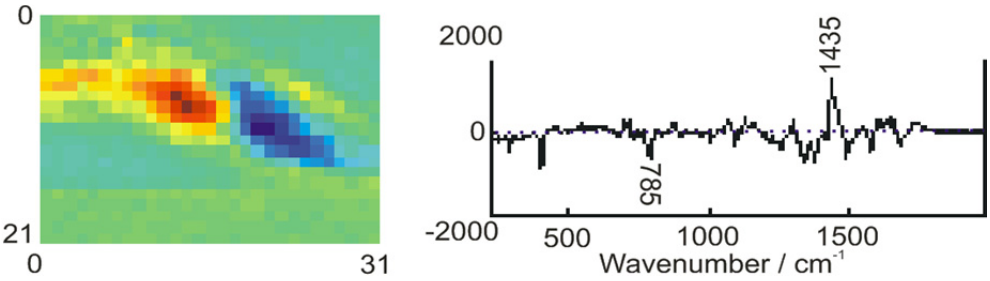

Fig. 3. Scores plot and loadings plot of PC4.

The loadings plot shows positive and negative peaks, with corresponding false colors: red for positive peaks and blue for negative peaks. The negative peak at 785 cm^{-1}, can be considered representative for the negative part of the loadings plot of PC4, being assigned to the stretching vibration of the O-P-O bond, which can be found in the DNA backbone. As such, this peak is a great indicator of DNA, and can be used as an extremely accurate method of pinpointing the nucleus, the main part of cellular DNA being stored in this component. Given that the peak is below the zero line within the PC, the nucleus is represented by the color blue (which sits at the bottom of the color scale) in the PC4 image.

The PC4 image also shows another main component that is inversely proportional to the DNA peak. This red region expresses an intense peak at 1435 cm^{-1}, which usually represents the same vibration that contributes to the peak at 1446 cm^{-1} (Fig. 2) but is shifted to a lower wavenumber because of the specific bonds the CH_2 molecule makes within lipids. Given that the endoplasmic reticulum is strongly involved in the synthesis of lipids, it is highly apparent that the region highlighted in red is in fact the endoplasmic reticulum [10]. The spectral component of this region is inversely correlated to the spectral component of the DNA, and is therefore represented by the color red, which sits at the top of the color scale.

This PC only has one defining characteristic, which is the peak at 813 cm^{-1}. This peak is important as it is the main indicator of undifferentiated MSCs. This peak appears as a result of the stretching vibration of the O-P-O bond within the RNA backbone, the same vibration that causes the appearance of the 785 cm^{-1} of the DNA, but is shifted to a higher wavenumber because of the single stranded nature of RNA, as opposed to DNA.

The last and most important PC of the ones built from the MSC Raman spectra is PC6 (Fig. 4).

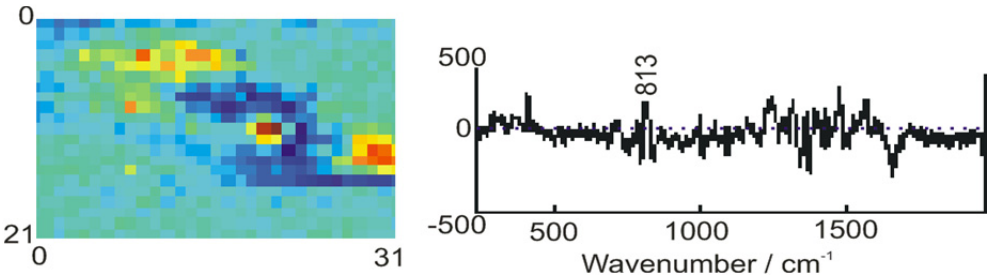

Fig. 4. Scores plot and loadings plot of PC6.

The PC6 scores plot from Fig. 4 shows the cytoplasmic RNA highlighted in red and yellow, but also an intense red point which can be distinguished in a region which was assigned to the cellular nucleus in PC4 (Fig. 3). This point is most likely indicative of the cellular nucleolus, whose one main component is RNA. Given that stem cells are in a state where they are always ready to undergo transcription, a large concentration of RNA is found in the cytoplasm of these cells, as they rapidly need to produce proteins that specialize the cell during differentiation.

As cells that are already differentiated do not present such an intensive transcription process, the concentration of cytoplasmic RNA is obviously lower, below the detection limit of conventional Raman spectrometers.

CONCLUSIONS

Raman mapping is a technique that is capable to provide spectacular results as it combines both spatial and spectral information. The Raman maps obtained from dental follicle MSCs show various cellular components represented in false colors as a function of their respective PCA scores. The most important investigated cellular component was the high, detectable concentration of cytoplasmic RNA, which is an accurate indicator of undifferentiated MSCs. Thus, we believe that Raman spectroscopy has the potential to become a leading method in the investigation of stem cells as it provides accurate, highly specific results, with a good spatial resolution and, more importantly, in a non-invasive manner. Therefore, Raman investigated cells can still be used in biomedical applications after their identification and, as such, Raman is the ideal discriminating method between usable stem cells and unusable differentiated cells, on a per application basis.

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