RAMAN SPECTROSCOPY: A NOVEL TOOL FOR LABEL-FREE AND NON-INVASIVE CELL ANALYSIS AND QUALITY ASSURANCE IN 3D-SKIN GRAFTS

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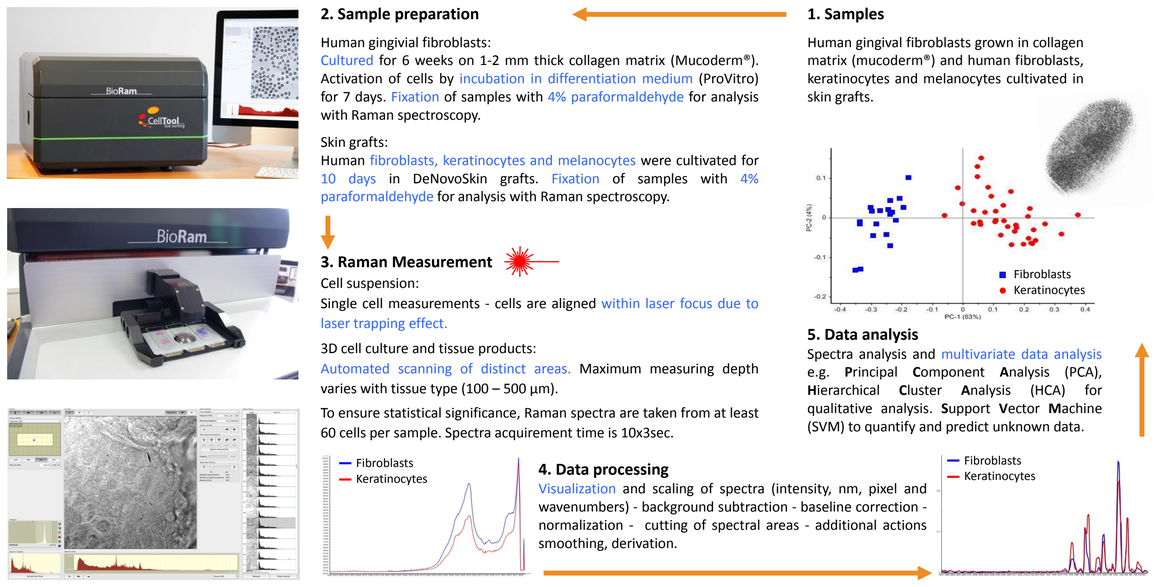
Introduction

Raman trapping microscopy is an innovative bio-analytical method for label-free identification of living cells purely based on their interaction with laser light. A 785nm laser coupled into an inverted microscope and focused through the objective is used to excite intracellular molecules. The resulting molecular vibrations cause a frequency shift of the laser light to longer wavelengths – called Raman spectra. Just like 'photonic fingerprinting' Raman spectroscopy (RS) provides specific information about the overall chemical composition of the specimen. It enables reliable identification of different cell types, discrimination of cell phase and even monitoring of stem cell differentiation without any biochemical labeling such as antibody-based markers or fluorescence molecules.

Here we present the feasibility of Raman spectroscopy for cell analysis and quality control in 3D cell cultures and engineered tissues.

Methods



2. Sample preparation

Human gingivial fibroblasts:
Cultured for 6 weeks on 1-2 mm thick collagen matrix (Mucoderm®). Activation of cells by incubation in differentiation medium (ProVitro) for 7 days. Fixation of samples with 4% paraformaldehyde for analysis with Raman spectroscopy.

Skin grafts:
Human fibroblasts, keratinocytes and melanocytes were cultivated for 10 days in DeNovoSkin grafts. Fixation of samples with 4% paraformaldehyde for analysis with Raman spectroscopy.

3. Raman Measurement

Cell suspension:
Single cell measurements - cells are aligned within laser focus due to laser trapping effect.

3D cell culture and tissue products:
Automated scanning of distinct areas. Maximum measuring depth varies with tissue type (100 – 500 µm).

To ensure statistical significance, Raman spectra are taken from at least 60 cells per sample. Spectra acquirement time is 10x3sec.

1. Samples

Human gingival fibroblasts grown in collagen matrix (mucoderm®) and human fibroblasts, keratinocytes and melanocytes cultivated in skin grafts.

5. Data analysis

Spectra analysis and multivariate data analysis e.g. **P**rincipal **C**omponent **A**nalysis (PCA), **H**ierarchical **C**luster **A**nalysis (HCA) for qualitative analysis. **S**upport **V**ector **M**achine (SVM) to quantify and predict unknown data.

4. Data processing

Visualization and scaling of spectra (intensity, nm, pixel and wavenumbers) - background subtraction - baseline correction - normalization - cutting of spectral areas - additional actions smoothing, derivation.

Results

Raman spectroscopy is a fast and non-destructive method to characterize cells and cell states and to check cell functionality in 2D setups. Furthermore, RS can be used to follow stem cell differentiation, identify pathogens, analyze diseased cells and monitor cell reaction on drugs and toxin. The focus of the present work was to show that Raman spectroscopy is also a valid tool for cell analysis and quality assurance of 3D cell based products. Raman spectra provide meaningful data about the metabolome of cells even in a depth of up to 300µm.

Analysis of fibroblasts in Mucoderm® - Fibroblasts grown in Mucoderm® for 6 weeks under normal or differentiation conditions were analyzed using Raman spectroscopy. Subsequent analysis of Raman spectra using PCA analysis enabled clear discrimination between cells and Mucoderm® matrix as well as between fibroblasts growing in normal and differentiation medium (see Fig. 1). Discrimination of fibroblast cells was mainly due to differences in amounts of collagen type1, proline, lipids and amids.

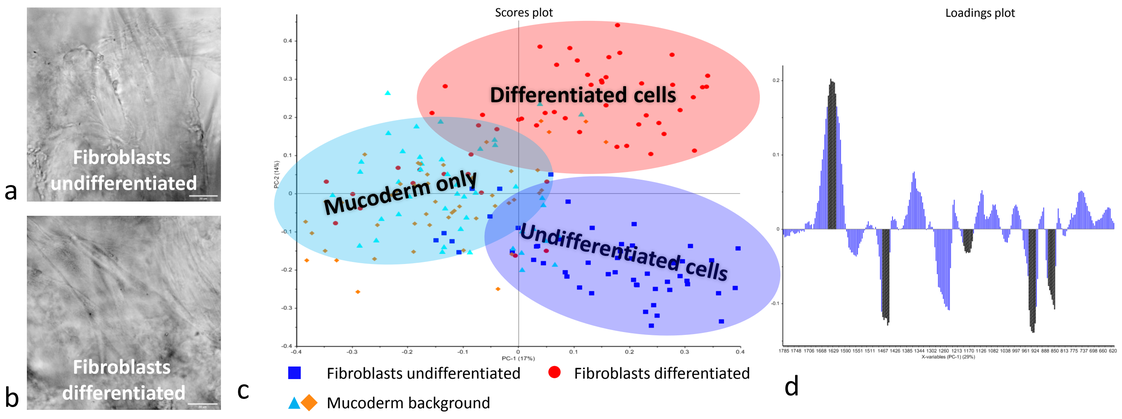
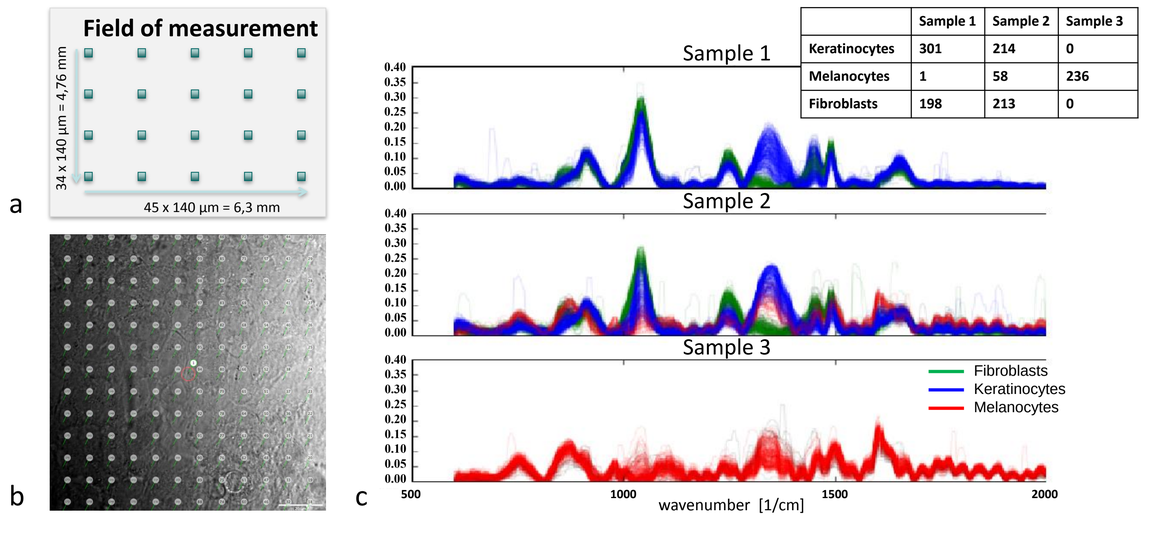


Figure 1: Monitoring of cell differentiation in 3D matrix. a, b: Representative bright field microscope images of fibroblasts. c: PCA Analysis of Mucoderm® only, treated and untreated fibroblasts. Clear differences between treated and untreated fibroblast can be observed in the scores plot. d: Loadings plot depicting wave number areas relevant for discrimination of fibroblast cells (black bars).

Discrimination of keratinocytes and fibroblasts in autologous skin grafts - Keratinocytes and fibroblasts were cultured on DeNovoSkin grafts for 10 days and subsequently analyzed using Raman spectroscopy. Raman measurements enabled clear differentiation of keratinocytes and fibroblasts, which was already observable in mean spectra of the two cell types (see Fig. 2c). Subsequent PCA confirmed these data (see Fig. 2d). Raman spectra of fibroblasts were clearly retrievable also in a depth of up to 300 µm within the skin graft, although microscopic visualization was no longer possible.



	Sample 1	Sample 2	Sample 3
Keratinocytes	301	214	0
Melanocytes	1	58	236
Fibroblasts	198	213	0

Figure 3: Results of blinded sample analysis. a: Schematic drawing of pattern for Raman spectra acquirement. b: Representative picture of Raman measurements on DeNovoSkin grafts. Grey pins indicate points of measurement. c: Spectra acquired in the different skin graft samples (green - fibroblasts, blue - keratinocytes, red - melanocytes). Measured cell numbers within each blinded sample are summarized in the inserted table.

Blinded sample analysis – Raman spectra of patient derived keratinocytes, fibroblasts and melanocytes in cell culture (data not shown) were used to determine the amount (ratios) of keratinocytes and melanocytes on the surface of DeNovoSkin samples. Up to 500 cells on top of the graft models were pin marked and automatically processed to Raman analyses. For identification and quantification of cell types, an Agglomerative Hierarchical Clustering with Cosine Distance was used. With this algorithm, Raman spectra allowed to identify 1 to 3 clusters in each of the three blinded samples that differed considerably in their peak positions. Comparison of the characteristic spectra of each cluster with known spectra of the different cell types allowed to unambiguously determine the fractional composition of the samples (see Fig. 3).

Raman spectroscopy allowed identification of cell populations within all analyzed samples (see table within Fig. 3). Unexpectedly, besides keratinocytes and melanocytes, another cell type was detected at the graft surface. Comparing spectra, those cells could be identified as fibroblasts lurking through the of keratinocyte/melanocyte layer. Final cell ratios were 99% keratinocytes, and 1% melanocytes in sample 1; 75% keratinocytes 25% melanocytes in sample 2; and 100% melanocytes in sample 3. The obtained results were in accordance with the plated cell numbers.

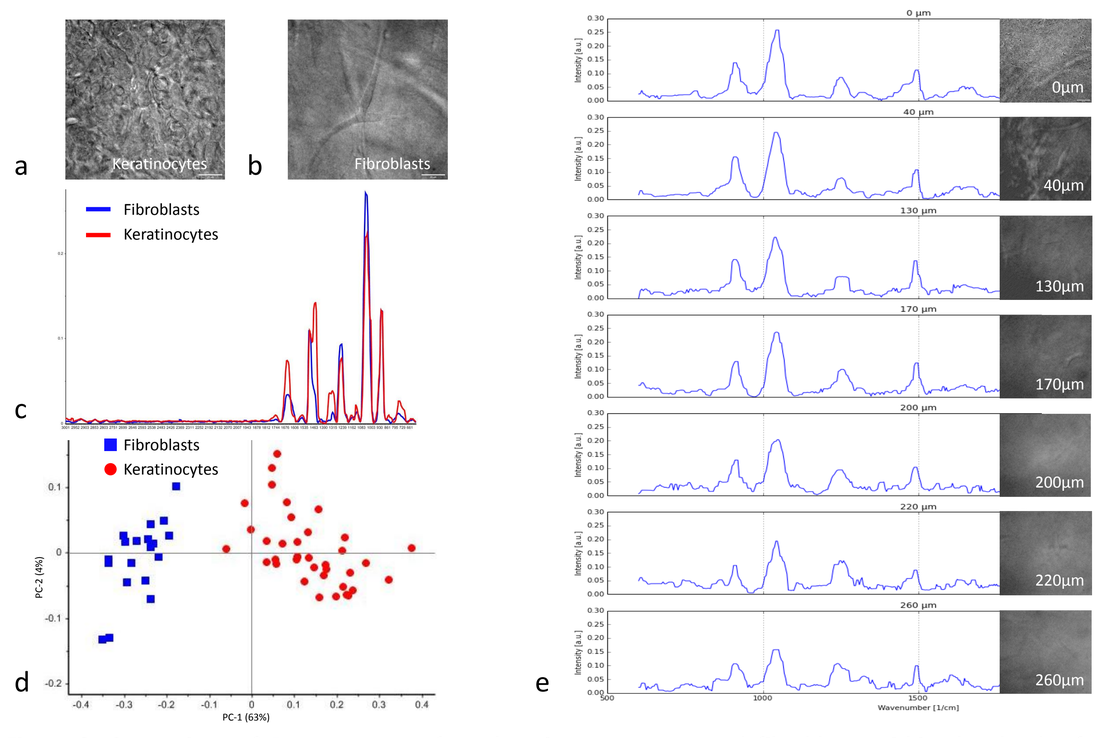


Figure 2: Comparison of Raman spectra from keratinocytes ontop and fibroblasts within the depth of skingrafts. a, b: Representative pictures of (a) keratinocytes and (b) fibroblasts within DeNovoSkin matrix. c, d: Mean spectra (c) and scores plot (d) of fibroblasts and keratinocytes. e: Z-stack Raman measurements of fibroblasts within DeNovoSkin matrix – maximum thickness of the skin graft = 300µm. Also in this depth specific Raman spectra could be acquired.

Discussion

Raman spectroscopy is a powerful tool for analysis of cells in 3D cell culture and tissues. In combination with multivariate data analysis the technology is highly accurate and sensitive, and applicable in a wide range of biomedical research and analysis. Raman spectroscopy works label-free and non-invasive providing highly specific molecular information about the entire metabolome of a single cell. Raman analysis provides spectra of cells that are as characteristic as a "fingerprint" without impairing cell viability. With this, Raman spectroscopy is a suitable tool for analysis and quality assessment of 3D cell cultures and engineered tissue to ensure quality and safety of those cell based products.

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