Cell analysis re-defined

Despite of a huge amount of different analysis methods, many questions regarding cell applications are still open and can only be answered with enormous efforts. In this respect a newly developed Raman microscopy system can help, as it combines noninvasive cell analysis under physiologic conditions with a data analysis using less than hundred measured cells.

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We are very fortunate to live in times where findings in biomedical and biotechnical research are more and more transferred into practical applications. They enable us to improve the treatments of many different diseases, optimize manufacturing procedures or to make our life more pleasant in general. Numerous methods in the basic biological research or the biotechnological manufacturing are based on cell systems of various kinds. With this, fast and reliable analysis of the cells, monitoring of cell-based procedures and quality control of the products are now more important than ever. The great challenge is to find a technology that allows efficient and cost-effective analyses of all cell-based systems, manufacturing steps and products.

The cell analysis methods presently used are as manifold as the cell systems to be analyzed themselves and in many cases they do a good job. However, there are fields in which the so-called "gold standards" reach their limits. Many methods, e. g. immunostaining or flow cytometry, are based on the application of antibodies or color markings. This makes these methods not only cost and time consuming but also limited in their use. There are by far not as many antibodies as there are cell types or cell conditions and many antibodies are simply not specific enough. Moreover, antibody-marked cells for instance may not be used for the treatment of patients. Other methods, such as the fluorescence-activated or magnetic-activated cell sorting (FACS, MACS), as well as various methods for the analysis of proteins and nucleic acids (DNA/RNA) require large cell amounts (> 100,000 cells) in order to deliver conclusive results. Furthermore, most of these methods are so-called "endpoint analyses" as they involve the destruction of valuable cells. Both are exclusion criteria for applications, where only little sample material is available or where the cells or cell products shall be used for the treatment of patients after the analysis.

Raman spectroscopy as the method of choice

A method that is cell-preserving as well as time and cost-saving is the Raman spectroscopy. Originally found in physics, chemistry and material sciences, this technology has now made its way into life sciences. With the newly developed BioRam system (**figure 1**), specialized in the needs of biologists and physicians, a high-precision and nevertheless easy-to-use Raman microscope is now available for the first time. The integrated optical tweezers hold the cells within the laser focus during Raman measurements, so that reliable spectra can be taken even of moving samples. The Raman microscope is so versatile that besides of single cells in solution, it can also be used for analyses of adherent cells in 2D and 3D cell groups and entire tissues. The system is designed for the observation of differentiation and treatment processes, the monitoring of production processes as well as for the quality control of cell-based products.

The Raman technology is based on the interaction of the laser light and the molecules within the cell. The molecular compositions of individual cells are translated into specific Raman spectra. Thus, each cell provides a unique "photonic fingerprint" that distinguishes it from other cells. With these "finger prints" it is possible to differentiate various cell types, to follow up cell differentiation or monitor specific cell reactions. The system also allows the quality control of cell-based products or production steps and this above all because the BioRam technology can not only be applied in 2D cell cultures but also in 3D tissues. In combination with the optical tweezers even cells in solution or freely moving bacteria can reliably be analyzed. Only 100 cells are sufficient to obtain conclusive analyses. Therefore, this method is also suitable for issues where only a few cells are available or where no material can be wasted. The fact that the cells are not modified or damaged in any way allows their use for further analyses or patient treatments. Due to the easy handling of the BioRam and the high specificity of the Raman spectroscopy, the possible applications are manifold. From this not only the basic research but also the applied research and life science production benefit. In the following, four application areas are described.

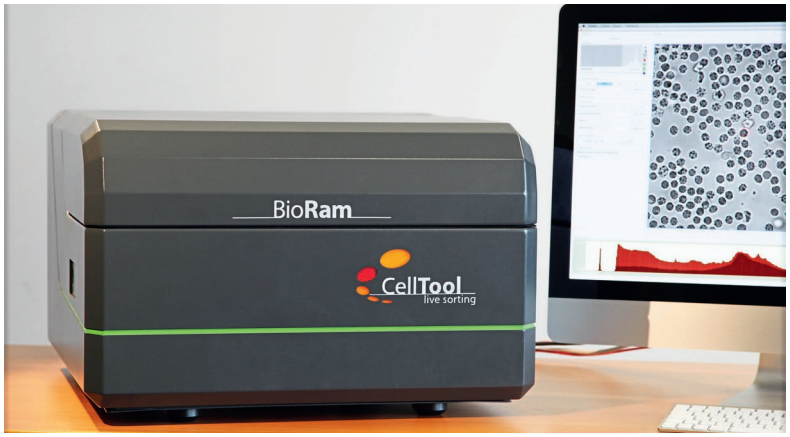

Figure 1: BioRam Raman microscopy system

Image: CellTool

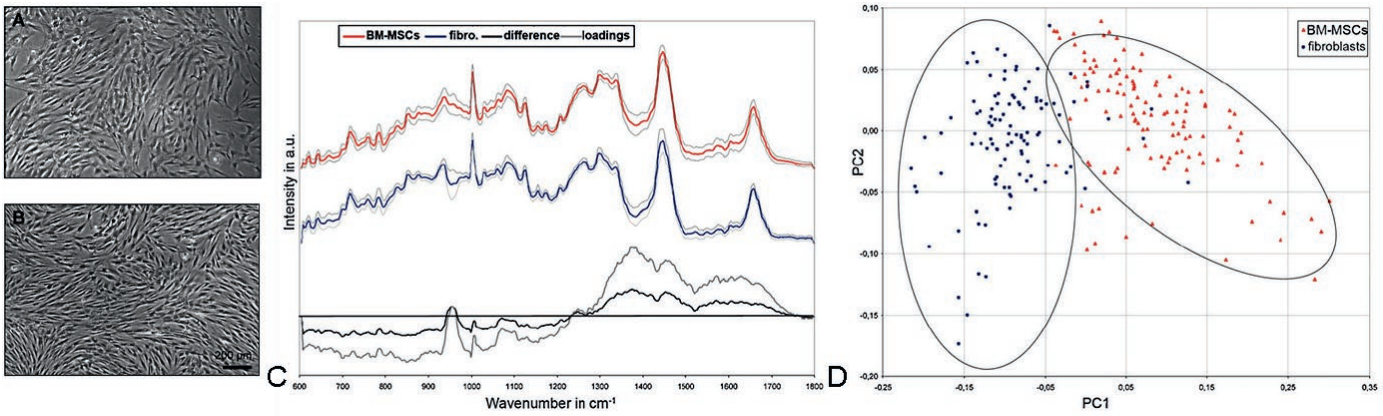

Figure 2: Raman spectroscopy for cell differentiation: (A-B) Bright field microscopy images of human mesenchymal stem cells (A) and fibroblasts (B)
(C) Raman mean spectra of human stem cells (red, n = 122) and fibroblasts (blue, n = 109) with difference spectrum (black line) and loadings values (grey line) (a. u. = arbitrary units)
(D) PCA-Scores plot (modified figures of [1])

Sensitive monitoring of cell development and differentiation

In science as well as in the biotechnology industry the monitoring of cell cultures is essential. The BioRam technology provides a quick, simple and non-destructive method for the in-process monitoring. This could be shown with mesenchymal stem cells from bone marrow and fibroblasts. Compared to microscopic and flow-cytometric approaches or to *in-vitro* differentiation assays, the Raman spectroscopy, in combination with a statistical evaluation of the measured values by principal component analysis (PCA), has proved to be extremely fast and efficient in distinguishing stem cell cultures from fibroblasts (**figure 2**) [1].

Further analyses in this application field showed that Raman spectroscopy is capable to differentiate between various cell conditions, e. g. apoptosis or necrosis [2], and to characterize the effects of various culture conditions of different culture media, oxygen content etc. Moreover, first analyses of cell culture supernatants show that even specific substances, e. g. insulin or other cell excretion products, can be detected.

Efficient quality control of cell-based products

Biotech companies use a wide range of different cell systems to control production. These production processes require frequent and reliable quality controls. However, due to the specific process requirements, time for quality measures is often very restricted and thus the number of appropriate analysis methods is limited. Initial tests for the quality control of blood products have shown that the Raman spectroscopy could prove the aging process of products like thrombocyte or erythrocyte concentrates. With the spectra it was possible to determine the date when the blood products were no longer usable, what absolutely coincided with the present empirically determined storage life limits. For erythrocyte concentrates could be shown that the storage life is donor-dependent. In this case, the immediate test of the blood product with BioRam shortly before transplantation could contribute to the patient's safety and additionally saves costs, given that blood products would no longer have to be discarded due to any preset cut-off [3, 4].

A special challenge is the quality assurance of 3D products and tissues, as many methods are not capable to analyze deeper lying layers of such samples. This is important insofar as cells around the edge of a cell group or tissue frequently behave differently from those situated in the middle.

The BioRam technology allows the data collection of significant spectra even in 3D cell cultures to a depth of 500 µm and more. Fibroblasts cultivated alone in a hydrogel showed other Raman spectra than fibroblasts co-cultivated with tumor spheroids formed by breast cancer >

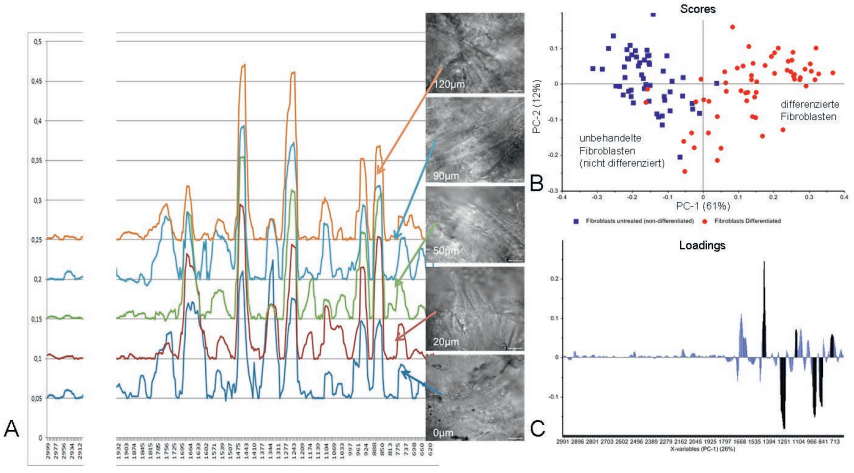

Figure 3: Raman spectroscopy in 3D cultures:
(A) Mean spectra and related microscope images of untreated fibroblasts in different depths of a mucoderm matrix
(B) PCA-Scores plot of untreated fibroblasts (blue) and differentiated fibroblasts (red)
(C) PC-1 Loadings plot. Black lines indicate in which areas of the spectrum the cell populations differ most significantly (modified figures of [5])

Images: CellTool

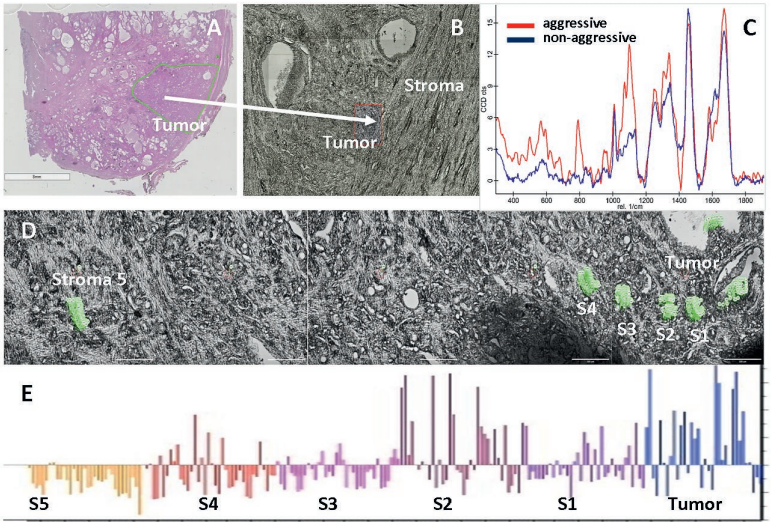

Figure 4: Raman examination of prostate tumors:
(A) H&E stained tissue section of a prostate tumor with the tumor area marked
(B) Dewaxed, 10 µm thick, unstained tumor section for the Raman measurement
(C) Raman spectra of aggressive (recidivism after 6 months) and non-aggressive tumor (no recidivism > 5 years).
Mean spectra show significant differences – the accuracy of differentiation aggressive/non-aggressive was 91 %
(D) Overview figure of measurements in the tumor and stroma (marked green)
(E) Results of the measurement after statistical analysis shown in a line plot. In the stroma the prediction accuracy aggressive vs. non-aggressive tumor depends on the distance from the tumor, and was 94 % at a distance of 100 µm and 80 % at 500 µm

cells. The difference could be assigned to the molecule collagen. Using the Raman spectroscopy, the tumor spheroids themselves could be dimensioned in x/y-direction along the "equator" as well as in z-direction. Even in a compact mucoderm-matrix, spectra of fibroblasts cultivated in there could be detected (**figure 3a**). Spectra at a depth of 120 µm were as significant as those detected at the outside of the matrix. Even in such a depth the BioRam could distinguish untreated cells from those that were treated with differentiation factors (**figure 3b, c**). Light-microscopic standard tests were not capable of doing that. In addition, the Raman spectra obtained allowed the statement that both cell populations mainly differed in the biomolecules collagen, lipids and primary amides [5].

The BioRam technology was especially convincing in the quality assurance of autologous skin grafts. During the production process, the contamination degree of fibroblasts and keratinocytes cultivated from the undamaged patient skin can be determined in the cell culture. After completion of the graft, the Raman microscope system can be used for purity and functionality tests directly in the 3D tissue. Compared to the FACS or DNA count procedures applied so far, the quality control with the new system saves times and material costs.

Early diagnosis of diseases and infections

Present procedures in tumor diagnostics are either based on insensitive, spectroscopic technologies or strongly depend on the quality of biopsies taken. Furthermore, biomarkers are often not available in order to reliably assess the aggressiveness of the tumors detected. Also in this case the new Raman system provides a remedy: It is indeed possible to examine fixed cells or fixed human tissues. The latter are mainly used in human diagnostics as a substrate for disease assessments. In the clinic-diagnostic routine, biopsy samples or tissue pieces taken from surgically removed organs are fixed in formalin and embedded in paraffin (wax) and are thus preserved unlimited. The tissue structure remains intact and after being stained with current dyes, microscopic sections are examined for disease characteristics by the pathologist. BioRam allows tissue examinations independent from dyes with the advantage that conclusive data can be supplied fast and comparatively cost-effective. In a clinical pilot study it could be proved that it is not only possible to diagnose prostate cancer with this method but also to distinguish the aggressive from the non-aggressive form (**figure 4**). This is particularly important in patients with prostate cancer of Gleason grade 6 and 7a – i. e. a classification according to severity and aggressiveness, deciding either on the removal of the prostate or a conventional treatment. Furthermore, the Raman method is independent from current biomarker-based approaches and thus independent from the individual genetic differences or factors leading to carcinogenesis. It is also possible with this physical method to analyze prostate cancer in tissue samples, even if the biopsy punch missed the tumor. In other words, Raman spectra measured outside the tumor, i. e. in the surrounding stroma, are equally conclusive and can differentiate aggressive from non-aggressive prostate tumors. The prediction accuracy depends on the distance from the tumor and was 94 % at a distance of 100 µm and 80 % at a distance of 500 µm from the tumor.

Another advantage of the method is that the measurement results are projected to digital microscopic tissue patterns and thus can be evaluated in combination with the traditional histologic methods. This provides a second confirmation of the diagnosis and consequently contributes to the patient safety considerably. The Raman spectroscopy could differentiate Hodgkin lymphomas from non-Hodgkin lymphomas or follow up the penetration of glioblastoma cells into nerve tissue [6]. Furthermore, the Raman spectra of the cerebral tumors meningeoma and astrocytoma are significantly different form each other [3]. Initial measurements on circulating tumor cells showed that the Raman spectroscopy can also be used for the identification of circulating tumor cells in patient samples. Another possible application area of the Raman spectroscopy is the follow-up and control of the therapeutic process and success. For instance, it is possible to follow up the effects of an active substance within cells. This could be proved on breast cancer cells of line SKBR3 which were treated with Herceptin. The process

Image: CellTool

of this treatment could be followed up in a simple and precise way (**figure 5**).

Also other diseases can be detected and monitored using Raman spectroscopy. So it is not only possible to differentiate individual bacteria stems [7], but also to prove the presence of bacterial and viral infections in human cells [4]. Furthermore, the percentage of human monocytes infected by the pneumonia germ Chlamydia pneumoniae could be determined with Raman spectrocopy and this considerably faster than with traditional methods, like DNA microarray or immunofluorescence staining [8]. Besides that, first experiments in the area of the neurodegenerative medicine show that Raman spectroscopy could help to detect diseases like Alzheimer's and Parkinson's at an earlier stage [9, 10].

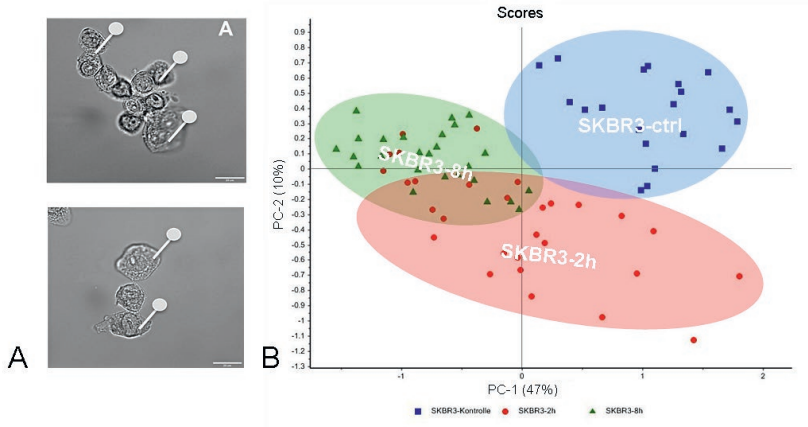

Figure 5: Follow-up of the Herceptin uptake in SKBR3 tumor cells: (A) Representative microscope images. Pins mark the areas of Raman measurements. (B) Scores plot of the PCA. It can clearly be differentiated between the various samples.

Detection of cell reactions on active substances and toxins

Toxicity tests are an obligatory requirement of the authorities for active substance testing and drug development. These tests are often associated with animal tests which are to be reduced to a minimum for ethical as well as economical reasons. This however, means a major challenge for pharmaceutical companies, cosmetics manufacturers, the chemical industry and last but not least for biomedicine. Human tissue and organ cultures nowadays represent a good alternative to animal tests, however, their analysis is often time-consuming and difficult.

Also in this area the newly developed Raman microscope system is able to support science and development. One example for that is the successful analysis of a 3D model of the human trachea mucous membrane in order to examine the infection with whooping cough. Therein, different cell types could be analyzed and it was possible to ensure that the models were not contaminated with tumor cells [11]. With this, the BioRam system provides a simple method for a fast and reliable analysis of cell reactions and toxicity. In combination with microwells, small cavities applied on a chip in which only one or few cells fit in, it is even possible to observe the behavior of individual cells over treatment periods.

Chances and possibilities of the Raman spectroscopy

After being used in chemistry, physics and material sciences, Raman spectroscopy has finally found its way into life science research and application. Thereby, the method proves to be so versatile that its possible applications have by far not been exploited yet. Above all, the cell-protecting technology, the avoidance of extensive preparation and staining procedures and the possibility to obtain conclusive results from less than 100 cells, makes this method a trendsetting cell analysis procedure.

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