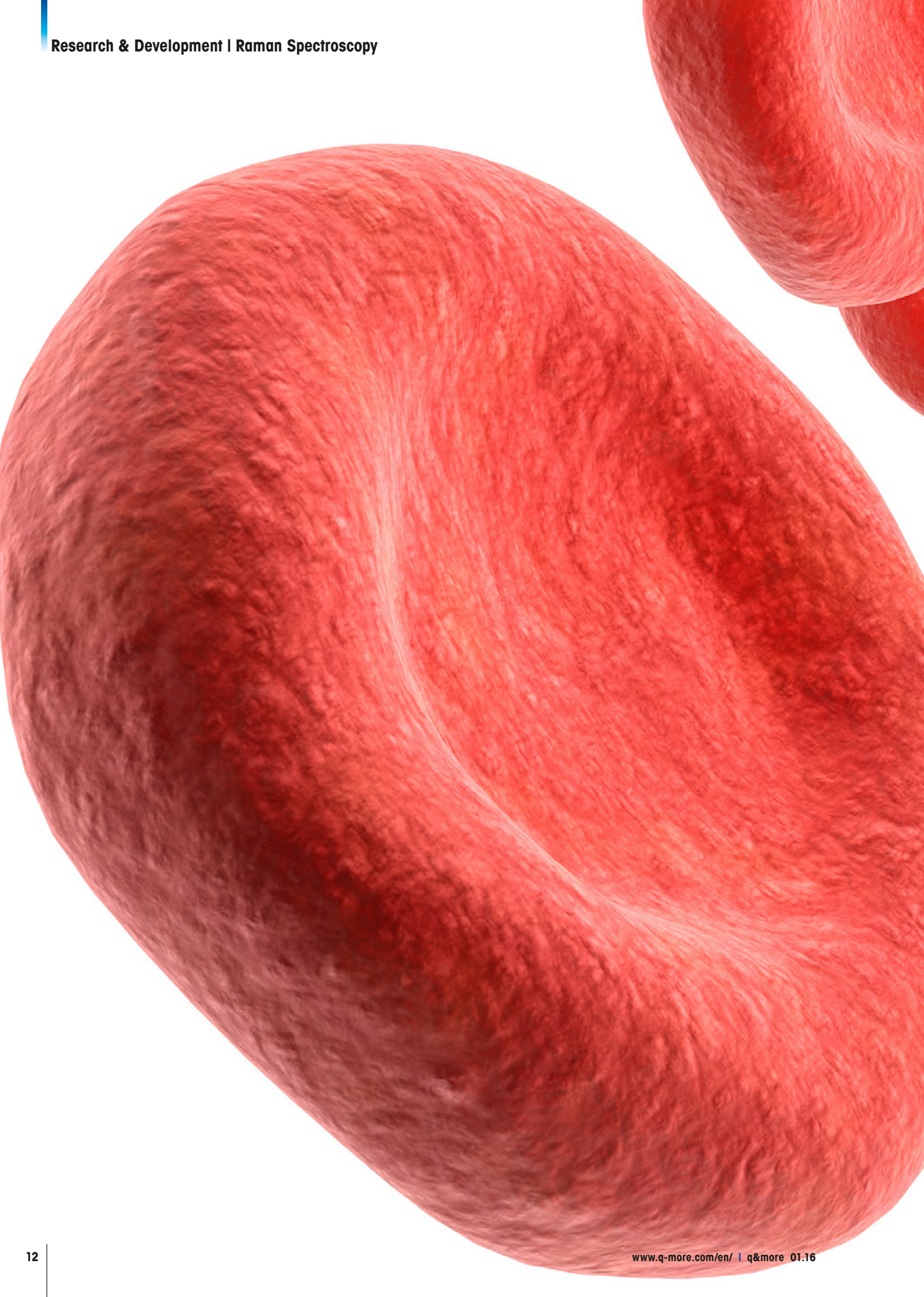

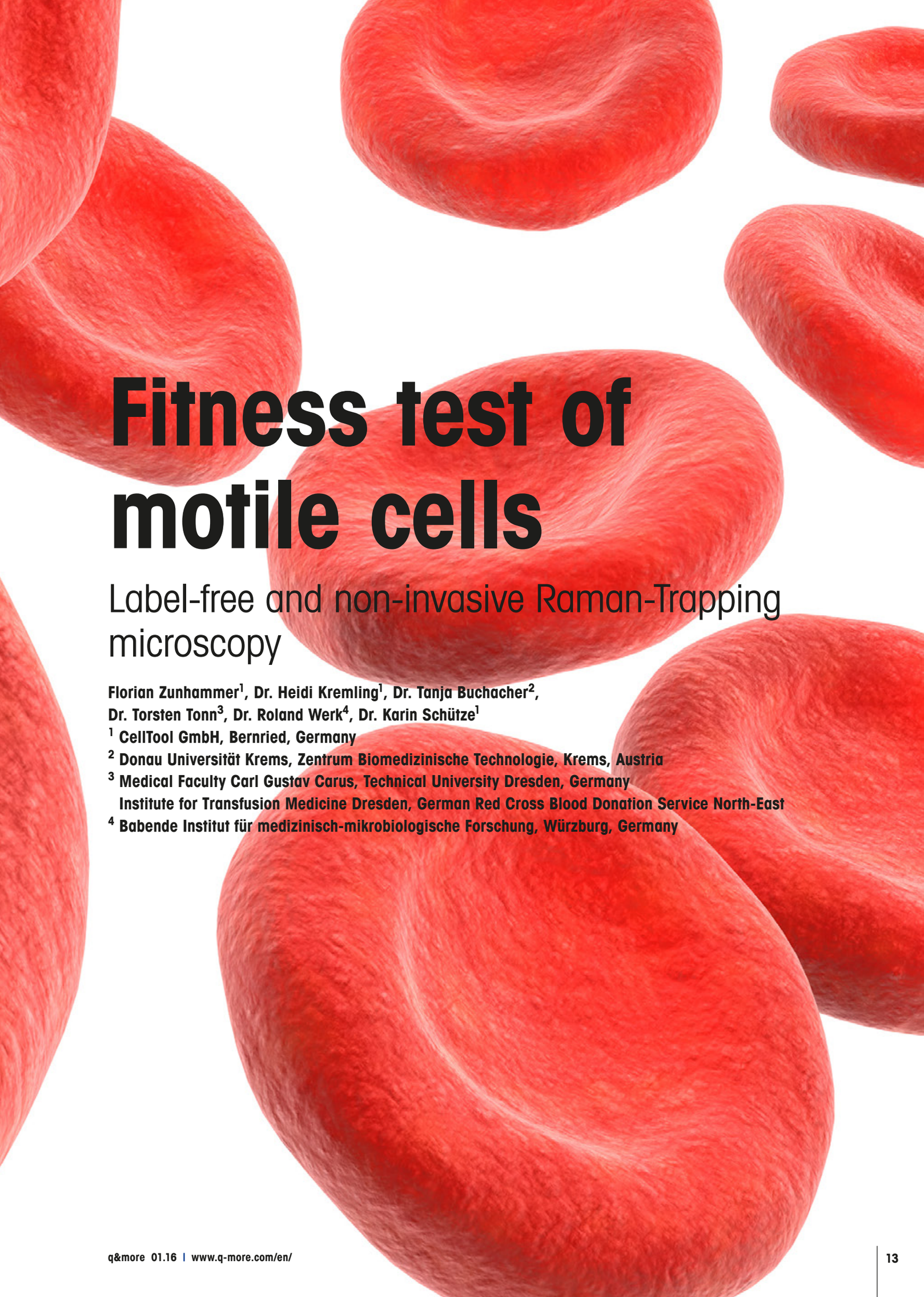

Fitness test of motile cells

Label-free and non-invasive Raman-Trapping microscopy

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Analysis of single cells is never easy – and it gets even more difficult when cells are not fixed at a surface but freely moving. In this case, Raman-Trapping-Microscopy arrests motile samples during spectral analysis. These most powerful devices enable novel insights into characteristics and behavior of eukaryotic cells and microorganisms.

Raman spectroscopy is an analytical method solely based on the interaction of molecules with light. Specifically, it generates a vibrational fingerprint, which is unique for every cell type. Optical trapping allows retaining and manipulation of small particles and motile samples. Combining Raman spectral microscopy with optical trapping allows efficient analysis of motile eukaryotic cells and microorganisms [1] – label-free and non-destructive.

There are two types of trapping possibilities:

I. Particles can be trapped in a stretcher, where two divergent counter-propagating laser beams form optical tweezers [2]. This kind of trap does not require high grade optics and passes only little energy into the trapped particle [3].

II. A single beam optical trap provides a force strong enough to hold and move small particles, like viruses, bacteria and organelles without destroying the trapped specimen [4,5].

The latter setup is realised in the BioRam® system (CellTool, Bernried, Germany), where the Raman excitation laser is set to allow simultaneously trapping during Raman spectra acquisition. As the combined Raman-Trapping-Microscope works non-invasively and under physiological conditions, cells remain vital for repeated use.

Challenge 1 – Identification of bacteria subtypes

Increasing occurrence of antibiotic resistant bacteria is a heavy threat in modern hospitals. In order to ensure safety of patients it is essential to check for and characterise remaining microorganisms on a routine basis. Current methods like mass spectrometry analyse entire bacterial colonies. Thus, only an overall picture of the bacterial culture can be shown, whereas subpopulations may be under-represented or even be masked within the results.

To show the large potential of the BioRam® system in this field, we analysed three different strains of *Pseudomonas aeruginosa* and *Staphylococcus aureus*, which were previously fixed with ethanol. During measurement,

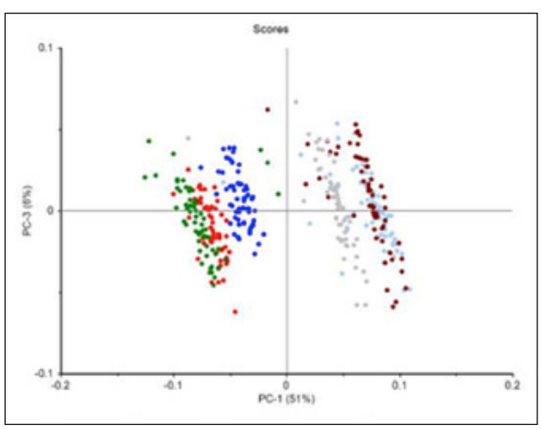

Fig. 1 Results of Raman spectroscopy and subsequent Principal Component Analysis (PCA) of *Pseudomonas* and *Staphylococcus* samples. Scoreplot (PC-1/PC-3) shows Raman spectra of different *Pseudomonas* ● ● ● and *Staphylococcus* ● ● ● strains. Every dot in this score plot represents one measured cell.

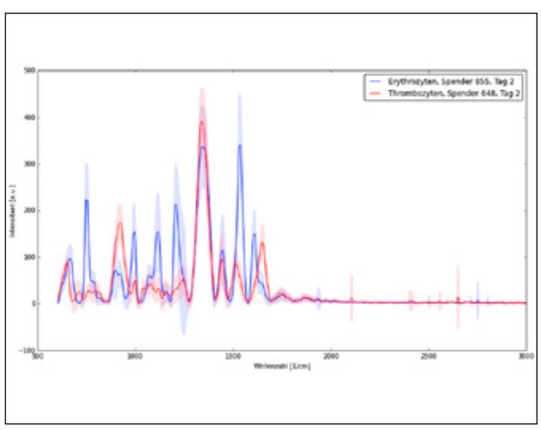

Fig. 2 Raman spectra of erythrocytes and thrombocytes: mean spectra with standard deviations of erythrocytes ● and *thrombocytes* ●. clearly differ. Marked peak differences are mainly related to the haem-group.

single cells were kept in position by optical trapping, enabling the acquisition of significant Raman spectra. Subsequent analysis of Raman spectra using Principal Component Analysis (PCA) – a standard method for reducing dimensionality in multivariate datasets – allowed the separation of bacterial species as well as of different strains. *Pseudomonas aeruginosa* and *Staphylococcus aureus* samples differed in their specific Raman spectra (see Fig. 1). A more detailed analysis of *Pseudomonas aeruginosa* and *Staphylococcus aureus* data revealed even more prominent differences between the single strains (data not shown).

Besides the measurement of ethanol fixed bacteria, it is also possible to perform Raman spectroscopy of living microorganisms taken e.g. directly from colonies of an agar plate that had been exposed to room air [6].

One of the huge advantages of Raman spectroscopy compared to methods like plaque assays, PCR or mass spectroscopy is its single cell resolution. With this, Raman spectroscopy allows detection of rare cells in bacterial colonies otherwise hiding within overgrowing bacterial subtypes.

Challenge 2 – Quality control of blood products

Even today, guaranteeing the quality of all blood products is difficult to achieve. Only about 1 % of the blood bags are actually tested for quality and purity. These tests were furthermore conducted during the production of the blood products and the tested bags need to be discarded afterwards. This means that there is so far no control of blood products immediately prior to transfusion. The combination of Raman spectroscopy with optical trapping provides a non-invasive, fast and easy approach for ensuring quality of blood products directly before use. This on one hand, enhances patient safety but also saves money as blood products can be tested individually and do not need to be discarded due to empirically determined dates.

In a first experiment, we took Raman spectra of living erythrocytes and thrombocytes using the BioRam® device. Like bacteria, blood cells were kept in the laser focus due to the trapping effect, allowing acquisition of high-quality spectral data. Spectra of different cell types significantly differed, mainly due to the presence of the highly Raman-active haem group in erythrocytes, which allowed easy discrimination of the cells (see Fig. 2).

In a further set of experiments we used Raman spectroscopy to follow the aging process of different blood products using thrombocyte and erythrocyte concentrates (Fig. 3).

Analysis of thrombocyte concentrates from three different donors up to 21 days after preparation, showed that platelets start to differ in their Raman spectra at day 8, whereas the largest variations were observed at a wavenumber range of 1,296 cm^{-1} – 1,333 cm^{-1} (see Fig. 3a). Differences in this range are well known to be associated with apoptotic cell death [7, 8]. Analysis of red blood cell

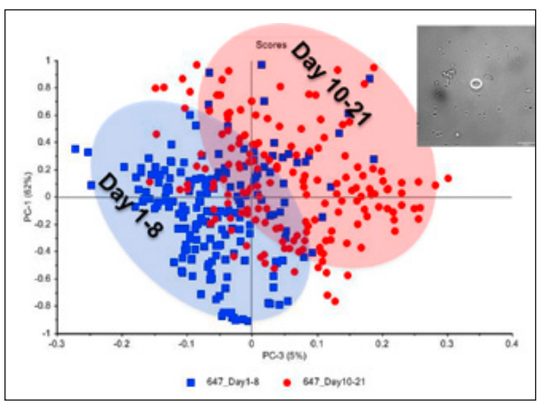

Fig. 3a Score plot of thrombocyte measurements: spectra obtained from cells between day 1 and 8 differ from spectra obtained between days 10 and 21, indicating changes in the thrombocytes after day 8.Picture at the upper right shows representative light microscopy picture. The circle depicts the site of Raman measurement.

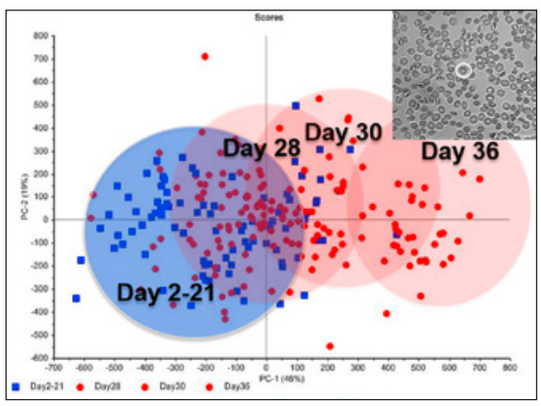

Fig. 3b Score plot of erythrocyte measurements: PC-1 scores correlate with cell states of erythrocytes. Up to day 21 all donors are identical. Thereafter changes in spectra seem donor dependent – "aging" occurs at different time points: day28, day 30 and day 36, respectively. Picture at the upper rightshows representative light microscopy picture. The circle depicts the site of Raman measurement.

Florian Zunhammer acquired his Bachelor's degree in Molecular Biotechnology and completed his Master's studies in Industrial Biotechnology. Since 2015, he works at CellTool GmbH as Application Specialist. His job there is the refinement and enhancement of the BioRam® System, as well as the development of robust and reliable statistical data analysis methods to be used in quality control applications.

Heidi Kremling studied biology at the Julius-Maximilians-University in Würzburg and is a biologist and expert in 2D- and 3D-cell culture, tumor biology and cell analysis. In 2014 she finished her PhD, which she performed at the Klinikum Großhadern Munich. Since February 2015, Dr. Kremling is working at CellTool where she heads the Demonstration- and Service-Lab and manages scientific research collaborations.

Tanja Buchacher obtained her Master's degree in Biotechnology from the University of Natural Resources and Life Science, Vienna, Austria. She received her PhD in Biochemistry from the same university. During this time, she worked closely together with immunologists at the Medical University of Vienna and scientists at the Austrian Institute of Technology. Currently, she works as a PostDoc researcher.

Torsten Tonn is Professor for Transfusion Medicine at the Medical Faculty Carl Gustav Carus, Technical University in Dresden. He is also CEO of the German Red Cross Blood Donation Service North/East. Prior to this position, he served as head of the cell and gene therapy unit of the Institute for Transfusion Medicine in Frankfurt/M. His interest is the improvement of quality and safety of cell based products and the translation of advanced cell therapies into the clinic.

Roland Werk gratuated in microbiology and medicine. He is clinical microbiologist at the BABENDE Institut and concerned with a systems biological approach to gut microbiota.

Karin Schütze studied biology and sports in Heidelberg and did her PhD work in the Institute for Applied Physical Chemistry in Heidelberg. As a postdoc at the University of California in Berkeley, USA, she assembled her first Optical Trap. In 1993 she and her husband Raimund Schütze founded the former PALM company which was successfully sold to Carl Zeiss in 2004. In 2008, she and her husband founded CellTool GmbH, focusing on engineering Raman systems for biomedical applications. In 2006 they received the "Berthold Leibinger Innovationpreis" and were nominated for the "Deutscher Zukunftspreis" of Germany's Federal President.

concentrates over a period of 36 days after donation, yielded comparable results. However, here the onset of cell decay was donor-dependent and started at different time points (see Fig. 3b). Taken together, the obtained results show that Raman spectroscopy in combination with optical trapping is a valuable tool for monitoring the quality of blood products in an easy, quick and non-destructive way directly before use. Thus Raman spectra survey could help to increase safety of blood transfusions.

Challenge 3 – Detection of infection

The combination of Raman spectroscopy and laser trapping can be used as a rapid diagnostic tool for the detection of infected cells. The obligate intracellular pathogen *Chlamydia pneumoniae*, responsible for respiratory infections and a wide range of chronic diseases, uses circulating monocytes as host cells to promote its own replication and dissemination. [9].

In order to demonstrate the power of BioRam® to detect cellular infection, we conducted an experiment, in which monocytes were infected with *C. pneumoniae* and recorded Raman spectra after 6 h and 48 h post infection. The PCA score plot shown in Figure 4 depicts distinct separation between healthy and infected monocytes after 48 h. Monocytes infected with C. pneumonia for 6 h did not differ from healthy monocytes, while after 4 h post infection, about 50 % of the infected cells showed differences in their Raman spectra, which could be associated with *C. pneumoniae* infection (see Fig. 4). Major differences between healthy and infected monocytes could be found in the regions 1,645 cm^{-1} – 1,660 cm^{-1}, 1,430 cm^{-1} – 1,451 cm^{-1}, 1,327 cm^{-1} – 1,356 cm^{-1}, 1,290 cm^{-1}– 1,306 cm^{-1} and 888 cm^{-1}– 934 cm^{-1} of the Raman spectrum, indicating changes in lipids, fatty acids, and nucleic acids [7]. Results from Raman spectroscopy could be verified using an oligonucleotide DNA microarray [10], quantitative real-time PCR and immunofluorescence. Raman analysis combined with optical trapping could therefore become superior to currently used blood cultures for the detection and characterization of cellular infections, as it enables highly sensitive determination of pathogens and provides immediate results.

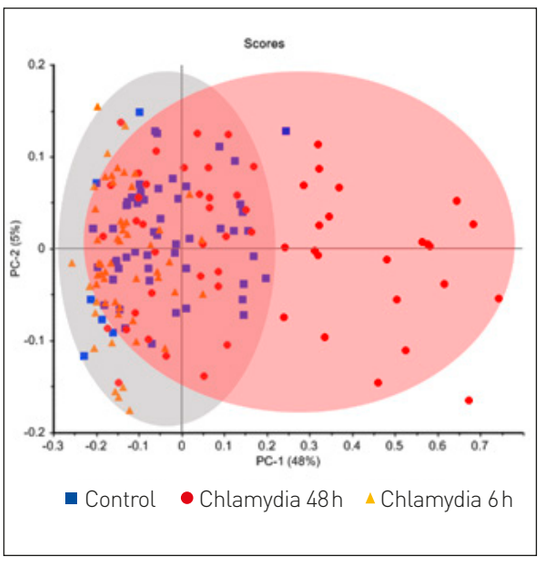

Fig. 4 PCA Scoreplot of *C. pneumoniae* infected monocytes after 6 h ▲, after 48 h ● and the control monocytes ■. About half of the infected monocytes after 48 h appear on the far right side of the Scoreplot due to the infection.

Conclusion

Confocal Raman-Trapping-Microscopy, a combination of Raman spectroscopy and the optical trapping of individual cells, is a highly sensitive method for visualising small differences between cell populations in a large variety of samples. We were able to demonstrate that Raman spectroscopy serves as an ubiquitous 'photonic marker' for the fast and efficient identification of pathogens, survey of cell 'fitness' (i.e. functionality) and early detection of infection. In addition, BioRam®'s non-destructive data acquirement under native cell conditions could become an important device for biologists and physicians to facilitate stem cell identification, to assure quality of cell based therapeutics, or to support cancer diagnosis [6].

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