QI™ mode- New perspectives in Microbiology

The research topic of microbiology covers a whole universe. Every organism that cannot be seen by eye belongs to this part of biology. This includes eukaryotic organisms like yeast cells, fungi and algae as well as prokaryotic organisms like bacteria. Virology can also be understood as a part of microbiology studies, although viruses are not strictly considered to be living organisms. The heterogeneity of the microbial universe is one reason for the huge number of different applications and investigations in this field. Microbial cells strongly differ in their physiology, their genetic composition and biochemistry [1]. There are important modern research issues are in the area of medicine, due to the potential of microbial pathogens to cause diseases, and increasing antibiotic resistance. Microbes are also used productively for fermentation, for instance, so other major research areas are in industrial systems or biotechnology [2].

The field of microbiology is advancing continuously with the development and application of new techniques. Nevertheless we are still at the beginning of the investigations of the microbial universe. Up to now it is not possible to estimate the total number of microbial organisms and so far only one percent of the microorganisms in the environment are culturable [3]. Direct observation of microbial cells started with the development of optical microscopes in the 17th century; the first bacteria were visualized by Antony van Leeuwenhoek 1676. Since then, the field of microbiology has been strongly connected with optical microscopy and has benefited from the enhancements in microscopy techniques. Despite the improvements of optical microscopy, the resolution in classical microscopy is physically limited to the wavelength of light, which makes the investigation of smaller particles like viruses impossible. Newer developments like confocal and electron microscopy in the last century or recently developed optical techniques like STED microscopy reach higher resolution, but there is still an increasing need to investigate microorganisms under physiological conditions without any difficult sample preparation in high resolution.

Fig. 1: *NanoWizard® 3 AFM in combination with a Zeiss AxioObserver inverted microscope. Perfect integration is achieved by JPK´s patented DirectOverlay™ approach.*

Atomic force microscopy (AFM) is a technique that enables a variety of interesting measurements for microbiology research. The microorganism can be measured in their native environment and the microbial surface structure can be measured down to the nanometer scale. AFM imaging can be used to investigate the surface architecture as well as the surface changes due to interactions with drugs and the environment [4]. In addition to information about the microorganism itself, the interactions between different microorganisms or between a surface and a microorganism can also be investigated [5].

Alongside AFM imaging it is also possible to use AFM force spectroscopy, which provides information about biomolecular forces and mechanical properties. It is possible to quantitatively measure the elasticity of microorganisms, to give a closer look into the mechanical properties in the living state. In addition to the surface stiffness it is also possible to observe tip-surface adhesion as well as cell adhesion and molecular recognition [6].

AFM imaging and AFM force spectroscopy offer complementary information about microbiological organisms and it was always a wish to create high resolution images and obtain the mechanical properties in parallel in a reasonable time. Quantitative Imaging, QI™, is a force curve based imaging mode which was recently developed by JPK Instruments. This makes it easier to image difficult samples in microbiology without the need of set-point or gain adjustments while scanning. QI™ Advanced is a software extension that enables nanomechanical measurements in parallel with high resolution imaging and perfectly fulfills the wish of a combination of AFM imaging and AFM force spectroscopy.

Quantitative Imaging – QI™

QI™ has been designed to enable even AFM beginners to quickly and intuitively image challenging samples [7]. In traditional imaging modes like contact mode or AC mode the set-points and the feedback loop must be adjusted properly. A wrongly selected set-point can damage the sample or loosely attached samples like round yeast cells or virus particles can be pushed away, which makes imaging impossible [8].

QI™ is a force-curve-based imaging mode. Therefore the user has the full control over the tip-sample force at every pixel of the image. The novel QI™ tip movement algorithm prevents lateral forces and controls the vertical forces for nondestructive imaging. Samples with soft material (e.g. biomolecules), sticky samples (e.g. bacteria), loosely attached samples (e.g. virus particles or yeast cells) or samples with steep edges (e.g. diatoms) can be easily measured using standard cantilevers.

QI™ Advanced – High resolution nanomechanics

The QI™ Advanced mode allows a full nanomechanical analysis of the measured sample. A preliminary view of stiffness and adhesion are available online and all data can be saved with a high sample rate up to 800 kHz for post-processing with custom settings. The schematic view in figure 2 illustrates the parameters that can be extracted during online analysis of an example force-distance view.

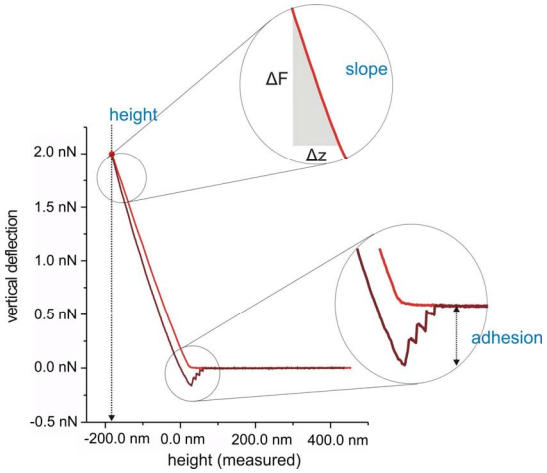

Fig. 2: *Schematic view of online analysis of force distance curves. Besides height information, the maximum unbinding force (adhesion) and the slope/stiffness of the sample are measured.*

For quantitative elasticity values, the Young´s modulus must be calculated for the particular cantilever shape. The most common elasticity fit for this kind of applications is the Hertz fit which is also implemented in the JPK Data Processing software. All implemented fitting operations can be done automatically using a batch processing that allows a quick and convenient processing of all the curves. Other analysis is also possible, for example to calculate the height at zero force (at the contact point) and display specific user defined adhesion events. Analysis routines can be saved and reloaded for all QI™ data files. There is no limitation of post processing analyzing and user-written software can also be used.

For a deeper view into QI™ and further details about the new imaging mode please refer to our technical report: QI™ mode – Quantitative imaging with the NanoWizard® 3 AFM.

Viruses

A virus is an infectious agent that can infect all types of organism. A virus itself can only proliferate inside a host cell and has no self-contained metabolism, which is the reason why a virus particle is not defined as a living organism. A virus consists of an outer protein coat that protects the inner genetic material (DNA or RNA). The outer protein coat is also called a virus capsid and is sometimes studied separately in order to investigate the viral properties without any danger of an infection. Viruses display a wide diversity in size and shape, but in general most viruses cannot be optically visualized due to their size of 20-300 nm. Therefore other microscopy techniques like AFM have to be used to investigate virus particles [9]. The most common virus capsid shapes are the helical and the icosahedral type. The tobacco mosaic virus is a prominent example for a helical capsid virus type, which has also been intensively investigated by AFM [10].

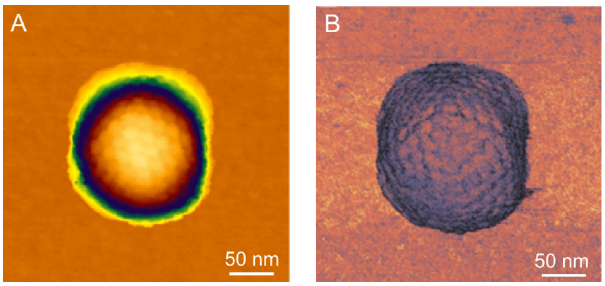

Fig. 3: *Herpes Simplex Virus capsid imaged in liquid, A) Height image (z-range: 100 nm) shows substructure of the virus. B) The substructures can be also recognized in the elasticity image.*

The icosahedral capsid virus type is the most common type and is seen in nearly all animal viruses and half of all virus families. The capsid consists of repeated identical protein subunits (capsomeres) and an icosahedron is the optimum way of forming a closed shell from these capsomeres [11]. A prominent example for this type of virus is the herpes simplex virus. The relatively large herpes simplex virus particles (similar to a 120 nm sphere) have generally been challenging to image. Lateral forces must be avoided to be sure that the virus is not pushed away while imaging. The vertical force must also be minimized, to be sure that the virus capsid is not accidentally broken [12]. Furthermore it is interesting to get more information about the stability and the nanomechanical properties of the virus capsids. It was shown for example in nanoindentation experiments on retroviruses (murine leukemia virus and HIV) that the virus particles soften during mutation [13].

Using QI™ it is possible to get a high resolution image of the virus capsid structure due to the controlled small vertical force (<100 pN) and the prevention of any lateral force. In parallel the information about the stability of the capsid can be measured and analyzed. Figure 3 shows a 300 nm x 300 nm image of a single herpes simplex virus capsid, where the subunits of the virus are clearly seen. In parallel it is possible to obtain information about the elasticity of the virus and the subunits (shown in figure 3B).

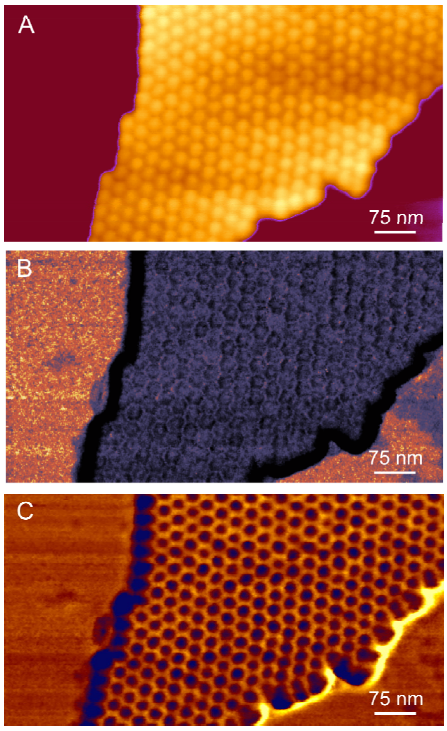

Fig. 4: *Genetically modified Tomato Bushy Stunt Virus capsid, A) Height image (z-range: 30 nm) shows the orientation of the viruses, B) the viruses can be also recognized in the elasticity (range: 3GPa) and C) the adhesion image (range: 2nN).*

Virus particles are not only interesting in medical research – there are also many applications in nanotechnology. Spherical, icosahedral plant viruses like the tomato bushy stunt virus (TBSV) are an ideal candidate for bottom-up technology. The idea is to use small building blocks to form larger elements through self-assembly, e.g. to create electrical components with very small dimensions. Viruses fulfill the requirements for such building blocks. They can be produced with a well-defined size and structure and it is possible to vary their chemical and physical functionality by modifying and changing their amino acid sequence [14].

TBSV is a small virus with a diameter of about 30 nm and belongs to the family Tombusviridae. It is non-human pathogenic and the molecular and biological functions are well characterized. This virus type has been used to investigate the self-assembly and building of homogeneous monolayer on surfaces. The TBSV wild type was genetically modified to generate two different types of TBSV by extending coat proteins at the carboxylic termini with positively and negatively charged amino acids (aspartic acid and histamine) by point mutation [14]. The virus capsids containing negatively charged extended side chains have the ability to form homogeneous, regular surface coverage, as shown in figure 4.

The genetically modified TBSV was measured on a mica surface using QI™. A self-assembled monolayer with highly ordered areas can be seen. Each virus capsid is symmetrically surrounded by six neighbours to form a hexagonal close packed structure. This regular surface coverage can only be seen if the negatively charged amino acids are present on the virus surface and is not visible in this form for the wild type or the positively charged genetically modified equivalent [14]. The Young´s modulus image and the adhesion image underline the ordering tendency and give further information about the genetically modified viruses. To get chemically relevant information about virus particles it is also possible to combine AFM measurements with Raman spectroscopy [15].

Algae - Diatoms

Diatoms are unicellular microalgae and are a common group of phytoplankton. They can be found in all aquatic and moist environments and first appeared more than 180 million years ago. A characteristic feature of diatoms is that they are encased within a unique cell wall consisting of silica. The siliceous skeleton is called a frustule, consists of two valves and is enveloped by organic material including proteins and polysaccharides. The two main types of diatom are the centric diatoms, with circular symmetry and the pennate diatoms with a bilateral symmetry [16].

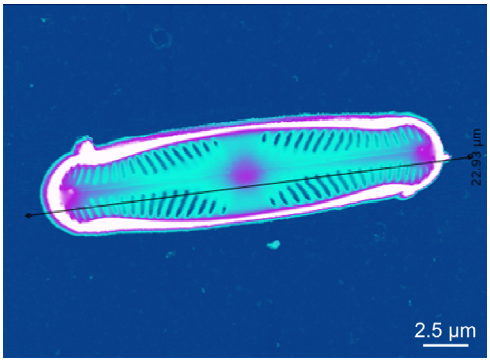

Fig. 5: *Ashed diatom height image (z-range: 1.5 µm) shows the complex silica skeleton of* CCCryo 272-06.

The frustules are especially interesting in nanotechnology research. The complex, precise and reproducible nanometer scale features made of silica are unique in biology and can have a wide range of applications. So far, siliceous structures with the hierarchical structure of the diatom skeleton can hardly be produced by chemical synthesis, which make them very interesting for nanotechnology applications [17].

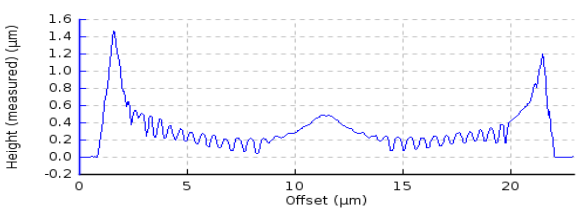

Fig. 6: *Cross section corresponding to the marked line in fig. 5 of CCCryo 272-06 underlines the sharp edges and small substructures of the frustule.*

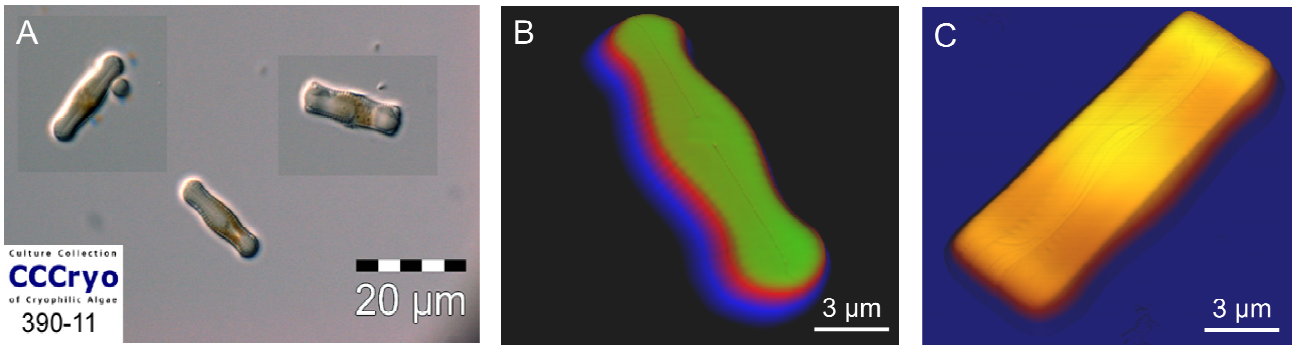

Fig. 7: *Living diatom strain* CCCryo 390-11*in liquid. A) The optical microscopy image shows the two different orientations of the diatom. These orientations were searched and imaged with QI™ (B and C). B) QI™ disclose the* frustule structure that can be seen in the three dimensional topography image (z.range: 6 µm), while C) the three dimensional image (z.range: 4 µm) of the other orientation disclose smaller surface characteristics.

The first diatom strain, CCCryo 272-06 (*Pinnularia subrostrat),* was collected by researchers from the Fraunhofer IBMT in a freshwater lake on King George Island, which belongs to Antarctica. The diatoms were ashed for a better investigation of the silica frustules and for better characterisation of the strain. The ashed diatom structure is presented in figure 5. The diatoms were measured in liquid on a coverslip to combine the AFM measurements with high magnification optics and DirectOverlay™ was used for a perfect integration of both techniques (for further information please refer to our technical report: Perfect Optical Integration with JPK´s DirectOverlay™). For a high resolution imaging on a coverslip the JPK Biocell™ was used.

The diatom skeleton that is shown in figure 5 has very sharp step edges of about 1.5 µm in height and is therefore very tricky to measure with standard AFM techniques. The cross section of the diatom skeleton shown in figure 6 gives a clear impression of the edges and the characteristic smaller substructures. QI™ offers the opportunity to measure without any lateral forces. Therefore it was possible to measure the ashed diatoms structure without any fixation (just resting on the surface). The silica structure demonstrates the characteristic frustules shape of the CCCryo 272-06 strain and underlines the ability of diatoms to make lightweight but strong structures. This knowledge about the exact frustule structure can be used e.g. to create lightweight constructions for different industrial fields.

As well as the skeleton structure itself it is also important to understand the process involved in the biomineralization of the frustules under living conditions. By using QI™ Advanced it is possible to get micromechanical information about the cell surface and get a deeper view into the elasticity and adhesion of diatoms. In particular the investigations of adhesion forces of diatoms can be very interesting. Diatoms can produce adhesives that are extremely strong and robust in sea and fresh water. This information can be used to engineer stable underwater adhesives. To get more information about living diatoms a second strain, CCCryo 390-11 (*cf. Pinnularia cf. Krockii)* was measured. This living diatoms strain was cultured in Spitsbergen and measured under liquid conditions.

Figure 7A) shows an optical image of the CCCryo 390-11 diatom strain. Here it is visible that the diatom can have different appearances depending on their orientation. This could be also measured by AFM and is presented in figure 7 B/C. Figure 7B demonstrates the bilateral symmetry of the diatom. In the AFM QI™ image it is possible to see the middle line (symmetry axis) and the frustule, skeleton structure underneath. The second image in figure 7C

shows the other orientation and underlines the three-dimensional shape of the diatom. Furthermore characteristic surface substructures can be identified. These structures were additionally investigated as shown in figure 8. High resolution images of several parts of the surface were measured with focus on the individual surfaces structure and nanomechanical properties.

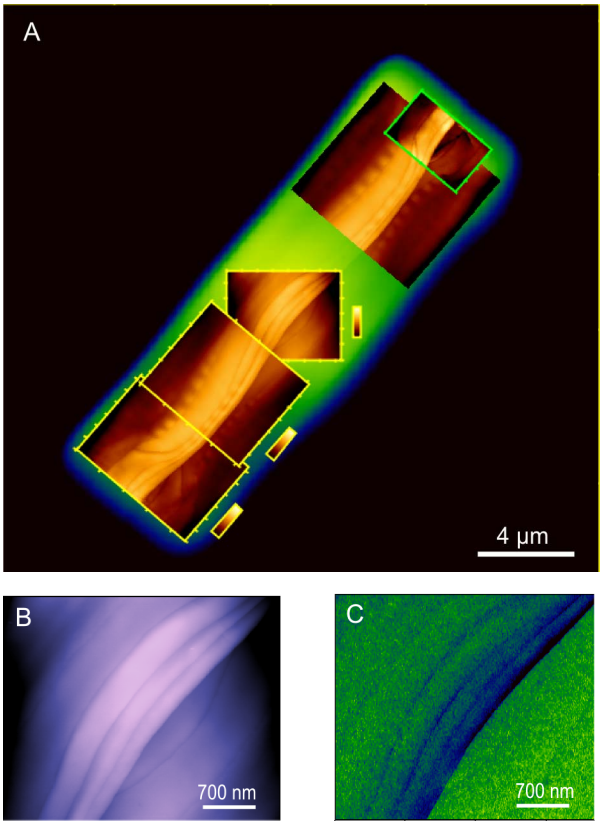

Fig. 8: *A) Overview image with several smaller height images (z-range: 300 nm) on top of the diatom CCC390-11 shows the characteristic surface structure. B) The height image (z-range: 300nm) and C) the Young´s modulus image (z-range: 200kPa) underline the shape-function relation.*

Bacteria

Bacteria are prokaryotic microorganism that can be found in nearly all environments on earth. On the one hand they have significance in biotechnology and industry. They can be used for the production of medicine like antibiotics and also for biomediation of industrial toxic waste. On the other side they can be pathogens and cause diseases like pneumonia and food poisoning. For AFM measurements, living bacteria were always an interesting research object to get a deeper impression about the surface structure and about the nanomechanical properties. Nevertheless it was always tricky to image living bacteria and only possible with special fixation methods [18,19].

One of the most commonly used laboratory bacteria is Escherichia coli (*E. coli*). This gram-negative, rod-shaped bacterium is between 2 µm and 4 µm long and 0.5 µm to 1 µm in diameter. *E. coli* is a model organism for microbiology and has a certain importance in biotechnology. An introduction of genes into the bacteria allows a mass production of proteins in industrial fermentation process. The bacteria have plasma membrane, surrounded by a periplasmic space in which there is a rigid but highly porous cell wall of peptidoglycan.

Figure 9 shows the height and Young´s modulus image of two living *E.coli* bacteria in buffer solution. Different development stages can be the reason for the different lengths of the bacteria. The single bacteria appear smooth and present a homogeneous surface structure. In addition it is possible to visualize the Young´s modulus in parallel (figure 9B). The Young´s modulus image shows the soft bacteria (around 40 kPa) against the hard glass surface. Stiffer and softer areas within the bacteria surface can be measured and correlated to height features or development stages during the *E.coli* ontogeny.

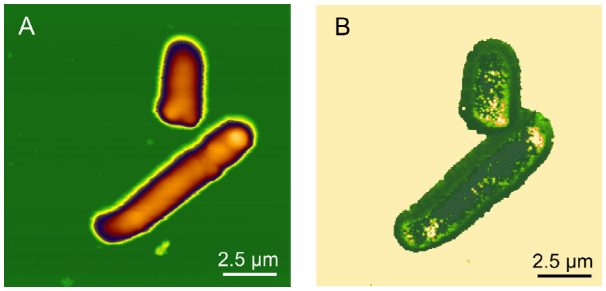

Fig. 9: *Living E.coli bacteria imaged in liquid, A) Height image (z-range: 2 µm). B) Elasticity image (range:40 kPa).*

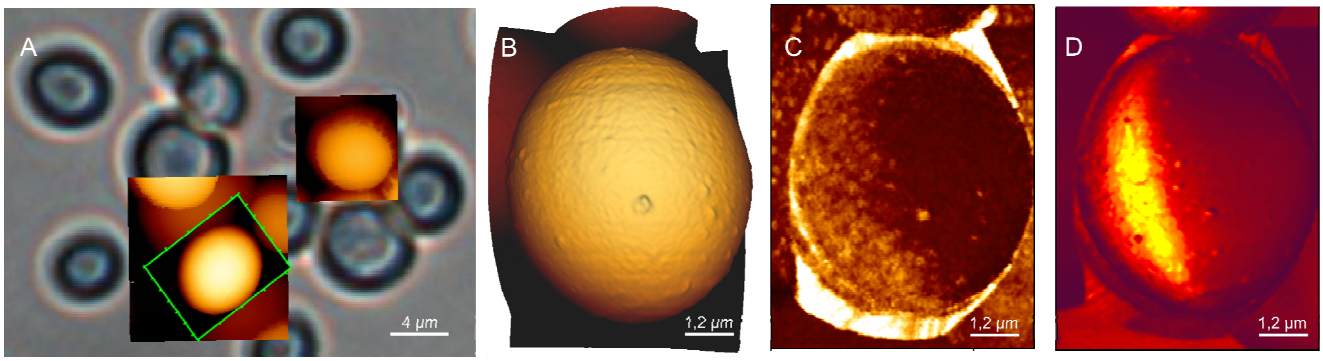

Fig. 10: *Yeast cells in buffer solution. A) The optical phase image is overlaid with different AFM images. B) The three dimensional topography image (z-range: 5 µm) of one yeast cell shows the surface structure. Additional information can be obtained by the C) adhesion image (range: 220 pN) and D) elasticity image (range: 1.6MPa)*

As well as the height image at the set-point force it is also possible to create height images at zero force (at the contact point) or at specific force values and indentation depths. This can be used to investigate the influence of external forces on the surface or for AFM tomography measurements [20].

Yeast cells

Yeasts are unicellular, eukaryotic microorganism that belongs to the classification of fungi. The yeast *Saccharomyces cerevisiae* is used in the baking and brewing industries and is a model organism for eukaryotic cells in biology. Their shape is oval to round with a diameter of 5-10 µm. *Saccharomyces cerevisiae* has the capability of haploid and diploid growth and rapid doubling time of a few hours [21]. The round shape and the large diameter make an investigation of yeast cells with AFM difficult. To be sure that the yeast cell is not pushed away by lateral forces that can be applied in traditional image modes it was common to immobilize the cells by mechanical trapping in a polycarbonate millipore membrane [22]. With the use of QI™ it is possible to adjust the separation length between tip and sample and avoid any lateral force. This allows the yeast cells to be measured without mechanical trapping, resting on a glass surface so that simultaneous high resolution optical imaging is possible.

In addition to the topography imaging also the mechanical properties of yeast cells are quite interesting. The yeast cells from *Saccharomyces cerevisiae* is surrounded by a thick, mechanically strong cell wall composition of proteins, polysaccharides and small amounts of chitin. This cell wall determines the cellular shape and enables the yeast cells to establish turgor pressure and resist osmotic changes. The interesting thing is that the yeast cells can show a local variation of the elasticity in the cell wall. During cell division (budding), chitin is accumulated in bud scar region, which lead to a significant stiffer cell wall region [23].

In Figure 10 the results of QI™ yeast imaging are presented. By using DirectOverlay™ an interesting region that contains a couple of yeast cells in different development stages was selected. Figure 9A shows an overlay of the optical phase image with several AFM QI™ images on different position. For a further investigation one yeast cell was chosen to obtain high resolution imaging and the mechanical properties. The surface structure seems relatively flat, with a few characteristic spots (figure 10B). This small inhomogeneous surface spots can also be recognized in the adhesion (figure 10C) and the Young´s modulus image (figure 10D). Interestingly, one part of the yeast cell is significant stiffer than the rest of the cell. This can be easily seen in the Young´s modulus image and cannot be connected with any specific height feature. The stiffer part of the yeast has an elasticity of about 1.6 MPa,

while the rest of the yeast cell shows an elasticity of 300 kPa. The additional mechanical information can be used to identify specific stages of the development of yeast cells.

Conclusions

The NanoWizard® 3 AFM together with the new unique QI™ mode offers new perspectives in microbiological research. The application note has shown a huge diversity of microbial samples with different sizes, shapes and nanomechanical properties. The presented viruses and yeast cells are similar in shape, but completely different in size and stability. While virus capsids are often very fragile, yeast cells show a robust appearance. Diatoms are relatively large with a complex substructure and the silica skeleton is very stiff compared to the investigated living *E. coli* bacteria. All of the different microbiological samples that were difficult to image in former times can now be easily imaged with QI™ by controlling the vertical force and avoiding any lateral force. In addition it is possible to analyze the collected force distance curves with regard to different aspects like Young´s modulus, adhesion, recognition events or electrical properties. Hence, QI™ allows a new view inside the universe of microbiology.

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Literature

[1] Dupres V., Alsteens D., Andre G., Dufrêne Y. F.: Microbial nanoscopy: a closer look at microbial cell surface, Trends in Microbiology 18:397-405, 2010
[2] Alsteens D., Dague E., Verbelen C., Andre G., Francius G., Dufrêne Y. F.: Nanomicrobiology, Nanoscale Res. Lett. 2:365-372, 2007
[3] Nitesh R.A.I., Ludwig W., Schleifer K.H.: "Phylogenetic identification and in situ detection of individual microbial cells without cultivation". Microbiology Rev. 59: 143–169, 2011
[4] Liu S., Wang Y., Application of AFM in Microbiology: A Review, Scanning 32:61-73, 2010
[5] Dufrêne Y. F., Atomic Force Microscopy, a Powerful Tool in Microbiology, Journal of Bacteriology 184:5205-5213, 2002
[6] Dufrêne Y. : Towards nanomicrobiology using atomic force microscopy, Nature Rev. Microbiology 6:674-680, 2008
[7] Haschke H., Jähnke T,: Nanometrology-QI™ offers next-generation AFM imaging mode for nanometrology, Laser Forcus World, 48, 2012
[8] Gadegaard N.: Atomic force microscopy in biology: technology and techniques, Biotechnic & Histochemistry 81: 87-97, 2006
[9] Kuznetsov Y.G., Malkin A.J., Lucas R.W., Plomp M., McPerson A.: Imaging of viruses by atomic force microscopy, Journal of General Virology, 82:2025-2034, 2001
[10] Cialla D., Deckert-Gaudig T., Budich,C., Laue M., Möller R., Naumann D., Deckert V., Popp J.: Raman to the limit: tip-enhanced Ramanspectroscopic investigations of a single tobacco mosaic virus, J. Raman Spectrosc., 40:240–243, 2009
[11] Carvalho F.A., Carneiro F.A, Martins I.C., Assunção-Mirand I., Faustino A.F., Pereira R.M., Bozza P.T., Castanho M.A.R.B., Mohana-Borges R., Andrea T. Da Poian A.T., Santos N.C.: Dengue Virus Capsid Protein Binding to Hepatic Lipid Droplets (LD) Is Potassium Ion Dependent and Is Mediated by LD Surface Proteins, J. Virol. 2012, 86:2096-2108, 2011
[12] Roos W. H., Radtke K., Kniesmeijer E., Geertsema H., Sodeik B., Wuite G. J. L.: Scaffold expulsion and genome packaging trigger stabilization of herpes simplex virus capsids, PNAS106: 9673-9678, 2009
[13] W. H. Roos, R. Bruinsma and G. J. L.Wuite Physical virology. Nature Physics 6:733-743 (2010)
[14] Lüders A., Müller C., Boonrod K., Krczal G., Ziegler Ch.: Tomato bushy stunt viruses (TBSV) in nanotechnology investigated by scanning force and scanning electron microscopy, Colloids and Surfaces B: Biointerfaces, 91: 154-161, 2011
[15] Hermann P., Hermelink A., Lausch V., Holland G., Möller L., Bannert N., Naumann D.: Evaluation of tip-enhanced Raman spectroscopy for characterizing different virus strains, Analyst, 136:1148-1152,2011
[16] Gebeshuber I.C., Kindt J.H., Thompson J.B., Del Amo Y., Stachelbergers H., Brzezinski A., Strucky G.D., Morse D.E., Hansma P.K.: Atomic force microscopy study of living diatoms in ambient conditions, Journal of Microscopy, 212:292-299, 2003

[17] Bradbury, J.: Nature´s Nanotechnologyists: Unveiling the Secrets of Diatoms, PLoS Biology, 2:347-350, 2004
[18] Meyer R.L., Zhou X., Tang L., Arpanaei A., Kingshott P., Besenbacher F.: Immobilisation of living bacteria for AFM imaging under physiological conditions, Ultramicroscopy 110: 1349-1357, 2010
[19] Chao Y., Zhang T.: Optimization of fixation methods for observation of bacterialcell morphology and surface ultrastructures by atomic force microscopy, Appl Microbiol Biotechnol 92:381–392, 2011
[20] Longo G., Rio L. M., Roduit C., Trampuz A., Bizzini A., Dietler G., Kasas S.: Force volume and stiffness tomography investigation on the dynamics of stiff material under bacterial membranes, J. Mol. Recognit. 25: 278–284, 2012
[21] Dague E., Bitar R., Ranchon H., Durand F., Yken H.M., Francois J.M.: An atomic force microscopy analysis of yeast mutants defective in cell wall architecture, Yeast 27: 673–684, 2010
[22] Elisabetta C., Walker G.M., Adya A.K.: Nanoscopic Morphological Changes in Yeast Cell Surfaces Caused by Oxidative Stress: An Atomic Force Microscopic Study, J. Microbiol. Biotechnol., 19, 547–555, 2009
[23] Touhami A., Nysten B., Dufrêne Y. F.: Nanoscale Mapping of the Elasticity of Microbial Cells by Atomic Force Microscopy, Langmuir 19:4539-4543, 2003