Investigation of living cells using JPK's QI™ mode

Although many adaptations and modes have been developed over the years, live cell imaging and characterization using atomic force microscopy (AFM) have remained challenging and reserved for experienced AFM specialists. Successful measurements needed a good understanding of the technique and also particular care because of the high features and soft surface of living cells. Imaging under physiological conditions can also contribute to thermal drift of the cantilever and bending due to adsorbed molecules. These factors made it difficult to maintain low imaging forces and to obtain images reflecting the actual surface of the cell.

JPK instruments recently launched its new QI™ (Quantitative Imaging) mode – a solution which makes imaging of challenging samples much easier and possible for all users. QI™ mode takes a force curve at every pixel using a unique tip movement algorithm and has two benefits. Firstly, very delicate samples such as living cells can be imaged without lateral forces. Secondly, the resulting data are much more versatile than just a few images. Each image pixel contains a force-distance curve which can be analysed to determine various values, like the adhesion force, contact point or Young's modulus. Here we present the use of the new JPK QI™ mode for the investigation of living cells. The principle of this imaging mode will be explained and different applications and the benefits for live cell studies will be discussed.

QI™ - fully compatible with life science systems

QI™ mode is fully compatible with life science systems. Accessories like the JPK PetriDishHeater™ or BioCell™, which allow for a controlled physiological environment, can be used without any restrictions. As a matter of course, QI™ mode integrates completely into standard transmission light microscopy techniques, such as differential interference contrast (DIC) or confocal laser scanning microscopy (CLSM), allowing for comprehensive investigation of living cells with complementary techniques.

Also the software has been optimized to overcome the difficulties involved in imaging living systems. The JPK ForceWatch™ technology plays a key role in using the QI™ mode in an automated way. Cantilever drift is corrected during the measurement automatically, which greatly facilitates live cell measurements.

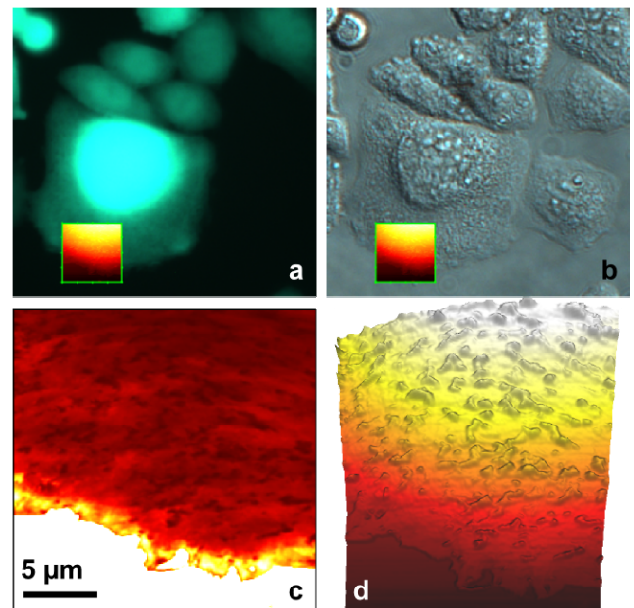

Fig. 1: *Overlay of optical (a: GFP fluorescence, b: DIC) and QI™ images (c: Young's modulus, d: contact point height) of a living CHO cell. QI™ image insert in optical images is 20x20 μm^2. (c) Young's modulus range = 200 kPa, (d) Height range = 4 microns).*

QI™ opens the AFM world to non-specialists

Imaging living cell using AFM requires particular attention to several factors, concerning the cell mechanics and topography as well as the physiological environment a cell needs to survive. The abrupt height changes of cells and the cantilever drift due to temperature changes in the physiological environment represent a major challenge for the AFM technique. Particular importance was attached to automatize this mode regarding these factors and to make this imaging mode easy to manage.
Beside the technical and experimental aspect, JPK dedicated itself to provide comprehensive processing of the

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resulting data without the need of any extra programming. Using the JPK batch processing feature, all force curves of an QI™ data file can be processed in an automated manner with all available operations (some examples are shown in fig. 2), such as the determination of the adhesion force, contact point or Young's modulus. All fit parameters can be adjusted, such as the indentation depth used for the Hertz model fit or the indenter geometry. At the end of the batch processing, a new image is assembled displaying the results of the single operations. Additionally, a histogram shows the distribution of the results and a results file lists the results of all operations in a table.

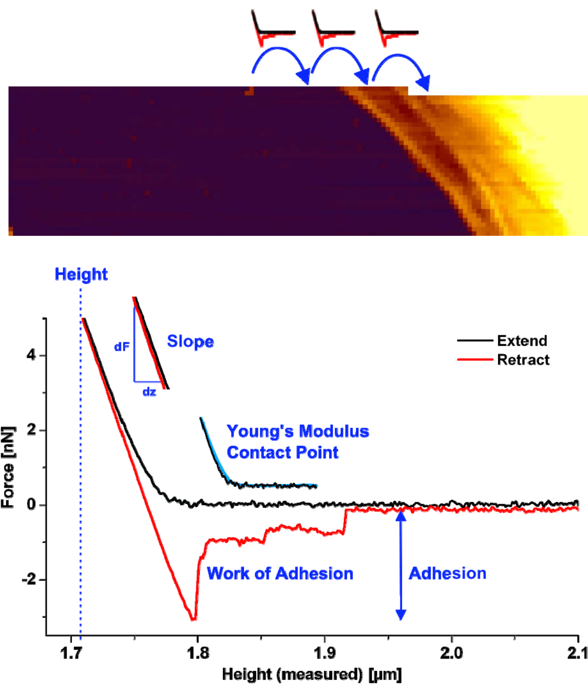

Fig. 2: *Principle of the JPK QI™ mode. A complete force distance cycle is performed at each pixel which provides real quantitative data. Any parameters like the Young's Modulus or Adhesion can be derived from the force curves and presented as images.*

Basics and imaging parameters for living cells

In QI™-Advanced mode, a complete force distance curve is acquired at each pixel of the scan region. All force curves are saved with the image and are accessible for offline analysis. Online analysis (during imaging) provides height, slope and adhesion channels. Offline analysis occurs with the JPK Data Processing software or with user specific software.

The transparency of the QI™ mode provides a high flexibility concerning imaging parameters. Especially for living cells, the imaging parameters, concerning the single force curves as well as the pixel to pixel movement, can be appropriately adjusted.

Depending on the purpose, the setpoint needs fine adjustment to very low forces (below ~100 pN) for very gentle imaging for instance, or higher forces to image sub-membranous structures or to yield sufficient indentation depth for high mechanical contrast. The continuous measurement and correction of the cantilever drift automatically ensures the maintenance of such low forces during the whole measurement. Adjustment of the tip velocity of course influences the duration of the image scan, but also strongly influences the mechanical response of the cell [12][13]. The stickiness of the cell membrane often requires pulling lengths of more than one micron for the cantilever to become free.

Aside from the force spectroscopy curves, the pixel to pixel and line to line movement of the cantilever can be adjusted to optimize imaging time and automation. The additional retract is very helpful for the large height changes typical for cells. It also provides the possibility to minimize the amount of data by giving an extra distance without recording more data. Figure 3 represents one of the main benefits of QI™ mode for live cell imaging. The freedom to adjust the pixel-to-pixel and line-to-line movement allows for imaging of whole cells, even if they show large height changes. Additionally different channels can be created, like the Young's modulus, which give an impression of the elasticity distribution of the cell surface.

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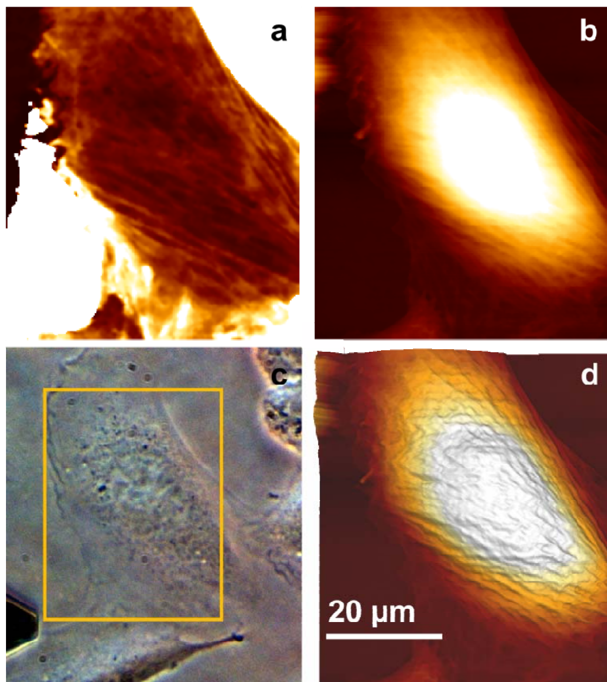

Fig. 3: *Young's modulus (a) and height image (b), phase contrast image (40x, Zeiss Axio Observer) and 3D illustration of the height image of a living CHO cell. Height range = 2.5 µm.*

Contact Point Imaging (CPI)

One of the parameters that can be determined is the contact point. The contact point is defined as the height when the cantilever just starts to touch the surface, i.e. when the cantilever starts to deflect. Consequently, the calculation of the contact point for the extend curve (fig. 2) provides images in terms of zero force. There are different approaches to determine this point [14]; here we used the contact point fitted with the Hertz model (fig. 2). Figure 4 shows the contact point and height image of the cell border of a living fibroblast. The height image shows recesses and rims resembling stress fibers and there are almost no cell projections visible (1 nN setpoint force). In contrast, the calculated contact point image shows a smoother surface and cell projections are clearly visible. In fact, this is the first time an AFM based imaging mode can provide such unique high resolution images which reflect the cell surface at nearly zero force. Additionally, this example shows how complementary information of the cell surface topography and sub-surface structure can be obtained using one imaging mode. For more information see product note "The new JPK Contact Point imaging (CPI) based on QI™ mode" under www.jpk.com.

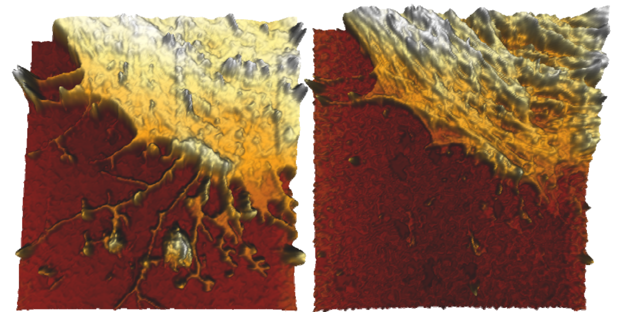

Fig. 4: *Contact point (left, 450 nm height range) and height image (right, 200 nm height range) of a living fibroblast cell. The scan range is 20x20 µm^2.*

Comparison to other AFM imaging modes

The standard AFM modes for live cell imaging are contact mode or imaging modes based on oscillations of the cantilever. Contact mode imaging is straightforward – an appropriate setpoint is determined (depending on cell and cantilever) and the cantilever height is continuously corrected to maintain the setpoint force during the image scan. The difficulties here are to find the appropriate setpoint and to counteract the thermal drift. As a rule of thumb, the setpoint should be as low as possible to minimize lateral forces. But it must also be sufficiently high to find and image the surface properly. The nature of the cell membrane can make it very hard to find this setpoint. The cell surface is not ideally smooth but very rough with its glycocalyx, which mainly consists of free floating polysaccharides. The resulting inhomogeneity of the surface condition requires much experience and exact fine adjustment to select an appropriate setpoint. When the setpoint is chosen, the experimenter must be able to assess the thermal drift of the cantilever and continuously adjust the setpoint to approximately maintain the imaging force. Additionally, lateral forces are constantly applied to the cell resulting in image artefacts. Oscillating modes cause less lateral forces, but also here it is crucial to find an appropriate setpoint through all the cell membrane components. The oscillation amplitude must not be too high to preserve the cell structure, but it must be sufficiently high to come free of the sticky glycocalyx. Indeed,

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the stickiness often requires very high amplitudes to free the cantilever, which can result in significant displacement of the cell membrane.

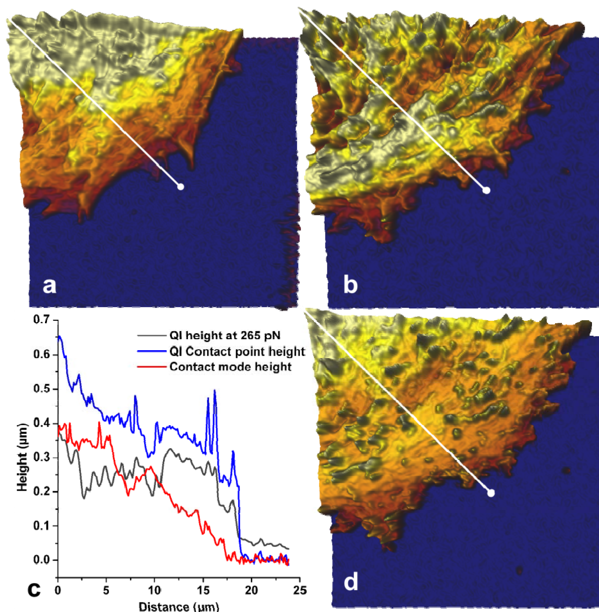

Fig. 5: *Contact mode and QI™ mode images of the same region of a living fibroblast cell (25x25 µm^2 scan). (a) Contact mode height (450 nm height range), (b) QI™ mode height (450 nm height range) and (c) contact point height (600 nm height range) calculated of the corresponding QI™ data. (c) The plot shows cross sections of the three images through the same coordinates (see white lines).*

QI™ mode provides a more comprehensive and easier solution. The force spectroscopy based vertical movement avoids lateral forces and the saving of the complete force curves allows for calculating different height values of the repulsive contact part of the force curve: from the height at the setpoint deflection of the cantilever down to the height of zero force, when the cantilever just starts to deflect. The automated drift compensation measures the thermal drift of the cantilever automatically and maintains the setpoint force exactly through the whole experiment. Once the imaging parameters have been set, imaging can be started and needs no additional adjustment.

As the cell membrane is extremely soft and the softest cantilevers available are never soft enough, it is nearly impossible to image the cell membrane without significant displacement. Figure 5 shows a contact mode and two QI™ images of the same region of a living cell. The contact mode as well as the QI image was taken with a setpoint force of 265 pN. Even if the setpoint force was the same for both imaging modes (Fig. 5 (a) and (b)), the two images display different morphologies. These differences could, for instance, be due to changes in cell morphology in between the two imaging processes. Additionally it is likely that some of the differences derive of the different cantilever movement. I.e. in contact mode, the cantilever scans the cell with constant force and stays in contact with the sample. The cantilever continuously applies lateral forces to the cell membrane and it is thinkable that cell features are moved during imaging. In contrast, in QI mode the cantilever is retracted from the surface between each pixel and nearly no lateral force is applied. Finally, the images as well as the cross sections through the three profiles show again the advantage of QI™ based imaging: The contact point image calculated of the QI™ data can give a more exact illustration of the cell surface than any image measured with a non-zero force.

Young's modulus images reveal elasticity of substructures

Nowadays, the Hertz model well established as a standard model to describe the mechanical properties of living cells [7][8][9][10][11] (see also the JPK application report: "Determining the elastic modulus of biological samples using atomic force microscopy"). Despite its limitations, Young's modulus has gained importance in different fields of cell biology like cancer research and developmental biology [1][2][3][4]. Cells are constantly varying their mechanical behavior; for instance, malignant cells often show a decreased stiffness [5] and the mechanical properties of dividing cells changes during the cell cycle [6].

The offline processing with the JPK Data Processing software provides Young's modulus images in high resolution which reveal differences in elasticity even of the cell sub-structure. Figure 6 b shows a Young's modulus image of a living fibroblast cell. The surface of the cell shows inhomogeneity of the Young's modulus, especially where the stress fibers are located. There are three

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prominent stress fibers (arrow in fig. 6 b, c and d) that are clearly visible in fig. 6 b and d, but not in c. The cytoskeleton is located directly below the plasma membrane and it is just logical that testing the membrane by indenting the cell for a few hundreds of nanometers visualizes the stress fibers in the height and finally in the Young's modulus image. And here again the contact point image shows a different topography without the underlying stress fibers.

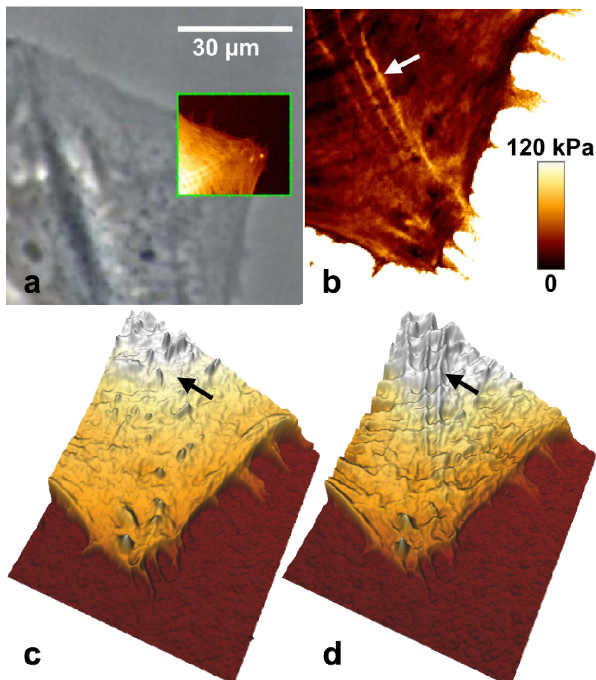

Fig. 6: Phase contrast image (a), Young's modulus image (b) and 3D illustration of the contact Point (c, 950 nm height range) and height image (d, 650 nm height range, 1 nN setpoint) of a living fibroblast cell. The arrows in (b), (c) and (d) indicate the same image position.

For the calculation of Young's modulus images, the QI™ based force curves are processed using the Hertz model [7][8]. Several fit parameters like the fitted indentation depth (fig. 7), contact point or indenter geometry can be adjusted. The curves in fig. 7 were taken with a high setpoint force of 1 nN to show the importance of appropriate parameter adjustment. For curves with ideal shape (fig. 7 right), the fitted indentation depth is not that critical. This curve was taken on a stress fiber and the calculated E-Module for the whole indentation depth (58.1 kPa) did not differ obviously from the modulus calculated for a limited indentation range (57.8 kPa). Curves taken on softer regions of the cell often contain breaks as the indentation increased, possibly deriving from indenting different sub-membranous structures or from penetrating of the cell membrane (fig. 7 left). Comparing the two fits – over the whole indentation depth and over the limited indentation range – results in two considerably varying Young's moduli. The value from the whole fit range (13.7 kPa) gives a much softer value and a rather mis-matching fit curve than the fit over the limited indentation range without the breaks (21.1 kPa).

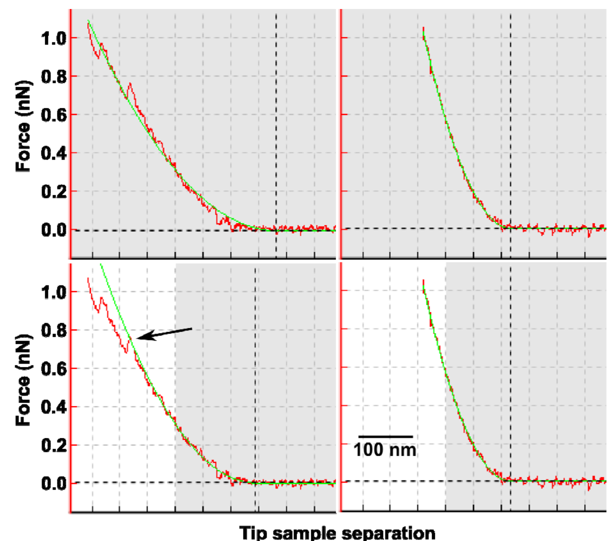

***Figure 7:** Two representative extend curves of the QI image in figure 3 fitted with the Hertz model. The upper curves were fitted over their whole indentation part, the bottom curves over an indentation of 100 nm. The arrow marks a break in the extend curve which could for instance derive from the movement of features below the tip.*

Conclusion

The new JPK QI™ mode not only provides a substantial progress for live cell imaging, it also offers new possibilities in the analysis of living cells. A full set of real force spectroscopy data is recorded for each image and provides the basics for comprehensive processing and information about the cellular structure and mechanics. High resolution images of any parameter that is determi-

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nable from the force curves, such as the Young's modulus or the adhesion force, can be calculated. With the QI™ mode, JPK opens this valuable tool to all scientists, especially to non-AFM-specialists, and facilitates the process of measuring and data processing considerably.

Outlook

QI™ was designed for a wide range of customers and the software contains many refinements to automatize and considerably facilitate the imaging process. Once all imaging parameters are optimally set, the measurement runs without the need of any readjustment, e.g. the measurement can be left unattended. With the JPK ExperimentPlanner™ even whole experiments can be created and executed automatically. The ExperimentPlanner™ can control the measurement itself, concerning imaging parameters, like different force setpoints or pulling speeds, as well as the movement to different scan regions, e.g. to different cells. Additionally, external devices, such as cameras, temperature control or the perfusion of the sample with different media can be triggered and their behavior can be programmed as well. Using the ExperimentPlanner™, QI™ mode facilitates the automation of imaging processes, even for living cells. Whole sets of experiments can be planned and executed automatically for high sample throughput and allow for highly significant statistics.

Literature

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