Using the CellHesion® module- a practical guide

Introduction

Cell-cell adhesion and the adhesion of cells to surfaces is a complex, yet fundamentally important process in multicellular organisms. Investigating this complex phenomenon is challenging because of the heterogeneity of adhesion molecules at the cell surface and the contribution to cell adhesion of both specific and non-specific interactions. Additionally, the trans-membrane proteins involved in cell binding are also often involved in signalling. Consequently, to generate detailed information on cell adhesion a technique should generate data on the component forces involved in, and cell response to, adhesion.

When studying cell adhesion, the application of a force large enough to break all bonds does not provide information about component parts of an adhesion system. As there is a mixture of specific and non-specific interactions, and given the fact that more than one specific ligand-receptor interaction will be involved it is difficult to determine the contribution of individual elements to the over-all binding. Despite this difficulty, many approaches have been developed to study cell adhesion ranging from cell binding assays to various techniques for quantifying the forces involved in ligand-receptor interactions.

Alternate methods

Cell binding assays are based on the determination of the percentage of cells attached to a substrate or monolayer after a set period of time. These assays provide information about the adhesion process as a whole, i.e. which surfaces a cell will adhere to, which cells may be more adherent to a particular cell line or substrate compared with other cells. There is no quantification of the binding forces involved and no way to distinguish the contribution of various elements to the adhesion process.

In order to quantify forces involved in cell adhesion, a number of advanced techniques have been developed. As well as atomic force microscopy (AFM) these techniques include optical tweezers, micropipettes, microplates, the parallel plate flow chamber and the biomembrane force probe.

For experiments with optical tweezers, cells in the early stages of adhering to a surface can be detached using a laser trap [1]. This technique is limited to applying forces in the pico-newton range, and therefore can only monitor cell interactions in the early stages of adhesion. Additionally, these experiments are designed such that a specific force is applied and the probability of cell detachment calculated. The detection of individual components of the cell adhesion is beyond the scope of the optical tweezers technique.

The micropipette technique has also been used to investigate the total force required to separate two cells [2]. Again, this technique does not separate out component forces that contribute to overall binding. Additionally, the technique can only investigate interactions between two single cells, not a single cell and a cell monolayer.

The parallel plate flow chamber, on the other hand, describes how shear forces effect the interaction of cells in suspension with a monolayer of cells seeded on the plates of the apparatus [3]. While this technique can be used to mimic the forces experienced by cells in the circulatory system, it requires advanced mathematics for data analysis.

The biomembrane force probe can detect the detachment of individual molecules in the context of cell binding, however, this technique measures the binding of a single cell to a coated bead, not another cell [4]. Therefore, despite being a powerful approach for the study of complex cell binding it is limited as it cannot be used to investigate cell-cell adhesion.

Force spectroscopy, using an atomic force microscope, is capable of resolving individual detachment events, as well as the overall force required to detach a cell [5, 6, 7]. This technique can be used to investigate cell binding to surfaces, a second, single cell or cell monolayers. While

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some interesting studies have been conducted using this technique, AFM based force spectroscopy has previously been limited in its application due to the insufficient effective pulling distances, the lack of superior optics and sample chamber of commercially available machines. We have developed the CellHesion® module to overcome this limitation.

How does force spectroscopy work?

At the heart of the atomic force microscope is the cantilever, which can be viewed as a flexible spring with a given spring constant. This means that the deflection of the cantilever can easily be converted into a corresponding force. The movement of the cantilever is detected by shining a laser off the back of the cantilever onto a detector. In such a manner, deflections of the cantilever (corresponding to applied force) can be detected.

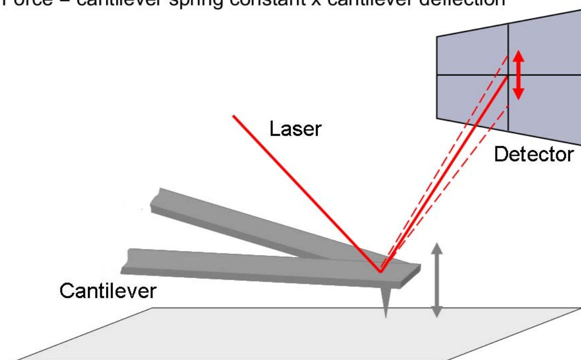

Fig. 1 Schematic of central components of an AFM. Movement of the cantilever causes movement of the reflected laser on the detector. As the cantilever can be viewed as a spring the amount of cantilever deflection can be used to calculate force applied.

To conduct force spectroscopy the cantilever is approached to and retracted from a surface using a piezo that controls movement in the z direction. The cantilever deflects as it comes into contact with the surface, a feedback system stops the piezo movement when a set deflection (and therefore force) is reached. After a set period of time the cantilever is retracted. If there is any interaction between the cantilever and the surface the cantilever will be pulled down toward the surface until the force required to disrupt the interaction between cantilever and surface is reached. If there are multiple interactions, multiple unbinding events can be detected as the cantilever is further retracted from the surface. For an in depth description of force spectroscopy experiments, please also see our technical report "A practical guide to AFM force spectroscopy and data analysis".

Fig. 2 The CellHesion® module. The CellHesion® module is an add-on to the NanoWizard AFM. The setup is integrated into an inverted light microscope for easy handling and to extend the scope of possible experiments.

The CellHesion® is designed for force spectroscopy experiments that measure cell detachment. In this case, a cell is attached to the flexible cantilever and brought into contact with either a second cell or a coated surface. As the cantilever-bound cell is retracted the bonds between the cell and the surface cause the cantilever to deflect toward the surface. As bonds between the probe cell and the surface break, the cantilever snaps up, allowing the force required for each unbinding event to be calculated. From such experiments, the maximal force required for detachment is recorded, along with events that correspond to detachment of single ligand-receptor couples. A value for the work required for detachment can also be calculated. To conduct such experiments, a large effective

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pulling range is required, as unbinding events can occur at a distance up to 100 µm from the surface.

CellHesion®- overview of instrumentation

The CellHesion® module is an add on to the JPK NanoWizard® that enables long distance pulling without sacrificing the imaging capabilities of the NanoWizard® scan head. The system is designed to be installed on an inverted microscope.

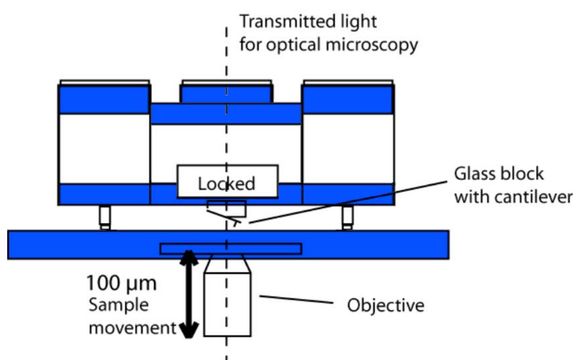

Fig. 3 Schematic of the CellHesion® module setup.

When operated in CellHesion® mode, the z-piezo of the NanoWizard® scan head is disabled and the sample stage piezos are responsible for the movement. The sample stage piezos have a range of 100 µm, to allow long distance force spectroscopy. The movement of the piezos is controlled by a closed loop feedback system. As such, the movement and positioning of the stage is accurate to sub-nanometer precision, and not subject to inherent piezo hysteresis. The objective can also be fitted with a piezo focussing device and moved in parallel with the sample stage- allowing cell responses to be monitored with conventional light microscopy techniques.

Conducting experiments using the CellHesion®

There are a number of technical considerations for conducting experiments using force spectroscopy. The nature of the cantilever, how the cell is bound to the cantilever, speed of cantilever movement, temperature, contact forces and contact times applied can all effect the experimental outcome.

Cantilever selection.

With a cell bound to the cantilever it is the cell that contacts the surface and compresses as the cantilever defects. As cells are very soft, the cantilever to which they are bound must also have a low spring constant, i.e. be very flexible. Cantilevers that are suitable for cell force spectroscopy experiments are those that are used for contact mode imaging on cells, i.e. those with spring constants in the range of 0.01-0.06 N/m.

Coating cantilevers

An important step in conducting cell force spectroscopy is the binding of the cell to the cantilever. The cantilever must be coated with a molecule that will allow binding of the cell with a bond-strength greater than the strength of the bonds that will be formed between the cantilever-bound cell and the target surface. One approach is to coat the cantilever with the lectin Concanavalin A (ConA) which binds D-glucose and α-D-mannose sugar moieties at the cell surface.

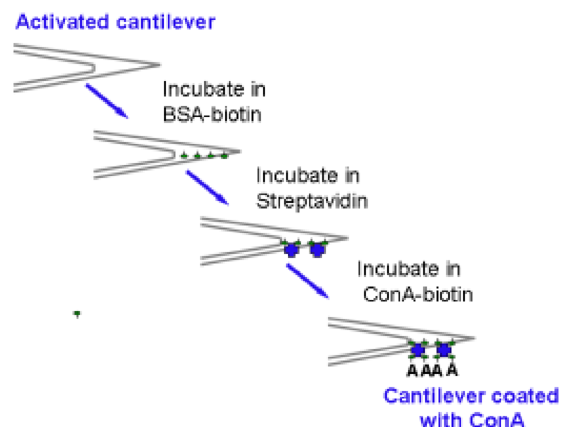

Fig. 4 Coating the cantilever.

Cantilever functionalization with ConA is a multi-step process. Initially the cantilever should be activated, either by UV treatment or with a residual air plasma cleaner. Cantilevers are then successively coated with biotinylated BSA, streptavidin then finally biotinylated ConA (see Figure 4) [8].

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Calibrating the cantilever

For force spectroscopy measurements it is important to calibrate the cantilever, such that the JPK SPM software can display cantilever deflection in terms of force. Calibrating the sensitivity of the system and the spring constant of the cantilever is made simple by in-built routines in the JPK SPM software. This calibration step should be conducted before the cell is bound to the cantilever. After calibration, the deflection of the cantilever will be expressed in newtons.

Attaching cells to the cantilever.

There are a number of points to consider when preparing a suspension of cells from which one can be attached to the cantilever. If the cells are already in suspension, they can be added straight into the sample chamber. If the cells of interest are cultured in 2D culture they must be released from the plastic culture dishes first.

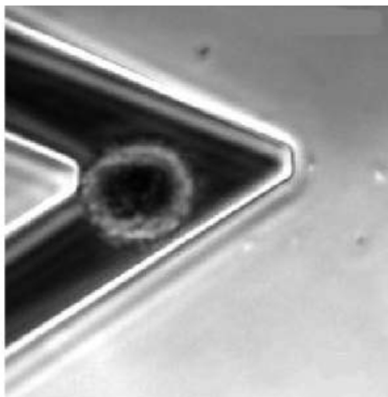

Fig. 5 Phase contrast image of a cell attached to the cantilever

There are two means by which cells can generally be released from plastic dishes a) treatment with trypsin, b) incubation in the absence of divalent metal ions. However, care must be taken with both of these methods. Trypsinisation leads to the cleavage of membrane proteins at the cell surface. Some of these same membrane proteins will be involved in adhesion and over-digestion with trypsin may remove adhesion proteins of interest. On the other hand, incubating cells in the absence of Ca^{2+} and Mg^{2+} will release cells from the plastic, however, binding of the cells to ConA may be reduced. In practice, it is best to rinse the cells with trypsin and then allow them to float off of the surface in PBS without Ca^{2+} and Mg^{2+}. After harvesting by centrifugation, the cells should be resuspended in the relevant media and allowed to recover for a defined period of time (30 min is sufficient), consistent across all experiments in a set.

The suspended cells should then be introduced into the sample chamber in which the target surface and calibrated cantilever have already been mounted. The cells are left to settle but not attach to the surface. By using the optical microscope, the cantilever can then be oriented over the cell and gently brought into contact with the top of the cell. After one minute of contact the cantilever and surface are separated and the cell should be stuck to the cantilever. The cell is then left to relax and form a strong interaction with the coated cantilever before acquiring the first force curves.

Temperature and medium control

Samples can be mounted in the JPK BioCell™ sample holder (Figure 6), which enables acquisition of high quality optics like phase contrast, DIC or fluorescence and AFM simultaneously. The cells are prepared on a standard cover slip. The BioCell can control the temperature, perfusion of the liquid, or CO2. All parts are cleanable or autoclavable. This is very important for cell adhesion studies.

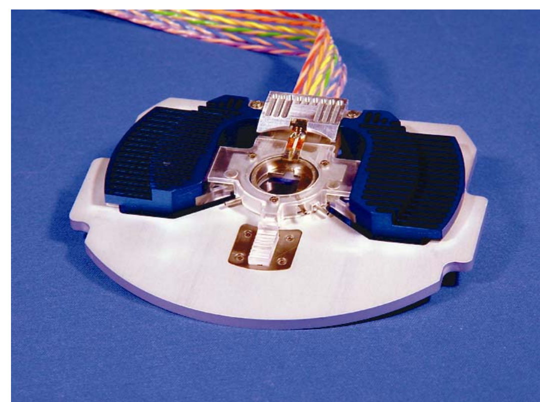

Fig. 6 The Biocell™ is designed to optimise image quality during experiments combining AFM and light microscopy while at the same time allowing temperature control. Peltier heating and cooling elements allow rapid adjustment of temperature.

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By controlling the temperature during an experiment the user could choose to either a) more accurately reflect physiological conditions, b) slow down the adhesion process by setting a lower temperature or c) conduct in situ experiments on temperature sensitive mutants by collecting a set of force curves at the permissive temperature, before adjusting the temperature and collecting a second set of curves at the non-permissive temperature.

Parameter selection

There are a number of critical parameters that need to be considered when conducting force spectroscopy experiments. These include pulling distance, contact force and time and the speed of approach to and retraction from the surface. When conducting force spectroscopy with a cell attached to the cantilever, one must also consider whether to include a relaxation time between each curve, and how many times to bring the cell into contact with the same region of the surface.

If conducting experiments measuring the adhesion of a cantilever-bound cell to a coated surface, a pulling range of 15 µm will usually suffice. However, when a cantilever-bound cell is to be brought into contact with a second cell, a significantly longer pulling distance is required. Due to the formation of membrane tethers, a separation distance of more than 60 µm may be required to break all the bonds between the cells.

The force applied to the cantilever-bound cell when it is in contact with the surface will determine the amount of cell spreading as the cell is compressed underneath the cantilever. An increase force may lead to increase surface area in contact and increased number of bonds to be broken. As for contact time, obviously more specific bonds between the cantilever-bound cell and the target surface will form over longer contact times. Approach and retract speeds must be carefully controlled to avoid excessive hydrodynamic drag.

The repeated acquisition of force curves on the same region by the same cell can be problematic due to passivation or deformation of the cantilever-bound cell or passivation of the surface. As the cell is living there will be some turnover of receptor at the surface of the cell, which may be upregulated when the cell is repeatedly brought into contact with the surface. Additionally, compression of the cell beneath the cantilever, which in contact, may lead to the cell flattening on the surface of the cantilever. By allowing a pause between the acquisitions of curves, the cell can relax and the user can perform the experiment in the same area.

The CellHesion® has capacitive sensors that allow accurate x, y and z positioning when the instrument is used in closed loop mode (i.e. when the feedback from the capacitive sensors is on). With the closed loop, experiments can be conducted either in "constant height" or "constant force" mode. Constant height and constant force refer to how the piezo responds to cantilever movement after the cell has come into contact with the surface. To reiterate, the cantilever-bound cell and the surface are brought into contact at a defined speed, as the cell comes into contact with the surface the cantilever will deflect, thereby applying a given force to the cell. At a user defined force the piezo controlling the z-movement stops. At this point how the system responds during the contact time is dependent on whether the system is operated in constant height or constant force mode.

When in constant height mode, during the contact time the z-piezo remains at the same position, regardless of how the cell responds to being in contact with the surface. Indeed, what usually happens when a cell is brought into contact with the surface is that the cell deforms elastically on contact, and then during the delay before retraction, the cell will relax, in the manner of a viscous material. This relaxation of the cell reduces the force applied to the cell, and pulls the cantilever toward the surface. This can be seen in a real time scan of cantilever deflection, showing the cantilever movement over the course of a force curve (Figure 7).

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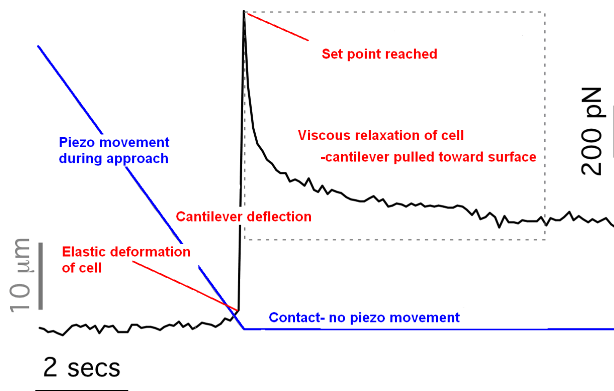

Fig. 7 Real-time curve of piezo movement (blue) and cantilever deflection (black) in constant height mode.

If the system is being operated in constant force mode, as the cell relaxes and the cantilever deflection (and concomitantly applied force) changes, the z-piezo will adjust. The piezo continually adjusts during the contact time to keep the deflection of the cantilever, and the force constant.

What do the results look like?

To analyse forces involved in the unbinding of the cantilever-bound cell from the surface, cantilever deflection (in Newtons) is plotted against piezo position.

This is known as a force-distance curve (Figure 8). There are four regions to a force distance curve. The regions marked 1 and 2 correspond to the approach phase, whereas regions 3 and 4 correspond to the retraction. In step 1 the piezos move the sample surface toward the cell bound cantilever. The curve is flat in this region as there is no contact between the cantilever-bound cell and the surface, hence the cantilever has not been deflected. In region 2 the cell comes into contact with the surface. The cell will elastically deform and the cantilever will deflect.

The piezo movement stops when the set point is reached. The force distance curve does not display what happens during the contact time (see real time curve above for cantilever behaviour during the contact time). Region 3 of the force curve corresponds to the retraction of the surface away from the cantilever-bound cell. As the surface is retracted the cantilever bends toward the surface, due to the interactions between the cell and the surface. The jumps in the curve in this region correspond to bond disruption. As a particular force is reached, due to cantilever deflection, an unbinding event will occur, the force required to effect this unbinding can be read directly from the force-distance curve. As the surface is retracted further all interactions between the cantilever-bound cell and the surface will be disrupted. Region 4 of the force-distance curve corresponds to the end of the retraction step, where the cantilever-bound cell and the surface are no longer in contact with each other.

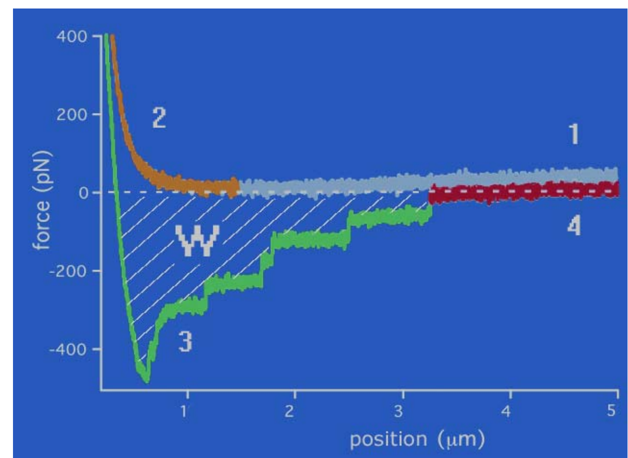

Fig. 8 Force-distance curve of Zebrafish binding to a fibronectin coated surface. Kindly provided by Dr. P-H Puech, TU Dresden.

There are a number of pieces of data that can be extracted from the force distance curve. From the slope of the curve in region 2, information about the elasticity of the cantilever-bound cell can be extracted. However, this is only relevant if the cantilever bound cell is being brought into contact with a hard surface. If cell-cell adhesion studies are being conducted, the slope in region 2 will depend on the elasticity of both cells. Elasticity measurements can be important in studies of cancer cells. The disruption of the cytoskeleton that can occur with cellular transformation has been found to lead to an increased elasticity of cancer cells in comparison with their non-transformed counterparts.

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The maximal force required to separate the cantilever-bound cell from the target surface can be determined directly from the force-distance curve. This corresponds to the difference between the baseline (undeflected cantilever) and the point of largest negative deflection of the cantilever (F in figure 9). Though the contribution of various interactions to this maximal force required for detachment will vary between cell lines, it represents the breaking of both specific and non-specific interactions.

After the initial unbinding event there follow a number of smaller force jumps. These are proposed to correspond to the rupture of specific ligand-receptor interactions. These unbinding events fall into two classes. Those that are preceeded by a previous bond rupture (J) and those that are preceeded by a segment of the curve where there is an increase in separation distance without and increase in cantilever deflection (T). The J events correspond to the unbinding of ligand-receptor interactions without a preceeding membrane deformation. The T events correspond to instances where a membrane tether has been extruded before the unbinding of the ligand-receptor complex. In such a manner are multiple components of cell binding distinguished in the one curve.

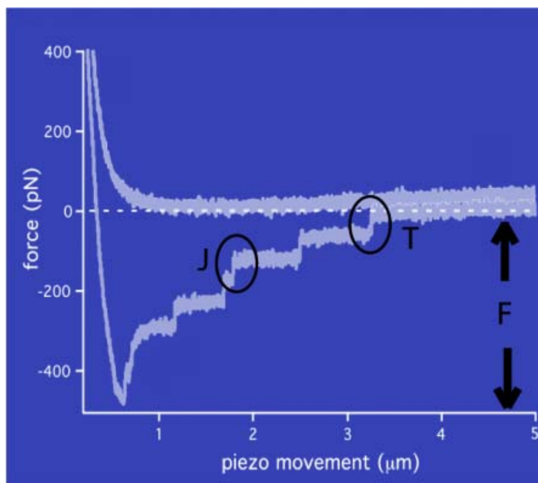

Fig. 9 Force-distance curve of Zebrafish binding to a fibronectin coated surface. From the force distance curve the maximal force required for unbinindning (F) can be determined. The unbinding of individual protein interactions fall into two categories, J events and T events (preceded by the extrusion of a membrane tether). Curve kindly provided by Dr. P-H Puech, TU Dresden.

The cell attached to the cantilever can then be used to collect multiple force-distance curves. It is critical that under all conditions in an experimental set a number of force-distance curves are acquired as there will be some variability between the curves under each condition. However, if sufficient force-distance curves are acquired then a comparison between different conditions can be made.

When cell-cell adhesion is studied, the extrusion of membrane tethers is often observed (Figure 10). In this case it is important that the effective pulling distance is set to at least 60 µm. If an insufficient pulling distance is used the cells will not be completely separated between acquisition of consecutive force-distance curves

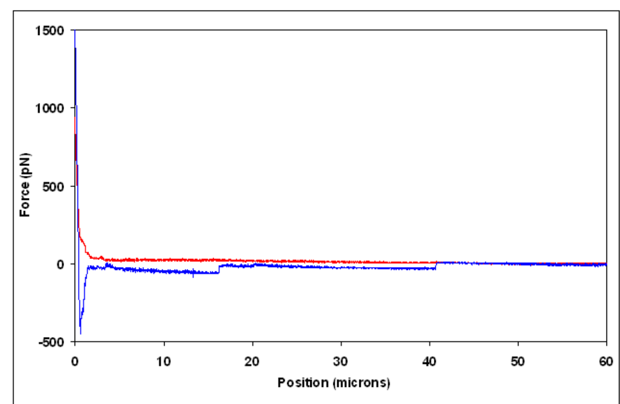

Fig. 10 Cell-cell adhesion studies require a long effective pulling range. In this case a melanoma cell was attached to the cantilever and brought into contact with an endothelial cell monolayer.

By conducting experiments in the Biocell™, after the acquisition of a set of control curves, a specific ligand-receptor interaction can be blocked by injecting a blocking agent into the Biocell™ and acquiring a second data set with the same cell. For instance, an RGD peptide could be introduced to block integrin interactions. By comparing the events in control force-distance curves with curves acquired after blocking it could be determined whether integrins are involved in the interaction between the cantilever-bound cell and the target surface.

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The CellHesion® is designed such that light microscopy data can be collected simultaneously with the force-distance curves. The objective can be fitted with a focusing device so that it moves in parallel with the sample stage. Alternatively the objective could remain in focus on the cantilever-bound cell. As both transmission and fluorescence optical microscopy techniques are available data could be collected on cell morphology changes, specifically labelled areas of either the cantilever-bound cell or target cells at the surface.

Conclusions

With the CellHesion®, cell adhesion to coated substrates, single cells or monolayers can be analysed in a manner that allows dissection of the contributing interactions between the cell and the target surface. The integration of the CellHesion® module into an inverted optical microscope extends the scope of such experiments further by enabling the response of either the cantilever-bound cell or the target cells to be monitored by light microscopic techniques. Such features make the CellHesion® a powerful new tool for the investigation of cell adhesion and cell mechanics.

Acknowledgements

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