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An atomic force microscope operating at hypergravity for *in situ* measurement of cellular mechano-response

J.J.W.A. VAN LOON*, M.C. VAN LAAR*, J.P. KORTERIK†, F.B. SEGERINK†, R.J. WUBBELS‡,§, H.A.A. DE JONG‡ & N.F. VAN HULST†,¶

**DESC @ OCB-ACTA, UVA-VU, van der Boechorststraat7, Amsterdam, Netherlands*

†*Optical Sciences group, MESA+ Institute, Fac. Science & Technology, University of Twente, Enschede, Netherlands*

‡*Vestibular Department ENT, AMC, UvA, Amsterdam, Netherlands*

¶*ICFO – Institute of Photonic Sciences, Castelldefels, Barcelona, Spain*

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Summary

We present a novel atomic force microscope (AFM) system, operational in liquid at variable gravity, dedicated to image cell shape changes of cells *in vitro* under hypergravity conditions. The hypergravity AFM is realized by mounting a stand-alone AFM into a large-diameter centrifuge. The balance between mechanical forces, both intra- and extracellular, determines both cell shape and integrity. Gravity seems to be an insignificant force at the level of a single cell, in contrast to the effect of gravity on a complete (multicellular) organism, where for instance bones and muscles are highly unloaded under near weightless (microgravity) conditions. However, past space flights and ground based cell biological studies, under both hypogravity and hypergravity conditions have shown changes in cell behaviour (signal transduction), cell architecture (cytoskeleton) and proliferation. Thus the role of direct or indirect gravity effects at the level of cells has remained unclear. Here we aim to address the role of gravity on cell shape. We concentrate on the validation of the novel AFM for use under hypergravity conditions. We find indications that a single cell exposed to 2 to 3 $\times g$ reduces some 30–50% in average height, as monitored with AFM. Indeed, *in situ* measurements of the effects of changing gravitational load on cell shape are well feasible by means of AFM in liquid. The combination provides a promising technique to measure, online, the temporal characteristics of the cellular mechano-response during exposure to inertial forces.

§Current address: The Rotterdam Eye Hospital, Rotterdam, Netherlands

Correspondence to: Jack J.W.A. van Loon. Tel: +31 (0)20-4448686; fax: +31 (0)20-4448683; e-mail: j.vanloon@vumc.nl

Introduction

Conceptually the effect of gravity on single cells is expected to be limited. In fact, based upon a theoretical comparison of various forces within the cell it has been predicted very unlikely that non-specialized mammalian single cells would display any discernible response to a (relatively moderate) change of gravity (Pollard, 1965, 1971; Albrecht-Buehler, 1991; Todd *et al.*, 1996). However, at the same time it has been shown in numerous studies that cells do behave differently under conditions of hypergravity (centrifuges), simulated hypogravity (clinostats, random positioning machine) and real microgravity, compared to their appropriate 1 $\times g$ controls. For an overview of gravity related cell studies, see Moore and Cogoli (1996), Mesland & Brillouet (1987), Brillouet (1995), Brinckmann & Brillouet (1999), Gaubert *et al.* (1999), Cogoli (1996, 2002), Haeder *et al.* (2006) and Clément & Slenzka (2006).

In vitro, gravity can act on single cells indirectly by changed fluid dynamics via Rayleigh or density-driven convection of the surrounding media, or directly via mass displacements of specific intracellular components or the whole cell volume. Thus we hypothesize that gravity affects cells, resulting in small changes of overall cell shape. To explore such small changes we have set out to measure cell height at various gravitational loads using atomic force microscopy (AFM) in liquid.

AFM has been introduced in 1986 by Binning and co-workers (Binning *et al.*, 1986). Since the early days quite some improvements have been made to make AFM applicable to the biological domain and allow mapping of cells. Particularly after the introduction of 'tapping mode' AFM (Zhong *et al.*, 1993) and its operation in liquids (Hansma *et al.*, 1993; Putman *et al.*, 1994) living cells can now be depicted almost unperturbed in their natural environment at high

spatial resolution (Putman *et al.*, 1994; Davis *et al.*, 1997). These developments have paved the way to now use AFM in conjunction with increased mechanical stimulation by exposing cells to hypergravity conditions. As a starting point, we used our custom-built AFM system (Van der Werf *et al.*, 1993) with a number of modifications in order to enable operation at hypergravity conditions.

We focus on the technical adaptations; necessary to enable AFM operation in liquid at variable gravity conditions while still preserving the high resolution capacity of AFM. In a first application we show that cells do change their shape as a function of gravitational load. For the first time, it is demonstrated that real time changes of the shape of a single cell do occur when exposed to different gravity levels.

Control of vibrations while operating at hypergravity

We mounted a custom-built compact stand-alone AFM system (Van der Werf *et al.*, 1993), adapted for wireless remote control, into a centrifuge (Fig. 1). This 3.6-m-diameter centrifuge provided ample room to accommodate the AFM scan head and all associated electronics. For details see Wubbels & de Jong (2000). An additional advantage of a large-diameter centrifuge is that inertial shear effects during these experiments were negligible (van Loon *et al.*, 2003). In Fig. 1B, the electronics on the central axis are clearly visible. Remote control was established by using a standard wireless remote desktop, in combination with computer controllable AFM height feedback settings. The total system, AFM head, electronics and sample holder were validated for mechanical integrity and reproducible measurements ranging from 1 to $3.5 \times g$. Due to constraints in the hardware used the load that could be applied during the actual cell measurement was limited to $3 \times g$. A special sample fluid cell was developed (Fig. 2B) to keep the cells in physiological culture medium during the measurements. An inverted microscope (Fig. 3, partly visible in the lower right corner) was used to visualize the position of the AFM cantilever and tip with respect to a selected cell, before accommodating the whole AFM assembly into the centrifuge gondola. The experiment was performed

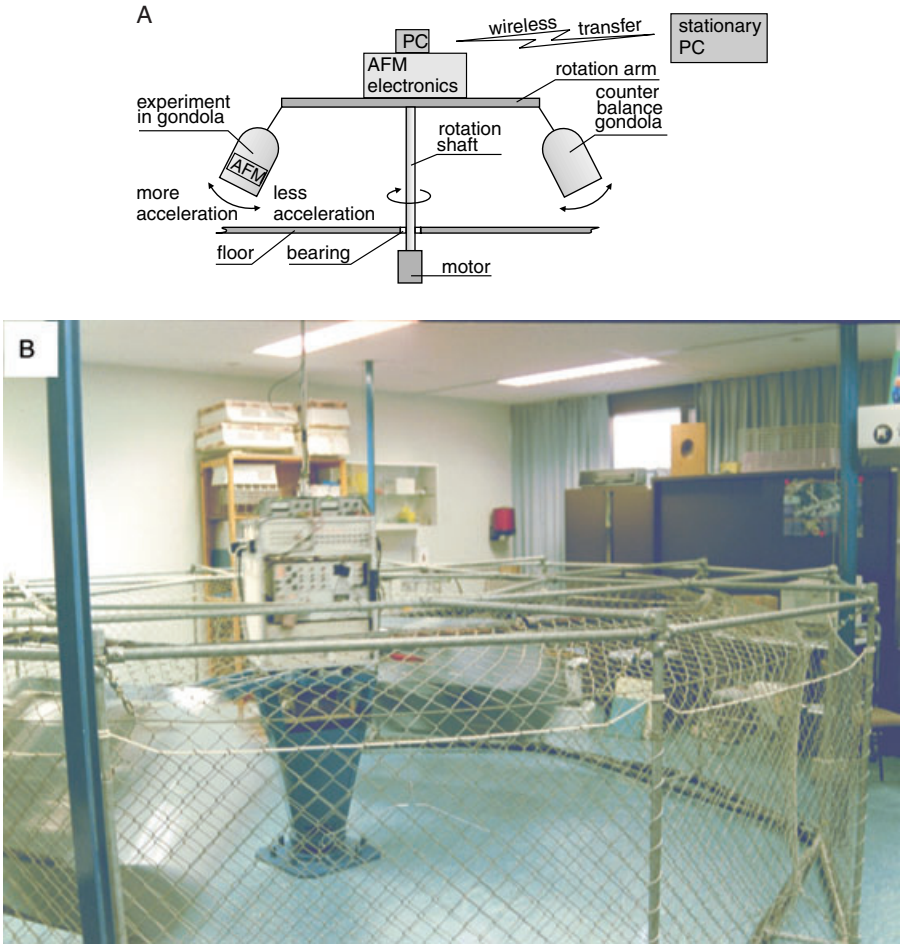

Fig. 1. (A). Schematic set-up of the large centrifuge fitted with a wireless operated AFM in one of the gondolas. The control electronics were mounted at the centre shaft of the centrifuge. (B) The 3.6-m-diameter centrifuge during measurements.

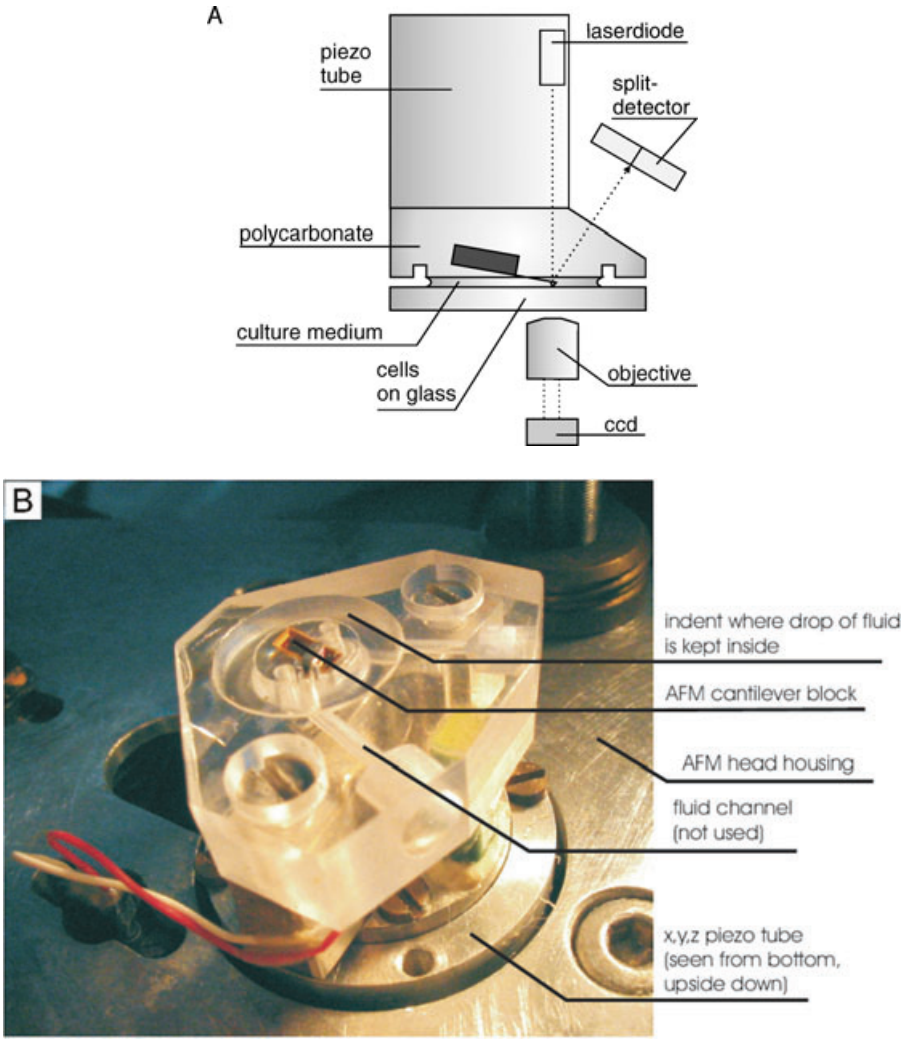

Fig. 2. (A) Schematic set-up of the AFM with fluid cell. The cells were maintained in a CO_2-independent culture medium during the measurements. The objective and CCD depicted at the bottom of the figure represent an inverted optical microscope used to align the AFM tip with the cell before mounting the AFM head into the centrifuge. Dimensions in this drawing are not to scale. (B) Detailed picture of the actual fluid cell.

measuring a selected single cell while being exposed to one, two and three times Earth's gravity.

Our main challenge was to operate an AFM in a 3.6-m centrifuge without sacrificing the nanometric accuracy. To estimate the feasibility of this project, an initial vibration measurement was performed to assess the typical centrifuge noise levels, before entering in AFM height measurements. To this end a fairly simple set-up was built. Inside one gondola of the centrifuge, a rigid brass plate of 3.4 kg ($5 \times 2 \times 40$ cm) was placed on top of 2 cm of damping material, see Fig. 4. On the side and top of the brass plate two accelerometer sensors (Brüel & Kjær, Nærum, Denmark) were mounted. Accelerometers are calibrated for a certain amount of electrical charge as function of acceleration. Here we used small sensors (small in relation to the mass of the brass plate) with sensitivities of 3.1 pC/g (Coulomb per acceleration g caused by the gravity of the Earth). The sensor outputs were amplified by Brüel & Kjær 2626 amplifiers, bandwidth 3 kHz, sensitivity 1V/3.1pC. The signals were digitized with SIGLAB and signal processing was done using MATLAB 5.3 and SIGLAB 3.2.4. The gondola at the other end of the centrifuge rotation arm was counterbalanced for proper functioning of the centrifuge. Both sensors were used to measure the acceleration levels at 1, 2.3 and $5 \times g$ as function of frequency. Next, the spectral displacement level can be calculated by dividing the acceleration data by ω^2. For our AFM experiments the vibration-limited height variation in nanometres is relevant. A simple second-order mass-spring system model was used, which mimics our AFM head consisting of a rigid body containing an x, y, z piezo scanner. This piezo scanner can be dimensioned from nm ranges to a few hundred micrometres and modelled as three orthogonally placed coupled mass

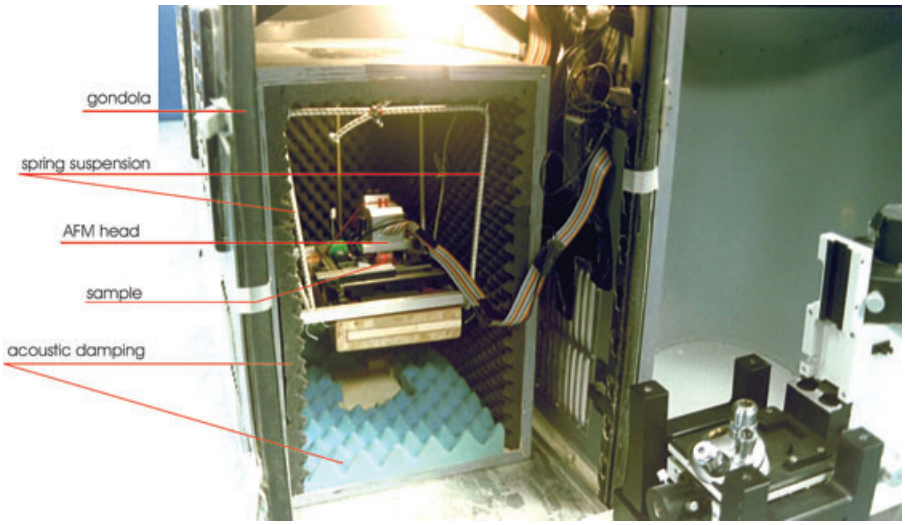

Fig. 3. shows the AFM head and base plate (plus sample) mounted in an acoustically damped box. Inside the box, the spring suspension is visible. The inverted microscope on the lower right was used to preselect the cell to be investigated before mounting the AFM assembly into the centrifuge.

Table 1. Results of vibration measurements in the large-diameter centrifuge. The column total noise displays the total noise up to 100 Hz in nm (rms), i.e. tip–sample distance variation.

Measurement description	Sensor	Total noise (nm)
Gondola at rest	Vertical	2.9
Gondola at rest	Horizontal	0.74
Gondola at 2.3 × *g*	Vertical	4.2
Gondola at 2.3 × *g*	Horizontal	3.1
Gondola at 5.0 × *g*	Vertical	4.4
Gondola at 5.0 × *g*	Horizontal	2.0

spring systems. For calculating the noise levels in the vertical direction, the vibration measurement in vertical direction and the lowest vertical resonance frequency of 1 kHz were used. A similar approach was applied for calculating noise levels in the horizontal direction. For very low frequencies the mass-spring system is very stiff, such that any vibration of the base plate (plus sample) in this frequency range results in a similar vibration of the tip and the tip–sample distance is not significantly changed. At frequencies well above the primary resonance frequency, the vibrations of the base plate do not lead to significant vibration of the tip and as a consequence the tip–sample distance does vary. This frequency dependence was taken into account when calculating the expected total noise. Finally, we took into account that the AFM height feedback only acts on frequencies below approximately 100 Hz, such that higher frequencies are attenuated when measuring the piezo height signal. Table 1 provides an overview of the total noise due to vibrations.

From Table 1 it can be concluded that the vibration levels remain in the low nanometre region. The worst-case estimated tip–sample distance variation is 4.4 nm (root mean square, rms) for gravity conditions of 5 × *g*. The low vibration levels are a very encouraging result from an instrumentation point of view. Clearly the vibration conditions in our centrifuge can be sufficiently controlled by a passive damping support and the stiffness and frequency response of our AFM head, to proceed towards AFM operation at hypergravity conditions.

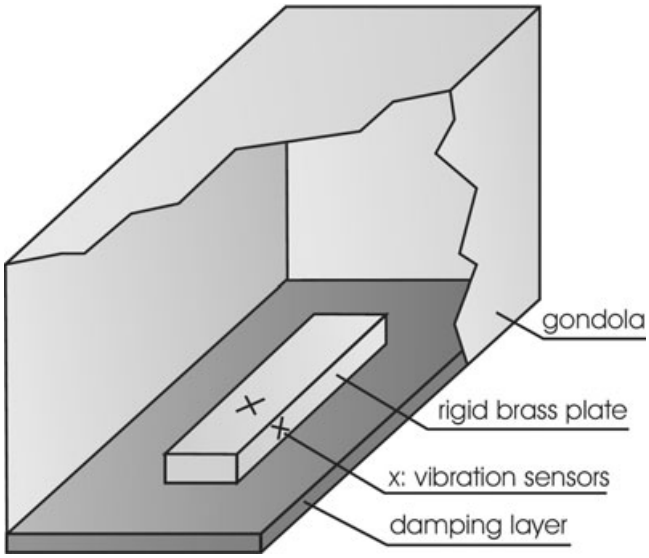

Fig. 4. Schematic drawing of the gondola containing the vibration measurement equipment. The other gondola (not drawn) was balanced for weight.

AFM operation in hypergravity

Next the AFM head was mounted in the centrifuge. To remain on the safe side regarding vibrations and acoustics,

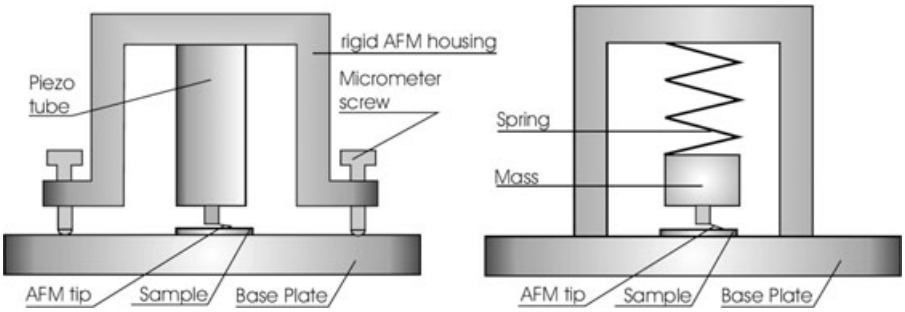

Fig. 5. shows a schematic representation of the AFM head and its equivalent mass-spring system. The AFM head consists of a rigid box that stands on three micrometer screws. The $x/y/z$ actuator, the piezo tube, is mounted inside this box. The AFM tip is mounted on the bottom side of the piezo tube. The sample is mounted on the base plate.

we mounted the AFM assembly in a spring suspension system in an acoustically damped box (Fig. 3). The inexpensive and simple spring suspension system acts like a vibration isolated optical table and can easily be dimensioned for different sizes and masses. It can be modelled as a second-order mass spring system with a vertical resonance frequency around 3 Hz and a low Q-factor (approximately 2) in the height direction. As a result, vibrations at frequencies above 3 Hz are reduced, whereas only a very minor increase in vibration level will occur around 3 Hz. In horizontal direction, the resonance frequencies are around 1 Hz with a Q of about 10. The AFM head is considered to be very stiff at 1 Hz, making the effect of the high Q negligible. The mass spring system of the combined damping material and brass plate (see Fig. 5) have a much higher resonance frequency than the AFM head in the spring suspension mount, therefore the spring suspension system is expected to reduce the noise levels of Table 1 even further.

We started imaging a hard surface, an uncoated DVD area, as reference test sample to assess the mechanical alignment and stability during high g AFM measurements. Figure 6 shows AFM topography images of the same DVD area at

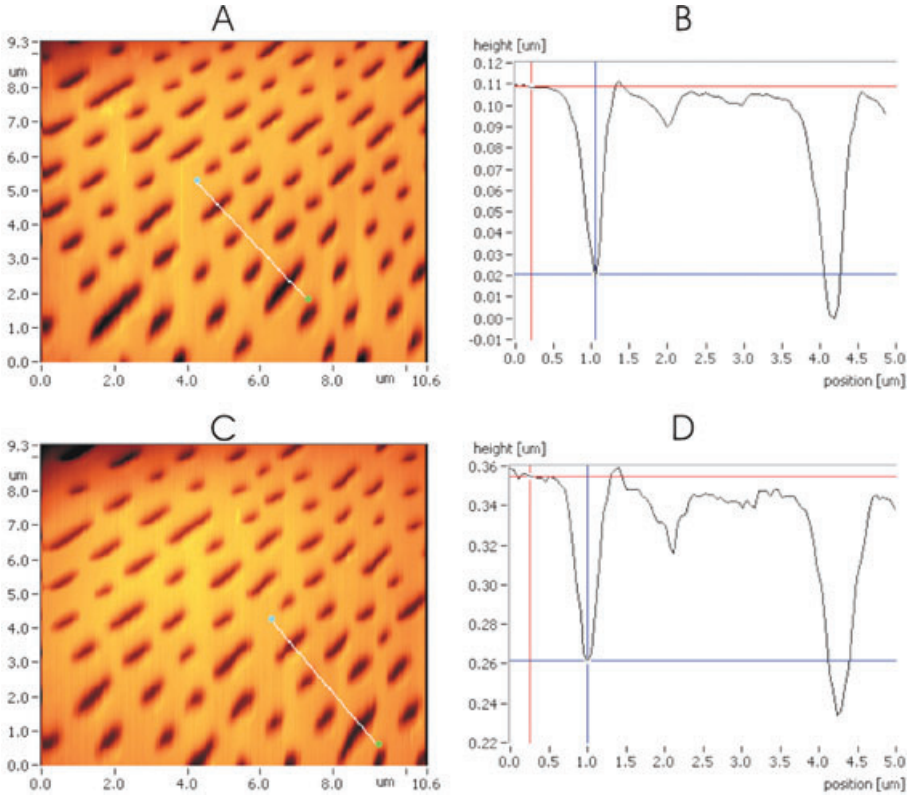

Fig. 6. Stability test for the AFM system under hypergravity, immersed in water. (A) Bare DVD surface topography at $1 \times g$. (B) Line trace at line in (A). (C) Same DVD surface topography as in A but now at $3.5 \times g$. (D) Line trace at line in (C).

$1 \times g$ and $3.5 \times g$. The images are largely similar; however, upon detailed inspection, comparing measured depth of the same DVD-pit, we found an indication of static deformation of the AFM as a function of gravity. Line traces in Fig. 6 show a measured pit depth at $1 \times g$ of 89 ± 1 nm and 93 ± 1 nm at $3.5 \times g$. So we have to take into account about 6% increased height when shifting from 1 to $3.5 \times g$ due to mechanical adaptation of the AFM head–sample configuration at increased acceleration.

The AFM topography data of the DVD surface provide a direct recording of the noise in height measurements. The measured height variation on the DVD surface between the lines of data pits is 0.5 to 0.6 nm rms in a bandwidth of 10–100 Hz, both for 1 and $3.5 \times g$. This height variation consists of the real DVD surface roughness plus the noise in the AFM height measurement. As these are not correlated, the noise in the AFM height measurement must be equal or smaller than these values. The 0.5 to 0.6 nm rms is even below the noise level predicted from the vibration analysis. Clearly the addition of a spring suspension and acoustical damping material inside the gondola has improved the sensitivity in height direction.

As our AFM head was not a priori designed to operate above $1 \times g$, it is not strange to find that the AFM-head assembly does show some deformation. The experiment on the hard DVD surface quantifies this deformation for height measurement in the 100 nm range up to the applied $3.5 \times g$. At the same time, the quantification of the alternating current (AC)-noise level in the height direction sets the minimum detectable feature height at 0.5 nm.

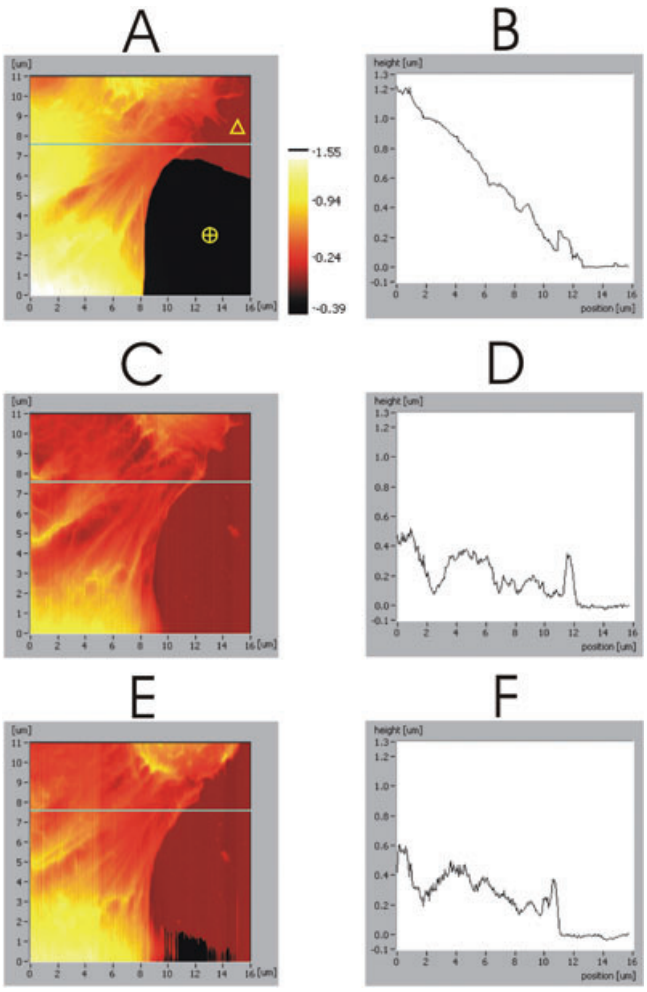

Fig. 7. Part of the same single cell at 1, 2 and $3 \times g$ in total scan area of 11×16 μm. In the AFM cell topography in (A), (C) and (E) we see part of the cell in the left and upper part of the picture. The dark brown area in (A), upper right part marked with arrow-head Δ, is the glass surface to which the cell is attached. The measurements were out of range in the black area in (A), the lower right hand corner at ⊕. The glass surface is used as reference plane and all cell heights are related to this value. (B), (D) and (F) show the line plots over line 177 (position 7.55μm) at 1, 2 and $3 \times g$, respectively. The higher parts of the cell, about 1.2 μm, are in the bright yellow domains. Time between each measurement was 4 min and 16 s, this is the time needed to scan one frame. Calibration bar in μm units.

Cell imaging in hypergravity

To demonstrate the capabilities of the AFM under hypergravity on single cells *in vitro*, we decided to image mouse osteoblast like cells in liquid at one, two and three times Earth's gravity. For cell sample preparation, we exposed mouse osteoblast like cells, MC-3T3, attached on a Termanox slide in a CO_2 independent buffered alpha-MEM medium (Gibco, Grand Island, NY, USA) supplemented with 10% foetal calf serum, L-glutamine, beta-glycerophosphate, Vitamin-C, fungizone and gentamicin.

One isolated cell, not touching neighbouring cells, was selected on the basis of normal light microscopy, and positioned in the scan area of the AFM. A recurring point of concern is the influence of the AFM on the soft surface of the cell, as the force of the AFM tip will deform the surface to some extent. To avoid variations of the tip–sample interaction force due to changing g levels, the AFM height feedback set point was checked every time a different g level was established. Figure 7 shows AFM images of the same MC-3T3 cell at 1, 2 and $3 \times g$. Due to the limited scan range of the AFM actually only part of the total cell is visible. Figure 7A–E shows the height changes as function of the increased gravity over time. The scans were taken consecutively with a total scan time of about 13 min for the three images shown. The cell changes both in shape and height going from 1 to $3 \times g$. It is clearly visible how cell cytoskeletal components, most likely stress fibres or microtubules, remain visible whereas the main cell height reduces or mass is shifted to other parts of the cell outside the scanning range. The average cell height within the scan range is 798, 348 and 515 nm at 1, 2 and $3 \times g$, respectively. The reduced height might indicate cell lowering.

However, this measured height can still be affected by shifted AFM response upon increased gravity.

From these first data presented it is clear that cell shape can be monitored in detail at increased g levels. This proof of principle opens the way for future detailed and quantitative hypergravity experiments on cell response using AFM (Fig. 7).

Discussion

We have adapted and validated an existing stand-alone AFM system (Van der Werf *et al.*, 1993) for hypergravity operation by implementing operation inside a centrifuge, inserting a passive damping suspension system and adding remote control to the electronics and software. After estimating noise levels and testing of stability, we have shown that the resulting AFM system in the centrifuge is suitable to measure the topology of living cells online in culture medium at hypergravity up to $3 \times g$.

One of the obvious changes in liquid bodies exposed to hypergravity is the increased hydrostatic pressure (van Loon 2007). Fortunately, for a fluid cell height of only 1 mm the pressure increase due to the applied levels of hypergravity is rather small, typically a pressure of 0.01 Pa at $1 \times g$ and 0.03 Pa at $3 \times g$. Another and even larger effect on the pressure in the small fluid cell is the capillary force. However, the capillary force is not influenced by hypergravity and therefore expected to be constant during the measurement. The changes in pressure might in theory influence the shape of the cell but are in practice negligible as even the atmospheric pressure can vary a few mbar in 1 h. Biologically significant pressures on osteoblasts are orders of magnitude higher, ranging from 1.3 kPa (Ferraro *et al.*, 2004) to even 200 kPa (Glanstchnig *et al.*, 1996; Kim *et al.*, 2007).

Our initial results show that cells do change height and shape at different g levels. However, at a (sub-)cellular level the force of gravity seems quite insignificant, compared to other forces that govern inter- and intracellular molecular interaction. Non-gravity related phenomena like thermal noise (kT) or chemical energies are orders of magnitude larger than a $1 \times g$ acceleration (Pollard, 1965, 1971; Albrecht-Buehler, 1991; Todd, 1989, 1990; Todd *et al.*, 1996). The main difference of a gravitational load as compared to the random Brownian motions is, however, that gravity acts continuously in the same direction. It has been postulated that the seemingly insignificant magnitude of gravity might assert its effect by integration and augmentation through mechanisms like reaction diffusion (Turing, 1952; Papaseit *et al.*, 2000), stochastic resonance (Pierson & Moss, 1995; Greenwood *et al.*, 2000) or 'signal averaging' of a constant small stimulus (Kondepudi, 1991).

The change in cell shape measured *in situ* and online under varying gravity levels supports the paradigm of direct effects of gravity onto cells. In fact indications for change of cell shape and associated cytoskeletal architecture have been observed on cells grown under real or simulated microgravity conditions and chemically fixed for subsequent cytoskeletal analysis (Boonstra, 1999; Vassy *et al.*, 2001; Uva *et al.*, 2002; Hughes-Fulford, 2003). Our observations are also in line with speculations by Vassy *et al.* (2001), based on tubulin configurations in cells exposed to microgravity as well as with more recent data by Searby *et al.* (2001, 2005).

Changes in cytoskeletal components concentration or arrangements will be reflected in changed cell morphology and cell height. These changes can be quite fast. For physiological concentrations, the typical turnover time of cytoskeletal actin filaments appears to be around 6 min (McGrath *et al.*, 1998). Using articular chondrocytes, Guilak *et al.* (2002) reported a viscoelastic solid creep behaviour followed by a decreased rate of deformation that resulted in an equilibrium displacement within 120 s after application of stress via micropipette aspiration. Also, the re-arrangement of peri-nuclear actin cytoskeletal components happens even within 18 s after a mechanical stimulus by poking with a micropipette tip (Heidemann *et al.*, 1999). And finally, it has been shown in microgravity experiments that cells do adapt their signal transduction pathways but also their cell shape within minutes in real and simulated microgravity after growth factor stimulation (de Groot *et al.*, 1990, 1991; Rijken *et al.*, 1991, 1994). Here we show cell adaptations at least within the 4-min time between AFM images. Future developments should increase scan rates to enable monitoring changes at a higher frequency.

Cell mechanical properties are, to a large extent, governed by the cell membrane, cytoskeletal components, and their architecture and interconnections. Cell shape and processes such as mass displacement also depend on these factors. Current techniques to determine cell mechanical properties can be broadly divided in two categories: (1) methods that apply a stimulation over the whole cell or a monolayer of cells such as substrate deformations (Neidlinger-Wilke *et al.*, 1994), squeezing cells between parallel plates (Thoumine & Ott, 1997), fluid shear (Bacabac *et al.*, 2004), vibrations (Bacabac *et al.*, 2006), the application of modelled surfaces (Tan *et al.*, 2003; Loesberg *et al.*, 2007) or intermittent hydrostatic pressure (Burger *et al.*, 1989); (2) methods that stimulate only parts of the cell body such as micropipette aspiration (Guilak *et al.*, 1999), optical tweezers (Ashkin *et al.*, 1987), magnetic forces applied linear (Glogauer & Ferrier, 1998) or as twisting torque (Hu *et al.*, 2003), and the use of specific AFM tips (Mahaffy *et al.*, 2004). Exploiting these techniques a rather broad scala of Young's moduli of cells have been reported. Estimated values range from 2.8 kPa for bovine chondrocytes (Knight *et al.*, 1996) to 12 kPa for TB/C3 hybridoma cells (Zang *et al.*, 1991). There are also significant modulus variations within the cell that may range from 0.5 up to 84 kPa (Grattarola *et al.*, 1996; Yoshikawa *et al.*, 1999). The centrifuge AFM set-up where, in advantage to other techniques, the applied gravitational force acts on

the whole cell mass, can contribute to the determination of cell mechanical properties. Moreover gravity combined with local AFM probing or stimulation, e.g. using ball-shaped tips (Mahaffy *et al.*, 2004), provides an easy way to apply the force to a single cell and simultaneously measure its visco-elastic properties under changing gravitational loads *in situ.* Recent studies suggest that not only cell surface and bulk properties but also the cell internal morphology might be monitored using AFM with scanning near-field ultrasound holography (Shekhawat & Dravid, 2005).

Our current exploratory study provides some clear guidelines towards improvement of a specialized AFM for cell studies in hypergravity. First of all, since our current AFM features open loop piezo actuators in x, y, z-direction, it has not been possible to obtain absolute height measurements, as both cell and AFM head are affected by the gravity load. This should be solved by placing position sensors on the piezo actuator system which are not significantly influenced by changes in g levels. These sensors should be used to apply position feedback on the x, y, z actuator or simply as a position monitor while performing the AFM height measurement on the cell surface, such that any gravity effect on the AFM can be corrected afterwards by data processing. Secondly, unfortunately our current AFM piezo actuator did not have a travel range in x,y and z direction big enough for a complete cell to be measured. Therefore, a number for the total height of a cell could not be given. Finally the choice of the type of AFM cantilever is only very marginally affected by gravity, due to its very low mass compared to its stiffness.

For future studies using AFM on cells in hypergravity, it would be interesting to increase the g level to comparable mechanical load levels as are used in techniques such as micropipette aspiration or cell poking. Experiments applying fluid shear stress or tethered beads exhibit a threshold value of $\sim$1 nN (Huang *et al.*, 2004). The force due to the weight of an attached model cell, i.e. half sphere with diameter of 10 μm and $\rho \approx 1.1 \cdot 10^3$ kg/m^3, is about 5 pN, the apparent weight (taken into account buoyancy) is about 0.24 pN. Therefore simple Newtonian mechanics imply that 1 nN is equivalent to about 200 times the weight of a cell at unit gravity. To achieve gravity levels of $200 \times g$, adaptations to existing AFM systems are not expected to be sufficient. A redesign of the system will be needed, with emphasis on a very rigid and lightweight scanner. Titanium is a good choice for a frame to achieve better stability. The electronics should also be redesigned for low weight, but this is relatively simple. Furthermore, position feedback should be implemented on the x-, y- and z-axis of the scanner to improve measurement accuracy.

Using combined AFM and hypergravity provides a novel tool to study dynamic cell mechanical adaptations in more detail. It could shed new light on changes of a whole single cell shape or visco-elastic responses in relation to, e.g. cytoskeletal drugs or cell membrane modulators.

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