Probes miniaturisation for improving the resolution of atomic force microscopy for soft samples in liquid

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INTRODUCTION

The atomic force microscope (AFM) is a unique tool for visualising and probing biological specimens in their natural environment, i.e. in liquid. The AFM produces an image by raster scanning a nanometre-sharp tip mounted at the end a flexible cantilever over the sample of interest. The deflection of the flexible cantilever thereby provides a direct measure of the compressive force exerted on the sample, which is in turn controlled and maintained constant by a feedback mechanism. This is known as the constant force, contact mode AFM operation.

Although conceptually the least elaborate mode of operation, it has yielded some of the highest resolution images of biological samples, such as 2D protein crystals. However, the application of contact mode imaging is limited because the raster scanning action of the tip induces a lateral drag force on the sample, which is often destructive to soft samples such as biological molecules. Another complicating factor that limits the application of contact mode AFM is that many biological samples, including proteins, have complex three-dimensional topographies while being only weakly attached to the underlying substrate.

For these reasons, other operation modes have been developed to reduce the lateral forces compared to contact mode AFM. These are broadly termed dynamic mode AFM. In these modes, the flexible AFM cantilever is mechanically oscillated (usually by means of a piezo transducer) near its resonance frequency during the raster scanning motion, and by doing so, the lateral drag force is greatly reduced because the probing tip spends only a fraction of its time in direct contact with the sample. The remaining compressive force can be controlled and maintained by means of a feedback mechanism as usual.

MINIATURISED CANTILEVERS IN DYNAMIC MODE AFM – SMALLER IS BETTER

While much effort has been focused on the development and improvement of different dynamic AFM modes, the miniaturisation of AFM cantilevers has been comparatively slower, principally because of the inherent practical difficulties and the initial scarcity of the necessary hardware to operate with them.

Figure 1 illustrates a comparison between a conventional (NCH type) AFM cantilever and a custom fabricated AFM probe with physical dimensions reduced approximately by an order of magnitude. There are several ways of explaining the advantages of miniaturising AFM cantilevers:

- **Resonance frequency** – By reducing the dimensions of the cantilever, its resonance frequency is increased. In practice, this implies that the oscillating cantilever interacts with the sample more times per pixel (i.e. more averaging of the data), which facilitates lower noise measurements. Alternatively, working with higher resonance frequencies allows for faster scanning, which is necessary for visualising biological processes in the ms time scale.
- **Thermal noise** – The thermal fluctuations at the cantilever resonance largely determine the ultimate resolution of dynamic mode AFM. The higher resonance implies that this thermal noise is spread over larger bandwidth, such that the magnitude of the thermal noise is decreased for a given measurement bandwidth. This contributes to improving the resolution of the AFM measurement.
- **Force sensitivity** – For dynamic mode AFM measurements, the above arguments can also be articulated via the noise in the force detection due to the thermal fluctuations at the cantilever resonance, as expressed by the minimum detectable force

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F_{\text{min}} = \frac{2k_B T}{\pi f_0 Q} \sqrt{\frac{1}{B}}
\]

where \(k\) is the spring constant of the cantilever, \(k_B\) is the Boltzmann constant, \(T\) is the temperature, \(B\) is the measurement bandwidth, \(f_0\) and \(Q\) are the resonance frequency and quality factor of the cantilever respectively, and \(\gamma\) is a coefficient of viscous damping.

Therefore, the minimum detectable force can be decreased, and the force sensitivity correspondingly increased, by increasing the resonance frequency and quality factor of the cantilever, while decreasing the spring constant (or while at least keeping it constant). This can be achieved by cantilever miniaturisation. In an alternative view, the friction (\(\gamma\)) due to viscous damping can be reduced by reducing the area of the cantilever that is moving through the surrounding medium, which again implies cantilever miniaturisation. These considerations become especially important for highly compressible samples such as biological molecules.

TOOLBOX FOR WORKING WITH SMALL CANTILEVERS

In our approach to improve the resolution of the AFM, we have combined some of the most promising developments in AFM technology to work with miniaturised cantilevers in a home-built setup. Referring to Figures 1a and 1b, it is evident that the first obstacle to overcome for using small cantilevers is the detection scheme. Conventional AFM systems use an optical beam deflection scheme with a laser spot size usually in the 20-50 \(\mu\)m range, which works well for conventional cantilevers but is clearly inadequate for the miniaturised cantilevers shown here. For our set-up, we have designed a Fabry-Perot type interferometer (with the reflective backside of the cantilever acting as one end of the optical cavity) for tracking the deflection of the cantilever (Figure 2).

The advantages of using an interferometric...
The interferometric approach also allows the possibility of performing both the detection of the cantilever deflection as well as the actuation of the cantilever simultaneously. In our home-built AFM, a second laser source (λ = 635 nm) is coupled into the same fibre via a wavelength division multiplexer and the power of the actuation laser is modulated to induce an oscillation of the miniaturised cantilever via the photothermal effect. The five-lens element system of the interferometer was corrected for chromatic aberrations to focus the actuation laser (~1 mW peak to peak) on the same ~3 μm spot as the detection laser for the interferometer (λ = 785 nm). A laser actuation scheme directly excites the cantilever and results in a less polluted resonance curve compared with the traditional piezo-acoustic actuation in which the response of the cantilever strongly depends on mechanical coupling with the cantilever chip and the fluid cell. The reflectivity of the backside of the cantilever can also be tuned with a composite metallic coating to provide a balance between the adsorption required for photothermal actuation and the reflectivity for the interferometric detection.

Another crucial piece of the puzzle for exploiting the advantage of small cantilevers is the controller electronics. Since smaller cantilevers have resonance frequencies an order of magnitude higher compared to conventional cantilevers, a controller with higher bandwidth electronics is essential.

In our system, we used a commercial Vortis Advanced SPM controller (JPK Instruments, Berlin, Germany) for feedback and image acquisition (Figure 3). The controller has the option for feedback operation in several dynamic AFM modes, including amplitude modulation (AM), phase modulation (PM) [10] and frequency modulation (FM) [11-13] with optional Q control or self-excitation loops. In our experiments with miniaturised cantilevers, we usually operate in constant amplitude phase modulation (CA-PM), which is similar to frequency modulation but does not require an additional feedback loop for phase to frequency conversion via a phase-locked loop. In CA-PM, the instantaneous phase of the cantilever as it interacts with the sample is determined from the digital lock-in amplifier and used as the feedback signal for the Z-feedback. A second feedback loop maintains the amplitude constant. This is different from the AM configuration (or tapping mode) whereby the amplitude signal as read from the lock-in amplifier is used for Z-feedback.

**DYNAMICS OF MINIATURISED CANTILEVERS ON APPROACH**

In general, the coarse approach of the cantilever probe to the sample surface must be handled with caution in order to preserve the tip sharpness. Well before contacting the surface, the cantilever experiences an increase in the long-range hydrodynamic interactions with the surface: there are noticeable changes in the cantilever resonance as the cantilever moves to within micrometre-scale distances of the surface (Figure 4a). The gradient of this hydrodynamic interaction scales inversely with the width of the cantilever. Subsequently, for miniaturised cantilevers, we have observed a drastic decrease in resonance frequency in the last few micrometres before the tip makes contact with the surface (Figure 4b, Figure 4a), usually accompanied by a corresponding decrease in the quality factor of the cantilever (Figure 5b). In comparison, the frequency shift for conventional cantilevers occurs gradually over tens of μm (Figure 4a).

When the tip makes contact with the surface, there is a noticeable upturn of the resonance frequency due to the short range (~1 nm) repulsive force. For a finite time the baseline frequency (and phase) drops rapidly over the final few micrometres prior to contact, conventional setpoint algorithms would result in a large frequency shift with respect to the cantilever resonance, and thereby a potentially
destructive tip-sample force. We overcome this by using an algorithm that continuously adjusts the baseline frequency during the tip-sample approach, such that the frequency shift in contact is measured with respect to the resonance frequency only a few nanometres above the surface. This approach also works well to ensure a gentler tip-sample approach with conventional cantilevers.

**IMAGING BIOMOLECULES IN CONSTANT-AMPLITUDE PHASE MODULATION**

Using constant-amplitude phase modulation mode (with a phase setpoint of ~20 degrees, and an amplitude setpoint between 1.0 and 1.5 nm), we have obtained high resolution images of the cytoplasmic side of the purple membrane protein, bacteriorhodopsin, crystallized in 2D lattices (Figure 6). The well-characterised trimeric structure can be clearly resolved both in the height image and the corresponding phase image (error signal channel). The images are in good agreement with the highest-resolution data available in the literature [2].

We also demonstrate the advantage of CA-PM for imaging biomolecules of non-homogeneous topography which can be more challenging than flat and closely packed crystals of membrane protein. As a test sample, we used DNA plasmids adsorbed on mica [14]. DNA is one of the most imaged molecules by AFM and as such can be regarded as a benchmark against which any biological AFM development can be tested. Standard AFM measurements on such samples are analogous to raster-scanning a wiggling snake (DNA) with a blind man’s stick while at the same time trying to capture the (Ångström high) fine detail on its skin. The adsorbed DNA can also protrude far from the substrate and can be easily dislodged by the raster scan of the probe. For those reasons, AFM had yet to resolve submolecular details of DNA, in physiological conditions, with the DNA in its natively coiled 3D structure [12]. Most AFM images show DNA as a featureless polymer without any noticeable substructure.

Applying CA-PM mode to the imaging of DNA plasmids, we observed an immediate and tangible difference compared with AM mode using the same miniaturised cantilever probe (Figure 7) [1]. In AM mode, the average height of the molecule measures ~1.1 nm on relatively straight segments of the DNA. This is about half the expected diameter (or height in the case of an AFM experiment) of the known structure of DNA suggesting that the molecule is compressed significantly in AM imaging mode.

When the same DNA plasmid is imaged in CA-PM mode (using the same cantilever probe), the height agrees to within a ±10% uncertainty with the 2.0 nm diameter of the DNA molecule, suggesting minimal compression of the molecule. Furthermore, it is obvious in Figure 7 that the plasmid DNA appears laterally wider in CA-PM mode than the AM mode image. This suggests that even with an AFM tip of ~6 nm nominal tip radius, significantly broader than the cross section of the DNA, the cantilever probe is still capable of accurately tracing the true topography of the molecule in CA-PM.

Figure 8 provides a further snapshot of the breadth of information that can be obtained when imaging DNA molecules of typical corrugation and mobility in CA-PM mode [1]. In the zoomed out bottom image (Figure 8b), we can clearly distinguish the intricate twists adopted by the plasmid DNA on the mica substrate. Furthermore, when zoomed further onto the molecule (Figure 8c), we can observe a tilted double-stranded structure with a periodicity of 3.6 ± 0.4 nm superposed to the ~2 nm protrusion of the DNA above the substrate. This Ångström-high corrugation can be identified as the subsequent (deep) major and (shallow) minor grooves of Watson and Crick’s double right-handed double helix with an average helical periodicity of ~36 nm [18].

These CA-PM images have been obtained with the tip convolution still apparent in the AFM images, suggesting that the imaging is performed by asperities protruding from the tip, akin to the mechanism of high-resolution imaging in contact mode AFM.

**CONCLUSIONS**

The combination of miniaturised cantilevers with phase (frequency) modulation method (which is increasingly being implemented in commercial AFM systems) results in non-monotonic hydrodynamic interactions between the cantilever and the sample which can be destructive to the tip during approach. We describe a method to circumvent this issue and applied CA-PM AFM to resolve the DNA double
helix. This represents a step-change improvement in AFM technology that will benefit the imaging of soft and complex samples at unprecedented spatial resolution. One possible example could be the observation of different and possibly metastable structural conformations of DNA as a result from the DNA being constantly twisted, bent and stretched by proteins mediating genome transactions in the cell. Furthermore, given the high resonance frequencies involved, the CA-PM method with miniaturised cantilevers is also exquisitely suited for fast and high resolution visualisation of biomolecular interactions.

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ABSTRACT
We describe a method to reduce the invasiveness of the atomic force microscope (AFM), thereby improving the spatial and potentially temporal resolution on soft samples in liquid. This has been achieved by miniaturising the cantilever probes, thus decreasing the thermal noise in the force measurement, and by accurately tracking the tip-sample interaction via changes in the cantilever phase or resonance frequency.

While these methods are increasingly being implemented in commercial AFM systems, this combination is highly non-trivial due to the mesoscopic hydrodynamic interactions between the cantilever and the sample. We characterise and provide a solution to overcome this phenomenon and demonstrate the power of the miniaturised probes by resolving the DNA double helix [1].