FluidFM: Combining Atomic Force Microscopy and Nanofluidics in a Universal Liquid Delivery System for Single Cell Applications and Beyond

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ABSTRACT
We describe the fluidFM, an atomic force microscope (AFM) based on hollow cantilevers for local liquid dispensing and stimulation of single living cells under physiological conditions. A nanofluidic channel in the cantilever allows soluble molecules to be dispensed through a submicrometer aperture in the AFM tip. The sensitive AFM force feedback allows controlled approach of the tip to a sample for extremely local modification of surfaces in liquid environments. It also allows reliable discrimination between gentle contact with a cell membrane or its perforation. Using these two procedures, dyes have been introduced into individual living cells and even selected subcellular structures of these cells. The universality and versatility of the fluidFM will stimulate original experiments at the submicrometer scale not only in biology but also in physics, chemistry, and material science.

In the last two decades, atomic force microscope (AFM) has revolutionized surface science at the nanoscale, not only as a microscope with atomic resolution but also as a nanofabrication tool displacing atoms, manipulating and reshaping complex molecules, and delivering molecules extremely locally on a surface. AFM is also starting to contribute to cell biology, elucidating features of membrane morphology, mechanical properties, division mechanism, metabolism, voltage-induced deformation or adhesion, and potentially as a cancer detection tool. However, in these and similar experiments, the use of the AFM to manipulate cells remains largely limited to (sometimes crude) mechanical processes such as indentation, compression, stretching, and scraping. One notable exception to this list is the transport of molecules into a cell via reversible binding to an AFM tip. Here we present a novel use of AFM principles to manipulate living biological cells by stimulation through delivery of active agents directly from a solution; the fluidFM (for fluidic force microscope) combines the accurate force-controlled positioning of AFM with the versatility of fluidics. A microsized channel is integrated in an AFM cantilever and connected via channels in the AFM chip holder to a delivery system, thus creating a continuous and closed fluidic channel that can be filled with an arbitrary chosen solution and can be immersed in a liquid environment (Figure 1a). An aperture in the AFM tip at the end of the cantilever allows liquids to be dispensed locally. Force feedback is ensured by a standard AFM laser detection system that measures the deflection of the cantilever and thus the force applied by the tip to the sample during approach and dispensing.

While this approach is similar to microinjection using glass pipettes, there are a number of essential differences. Microinjection uses optical microscopy to control the position of the glass pipet tip both in the xy plane and in the z direction (via image focusing). As consequence of the limited resolution of optical microscopy, subcellular domains cannot be addressed and tip contact with the cell
membrane cannot be discriminated from tip penetration of the membrane. Cells are often lethally damaged and skilled personal are required for microinjection.\textsuperscript{19–23} The limited resolution of this method and the absence of mechanical information contrast strongly with the high resolution imaging and the direct control of applied forces that are possible with AFM. Precise force feedback reduces potential damage to the cell; the cantilever geometry minimizes both the normal contact forces on the cell and the lateral vibrations of the tip that can tear the cell membrane during microinjection; the spatial resolution is determined by the submicrometer aperture so that injection into subcellular domains becomes easily achievable.

The inclusion of force control in the dispensing/injection system also enables extremely local dispensing on surfaces. With force feedback, the dispensing tip can be brought into very close contact with a sample without risk of damage either to the sample or to the tip so that a minimal amount of solution can be released directly at the chosen spot. Local dispensing will allow precise stimulation of cells, applying biomolecules to a well-defined position on one cell that may either be isolated or part of an ensemble or network of cells. It will also open new possibilities in biochemical analysis, for example, allowing analyte solutions to be dispensed locally on an array of antibodies.

Microchanneled AFM cantilevers were produced using an original microfabrication process based on thermal fusion bonding of two previously etched silicon wafers to create cavities lined with silicon dioxide within the body of the silicon. This was followed by a selective silicon etch to produce free-standing silicon dioxide structures.\textsuperscript{24,25} The result is a hollow glass cantilever attached to a silicon chip (see Supporting Information Figures S1 and S2). The closed microchannel within the cantilever enters the silicon chip and ends in an

\textbf{Figure 1.} The fluidFM. (a) Diagram showing a microchanneled cantilever chip fixed to a drilled AFM probeholder. The system was shown to be watertight up to 4 bar internal overpressure but overpressures of the order of 10 mbars were usually applied. The fluidFM can be operated in air or with the whole system (probeholder and chip) immersed in a liquid. The external liquid or bath and the liquid in the microchannel may be the same or may be different. During liquid dispensing, the substrate can be simultaneously observed with an optical microscope either through the transparent probe holder or through the glass substrate. (b) Scanning electron micrograph of the aperture beside the apex of the pyramidal AFM tip for the intracellular injection experiments as in Figure 3. The tip is made of silicon nitride (\textit{Si}_3\textit{N}_4) with a wall thickness of 100 nm. The apertures are milled by focused ion beam with a diameter ranging from 1 \textmu m down to 100 nm. Thin \textit{Si}_3\textit{N}_4 walls are compulsory for milling such nanosized holes. Before the FIB milling, a metallic layer (gold or platinum) is deposited on both sides of the cantilever to avoid charging effects during milling and increase the reflectivity for the AFM laser. (c) Scanning electron micrograph of the aperture at the apex of the pyramidal AFM tip for the cell staining experiments by gentle contact as in Figure 4.
The force detection system of the fluidFM is so sensitive that the interactions between tip and sample can be reduced to the piconewton range enabling two different strategies when addressing fragile samples such as living cells. At small force set points, the hollow cantilever with the aperture beside the apex as in Figure 1b can be brought into gentle but close contact with cells without unwished puncturing of the cell membrane (see Figure 2a). On the other side, membrane perforation for intracellular injection is simply achieved by selecting a higher force set point taking advantage of the extremely sharp tip (radius of curvature on the order of tens of nanometers) as indicated by the typical indentation jump of the force distance curve of Figure 2a. If the aperture is milled at the tip apex as in Figure 1c, no indentation jump is observed in the force—distance curves (see Figure 2b) meaning that the tip with this configuration remains in “gentle contact” with the membrane without tearing it.

Intracellular injection (see Figure 3a) was demonstrated by using fluorescein isocyanate sodium salt (FITC) as dye because it neither binds to the cell membrane nor does it spontaneously diffuse across it. Figure 3b is an AFM topography image of a myoblast cell taken in the amplitude-modulation (AM) mode with the fluidic channel filled with a physiological solution containing the FITC dye. The AM mode decreases the force exerted on the cell because the effective stiffness of the probe becomes $k_{	ext{eff}} = k/Q$ with $Q$ as the quality factor of the cantilever in liquid environment (see Supporting Information, Figure S2b). The cell is resolved with a spatial accuracy down to 200 nm details confirming the ability of the microchanneled cantilevers to act both as scanning probe and as nanopipette. Upon completion of the AFM imaging, the probe was stopped and positioned with the AFM controller over a selected point of the cell. As we are relying on the force—distance curve of the tip approach to the cell in order to determine the set point necessary to penetrate into the cell membrane, we switched to the contact mode, introduced the extremity of the tip into the cell, injected the fluorescent solution by hydrostatic pressure for few seconds, and finally retracted the tip far away from the selected cell. From simulations of the flow rates through the aperture of such hollow cantilever (see Supporting Information, Figure S3), we can estimate that the injected amount of FITC solution is less than 10 fL. Figure 3c is a fluorescent image taken with a confocal scanning laser microscope (CLSM) of the same cell in Figure 3b. It can be seen that that the introduction of liquid into the cell has resulted in the appearance of a fluorescence signal without an important change in the cell volume. To demonstrate that the dye is inside the cell, $z$-stack images were taken. The corresponding cross section shows a fluorescent intensity not only at the cell membrane but also in the cytosol. The fact that the dye remains in the cell indicates that the membrane recovers and reseals tightly after the withdrawal of the tip. Figure 3d,e shows intracellular injection of FITC into three neurons one after the other with the same microchanneled cantilever proving that probes can be used for consecutive manipulation without clogging.

**Figure 2.** Contact regimes with the cell membrane. (a) Typical force distance curve on a myoblast cell for a cantilever with an aperture milled beside the pyramid apex like in Figure 1b ($k = 0.3$ N/m, approach of 50 nm/s); the arrow indicates the typical discontinuity corresponding to indentation of the cell membrane. The set point of around 3 nN thus separates the two contact regimes, the gentle contact on the cell membrane and the membrane perforation for intracellular injection. (b) Typical force distance curve on a myoblast cells for a cantilever with an aperture of 1 $\mu$m milled at pyramid apex like in Figure 1c ($k = 0.3$ N/m, approach of 50 nm/s); no indentation discontinuity appears with this aperture configuration.
Figure 3. Intracellular injection by force-controlled perforation of the cell membrane. (a) Diagram showing the intracellular injection procedure by membrane perforation (not to scale). The aperture is intentionally milled beside the apex in order to exploit the nanometric curvature radius of the pyramid to facilitate the membrane indentation (set point of 3 nN at least). (b) Differential interference contrast image of a myoblast and corresponding AFM image (amplitude of 30 nm, scan rate of 0.3 s, cantilever as in Figure S2). (c) CLSM stack image of the same cell for $z = 1 \mu m$ after FITC intracellular injection of 15 s and corresponding cross section along the dashed red line; the fluorescent thickness of 6 $\mu m$ is in accordance with the height of the cell measured by AFM. (d,e) Differential interference contrast image of neuroblastoma and corresponding fluorescent image upon FITC intracellular injection of the three ones in the middle.
The gentle contact procedure is demonstrated by staining live neuroblastoma cells as illustrated in Figure 4a. A microchannelled cantilever filled with CellTracker green, a membrane-permeant dye, was positioned over one cell using an optical microscope. It was then touched onto the cell and left in gentle contact until the dye diffused into the cytoplasm. CellTracker green was used because its fluorescence depends on the enzymatic activity of a live cell. CellTracker green is a nonfluorescent chloromethyl derivative that passes through the cell membrane into the cytoplasm where enzymatic activity converts it into a fluorescent membrane-impermeable species.

Figure 4b shows several neuroblastoma cells on a glass slide in a physiological buffer. The fluidFM cantilever filled with CellTracker green was touched onto the cell marked by the red circle and left for 15 min, which is the time necessary for the cell to metabolize the agent diffusing through the membrane and express the fluorescent product. The cell addressed with the tip became fluorescent whereas neighboring cells were not, demonstrating that the dye was indeed introduced into the selected cell, that the cell was not lethally damaged, and that the staining procedure does not affect nearby cells.

The precise delivery of a dye into a cellular substructure can also be achieved thanks to the imaging capacities of the AFM. A region is first scanned with fluidFM and the topographical information is used to access structures too small for optically controlled microinjection systems. Figure 4c is a differential interference contrast image of two neuroblastomas connected by a neurite forming a varicosity-like structure (red arrow) and corresponding AFM profile along the white dotted line (AM mode, amplitude of 50 nm, scan rate of 0.1 s, cantilever as in Supporting Information, Figure S2a). The black arrow indicates the point on the profile where the tip was precisely approached with the AFM control. (d) Corresponding fluorescence image after staining the varicosity-like structure with acridine orange for 1 min.

Figure 4. Staining living neuroblastoma cells by gentle contact on the cell membrane. (a) Diagram showing the staining procedure by gentle contact (not to scale). The hollow tip is maintained in contact with the cell membrane thanks to the force feedback (set point of less than 1 nN). The active agents dissolved in the solution of the microchannel spontaneously diffuse across the membrane into the cytoplasm. (b) Superposition of a differential interference contrast image and of the corresponding fluorescent one of a cell after staining with CellTracker green. The microchannelled cantilever filled with CellTracker green is positioned over the cell in the red circle using the optical microscope. The tip is then brought into gentle contact with the cell membrane by the AFM force feedback system and left there for 15 min, before taking the fluorescent and phase contrast image. (c) Optical image in differential interference contrast method of two neuroblastoma cells connected by a neurite forming a varicosity-like structure (red arrow) and corresponding AFM profile along the white dotted line (AM mode, amplitude of 50 nm, scan rate of 0.1 s, cantilever as in Supporting Information, Figure S2a). The black arrow indicates the point on the profile where the tip was precisely approached with the AFM control. (d) Corresponding fluorescence image after staining the varicosity-like structure with acridine orange for 1 min.
fluorescence intensity along the neurite. This demonstrates ease with which solutes can be delivered in a controlled manner into selected subcellular structures with the fluidFM.

Introduction of material into a cell in an AFM-based procedure has been achieved by other authors.\textsuperscript{15–17} In their approaches, the AFM nanoneedle was coated or functionalized with selected molecules and then pushed through the cell membrane into the cytoplasm where the molecules were released. However, this approach is limited to vanishingly small quantities of those molecules that can be reversibly bound to an AFM tip and then spontaneously desorb from the tip to the intracellular environment. On the contrary, the fluidic channel of the fluidFM represents an infinite reservoir for the active agent moreover in a form that is immediately available for repeated delivery as it is not bound to the tip.

Since the biomolecules to be introduced into the cell no longer have to be grafted or adsorbed to the AFM tip but are simply dissolved in a delivery solution, a much wider range of applications of intracellular injection becomes available than with the methods of Chen, Cuerrier, or Han. This range now spans from the insertion of particles to study how they move inside the cell according to their chemical functionalization, to the insertion of proteins, genes (eventually into the nucleus), enzymes, ligands for fundamental research of cell biology but also for applications like tissue engineering from single ad hoc infected cells. In addition, the gentle contact approach opens the door for studies such as the behavior of viruses at the cell membrane or the interaction of drugs with transmembrane proteins.

In conclusion, we have shown novel applications using hollow force-controlled AFM cantilevers. The microchanneled cantilevers were successfully connected to a delivery system via a modified AFM probeholder thus enabling force-controlled dispensing of a solution containing selected molecules into individual cells in a physiological environment. The cantilever geometry and the AFM force feedback allow very local as well as facile delivery of molecular species to cells either by gentle contact with their membrane or by perforation of the latter.

The experiments described here demonstrate the potential of the fluidFM in the field of single-cell biology through precise stimulation of selected cell domains with whatever soluble agents at a well-defined time. We confidently expect that the inclusion of an electrode in the microfluidics circuit will allow a similar approach to the microfluidics circuit will allow a similar approach for the active agent moreover in a form that is immediately available for repeated delivery as it is not bound to the tip.

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Supporting Information Available: Description of Materials and Methods. Figure S1: Micrographs of the microchanneled cantilevers. Figure S2: Resonance peaks of a microchanneled cantilever in different environments. Figure 3S: Water flow rates through the microchanneled cantilevers for different values of the aperture diameter at the tip apex. This material is available free of charge via the Internet at http://pubs.acs.org.

References


(32) The membrane-permeable CellTracker and acridin orange were used in combination with ethidium bromide to differentiate between living and apoptotic cells.

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