NADIS: A Novel AFM-based Tool for Dispensing Fluids into Single Cells

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INTRODUCTION
Over the last decade a number of adaptations of scanning probe methods have emerged that allow the deposition of small amounts of material onto a surface, such as Dip-Pen Nanolithography (DPN) [1,2], nanoscale dispensing (NADIS) [3] and ‘fountain pen’ type systems [4-6]. These techniques are all based on micromechanical probes similar to the cantilevers used in scanning force microscopy (AFM), but differ in their deposition mechanisms. Very small features can be written with DPN since molecules are directly transferred from a sharp AFM tip that has been dipped in a solution. In contrast, NADIS and fountain pens allow a steady flow of material along the cantilever by capillarity, often through closed channels in the cantilever arms to deliver material to a machined opening in the probe tip. The fact that the channels are closed is crucial since it allows the deposition of sub-attoliter volumes not only under ambient conditions [3], but in liquids as well [7] – an important advantage for work with biological samples, and a key objective when the development of NADIS was started at CSEM [8].

INJECTION INTO CELLS
From its earliest days, force microscopy has been applied to cell biology, first for imaging purposes, later for the investigation of mechanical properties and adhesion forces. The ability to measure a variety of parameters otherwise difficult to access, in combination with a gentle and in-situ type of operation have made AFM a powerful new tool in the life sciences.

More recently, AFM based techniques have been developed to introduce molecules into cells, mostly by depositing them onto the AFM probe or a nanoneedle attached to it, and then poking the tip or needle through the cell membrane and into its interior [9-11]. The main advantages over conventional micropipette-based methods [12] lie in the microscopic control of location and forces applied to the cell during perforation of the cell membrane. However, with these AFM based methods, the amount of matter transferred into the cells remains limited to the molecules that can be adsorbed to the tip surface. Furthermore, the transfer has to rely on reversible binding of the cargo to the tip.

The application of nanoscale dispensing (NADIS) to cell injection circumvents this shortcoming [13]. In NADIS, cantilever probes with integrated fluidic channels are used to deliver minute volumes of liquid to the sample, while keeping the high spatial control and imaging capabilities from force microscopy. Figure 1 shows the NADIS principle applied to injection into cells. The liquid (a green dye solution) is delivered to the probe tip by a hollow cantilever acting as a microfluidic core and a tip with an aperture at the apex. In this article we describe the technology of NADIS and give some examples showing its use in injecting into individual living cells and subcellular structures.

ACKNOWLEDGEMENTS
This project was partly financed by the Commission of Technology and Innovation (CTI) and the Swiss Federal Office for Education and Science (OFS) in the framework of the EC-funded project NaPa.

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Microscopy and Analysis 23(6):11-13 (EU), 2009

Figure 1:
The principle of nanoscale dispensing (NADIS) in a liquid environment. The AFM tip with integrated channels is brought into gentle contact with a soft specimen, such as a biological cell and an external pressure controller (not shown) allows a small quantity of liquid to be dispensed.
channeled cantilever coming in from the right. The tip is put in gentle contact with the cell surface, and the cell membrane is eventually penetrated. It is an advantage to control the tip-sample interaction force when approaching the cell, in order to avoid unnecessary loading and damage to the surface. When contact is established, liquid can be delivered by precise application of external pressure. The use of a closed fluidics circuit within the cantilever allows for virtually unlimited volumes of material to be dispensed in a liquid environment. The whole process is monitored by simultaneous (fluorescence) microscopy.

The NADIS technology has been integrated in our commercial AFM/fluorescence microscopy system using a homemade cantilever chip holder and an external tubing system with pumps (Figure 2). While the chip holder is especially adapted to the microscopes in the authors’ laboratories, it appears straightforward to design dedicated adapters for different AFM platforms without difficulties.

MICROFABRICATION OF THE NADIS SYSTEM
The cantilevers used for NADIS feature microfluidics channels inside the cantilever arms, connecting the probe tip to liquid reservoirs which are positioned back on the holding chip. These cantilever structures are created by precise alignment and fusion bonding of two pre-structured silicon wafers. During fusion bonding, an oxide layer grows on all silicon surfaces and covers the interior surfaces of channels embedded between the two wafers. Most of the silicon is etched away to ‘release’ the embedded channels with their oxide walls which then serve as the cantilever structure. A sharp probe tip is patterned at the end of the channel, and is later opened by Focused Ion Beam milling. Figure 3 shows some details of the microfabricated structures.

A simple dedicated liquid adapter connecting the on-chip reservoirs to a laboratory liquid supply system (as shown in Figure 2) is sufficient to allow work with the NADIS cantilevers on many different AFM platforms.

EXPERIMENTS
NADIS allows the manipulation of cell membranes in a force-controlled manner, which is an advantage for the cell injection process. The cantilever deflection recorded during the approach of the NADIS probe to the cell membrane is a reliable indicator of the first probe-cell contact, and of the moment when the cell membrane is perforated by the tip. Excessive forces can thus be avoided, and the cells generally survive this manipulation.

Figure 4a is an optical image of a single cell – a myoblast - seen in differential interference contrast microscopy. The adjoining image (Figure 4b) shows the same cell after injection of the dye, fluorescein isothiocyanate (FITC). FITC was chosen for this demonstration as it cannot cross an intact cell membrane except by injection. The NADIS tip was introduced into the cell using the cantilever deflection to monitor when the tip penetrated the membrane. Once in the cell, less than 10 fL of the FITC solution were introduced and the tip was then withdrawn. From Figure 4c it is clear that the fluorescence label has been successfully injected and that it remains inside the cell.

The design of the NADIS tip can be adapted to suit specific experimental requirements. For liquid dispensing on a surface an aperture at the very end of the tip - as shown in Figure 3 - is ideal. In contrast, for microinjection work, a sharp tip is required, and an aperture at the side of the tip facilitates membrane perforation. This design also preserves the high resolution AFM imaging capabilities of the probe. Figure 4c shows an AFM image taken of the same cell, demonstrating the broad imaging capabilities of the combined optical/fluorescence/AFM microscopy setup available to support the injection experiments.
Figure 4:
Injection into single living cells by membrane perforation. (a) A single, living myoblast imaged by differential interference contrast microscopy. (b) The same cell imaged using fluorescence microscopy after FITC injection by NADIS, a strong fluorescence signal is observed. (c) The same cell imaged by atomic force microscopy with a NADIS probe.

Force microscopy is a high-resolution imaging method, which allows NADIS to be performed with precise control of the location where the material is deposited, or injected. Figure 5 shows an example of highly localized staining of a subcellular structure. In the first image (5a), a group of living neuroblastoma cells can be seen, with a distinctive subcellular structure: an axonal varicosity. The second image (5b) shows the same group of cells, imaged using fluorescence microscopy after labeling the varicosity. The NADIS cantilever was used both to find the optimal position on the surface of the varicosity and to transport the dye solution to the cell. In the right image (5c) a single neuroblastoma cell has been injected with Celltracker Green, indicating that the cell is alive, as this dye only becomes fluorescent after metabolism by enzymes in the cell.

CONCLUSIONS
The microinjection of solutions into viable cells has been demonstrated using the NADIS technology with microchanneled AFM cantilever probes. The force control of NADIS and other cantilever-based approaches to single-cell manipulation and injection offer significant advantages over classical methods based on glass micropipettes, as they allow the improvement of the localization of the microinjection as well as cell viability.

This technology might open the way to extraction of cytoplasm, and potentially to automated patch clamping under force feedback. This would lead to a better understanding of cell processes under continued cell viability, with potential applications in biology and pharmacology.

Other applications of NADIS are mostly in the field of material science and surface chemistry, such as for the functionalization of protein microarrays [14].

REFERENCES

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