Nanofountain Probes for Single-Cell Transfection: A Comparative Study Assessing Invasiveness

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Abstract

This study examined the invasiveness of nanofountain probes (NFP) when used as a transfection tool for single-cell research. For comparison, the damage caused to cells by more commonly used commercial atomic force microscope (AFM) probes was also studied. Forces were applied to cells at increasing levels using both probes, and the effects were observed and recorded. Results demonstrate that NFPs can be used to transfect cells at higher forces and yet yield less damage than commercial AFM probes.

Introduction

The ability to inject materials such as proteins, DNA, and drugs into individual cells is applicable across a wide array of areas, especially therapeutic development. Injection technique enables research at the single-cell level due to its ability to target specific cells. A commonly used alternative to injection, for example, is vehicle-mediated transfer, which uses a liposome as a membrane to hold the desired material and allows it to be absorbed by the cell.[1] The main disadvantage of this technique over direct injection is the lack of selectivity:[1] a cell must be physically isolated in order to be transfected as a single entity. This method is also limited by the size of the molecule that can be carried by the liposome.[1]

In recent decades, a variety of methods have been developed for single-cell injection. Many of the currently available tools have limitations that prevent their use outside specific applications. One frequently encountered problem is the damaging effects of injecting a foreign tool into a cell.[1] For example, cells injected using commercial AFM tips showed only 30% viability following injection.[2] Cell viability is essential for analyzing how injected materials affect cells, so increasing cell survival is a goal of newer techniques.

Another tool, using the AFM as a control, is the nanofountain probe (NFP),[3] which functions as a highly miniaturized fountain pen with a volcano-shaped tip fed by enclosed microchannels that run from an on-chip reservoir where the ink (e.g., nanoparticles in liquid suspension or biomolecules in buffer solution) is stored. This design allows for the continuous flow of ink to the tip and onto a substrate.[4] Direct patterning of these inks onto substrates has been demonstrated with gold nanoparticles, DNA, and nanodiamond (ND) particles.[3–5] It has also been demonstrated that NFP is capable of injecting NDs into cells with high precision of both force and position.[5,6] Figure 1 illustrates the NFP's design.

The NFP's ability to continuously deliver molecules to a living cell is a considerable advantage over other techniques. However, while it was hypothesized that the damage to the cell from insertion of the NFP is minimal and reversible, this had not been verified experimentally. The purpose of this study was to demonstrate NFP's minimal damage to cells in order to further validate its use as an injection tool.

Background

Researchers have been developing different methods of material transfection, the artificial injection of a substance into cells,[7] for decades. The methods vary widely, each having advantages and disadvantages.

Carrier-mediated transfer uses a carrier (e.g., liposomes) to contain a desired material and pass it through the membrane of the cell. However, with this method it is difficult to target a specific cell within a culture.[8]

Protein transduction uses a medium to transfer proteins to cells.[9] In protein transduction, a protein enters the cell and triggers the cell's internal mechanisms to produce more of the protein to transfer to other cells. When introduced through the surrounding media, proteins are transduced to all cells equally. When one cell is targeted, the spread of the protein to other cells is correlated to those cells' distance from the originating cell.[9]

Electrical plasma membrane permeabilization is another method of inserting material into cells. It cuts the cell's membrane so that a material can be inserted. While this creates a precise insertion area, the degree of damage to the cell is not known.[10] Also unknown is how accurately the behavior of a partially intact cell represents that of an undisturbed cell.[11]

Direct injection techniques use an insertion tool to target a single cell for material insertion.[12] Micropipettes have been utilized, but the size of the tip (0.5–1 μM) is large relative to the size of the cell. This causes damage, often irreversible, to the live cell during injection. In

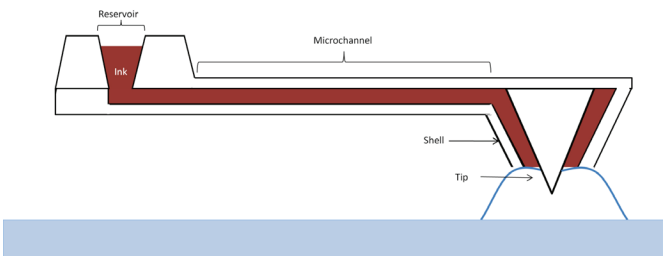

Figure 1. NFP schematic illustrating the molecular ink, held in the reservoir, that is fed to the tip of the NFP through microchannels.

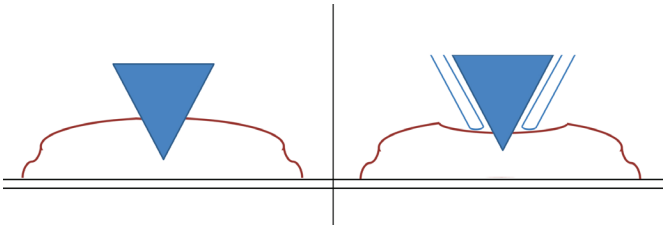

Figure 2. NFP and AFM probe cellular contact. The left drawing shows the AFM probe's entire outer surface penetrating the cell, while the right drawing outlines the NFP inner shell's expected puncturing of the cell as the outer shell rests on the cell surface.

addition, with this method it is difficult to measure the amount of material inserted into the cell.[1,12]

More recently, nanoscale tools have been designed to insert material into cells. While it seems evident that smaller tools will make smaller insertions when penetrating the cell membrane and thus cause less cell damage, there are also shortcomings. Nanoneedles, for example, cannot hold many particles. However, the benefit of using a nanoneedle (less than 400 nm in diameter) is that it will not cause irreversible harm to the cell and can be left in the cell for long periods of time.[13] In further tests, cells injected using nanoneedles were observed to live and divide, passing the injected protein to 74% of the divided cells. Another study reduced the size of the injector to 1–20 nm and controlled the needle position and insertion force using the AFM. This was successful due to the injector's precise control in the position of the delivery site and volume of material delivered. The injector could also be left inside the plasma for longer than one hour with no irreversible damage to the cell.[14]

NFPs offer a viable alternative to other techniques for cellular insertion of materials. The first-generation NFP was developed in 2005 at Northwestern University, and currently the third generation is in use.[4,15] A primary advantage of the NFP over similar tools is that it offers continuous flow of molecular ink to the tip due to a reservoir built into the body of the chip.[4] The probe tips have a 200 nm diameter, which is significantly smaller than the 400 nm diameter found to be the maximum size for avoiding irreversible cell damage.[15,16] This tip size should allow for longer duration of insertion into the cell without the permanent cell damage caused by micropipette tools.[12] The AFM's control of the NFP allows monitoring of the probe tip's force and position with nanoNewton and nanometer resolution, which is not true of several other nanoscale devices.[15] This is advantageous, because many similar tools do not offer this degree of control over the amount of force and the position where the tip enters the cell. In addition, AFM-based control allows the user to view the force exerted by the cantilever tip on the surface until it has made contact and punctured the cell; the contact force increases when contact is made and continues to increase until the NFP tip punctures the cell membrane.

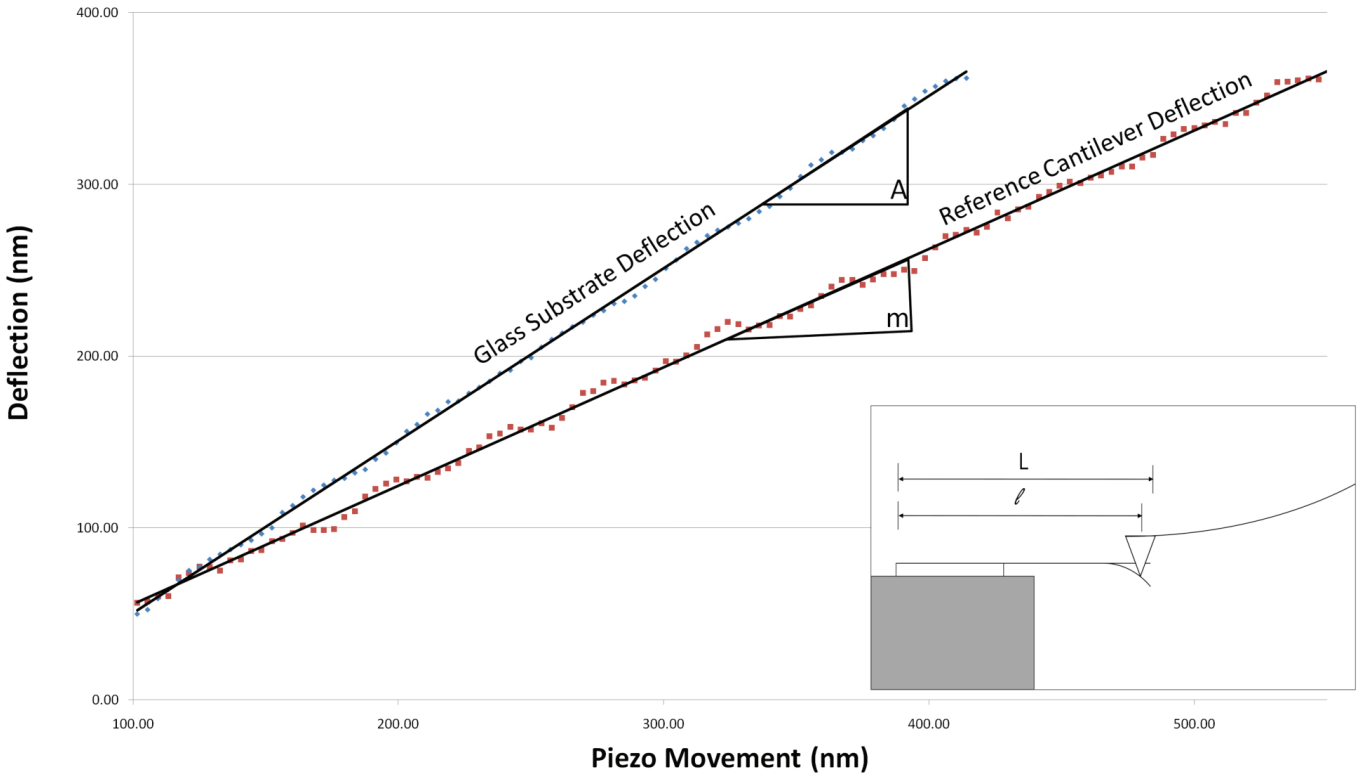

Figure 3. Probe calibration technique. Deflection of the cantilever when applied to the glass substrate is the slope defined as *A*. Deflection of the cantilever when applied to the reference cantilever is defined as the slope m. Inset: The probe is put into contact at length *l* with the beam of total length L.

To this point, the assumption that the NFP will not cause irreversible damage had not been proven. This study will document and compare the amount of damage that occurs to a cell after NFP and AFM probes penetrate the membrane. The hypothesis is that the unique core-shell tip geometry of the NFP will control the radius of the puncture, reducing damage to the cell. Because the NFP has a sharp inner tip and a rounded outer shell, the sharp tip should puncture the membrane first. Upon further insertion, the outer shell should come into contact with and deflect the membrane but prevent further puncture and widening of the hole. This concept is illustrated in Figure 2. Reducing the actual penetrating diameter of the tip should cause less damage to the cellular structure. Verification of this hypothesis will enable use of the NFP to conduct minimally invasive, direct in vitro single-cell injection of a continuous flow of molecules.

Approach

AFM probes and NFPs were used to puncture cells and the resulting damage compared. This allowed a direct assessment of the NFP as a transfection tool as compared with more commonly used commercial AFM probes. To ensure similar conditions, commercial AFM contact mode probes were selected with a specified stiffness (0.2 N m^{-1}) close to that of the NFP (0.175 N m^{-1}).[14] Each probe's actual stiffness can deviate, however, from the manufacturer's specification. For that reason, commercial probes were calibrated experimentally to find their actual stiffness. In order to calculate the stiffness, an AFM probe was mounted in the AFM (Veeco Dimension 3100) and configured to operate in contact mode. Equations 1 and 2 were used to calculate the probe's stiffness. The probe was initially brought into contact with a glass substrate (assumed to be perfectly rigid), and the resulting force-displacement response was recorded as seen in Figure 3. This measurement was recorded as the deflection sensitivity of the cantilever, A. A substrate containing reference cantilevers of differing known stiffness with lengths L was then placed under the AFM. The AFM probe was lowered to a reference cantilever until contact was made at a specified distance from the base of the cantilever, *l*. The slope of the resulting force-displacement curve m was then measured. Q' is the ratio of m and A. The stiffness of the AFM probe, k_2, was then calculated.

$$(1) \quad Q' = \frac{m}{A}$$

$$(2) \quad k_{2\,adj} = k_2 \left(\frac{L}{l}\right)^3$$

$$(3) \quad k_2 = k_1 \frac{Q'}{1 - Q'}$$

HeLa cervical cancer cells, used throughout this study, were probed with commercial AFM tips and NFPs to observe the cells' reaction to a given force of insertion. Prior to probing, individual cells were imaged in a Nikon Eclipse ME600 epifluorescence microscope and their location on the substrate noted. The cells were then moved to the AFM, where each cell in the selected series was individually punctured with a prescribed force by the AFM probe for 10 seconds. Probing experiments were conducted using a liquid cell, which maintains both the AFM probe and the sample in a continuous body of liquid. This allows the cells to be maintained in a natural buffer solution and also prevents the AFM probe from having to break through the liquid surface to reach the cells.

	AFM P. Force (nN)	NFP Force (nN)
Visible puncture	145.97 *(Figure 6)*	413.88 *(Figure 9)*
Morphology change	157.41 *(Figure 7)*	222.43 *(Figure 8)*
Detach	172.69 *(Figure 5)*	N/A
Rupture	172.69 *(Figure 4)*	N/A

Table 1. Cellular damage, type of probe used, and force applied to cell. The corresponding image for each entry is also noted.

To enable further investigation of the effects of probing on the cell structure, the cytoskeleton was fluorescently stained with Organelle Lights™ Actin-RFP. Imaging the cells before and after probing allowed assessment of cytoskeleton disturbance resulting from the probing.

Results

All probing experiments are summarized in Table 1, which lists the type of probe used (commercial AFM probe or NFP), the force applied, and the results. As a control, cells were first probed using commercial AFM probes. The severity of cellular damage increased with applied force. At relatively high forces of 172.69 nN, the cells burst or partially detached from the substrate. Figure 4 shows a cell that burst due to AFM probe contact. Figure 5 shows a cell that partially lost adhesion with the substrate due to probe insertion. At a lower force of 145.97 nN, the cell was not damaged to the point of rupturing, but lasting punctures in the membrane were visible. An example of this can be seen in Figure 6. In addition, at forces of 157.41 nN, cell morphology changed, as can be seen in Figure 7.

The NFP's impact on the cell was relatively low compared with that of the commercial AFM probes. Even at significantly greater applied forces, cells exhibited less visible damage than those pierced with commercial AFM probes. An example can be observed in Figure 8; in this image, the NFP was applied to the cell with a force of 222.43 nN. While the probed cell was clearly disturbed and shifted slightly relative to the neighboring cell, actin filaments are still intact across the gap between the cells, and no detachment or rupturing is apparent. In comparison, cells probed with 172.69 nN using the commercial AFM probes ruptured entirely. Forces as high as 413 nN were applied to the cells with the NFP. While this did cause a visible perforation in the cell (similar to that observed with the commercial AFM probes at 145.97 nN), no rupturing or other type of permanent damage occurred, as can be observed in Figure 9. Thus, as seen in Table 1, using the NFP results in relatively little cellular damage, even at greater forces than were applied using commercial AFM probes.

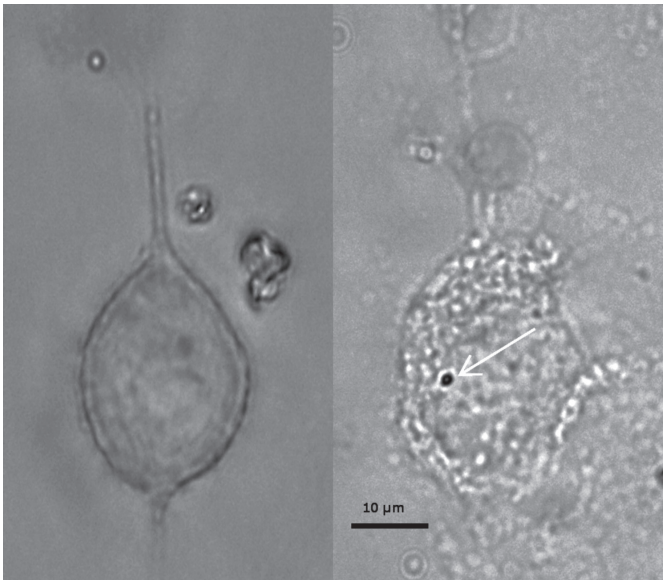

Figure 4. HeLa cell ruptured due to puncturing by commercial AFM probe at 172.69 nN (right). Images were taken before (left) and after (right) probe contact. Arrow indicates point of probe insertion.

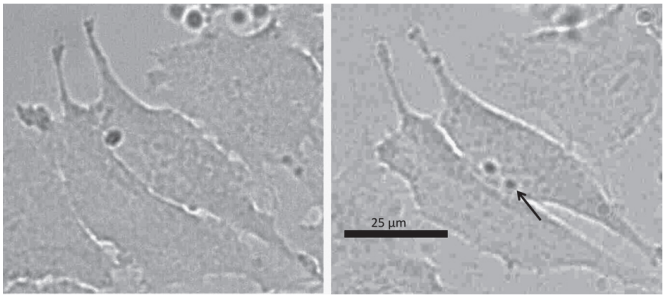

Figure 6. Visible hole in cell membrane after contact with AFM probe at 145.97 nN (right). Images were taken before (left) and after (right) probe contact. Arrow indicates point of probe insertion.

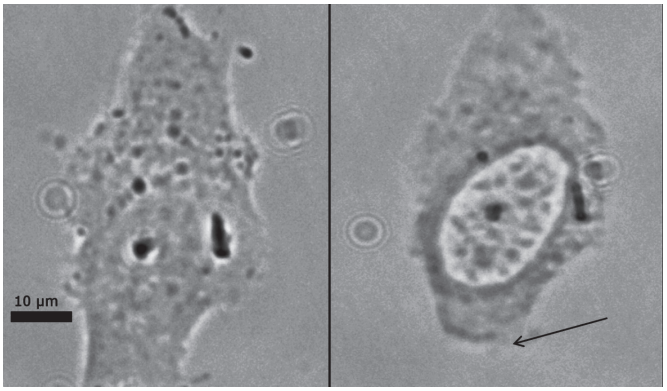

Figure 5. Detachment of HeLa cell due to AFM probe contact at 172.69 nN (right). Images were taken before (left) and after (right) probe contact. Arrow indicates region of ruptured adhesion.

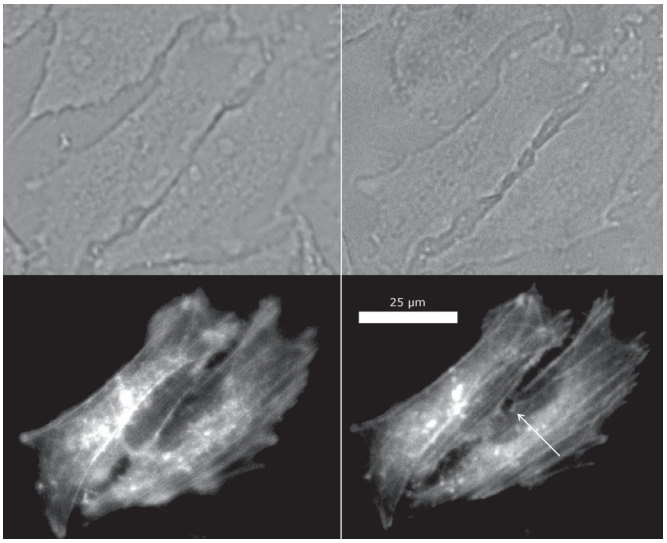

Figure 7. HeLa cell before (left) and after (right) AFM probe contact at 157.41 nN. Images were taken in bright field (top) and red fluorescence imaging (bottom). Arrow indicates point of contact.

Discussion

The main finding of this study is that the NFP does appear, as hypothesized, to qualitatively cause less harm to a cell during injection than commercial AFM probes. Following piercing of the cell with the NFP, actin filaments inside the contact area remained intact, indicating that disruption to the cell was not irreversible. In contrast, cells lost adhesion with the substrate or entirely ruptured at relatively low forces when commercial AFM probes were used (see Table 1).

Certain trends arose with respect to the force level and the type of probe used. For AFM probes, no visible cell damage occurred at forces below around 145 nN. The range for using an AFM probe at forces above that point, however, was narrow; permanent damage occurred around 173 nN. The NFP's effect on cells was different. Force was applied up to 222 nN without visible damage, and force was applied up to 413 nN without permanent damage. No cell rupturing or detachment by the NFP was observed even at high forces. This supports the hypothesis that the outer shell of the NFP tip moderates the depth of penetration into the cell membrane. Previous NFP transfection experiments showed that forces of only 27 nN were required to successfully transfect cells,[6] approximately 7% of the highest force applied without permanent damage in this study. However, the NFP's ability to apply significantly higher forces to cells without damage demonstrates its robustness for use in transfection. This capability allows flexibility in the amount of force applied to the cell, increasing the probability of cell survival and decreasing the likelihood of altering the cell's behavior.

Limitations of Methodology

The experiments reveal that cellular reaction to probing is diverse. For example, when pierced with the same force using commercial AFM probes, some cells partially lost adhesion with the substrate, while others ruptured entirely. The variation could be due to differences in cellular morphology or the relative location of the point of probing. Fluorescent staining aided visual observation of the probes' impact on cell structure. However, photobleaching (the bleaching of the stained cells due to light

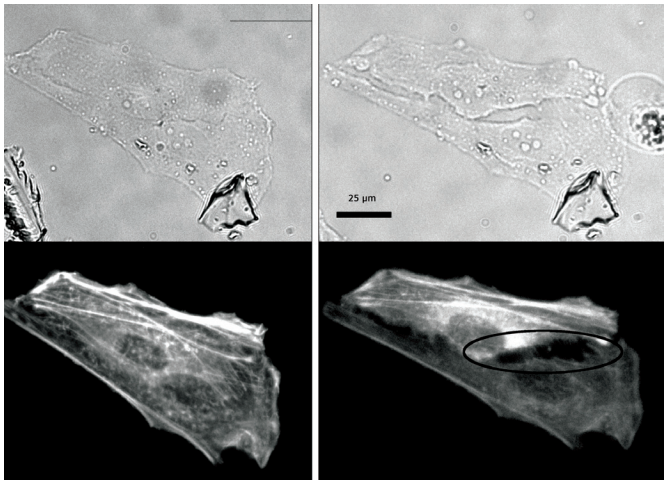

Figure 8. HeLa cell before (left) and after (right) NFP probing at 222.43 nN. Images were taken in bright field (top) and red fluorescence imaging (bottom). Area of NFP insertion is circled.

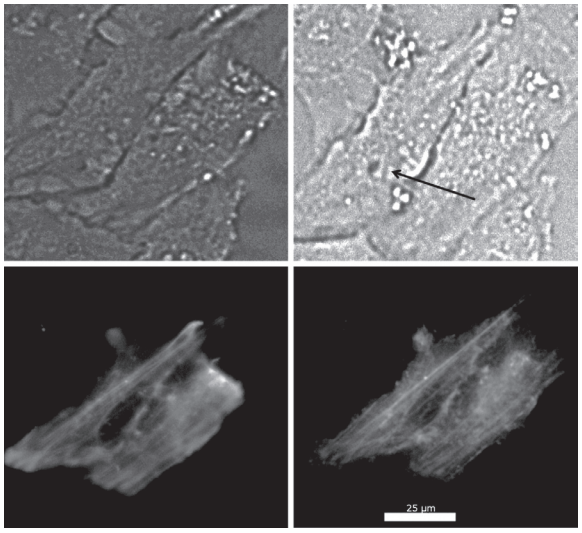

Figure 9. HeLa cell before (left) and after (right) NFP probing at 413.88 nN. Images were taken in bright field (top) and red fluorescence imaging (bottom). Arrow indicates point of NFP insertion.

exposure) of the stained structures limited the number of fluorescent images of a given sample that could be captured. In the future, the use of confocal fluorescence microscopy will provide higher resolution imaging and reduce the effects of photobleaching, allowing long-term imaging of stained cells.

Conclusions

This study examined the invasiveness of the NFP during the cell transfection process. By demonstrating that damage caused to cells by the NFP was relatively low compared with that caused by more commonly used commercial AFM probes, the major objective of this study was met. HeLa cells were more likely to be damaged, and damaged to a greater extent, when commercial AFM probes were used than with the NFP. Using the NFP, higher forces could be applied to the cells without loss of cell structure integrity, whereas cells were catastrophically damaged at relatively low forces when commercial AFM probes were used.

This investigation could be expanded to include an in-depth analysis of cellular structure and cell replication after probe-based injection. The use of confocal fluorescence microscopy, in place of epifluorescence imaging, to image stained cells before and after injection would be expected to reduce photobleaching and allow imaging of the long-term effects of probing on the cells. Finally, capturing the full force-displacement history of each injection with the AFM should allow more detailed analysis of the puncture event and ensure a more accurate measurement of the force applied during the moment of puncture.

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