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Optical manipulation of a single human virus for study of viral-cell interactions

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Abstract

Although Ashkin and Dziedzic first demonstrated optical trapping of individual tobacco mosaic viruses in suspension as early as 1987, this pioneering work has not been followed up only until recently. Using human immunodeficiency virus type 1 (HIV-1) as a model virus, we have recently demonstrated that a single HIV-1 virion can be stably trapped, manipulated and measured in physiological media with high precision. The capability to optically trap a single virion in suspension not only allows us to determine, for the first time, the refractive index of a single virus with high precision, but also quantitate the heterogeneity among individual virions with single-molecule resolution, the results of which shed light on the molecular mechanisms of virion infectivity. Here we report the further development of a set of microscopic techniques to physically deliver a single HIV-1 virion to a single host cell in solution. Combined with simultaneous epifluorescence imaging, the attachment and dissociation events of individual manipulated virions on host cell surface can be measured and the results help us understand the role of diffusion in mediating viral attachment to host cells. The establishment of these techniques opens up new ways for investigation of a wide range of virion-cell interactions, and should be applicable for study of B cell interactions with particulate antigens such as viruses.

Keywords

Optical trapping; HIV-1; manipulation; micropipette; single virus; single cell; virus-cell interactions

1. INTRODUCTION

Entry into the host cell is the critical first step in virus infection. Although a single virion can produce infection, the actual efficiency differs widely from virus to virus. Bacteriophage T4

6. AUTHOR CONTRIBUTIONS

W. C. directed the project; X. H. and M. C. D. conducted manipulation experiments; C. T. prepared and assayed HIV-1 virions; W.C. and X. H. wrote the paper.

has an efficiency of infection approaching 100%. However, this is not true for most animal viruses. Even under the most favorable culture conditions, the efficiency of infection typically ranges from 0.1% to 10%. Among many factors that contribute to this apparent low efficiency, a major determinant is virion entry. Different efficiencies of viral entry have been observed experimentally, but the molecular mechanisms that underlie these differences are not well understood. It has been postulated that a large proportion of viral particles may be inherently defective. Alternatively, the attachment of the virus to the host cell or the penetration into the host cell is rather inefficient process. The efficiency of viral entry is directly related to the infectivity of the virus. Therefore, to understand this process is of great medical importance.

Photons carry momentum and can therefore exert forces on matter. Although the typical force generated by photons is very small, the range of force (10^{-12} Newton, pN) is significant for microscopic objects at the nanometer scale. Optical tweezers exploit this fundamental property to trap micron-sized objects in a potential well formed by light, and allow manipulation of these objects in three dimensions (3D). Since the first demonstration of stable optical trapping of micron-sized dielectric objects by Ashkin and coworkers [1], this contact-free manipulation technique has found broad applications in physics, chemistry and biology [2]. With optical tweezers, small objects such as single cells or micron-sized particles can be positioned, transported or even sorted [3]. Owing to its unique capability to manipulate microscopic objects, apply and measure mechanical force, and quantify displacement at subnanometer resolution, optical tweezers have become an important tool to study the biochemical and biophysical properties of motor proteins and biopolymers at the single-molecule level with unprecedented scale and resolution. However, although Ashkin and coworkers first demonstrated optical trapping of a single tobacco mosaic virus [4], the application of optical tweezers to study virus-cell interactions has never been reported.

Using human immunodeficiency virus type 1 (HIV-1) as a model virus, we have recently demonstrated that a single HIV-1 virion can be stably trapped, manipulated and measured in physiological media with high precision [5]. This work represents the first demonstration of optical trapping and manipulation of a single human virus. Because a virus needs to enter its host cell in order to replicate itself and produce virion progenies, it is desirable to manipulate a single virus at the cell surface in order to understand this fundamental process and aspects of viral entry. To this end, here we report the development of a set of microscopic techniques in order to physically deliver a single HIV-1 virion to a single host cell in solution. This technique helps us understand the role of diffusion in mediating viral attachment to host cells, and opens up new ways for investigation of virion-cell interactions.

2. RESULTS

2.1 Micromanipulation of a single mammalian cell

In principle, one can use optical trap to ‘grab’ a single mammalian cell, and use a second trap to capture a single animal virus, and then bring them together to deliver the virus to the cell. However, for less exposure of the cell to laser irradiation and more flexibility in doing the experiments, we have adopted the micromanipulation technique developed by

biomedical engineers for manipulation of a single mammalian cell, and use this technique to conduct delivery of a single virion to a single cell.

A schematic of the microfluidic chamber that we used for optical trapping of single virions and single cells is shown in Fig. 1A. This chamber was constructed using two coverslips and a patterned plastic layer to form three parallel flow channels; the middle channel is connected with both top and bottom channels through capillary tubing so that contents from either top or bottom channel can be delivered into the middle channel as driven by either hydrostatic pressure or a syringe pump. The microfluidic chamber is installed on a 3D motion stage, and the front objective delivers 830 nm laser for trapping inside the microfluidic chamber (Fig. 1B). We typically prepare a suspension of single cells, and inject the cell suspension into the top channel. Single cells can then be optically captured in the middle channel at the exit of the top capillary tubing. Once a single cell is optically trapped, the immobilization of the single cell atop the micropipette can be achieved through precise control of the hydrostatic pressure at the pipette opening [6]. This pressure was adjusted by changing the vertical position of a syringe connected with the other end of the micropipette (Fig. 1A). To quantitatively test this hydrostatic pressure, we optically trapped a polystyrene bead (2.8 μm in diameter) directly over the opening of the micropipette (Fig. 1C).

The hydrostatic pressure established by the height difference between the pipette opening and buffer level in the syringe generates a flow in or out of the pipette opening. This flow in turn generates a Stokes force on the bead that can be directly measured by optical tweezers [7, 8]. Fig. 1D shows the result of such a measurement. For a fixed location of the optical trap, the force on the bead displays a linear dependence ($R^2=0.998$) on the height of the syringe. Importantly, there is a vertical position at which the net hydrostatic pressure is zero. Above this position, there is a positive pressure at the pipette opening while below this position, the pressure is negative. This result indicates that we can precisely control the pressure at the opening of the micropipette, which allows us to *reversibly* immobilize a single cell atop the pipette. This was further shown in Fig. 2 using a single SUP-T1 cell as an example, a T cell line derived from an eight-year-old non-Hodgkin's T cell lymphoma patient [9]. Initially, a single T cell was trapped by optical tweezers and transported to the micropipette opening (Fig. 2A). We then applied a negative pressure to immobilize the cell atop the micropipette (Fig. 2B). We found that there is a threshold of suction pressure below which the cell can be stably placed atop the pipette (Fig. 2B and C), and above which the cell will be continuously deformed and sucked into the pipette (Fig. 2D and E). This process, however, can be fully reversed if we apply a positive pressure, and the cell can recover back to its normal shape (Fig. 2F). We estimate the threshold pressure to be $\sim 20 \text{ N/m}^2$, which translates to a suction force $\sim 250 \text{ pN}$ for a micropipette of $4 \mu\text{m}$ diameter. This estimation compares very well with literature findings [10]. Furthermore, the cell remains alive atop the pipette for at least 30 min as indicated by fluorescent dye assays [11]. These results thus establish a regime for manipulating a live cell with minimal perturbation.

2.2 Optical delivery of a single virus to a single cell

We have recently developed the capability to optically trap a single HIV-1 virion in suspension [5]. The ability to manipulate a single cell further allows us to deliver a single

virus to a single cell using the microfluidic chamber design (Fig. 1A). To this end, we injected suspension of SUP-T1 cells into the top channel, and captured a single T cell in the middle channel at the opening of the top capillary tubing using optical tweezers. We then transferred the cell using the 3D motion stage and placed the cell on top of the micropipette in the middle channel as we illustrated in Fig. 2.

To deliver a single virion to the live T cell, we injected diluted HIV-1 virions into the bottom channel, and optically trap a single virion in the middle channel at the opening of the bottom capillary tubing. The single virion was confirmed by the virion diameter measured using the back-focal-plane interferometry as we described previously [5]. Aggregates of virions were discarded. The single virion was then transferred to the vicinity of the single T cell on top of the micropipette through the motorized chamber stage, and slowly brought into contact with the cell surface. The physical distance between the virion and the cell was judged by both the fluorescence image of the virion excited by a 488-nm solid state laser and the trapping laser deflection signal at the objective back focal plane. Typically for a single cell, we delivered a single virion to different spots on the cell surface: three deliveries from the right, three deliveries from the left and three deliveries from the top of the cell. The single cell was then replaced with a fresh new cell for more delivery and measurements. This delivery strategy was made possible by the 3-axis motorized stage that is controlled remotely through a computer (Fig. 1B). Two types of delivery experiments were conducted both at 20°C in PBS. In one case, the virion was slowly brought into contact with the cell surface. Upon virion and cell encounter, the optical trap was shut off immediately, and the virion was imaged via 488 nm epi fluorescence excitation. Out of a total of 73 trials, all virions dissociated from the cell surface upon their initial collision, represented by Video 1 and 2, which resulted in an attachment probability of 0%. In the second scenario, the virions were first incubated with 10 µg/ml Diethylaminoethyl-dextran (DEAE-dextran), which is a polycation known to enhance the attachment of HIV-1 virions onto host cell surface nonspecifically [12]. Out of a total of 77 trials, 59 virions attached to cell surface upon first collision, represented by Video 3 and 4, which resulted in an attachment probability of greater than 77%. This dramatic difference in the viral attachment efficiency indicates that to form a specific contact between the virus and the cell upon a single collision is a rather inefficient process. This low efficiency can be caused by either low envelope glycoproteins on virion surface, or low and perhaps heterogeneous receptor distributions on the host cell surface, which can be investigated in the future.

For those virions that were attached onto the cell surface in the presence of DEAE-dextran. They remained stationary on cell surface throughout our experimental time frame. No internalization of virions was observed. This is consistent with the fact that at 20°C, neither direct fusion nor endocytosis will occur under these conditions. These results demonstrate that one can precisely deliver a single HIV-1 virion to a single host cell at a desired location. In the presence of auxiliary agents, the single virion can be attached to the cell surface. Future experiments under conditions that are conducive to virion internalization may allow observation of those events and measurement of their efficiency relative to virion attachment.

2.3 Collection and recovery of a single cell after an experiment

The unique advantage of manipulation using micropipette is its reversibility: negative pressure allows immobilization of the cell atop the pipette while a positive pressure can be used to dislodge the cell. To take advantage of this reversibility, we installed a collection tube on the side of the micropipette (Fig. 1A) and tested our ability to collect the single cell after immobilization. We first applied a positive pressure inside the micropipette to release the cell, captured it using optical tweezers, and transported the cell to the opening of the collection tube (Fig. 3A). We then applied a negative pressure through the tube to collect the cell (Fig. 3B and C). As expected, the single cell was recovered and examined under a regular microscope as shown in Fig. 3D. A similar process was shown in Video 5 below. This single-cell collection technique is potentially useful for further assay of the single cell after an experiment, especially when additional culturing of the cell is desired.

2.4 Micromanipulation for imaging of a single mammalian cell

The placement of a single cell atop a micropipette offers additional technical capabilities in conjunction with optical tweezers. We demonstrated previously that the 830 nm trapping laser can be used for two-photon fluorescence (TPF) excitation and imaging of single molecules of enhanced green fluorescent protein (EGFP) in mammalian cells [13]. To further test this micromanipulation method for imaging of a single cell, we transfected 293T cells with a plasmid that expresses an EGFP-Vpr fusion protein [14] and then immobilized a single cell atop the micropipette with a suction pressure that is well below the threshold pressure. For imaging of EGFP fluorescence [13, 15], we scanned the micropipette through programmed movement of a 3D motion stage in x and y direction, and obtained the two dimensional two-photon fluorescence (TPF) image of the cell as shown in Fig. 4A–D. One may wonder that the optical trap will exert force on the cell during the scanning process, and this force may be sufficient to dislocate the cells even slightly as a result of this scanning procedure. Control experiments showed that the cell displayed no movement during the scanning process, which was necessary for construction of a cell image (Fig. 4E–H). As shown in Fig. 4A–D, cell boundaries were clearly distinguished from the background in these TPF images, and there were variations of fluorescence intensity across the entire cell. Moreover, there was a clear difference among individual cells in the amount of EGFP fusion proteins expressed, as shown by the variation of fluorescence intensities across the four panels. Control cells without transfection showed no fluorescence. The distribution of fluorescence throughout the entire cell is expected as this fusion protein has a weak nuclear localization sequence [14].

3. DISCUSSION

Despite the enduring importance of virus-host cell interactions, the way to study viral infection of host cells has been unchanged: a population of viruses and cells are incubated and measured. The complex pathways and interactions involved often prohibit a clear understanding of the mechanisms behind. In this work, we have developed a novel technique to study virion attachment to host cells. In this technique, a single virion was captured via an optical trap, and it was delivered directly onto the surface of a single host cell that was held

by a micropipette. This technique allows us to study virion interaction with its host cells, one at a time.

Brownian diffusion of a virion has been proposed to be one bottleneck in infection of host cells [12, 16–18]. Direct delivery of a single virion to host cell surface bypasses diffusion. However, even when diffusion is bypassed, the efficiency to form specific virion-cell contact is low, suggesting that other barriers instead of diffusion limit virion infectivity. These potential barriers include the envelope glycoprotein density on individual virions [19], and also the density and distribution of receptors on individual host cell surface, the impact of which on viral attachment can be investigated systematically using current experimental design.

The integration of optical tweezers [20, 21] with TPF imaging in a single instrument makes the system versatile. On the one hand, we can use optical tweezers for manipulation of single molecules [22], single viruses and single cells; on the other hand, we can use continuous-wave laser-excited TPF to image live cells without the need of additional laser source. To take advantage of both modalities, we developed a method for imaging of single live cells that grow in suspension (Fig. 4). Conventional fluorescence imaging methods developed for cultured cells are largely limited to cells that can grow on a coverslip surface. There are few reports on imaging of live cells in suspension due to the requirement for noninvasive immobilization. We use optical tweezers to capture a single cell from culture suspension, and developed a micropipette system controlled by hydrostatic pressure to immobilize a single cell atop the micropipette for TPF imaging. The unique advantage of using micropipette for manipulation is that the entire procedure is reversible. On the one hand, a single cell can be immobilized by a negative pressure; on the other hand, the cell can be easily released from the pipette by applying a positive pressure, and transported by optical tweezers for collection and recovery. No damage was incurred to the cell throughout the process. This eliminates the requirement to grow or fix cells on coverslip surface, and is well suited for imaging live cells that are cultured in suspension, such as T cells and B cells. Direct imaging of these cells close to physiological conditions is important. A specific example is provided by HIV-1. Current live cell imaging methods almost exclusively use a HeLa cell derivative that can grow on coverslips for imaging of virion-cell interactions [23], yet the major physiological targets of HIV are T cells. Because different cell types may directly influence virion-cell interactions [24], it is necessary to develop tools for direct imaging of these T cells that are cultured in suspension. As we have shown above, we can work with CD4⁺ T cells directly instead of engineered HeLa cells.

Lastly, our developments as reported here open up a range of possibilities for single cell-based experiments [25, 26]. For example, we can deliver a virion or a nanoparticle to a single cell using optical tweezers, and use TPF or epifluorescence imaging to follow the recognition or uptake of the particle by the single cell. Subsequent single cell collection and recovery allows further analysis using other techniques to diagnose the consequence of virion internalization. Similarly, we can use optical tweezers to manipulate a single receptor on the cell surface, use TPF imaging to measure reporter gene expression, and further collect cells and use PCR for gene expression analysis to probe the mechanisms of mechanotransduction. The unique feature of this experiment may allow dissection of

fundamental events without complication of multiple virions or multiple cells. Literally, this integrated system allows one to use single cells as a test tube, and optical tweezers as a 'pipetman', leading to a new approach for the study of biology at single-particle and single cell level.

4. MATERIALS AND METHODS

4.1 Cell culture and production of HIV-1 virions

SUP-T1 cells were cultured at 37°C with 5% CO₂ in RPMI supplemented with 10% FBS. HEK 293T/17 cells (ATCC, Manassas, VA) were cultured at 37°C with 5% CO₂ in DMEM supplemented with 10% FBS. The procedures to produce HIV-1 virions have been described in detail elsewhere [27]. Briefly, the EGFP-labeled HIV-1 virions were produced by transient transfection of 293T cells with two plasmids using Mirus TransIT LT-1 transfection reagent. One of the plasmids encodes HIV-1 proviral DNA pNL4-3 that contains an EGFP flanked in between the matrix and capsid domains of the Gag protein (iGFP) [28] and carries a premature stop codon in gp120/gp41 coding sequence. The other plasmid encodes a fully functional NL4-3 envelope glycoprotein gp120/gp41. At 24 hours post transfection, the supernatant from the cell culture was harvested. Viral particles were filtered through 0.45 µm low-protein binding filter, aliquoted, flash frozen in liquid nitrogen and stored in -80°C freezer.

4.2 Optical tweezers and epifluorescence imaging

The trapping of individual virions in solution was done as described previously [5] with a few modifications. Throughout, a home-made OTs instrument using a tapered amplifier diode laser at 830 nm (SYS-420-830-1000, Sacher LaserTechnik LLC, Germany) was used for optical trapping of individual HIV-1 virions [11]. Briefly, a laser power of 130.8 mW at the focus was used throughout for optical trapping and simultaneous TPF excitation. The live virus stock thawed from -80°C was diluted in PBS to a concentration of $0.6 - 1.6 \times 10^8$ virions/ml and injected into a microfluidic chamber for optical trapping. The diameter of individual virions was measured as described previously [5]. A Coherent OBIS solid state laser at 488 nm was used for epifluorescence imaging of EGFP-labeled virions. An electron-multiplying charge-coupled device (EMCCD) camera (Evolve, Photometrics) was used for all fluorescence detection including epifluorescence imaging. Individual HIV-1 virions were identified based on an EGFP positive signal and a measured diameter that lies between 96 and 216 nm as described previously [5]. All the trapping and imaging experiments were conducted at a constant temperature of 20.0 ± 0.2 °C.

4.3 Calibration of the micropipette system controlled by hydrostatic pressure

The height difference between the micropipette opening and buffer level in the movable syringe created a hydrostatic pressure at the pipette opening. We placed the optical trap at a distance away from the pipette opening. We tested the hydrostatic pressure quantitatively using an optically-trapped polystyrene bead (2.8 µm diameter). Due to the presence of the hydrostatic pressure, the trapped bead was displaced from its equilibrium position and we measured the force on the bead directly using optical tweezers [11]. For a fixed location of the optical trap, we changed the hydrostatic pressure gradually by adjusting the height of the

movable syringe. As shown in Fig. 1D, the measured Stokes force varied linearly with the syringe height, suggesting that the pressure generated at the pipette opening was proportional to height difference between syringe and the pipette opening. The threshold pressure for suction of a single T cell was estimated based on the hydrostatic pressure $p = \rho g h$ that we established in the system, where ρ is the density of the buffer, g is the acceleration of gravity, and h is the relative height of the syringe away from the balance point at which $p=0$.

4.4 Single cell collection and recovery

To collect the single cell atop the micropipette, we installed a collection tube ($\sim 40 \mu\text{m}$ inner diameter) on the side of the micropipette (Fig. 1A). After immobilization of a single cell atop the micropipette, we apply a positive pressure to ‘push’ the cell back to the media, capture it using optical tweezers, and transport it to the opening of the collection tube (Fig. 3A). This collection tube was also connected with a movable syringe so that we can apply a negative pressure to collect the cell into the tube (Fig. 3B and C). To recover the cell for further analysis, we found it necessary to approximately estimate the location of the cell inside the collection tube to facilitate its recovery. We estimated that the cell flew into the collection tube at a rate around $100 \mu\text{m/s}$ based on video microscopy. At this flow rate, it will take 300 – 400 s for the cell to travel through the collection tubing (3–4 cm long), and ~ 83 min to travel through 1 cm of the polyethylene tubing (0.28mm diameter) immediately at the end of the collection tube. Based on these estimations, we waited for ~ 10 min after cell suction, and directly recovered the cell from the portion of polyethylene tubing at the end of the collection tube. The recovered single cell was then deposited onto a coverslip for examination under a microscope (Fig. 4D). Control experiments show that the cell we collected was indeed the one we immobilized atop the pipette, as repeated mock collection without any cells recovered nothing under the microscope.

4.5 Production of fluorescent cells and TPF imaging

To produce fluorescent cells for TPF imaging using optical tweezers, 293T cells were transiently transfected with a plasmid encoding EGFP-Vpr fusion protein using calcium phosphate method. At 40 hours post transfection, medium was removed. Cells were washed gently with Dulbecco’s Phosphate-Buffered Saline (DPBS), and detached from surface using Trypsin-EDTA. At 2 min after trypsin digestion, complete medium (90% DMEM + 10% FBS) was added to stop the reaction and the cells were resuspended in DPBS and ready for experiments. For optical trapping and fluorescence imaging, cells were exchanged into phosphate buffer (100 mM, pH 7.2) by centrifuge, and flown into the upper channel of the microfluidic chamber. We used optical tweezers to capture individual cells near the opening of the upper capillary tubing (Fig. 1A), and placed the cell atop a micropipette (opening around $4\text{--}5 \mu\text{m}$) controlled by hydrostatic pressure. The hydrostatic pressure was generated by connecting the end of the micropipette with a syringe that can be adjusted vertically (Fig. 1A). This syringe and micropipette system was filled with buffer and we adjusted the pressure at the pipette opening through adjustment of the syringe height. When negative pressure was generated, a single cell could be stably immobilized atop the micropipette for fluorescence imaging. When positive pressure was generated, the single cell could be released from the pipette for capture by optical tweezers. To image the single cell

atop the micropipette, we placed the laser focus close to the cell, and used a custom-written LabView program to conduct scanning of the micropipette in two dimensions. TPF intensity emitted from the laser focus was recorded by the EMCCD with an exposure time of 1 s and cell images were constructed using Matlab image processing toolbox.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

(A) Design of the microfluidic chamber for single cell immobilization, single virion delivery, imaging and single cell collection. The chamber contains 3 channels: upper and bottom channels are used to flow in components such as cells or viruses; the center channel is used for experiments. Capillary tubings are placed between the center channel and the other two channels to deliver cells or beads into the center channel. A micropipette (4–5 μm diameter at the end of the opening) is installed to immobilize a single cell by the hydrostatic pressure established with a movable syringe filled with buffers. A collection tube ($\sim 40 \mu\text{m}$ diameter) is installed on the side of the micropipette to collect cells similarly using hydrostatic pressure driven by a second movable syringe. (B) Experimental setup for optical tweezers. The optical trap is focused at the center of the microfluidic chamber. (C) Test of the hydrostatic pressure at the micropipette opening using a polystyrene bead (2.8 μm diameter). The bead is trapped by optical tweezers and placed over the pipette opening. We systematically changed the height of the syringe connected with the micropipette and measured the corresponding Stokes force acting on the bead using optical tweezers. The

scale bar is 10 μm . (D) The Stokes force acting on the bead varied linearly with the height of the syringe, confirming the establishment of a hydrostatic pressure.

Figure 2.

Manipulation of a single T cell by a micropipette. (A) A SUP-T1 cell is trapped by optical tweezers and transported to the opening of a micropipette. (B) Upon release of the optical tweezers and application of a negative pressure below a threshold at the micropipette opening, the cell can be immobilized stably atop the pipette. (C) At the threshold negative pressure, the single cell is deformed to form a hemisphere inside the pipette. The diameter of the hemisphere approximately equals the diameter of the pipette opening. For both (B) and (C), the cell can be immobilized atop the pipette stably without damage. (D and E) when the negative pressure is further increased above the threshold, the single cell is continuously deformed and stretched into the pipette. (F) When a positive pressure is generated by moving the syringe, the process shown in D and E can be reversed and the single cell finally recovers its original morphology and can be released from the pipette. The scale bars are 10 μm each.

Figure 3.

A single cell was trapped by optical tweezers, and delivered to a collection tube (A). We switched off the optical trap, and the cell went into the collection tube due to the applied negative pressure (B–C). Finally, the cell was recovered and placed on a coverslip under microscope (D). The scale bars are 10 μm each.

Figure 4.

TPF images of individual 293T cells atop a micropipette. These cells were transiently transfected with EGFP-Vpr fusion protein as described in Materials and Methods. (A–D) show four individual cells with varied expression levels of the fusion protein. The scale bars are 5 μm each. (E–H) Bright field images of a single cell during the scanning of the micropipette. Panels E to H show four different locations of the micropipette relative to the laser focus, which was indicated by the red stars in each panel. No movement of the cell relative to the micropipette was detected during the scanning process, as confirmed by overlaying these bright field images using the contours of the micropipette as a reference. The scale bars are 10 μm each.