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Research Article

Keywords:

Posted Date: December 8th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-1141503/v1

Extending applications of AFM to fluidic AFM in single living cell and extracellular vesicle studies

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Abstract

In this article, a review of the application of atomic force microscopy (AFM) for the analyses of extracellular vesicles is presented. This information is then extended to include fluidic Atomic Force Microscopy (fluidic AFM) applications. Fluidic AFM is an offshoot of AFM that combines a microfluidic cantilever with AFM and has enabled the research community to conduct biological, pathological, and pharmacological studies on cells at the single-cell level in a liquid environment. AFM applications involving single cell and extracellular vesicle studies, colloidal force spectroscopy, and single cell adhesion measurements are discussed. In this review, new results are offered, using fluidic AFM, to illustrate (1) the speed with which sequential measurements of adhesion using coated colloid beads can be done, (2) the ability to assess lateral binding forces (LBFs) of endothelial or epithelial cells in a confluent cell monolayer in appropriate physiological environment, and (3) the ease of measurement of vertical binding force (VBFs) of intercellular adhesion between heterogeneous cells. Finally, key applications are discussed that include extracellular vesicle absorption, manipulation of a single living cell by intracellular injection, sampling of cellular fluid from a single living cell, patch clamping, and mass measurements of a single living cell.

1 Introduction

Cell biomechanical properties play an important role in regulating cellular activities, such as cell adhesion, migration, and barrier functions, and are related to intracellular structures, signal transductions, biochemical pathways, and metabolic functions. Modeling host-pathogen interactions in the cell surface microenvironment requires a platform in which to explore single cells if we are to gain deeper insights into the mechanisms that underlie infection and inflammation at the nanoscale level[1].

Invented by Binnig *et al.* in 1986[2], AFM has emerged as an indispensable technique for the study of host-pathogen biophysical interactions in real time[3]. AFM measures the force dynamics between a probe tip and a sample at the nanoscale level. AFM is capable of probing surface biomechanical features of single cells, including those of microbes[4], by measuring the interacting force between the probe and cell surface. Unlike other microscopy techniques, AFM captures high-resolution, three-dimensional (3-D) images of the cell surface without special sample preparation such as fixation, staining, or labeling, and therefore can be applied to models of living cells[4]. Furthermore, the probe tips can be functionalized with various biochemical materials, including recombinant proteins[5], antibodies[5], and cultured cells[6]. This affords AFM the unique capacity to directly measure protein-protein and protein-cell interacting forces at the single molecule or single cell level[5,6], thus allowing for the biomechanical characterization of target-specific cell surfaces.

In this review, we will build upon a recent review article by Bhat *et al.*[3] that presented a summary of AFM applications for the study of pathogens, and will expand it by providing a review of AFM use in the growing research field of extracellular vesicle (EV) biology. Our attention will be focused on the applications made possible by fluidic AFM, which is a relatively new nanofluidic extension of AFM[7,8]. We will review certain key applications that are relevant to the initiation of intracellular pathogen infection and host responses. These include biophysical characterization of single living leukocytes or bacterial adhesion to endothelial cells, viral probe-mediated single living cell-virion binding force measurements, EV cell surface dispensing and absorption, endothelial or epithelial cell barriers, and manipulation of single live cells by intracellular injection, sampling of cellular fluid from a single live cell, and single live cell mass measurements[9-13]. A faster method to sequentially measure adhesion with coated colloid beads using fluidic AFM is presented. Assessment of lateral binding forces (LBFs) of endothelial cells (ECs) on a mature monolayer and of intercellular vertical binding forces (VBFs) between heterogeneous cells is also shown.

2 AFM for EV analyses

Cell-to-cell communication is critical for maintaining mammalian homeostasis and responding quickly to environmental stimuli, including pathogens. Besides direct intercellular contact, this communication is often mediated by soluble factors that can convey signals to a large repertoire of responding cells, either locally or remotely. EVs transfer functional mediators to neighboring and distant recipient cells[14,15]. EVs are broadly classified into two categories, exosomes (50-150 nm) and microvesicles (100-1000 nm), distinguished by the cell membrane of origin[16]. Exosomes and microvesicles are also termed small and large EVs, respectively[17-20]. After the membrane of the late endosomes buds inward, exosomal biogenesis begins with the formation of intraluminal vesicles, which are the intracellular precursors of exosomes[16,21]. Before they are released into the extracellular environment as exosomes, the intraluminal vesicles are internalized into multivesicular bodies, transported inward, and fuse with the plasma membrane[16,21,22]. Microvesicles are rapidly generated at the plasma membrane by outward budding[16,21-23]. EVs contain many types of biomolecules, such as proteins and nucleic acids. Exosomes can convey signals to a large repertoire of recipient cells either locally or remotely by transferring functional cargos, thus contributing to disease pathogenesis, including infection and inflammation[21,23-30].

Differential ultracentrifugation was historically employed for exosome isolation, but it suffers from aggregation issues and decreased integrity of exosomes after resuspension[22,30-33]. It is essential to characterize the size and morphology of isolated particles because both quality and quantity are crucial for the outcome of downstream assays involving the functional roles of EVs

and their contents. Small EVs have a diameter of less than 200 nm, which prevents the use of optical microscopies for single particle characterization[34]. AFM and electron microscopy (EM) are the two nanoscale methods of choice to image and study EVs.

Tapping mode high resolution AFM to image EVs is a label-free and relatively quick technique that does not involve complicated sample preparation. Tapping mode AFM provides a 3-D image of surface structures and is commonly used to evaluate the integrity of EVs at the single particle level (**Fig. 1**). Using size-exclusion chromatography (SEC), small EVs (50-150 nm) were purified from *Rickettsia*-infected mouse plasma and culture media of primary vascular endothelial cells (ECs). Evaluation of single particle morphology using tapping mode AFM images verified the integrity of isolated exosomes from experimental specimens[35].

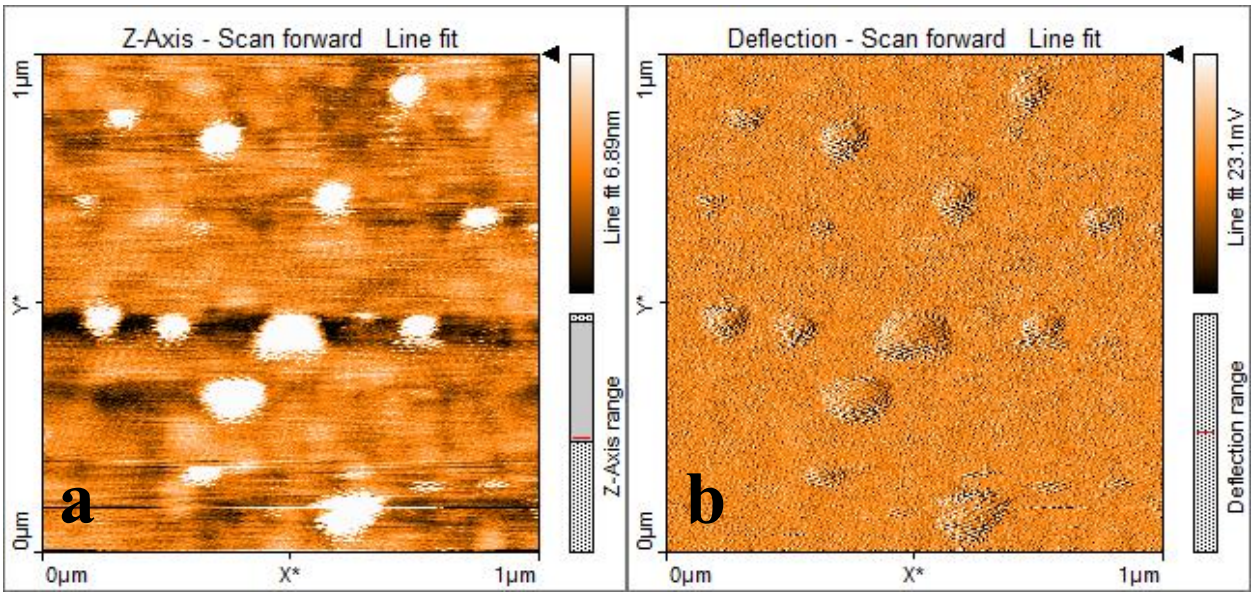

Fig. 1: AFM scanning images of EVs derived from human sera samples. **(a)** White spots are the EVs with diameter ranges between 50 and 150 nm. **(b)** Imaging constructs using deflection signals from a cantilever reveal the shapes of EVs.

Using AFM, researchers are able to focus on both the biochemical assessment of an EV particle surface[36] and are able to quantify the surface biophysical characteristics of EVs at the single particle level[37-40], beyond merely assessing the size and counting the particle number. Sharma *et al.* used force spectroscopy with AFM tips functionalized with anti-CD63 IgGs, and reported evidence of the presence of tetraspanin CD63, an endosomal marker[41], on the exosome surface, directly suggesting an endosomal origin of exosomes instead of a plasma membrane origin[36]. By simultaneously acquiring high-resolution tapping mode AFM scanning images combined with force spectroscopy in a liquid environment, the analysis of the mechanical properties of a single EV provides further insights into the biophysical changes between EV subgroups[39]. AFM offers features that enable the standardization of the functional analysis of EVs, such as label-free quantitative biomechanical profiles that address the regulation of EV uptake in recipient cells[34,42].

3 From AFM to fluidic AFM

Glass micropipettes are used in a number of applications in biology, such as intracellular injection[43] and patch clamping for electrophysiology measurements[44]. Near-field scanning optical

microscopy (NSOM), first reported in 1983[45,46], made use of micropipettes in a scanning arrangement. The pipettes were later combined with scanning probe microscopes such as AFM[47] and scanning tunneling microscopes[48]. Glass micropipette thermocouples were constructed and used with AFM for nanoscale temperature measurements[49]. Glass micropipettes of nanomolar size apertures combined with AFM were also used for nanoscale liquid and gaseous material delivery[50] and protein printing [51]. Francis *et al.* in 1987[52] measured single cell adhesion by applying suction with a glass micropipette and tracking the movement with an interference reflection microscope (similar to an AFM). Other detection schemes (non-AFM based) with glass micropipettes have been used for cell adhesion measurements[53-56]. Glass micropipettes are pulled individually using a mechanical pipette puller and heated in a serial process that is relatively cumbersome and often does not produce uniform results.

Micromachined (micro-electromechanical system, MEMS) fluidic cantilevers can be fabricated for AFM applications. MEMS cantilevers are mass-produced with minimal variations between devices, overcoming throughput and non-uniformity issues. In addition, micromachining offers greater control over the size and location of the aperture. Micromachined fluidic AFM cantilevers were first reported for fountain-pen lithography applications [57-60]. In 2009, Zambelli's group (ETH Zurich, Switzerland) introduced the fluidic force microscope (fluidic AFM)[61] that employed a micromachined fluidic AFM cantilever, similar to the ones reported for fountain-pen applications, for biological applications and demonstrated intracellular injections and staining with AFM force control. In addition, this technology was successfully commercialized by Cytosurge AG (Zürich, Switzerland) and SmartTip BV (Netherlands).

Fluidic AFM has been used for many biologically relevant applications, including single cell adhesion measurements (adherent or in suspension)[62], agent and biomolecule delivery[61], single cell content extractions[63], and patch clamping[64]. The use of fluidic AFM cantilevers overcomes many issues encountered in conventional AFM cantilevers. For example, in conventional AFM single-cell force spectroscopy, cells are irreversibly attached to an AFM cantilever via biochemical functionalization[6], which is labor intensive and time consuming. In addition, single-cell force spectroscopy requires that the cell-cantilever coupling force is stronger than the adhesion interaction force to be detected. The biochemical adhesive-mediated attachment of cells to the conventional AFM cantilever may be not strong enough[13]. Consequently, cell-to-cell binding force measurements become challenging, particularly when probing the lateral forces between cells in a monolayer[16]. Furthermore, biochemical-mediated immobilization techniques may perturb cells[6]. Using the fluidic AFM technique,[8] it is relatively easy to attach a cell using suction and bring it into contact with other cells or functionalized surfaces to measure adhesion[65], as shown in **Fig. 2**, importantly the device can be reused.

Commercially available fluidic cantilevers by Cytosurge are either tipless (with a micron-size aperture) or have a pyramid-shaped tip (with an aperture of a few hundred nanometers in diameter located on the side or the apex of the tip)[13]. Typically the nanometer size opening is fabricated using focused ion beam (FIB) technology[66]. The channel height ranges from 0.2 to 1 µm and the fluidic channel on cantilever chip is connected to a reservoir. The tipless cantilevers are utilized in applications such as exchangeable colloidal force spectroscopy[67,68], spatial manipulation of a targeted cell[69], and single cell or cell-cell binding force measurements[70]. The cantilevers with pyramid-shaped tips are mainly employed in applications that require delivery of biomolecules and sampling[63,71]. For dispensing material onto the apical surface of a cell, a tip with an aperture at the apex is commonly used. For intracellular experiments, an aperture on the side of the

pyramidal-shaped tips[13] is preferred to deliver or extract loads from a cell's plasma membrane or the cell's nucleus.

Various research groups have reported on the development of novel devices that have not been commercialized. Inspired by NSOM and fountain-pen lithography, Meister *et al.*[72] developed a nanoscale dispensing fluidic probes that includes a hollow Si_xN_y tip on a $Si–Si_xN_y$ cantilever. Hug *et al.* reported a fluidic cantilever entirely made of silicon oxide[57]; a version of this device was later used in fluidic force microscope work[61]. A hollow silicon nitride (Si_3N_4) tip on a silicon dioxide (SiO_2) fluidic cantilever and an array of these devices was developed for high throughput applications[73]. A silicon nitride fluidic cantilever without a tip was reported by Schön *et al.*[74] and in a later effort, a tip with a submicron aperture was included[75]. Other efforts included the use of flexible materials like SU-8 as a cantilever material[70,76-78]. Several of the fluidic cantilevers also included embedded sensing elements, eliminating the need for an AFM laser for deflection detection[76,79]. In a recent effort, a fluidic AFM cantilever was fabricated using 3-D printing[80].

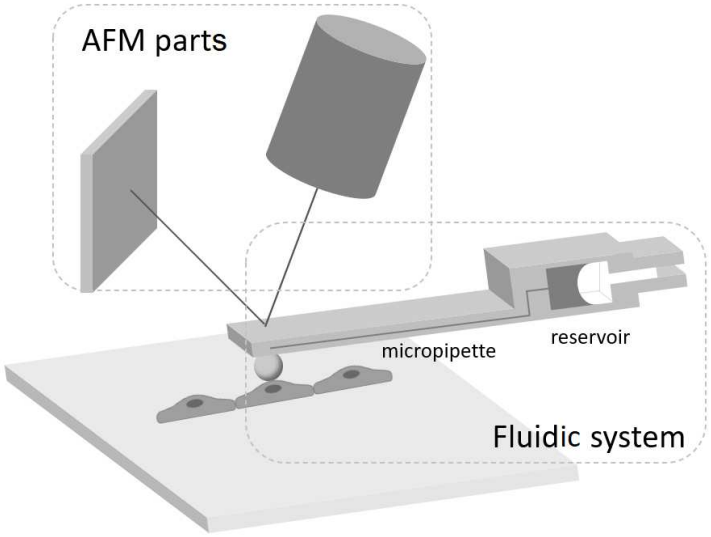

Fig. 2: A graphic representation of a fluidic AFM system designed to measure the adhesion force between a coated microsphere and a target cell. The coated microsphere to can be replaced with another coated microbead or other cell depending on the objective of the experiment.

4 Exchangeable colloidal fluidic AFM force spectroscopy and an example of colloids for fast cell adhesion measurements

To minimize potential mechanical perturbations on a target cell, AFM cantilevers with micrometer-level size spherical colloids are employed in place of a sharp tip for cell surfaces nanomechanics. Typical applications of colloidal probes (**Fig. 3**) include identification of specific biomolecules on targeted individual cell surfaces at the single molecule level[81], adhesion measurement[82,83,84], and the study of mechanical properties [85,86]. The procedure to attach the colloidal probe to a microcantilever has not seen significant advancement in the past decades and entails irreversibly immobilizing a spherical particle at the end of a tipless cantilever[87-89]. To obtain a dataset of sufficient statistical rigor requires the attachment of many colloidal probes to different cantilevers. Functionalization of a colloid to a cantilever allows for the probe of only one target. Thus, a new conventional AFM cantilever-colloid probe is required to measure different target

cells or different receptors, increasing the cost and labor. Also, calibration is required every time a cantilever is exchanged. Furthermore, differences in the mechanical properties between cantilevers that stem from variations in the fabrication process induce potential challenges to compare results from different cantilevers.

Unlike conventional AFM cantilevers, fluidic AFM micropipette cantilevers are reusable. It is possible to probe different targets by functionalizing and replacing microbeads[62,90]. Microbeads are functionalized by mixing with reagents following standard protocols. Microbeads are easily exchanged by applying negative and positive pressure using a fluid pressure controller (**Fig. 3**). Using exchangeable colloids and fluidic AFM force spectroscopy, we recently reported that the intracellular cyclic adenosine monophosphate receptor EPAC1 modulates rickettsial adhesion on host cell surfaces in association with Y23 phosphorylation of the bacterial binding receptor Annexin A2[68]. An example of fast serial adhesion measurements is described in the following section 4.1.

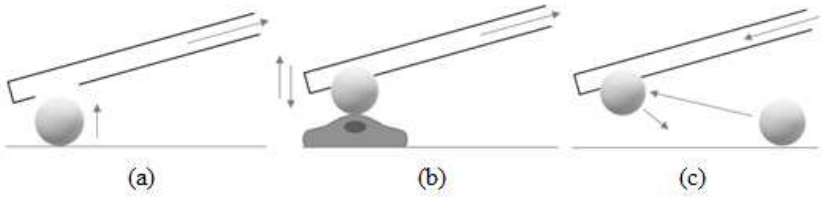

Fig. 3: **(a)** Negative pressure is applied within the microchannel of the cantilever aperture to absorb the bead to the cantilever. **(b)** A piezoelectric device is used to drive the bead and attached probe for force spectroscopy analysis. **(c)** Pressure on the microchannel is adjusted to replace the bead.

4.1 Example of colloids for fast cell adhesion measurements

In this section, we further describe a faster method to sequentially measure adhesion using coated colloid beads[62,91-93]. Aiming to increase adhesion speed without having to exchange beads after each measurement, the functionalized beads are dispersed onto a confluent or near-confluent cell layer in culture and allowed to adhere for a specific time. The fluidic microcantilever is used to apply suction and pull the beads sequentially to measure adhesion. The cells with beads are examined optically to ensure that the beads are adhered to the cells and are not over an area without cells.

An example of this methodology is described as follows. A human bone marrow fibroblast cell line (CRL-11882, ATCC) was cultured for 48 hours. Gold nanoshells on 10 µm diameter silica beads were functionalized with a water-soluble cross-linker (DTSSP; 3,3'-dithiobis [sulfosuccinimidyl propionate]). DTSSP adheres to the gold surface by disulfide linkage and covalently to the surface proteins of the cell membrane[94,95]. As a control, uncoated beads were used. These experiments were not used to derive any meaningful conclusions on the adhesion properties on the cell, but rather to demonstrate a pathway to conduct high speed sequential adhesion measurements. For fluidic AFM measurements, the protocol depicted in **Fig. 4** was used. The spheres were dispersed on a confluent layer of cells growing on a round coverslip and allowed to adhere inside an incubator (steps 1-2 in **Fig. 4,** and **Fig. 5a**). The AFM is used to guide the cantilever optically near the bead while applying suction (step 3). The AFM detects contact, and the bead is attached by the suction applied (step 4, detected by the force-distance curve). The cantilever then pulls the bead upward and away from the cell membrane to measure the force-distance curve. During this pulling phase, the adhesive force strength is measured and is shown in

the force-distance curve (step 5). Beads are discharged by applying positive pressure (step 6), and the tip is moved to another bead for another measurement. A cycle of measurements from step 3 to step 6 takes about 3 minutes to complete.

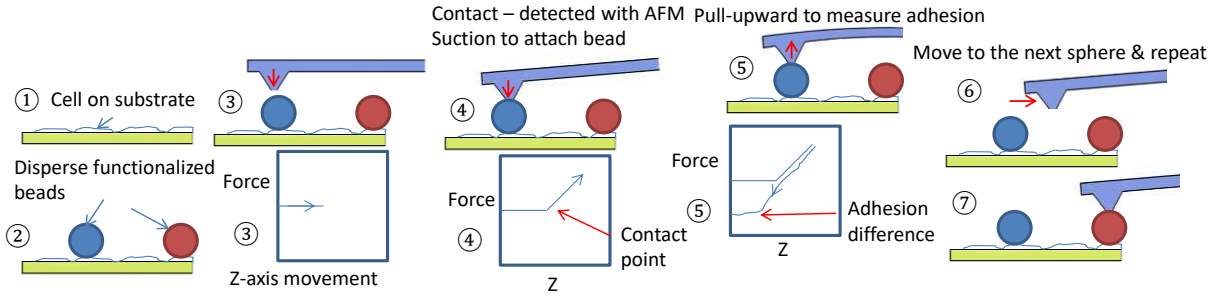

Fig. 4: Protocol for high-throughput adhesion measurements using fluidic AFM.

Cell adhesion studies used 4 µm aperture fluidic cantilevers (Cytosurge AG, Switzerland) (**Fig. 5b**). The spring constant was measured at approximately 1.33 N/m using the Sader method[96] (**Fig. 5c**). Before each experiment the cantilevers were plasma-cleaned. The fluidic cantilevers were mounted on a FlexAFM with a C3000 controller (Nanosurf AG, Liestal, Switzerland) and connected to tubing and a pressure controller (Cytosurge AG, Zürich). Following the experiments, software provided by the atomic force microscope manufacturer was used to analyze the adhesion measurements (Nanosurf AG, Liestal, Switzerland).

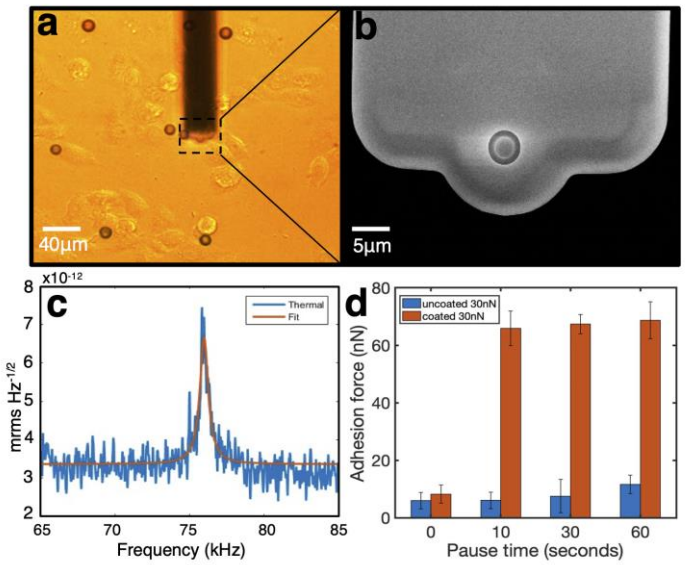

Fig. 5: **(a)** Optical image of fibroblasts with 10 µm beads and fluidic AFM cantilever. **(b)** Scanning electron microscopy (SEM) image of a fluidic AFM cantilever (from Cytosurge). **(c)** Resonance frequency measurement of the cantilever used. **(d)** Summary of results of the adhesion forces measured in different pause times, conducted with uncoated or coated beads; results are derived from force-distance curves.

The beads were aspirated onto the cantilever tip using a negative pressure (~ -700 mbar) (**Fig. 5a and Supplemental Video 1**). While maintaining negative pressure, the cantilever is pulled upwards generating a force-distance curve. During measurements, pressure is reduced to -500

mbar. Force-distance curves are acquired with a maximum force set-point of 30 nN with surface pause times of 0s, 10s, 30s, 60s (defined as how long the tip contacts the bead before moving upwards). Each measurement is an average of 5-10 force-distance curves per pause time. Beads are discarded by applying positive pressure, and new beads are aspirated and tested on different cells (**Supplemental Video 1**). DTSSP coated beads showed an ~10-fold increase in the adhesion force to fibroblasts versus uncoated beads at pause times greater than 10 seconds.

5 Single cell manipulation

5.1 Single cell treatment

5.1.1. Intracellular injection: Glass micropipettes have been used for intracellular injection[43,97]. However, conventional micropipettes lack force feedback for real-time sensing of cell membrane contact and rupture. In addition, delivering small loads in single cells or inside the cell nucleus is particularly challenging[98]. Fluidic AFM enables intracellular injection down to femtoliter level with force feedback, minimizing cell damage[13,71]. The load is released by passive diffusion or by applying a positive pressure using a nanopore fluidic AFM cantilever[71].

5.1.2. Cell surface dispensing: To precisely observe the initiation of virus particle infection of a single living cell, Stiefel *et al.* used fluidic AFM to position individual and multiple virions onto the cell surface[99]. By placing different numbers of virus particles on host cells, they showed that the infection rate grows superlinearly with the number of particles placed on a single cell. This points to a synergy between viral particles which impacts the early stage of the infection process[99]. Similarly, any small non-biological[9] or biological[100] particles, including EVs (discussed below), viruses, and bacterial pathogens, can be dispensed onto cellular surfaces. In another study, the fluidic AFM was combined with a fast-scanning confocal microscope to study host response to viral exposure in real-time[101]. In this work the fluidic AFM was used to attach nanogold particles (400nm diameter) functionalized with virions[101].

5.1.3. Single living recipient cell of EV: Transmembrane proteins from parent cells are present on both small and large EVs, while cytosolic proteins and genetic material are contained within the lipid bilayer membrane, which facilitates transportation to remote recipient cells without a loss of bioactivity[102,103]. There is growing evidence that a variety of cells can serve as parent cells of EVs. EVs have been recognized as cell-type biomarkers because they maintain the same topology of transmembrane proteins as the parent cell plasma membrane[104]. Once docked on the recipient cell apical surface, the adhesion and internalization are processed in a receptor-dependent manner, in which membrane fusion and endocytosis occur[16]. The level of uptake is proposed to be dependent on the recipient cell type because the nature of the endogenous membrane-derived surface enables exosomes to inherit cell-type surfaces from their parent cells, posing different affinities to different cell types[105,106]. For example, exosomes from primary neurons are only taken up by other neurons, whereas those from a neuroblastoma cell line bind equally to astrocytes[105]. Similarly, bone marrow dendritic cell exosomes were preferentially captured by splenic dendritic cells, rather than by B or T cells[106]. Exosomes from oligodendroglia precursor cells were taken up by microglia but not by neurons or astrocytes. Therefore, different cell-derived exosomes have different cell-type tropisms, potentially like some emerging viruses[107,108]. Therefore, direct evidence from a single living cell model is crucial to help identify the cell type of a recipient cell and characterize the mechanism of the cell-type tropism. Fluidic AFM is a powerful tool that can be used to assess purified cell-type EVs in various cell models at the level of a single cell by manipulating dispensing dose-dependent EVs.

5.2 Single cell sampling

Obtaining single cell content for downstream analysis without cell lysis, thus enabling for post-extraction monitoring, is challenging. In the past, AFM tips were used to extract mRNA from live cellos by chemically modifying the tip surface to immobilize gene-specific primers complementary to the mRNAs of interest[109,110]. Fluidic AFM enabled single cell content extraction[13]. Guillaume-Gentil *et al.* inserted a minimally invasive nanopipette cantilever into a single living HeLa cell for cell compartment-selective extraction of the native intracellular fluid[63]. The extractions were successfully used for downstream molecular analyses (transmission electron microscopy, enzyme activity assays, and gene expression studies). It is worth noting that cells were viable up to five days post-extraction and that viability was dependent on the volume extracted from cytoplasm and nucleoplasm. In an extension of this work, Chen *et al.*[111] developed a technique called Live-Seq. Standard single cell RNA sequencing involves lysis, thus providing a snapshot and endpoint measurement. In Live-Seq, repeated cytoplasm extractions from the same cell for downstream RNA sequencing was made possible. Using fluidic AFM, it is now possible to extract cytoplasm, treat the cells with agents of interest, and repeat the extraction after several hours[112].

6 Cell-to-cell force analysis

The spatial interaction between cells, hetero- or homogenous, plays a central role in the pathogenesis of infection and inflammation. Cell-to-cell lateral contacts are critical for tissue homeostasis. The paracellular pathway is an extracellular route across endothelia and epithelia that is generally used for passive transportation of water and small solutes; however, in some cases particles as big as leukocytes may cross it [113]. The epithelial or endothelial barrier is maintained by intercellular multi-protein junctional complexes, either adherent junctions (AJs) and tight junctions (TJs), functionally sealing the lateral space between cells against unbinding forces on the lateral contact sites[114]. The interplay between TJs and AJs regulates major rate-limiting paracellular pathways by allowing particles to permeate across the paracellular route[115] and establishing cell polarity[116]. Dysfunctions, ruptures, and breach of the epithelial or endothelial barriers are major causes of infection and inflammation. Therefore, measuring the lateral binding forces (LBFs) between homogenous cells in response to different stimuli is crucial to understanding the precise biomechanical mechanism underlying intercellular barrier dysfunctions, which is a major outcome of host responses to infections, including inflammation. Traditionally, paracellular permeability can be indirectly evaluated using two methods: measuring trans-endothelial electrical resistance (TEER)[117] and fluorescein tracers after passing through the monolayer[118]; both methods involve indirect measurements[119].

Specific interactions between microbial surface ligands and host receptors account for tissue tropism and influence microbial distribution at the sites of infection. Adherence, however, is also a virulence factor, which requires resistance to the shear stress exerted by flowing blood at the blood-endothelial interface, promoting microbial uptake by ECs to initiate infection[120]. In bloodstream infections, a major determinant in bacterial disease outcomes is the adherence to ECs by microorganisms that lead to the establishment of metastatic endovascular infections[121-127]. By activating the production of pro-inflammatory cytokines that cause local and systemic inflammation, the attachment of microorganisms to the mucosal surface is a key step in the successful establishment of mucosal infections[128]. Thus, the quantification of the adhesion between pathogens and epithelial/endothelial cells is vital, and may lead to notable advances in our understanding of the interplay between microorganisms and the host at the initial stage of

infection[129]. In addition, at the site of inflammation, various stimuli induce endothelia on the surface of the luminal blood vessel to become adhesive for leukocytes[128]. Following their initial contact with activated endothelia after margination, leukocytes roll along endothelial apical surfaces until they are captured. The adhesion of leukocytes to vascular endothelium is a hallmark of focal inflammation. A variety of methods have been developed to study pathogen–host and leukocyte adhesions, including *in vitro*[3], genetic[130], molecular, and animal methods[121,122,128]. However, direct evidence regarding the biomechanical nature of adhesion is still lacking. Vertical binding force (VBF) measurements between a single bacterium or leukocytes and the target EC reveal the fundamental biomechanical nature of adhesion and its underlying mechanism[128,131]. In conventional AFM-based, single-cell adhesive force assays, cells are attached to the AFM cantilever to probe adhesive forces with adherent cells or substrates. Various irreversible immobilization strategies have been introduced since the development of this technology[129]. Among these methods, the most straightforward consists of immersing the tip in a cell suspension in order to attach a single cell[129]. However, this might lead to the irreversible attachment of multiple cells. Furthermore, attaching the cells biochemically may result in weak immobilization of the cell to the cantilever.

Fluidic AFM can be used to capture single living cells for adhesion measurements[68]. This novel approach has been used to study endothelial barrier function by measuring the LBFs between ECs[132], and also to dissect the molecular mechanism of *Candida albicans* adhesion by measuring the LBFs between yeast cells[67].

6.1 LBFs involving paracellular barrier function

Conventional technologies to directly measure LBFs of living cell-to-cell contacts were not available until a recent report that used fluidic AFM for the direct measurement of LBFs on a cell monolayer[132]. Sancho *et al.* quantified and compared LBFs between L929 fibroblasts and human endothelial cells from an umbilical artery, and provided evidence that vascular ECs exerted strong intercellular adhesion forces, while fibroblast adhesion forces were not detectable. Furthermore, they reported on the dynamics of the LBFs during endothelial-to-mesenchymal cell transition[132]. This study demonstrated the ability to assess EC LBFs in a mature monolayer in physiological settings, providing further evidence that these types of tools can be used to enhance our knowledge of biological processes in developmental biology, tissue regeneration, and disease states like infection and inflammation.

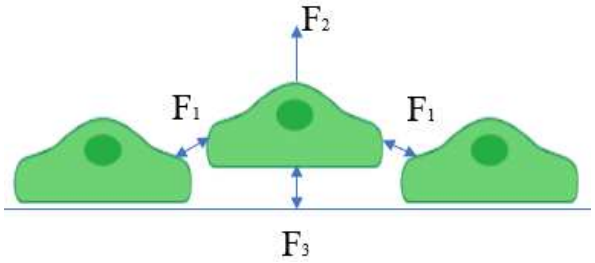

Fig. 6: A demonstration of the mechanical interaction between a cell and its surroundings when being pulled in the LBF assay.

Fluidic AFM presents a feasible solution for testing cell-to-cell LBFs. By measuring the probe lifting force for a specific cell, the cell-to-cell LBF can be obtained by using the equation:

$$W_3 = W_1 - W_2$$

where W_1 is the total lifting work when probe removes a cell in high cell density area or case (**Fig. 6**), W_2 is the total lifting work when probe removes a single cell in low cell density area or case, and W_3 is the proposed total work between cells[132].

In order to complete measurements of LBFs of ECs, a larger Z-axis travel range piezo is needed, because vascular ECs are exceedingly thin[133] with a relatively larger surface area compared with other epithelial cells[134,135]. A large travel range gives the cantilever the ability to move a further distance in the Z-axis direction in a more stabilized form and separate the captured cell from the monolayer, making measuring the LBFs of ECs feasible. A 150 µm Z-axis actuator (Core AFM, NanoSurf, Liestal, Switzerland) is used for the experiments shown in **Fig. 7** and **Movie 1**.

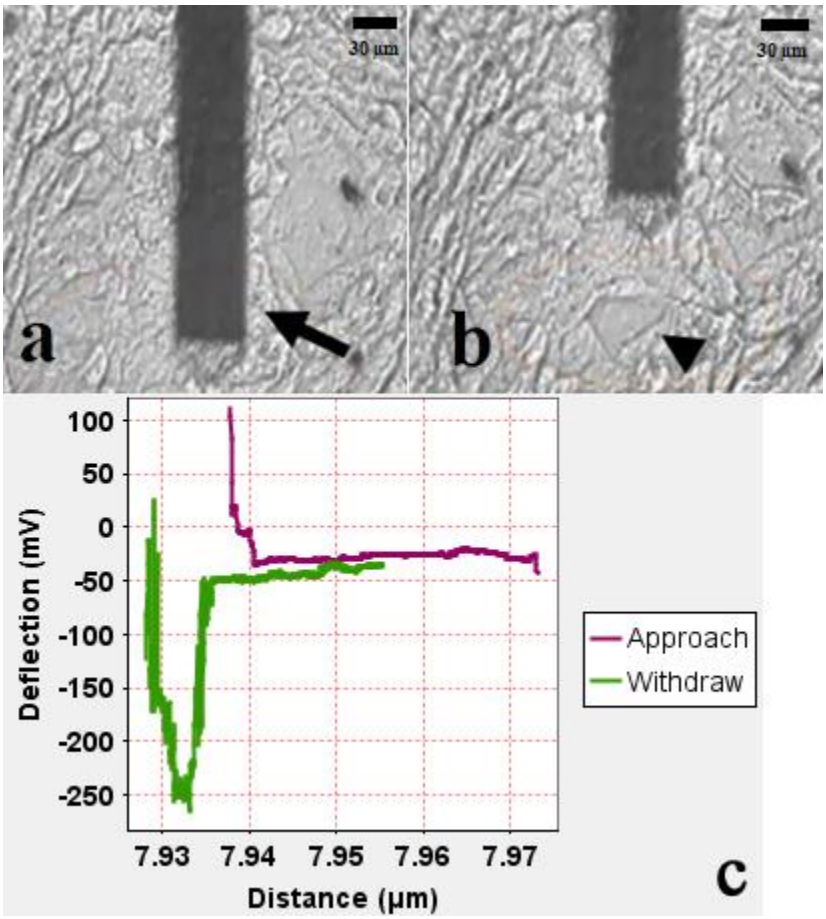

Fig. 7: Photomicrograph of fluidic AFM micro-cantilever capturing a living, adherent Vero cell. **(a)** The fluidic AFM system for measuring live cell LBFs employs micropipette (arrow) technology. **(b)** Empty space (arrowhead) where one cell is pulled by the fluidic AFM. **(c)** Representative force distance (F-D) curves of the LBFs of Vero cells are shown. Scale bars, 30 µm.

Movie 1.mp4

Movie 1: A single, living brain microvascular endothelial cell (BMEC) isolation from the monolayer using a fluidic AFM micropipette cantilever. Retraction forces that contribute to the final calculation of LBFs are measured by AtomicJ[136]. The video is displayed at 8X the original speed.

6.2 Intercellular adhesion VBFs between heterogeneous cells

The principal of applying fluidic AFM to study VBFs is that the micropipette acts as a cell probe by capturing a single living cell via negative pressure, which then interacts with a single living target cell in a liquid environment (**Fig. 8**). This specialized cantilever is connected to the fluidic pressure controller. The opening at the apex of the probe varies from 300 nm to 8 µm. A continuous flow from the fluidic pressure controller creates negative pressure after a cell attaches to the aperture of the micropipette probe, which then becomes a cell probe. After the cell probe is moved onto the apical surface of the target cell, single point force spectroscopy is performed, and the interacting force between the cell probe (i.e., the micropipette-captured cell) and the target cell is measured in nanonewtons (nN). Thus, VBFs between a single living bacterium or leukocyte and an epithelial cell or EC can be quantified.

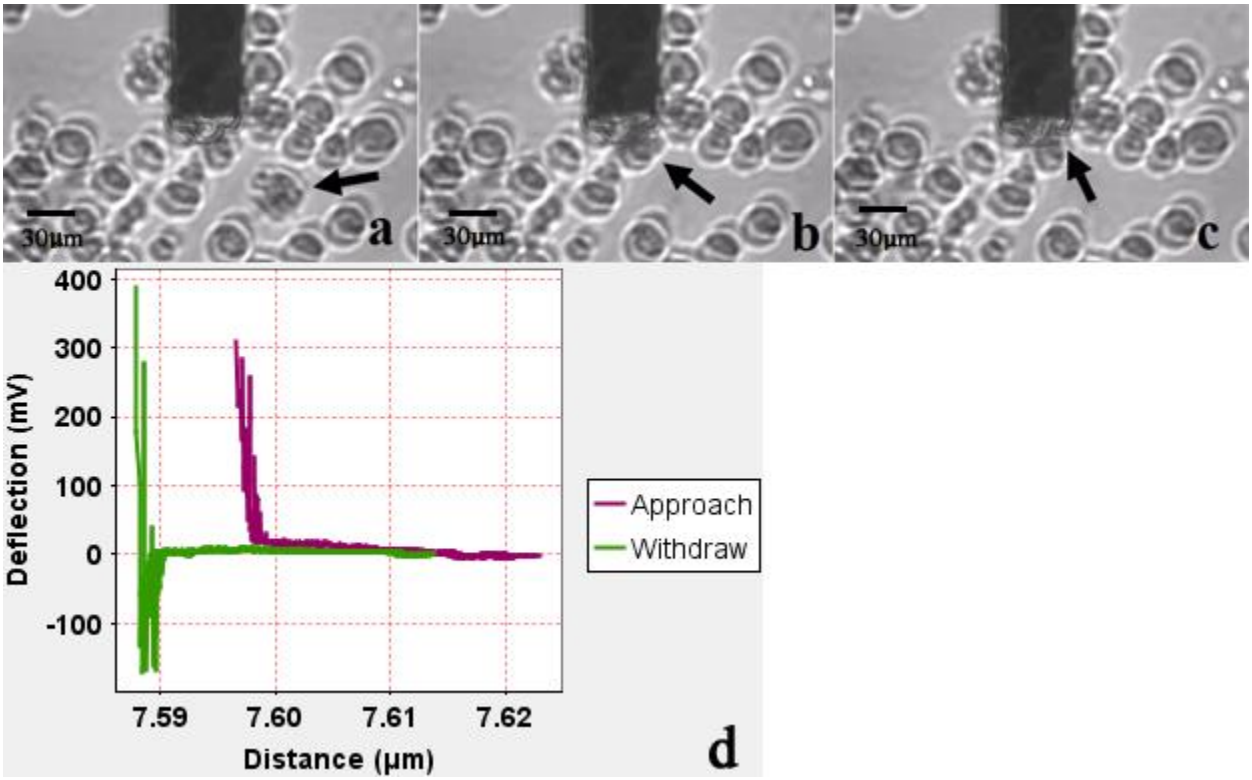

Fig. 8: Photomicrograph of a live monocyte captured on the micropipette probe in media to measure the VBFs in a co-culture of monocytes and BMECs. **(a)** Floating monocyte (arrow); **(b)** floating monocyte is being captured by the microcantilever; **(c)** monocyte is completely captured; **(d)** F-D curve approach and withdraw. Scale bars, 30 µm.

Movie 2.mp4

Movie 2: In a co-culture of BMECs and THP1 cells, a living THP1 is captured at the aperture of a micropipette cantilever and used as a cell-probe to measure the VBFs between living monocytes and BMECs. The video is displayed at 8X the original speed.

7 Single cell mass measurement

Cell mass is a critical parameter impacted by disease dysregulation[137] and is indicative of the quantity of fluids, biomolecules, macromolecules, amino acids, lipids, and nucleic acids within a cell[138]. Processes such as cell differentiation, gene expression[139], cell shape, metabolism, migration, and proliferation[140] can be investigated using measurements of cell mass to study regulation. Rapid cell mass fluctuations may provide insights into basic cellular processes, such as the response to growth stimuli, ATP synthesis, glycolysis, or the transport of water or other substances across the cell membrane[138]. Highly precise mass measurements are required due to the small masses involved, the irregular shapes of cells, and the need to study individual cell behavior. Such higher-resolution measurements are required to advance our understanding of cellular growth, as prior studies into cellular growth patterns have not been conclusive and have shown varying growth curves ranging from linear to exponential[141,142].

To date, research involving cell growth has mostly relied on volume measurements, as it has been extremely difficult to measure single cell mass[143]. However, cellular volume and mass may change at different rates, with mass being a better indicator to assay cell growth in single cells[142]. Changes in cell volume have a strong relationship with cell density, which is impacted by osmotic and other processes, whereas cell mass indicates growth as cells acquire new biomass, in particular the protein content[142]. Thus, there is a critical need for improved, highly precise methods of dynamic and continual cell mass measurements[142,143].

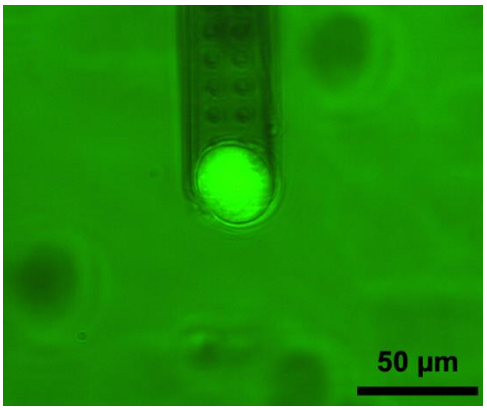

Fig. 9: Photomicrograph of a mammalian cell (HEK, ATCC CRL-12585) attached by applying 100 mbar of negative pressure during fluidic AFM. Image bright field and fluorescence using Celltracker Green (Invitrogen™).

Current promising approaches to detecting the mass of single cells that rely on microcantilever resonance frequency changes include microchannel resonators [144] and pedestal mass sensors[145]. A technique employing conventional AFM cantilevers, which are functionalized to pick up single cells in cell culture media, called inertial pico-balance resonators[138], have been used for mass measurements of single adherent cells and are shown to function from milliseconds to days[138].

There are several advantages to using fluidic AFM in analyzing the mass of single cells. Much like inertial pico-balance resonators[138], fluidic AFM achieves high temporal resolution and offers similar mass resolution. Importantly, cell attachment can be achieved without the need for antibodies, by physically grabbing the cell (**Fig. 9**) in an aqueous environment. This offers a major advantage, as it enables the study of non-adherent cells such as immune cells. Another advantage is that the device can be reused, enabling the measurement of several cells in a short period of time.

Potential challenges could be that the behavior of adherent cells (most mammalian cells) is affected if they are suspended, thus pipette attachment may not allow the cells to behave physiologically normal. In addition, the Q factor of the cantilever in liquids drops dramatically compared to air due to high damping in liquids, which decreases sensitivity. However, this is a common problem for all microcantilever techniques performed in a liquid environment (it is worth noting that this is not the case with microchannel resonators[144] that operate in air). In an initial proof-of-concept, fluidic AFM was used to measure the mass of yeast and beads in air, with the ability to catch and release at picogram resolution[146]. Nonetheless, techniques that employ fluidic AFM for single cell mass measurements require further study.

8 Electrophysiology

Patch clamping[44] is used in multiple areas of biology such as cardiology (cardiomyocytes), neurology/neuroscience (neurons), endocrinology (pancreatic beta cells), and myology (muscle fibers). Ion channels in immune cells play significant roles in directly or indirectly regulating intracellular signaling pathways, cell development, innate and adaptive immune responses, and autoimmunity[147]. Ion channels can potentially become pharmacological targets for autoimmune diseases[147]. Bacteria have many ion channels that respond to chemical and physical alterations[148-150]. Classical patch-clamping cannot be performed due to the structure of the bacterial cell wall and the small size of bacterial cells[148,151].

Patch clamping is the gold standard for electrophysiology and offers an accurate and unmatched measurement of ion currents and membrane potentials; however, it is labor intensive and time consuming, requiring an entire day to record two to four cells[152]. Furthermore, patch clamping requires lengthy training and expertise due to the difficulty in operating the pipette. Cells and pipettes are prone to damage, requiring frequent replacement. The pipette is guided visually under microscopic observation without force-feedback. Finally, excessive mechanical stress before or during a procedure may affect the results[153].

Combining AFM with a fluidic probe for patch clamping has many benefits. The entire measurement can be partially automated. The AFM force-feedback mechanism acts as a feedback touch sensor to detect contact with a cell, significantly reducing the likelihood of cell damage, training time, and time per measurement. This requires compliant cantilevers for nondestructive contact and direct measurements. Ossola *et al.*[64] reported that a fluidic AFM cantilever was used for a combination of patch clamping and contraction measurements of cardiomyocytes. The device's geometry and other factors did not allow for the formation of a GΩ seal (reported in the 10s of MΩ). Furthermore, the high value of the spring constant (1.8 N/m) exerts a force that could be damaging to the cells and interfere with the measurements. Therefore, while the combination of AFM and fluidic probes is very promising for automation of single cell patch clamping, a number of modifications are needed at the device level before patch clamping can be used by the wider user base. The use of MEMS fluid cantilevers and AFM may result in lower noise compared to conventional patch clamping, less cell content diffusion in the pipette, minimization of mechanical stress, less damage to cells, and device uniformity. In addition, it would enable additional modalities such as cell adhesion, cell contraction, and elasticity to be considered. While fluidic AFM is low throughput, it is capable of providing high content analysis. For instance, patch clamp AFM could be combined with single cell content extraction using fluidic probes for single cell sequencing, thus enabling single cell physiological and genotypic characterization.

9 Conclusion and future perspectives

In this review, we introduced the use of AFM in EV analysis for host–pathogen studies. We also provided a review of fluidic AFM development and discussed some new applications with a slight focus on cell biology applications in cell/pathogen adhesion and cell barrier function as a key host response. Fluidic AFM features a cantilever embedded with micromachined microfluidic channels. Through proper pressure control, targets such as cells or beads can be attached to the tip by suction to conduct desired measurements. Targets are subsequently repelled by overpressure, and the cantilever could be reused for subsequent experiments. The development of fluidic AFM enables various experimental directions, including fast adhesion measurements, single cell treatment and sampling, cell-to-cell LBF and VBF analyses, single cell mass measurements, and patch-clamping measurements. Some of the directions presented in this review are more widely explored, and yet some of the applications are still in their initial stage of development and require further scientific exploration to produce meaningful results. Given that fluidic AFM was invented relatively recently, it stands as an emerging and prominent candidate for single living cell and EV studies, and there is room for further improvement, optimization, and innovation. One direction of improvement is the development of additionally specialized fluidic cantilevers that serve specific applications such as patch clamping. Experimentation with new structural materials, such as polymers, may result in more compliant devices. Embedding sensing elements, such as deflection sensors and electrodes, on the cantilevers may enhance their functionality and perhaps even eliminate the need for the AFM optical lever. As AFM innovations are commercialized, they will also contribute to performance enhancement, such as the introduction of photothermal excitation[154-156] in commercial units that significantly improves the AFM performance in liquids.

Acknowledgements

We gratefully acknowledge Drs. Zhengchen Su and Edward Nelson for their contributions in helping to establish our fluidic AFM system capabilities. We also thank Dr. Nelson for assistance with measurements and for insights into the use of AFM and fluidic AFM. We thank Dr. Kimberly Schuenke for her critical review and editing of the manuscript. This work was supported by NIH grants R01AI121012 (BG), R21AI137785 (BG and AG), R21AI154211(BG), R03AI142406 (BG), R21AI144328 (BG), Sealy Center for Vector Borne and Zoonotic Diseases pilot grant FY21 (BG), and the UTMB Center for Biodefense and Emerging Infectious Diseases pilot grant FY21 (BG). The sponsors had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

supplemental video 1.mp4

Supplemental Video 1: The cantilever is moved above the target cell, which has a coated bead on its surface. Negative pressure is applied so that the bead is captured by the cantilever. Force spectroscopy is then used to determine the adhesion force between the bead and the target cell.

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