Journal of Visualized Experiments

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--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE59609R1
Full Title:	Functionalization of atomic force microscope cantilevers with single-T cells or -particle for immunological single-cell force spectroscopy
Keywords:	Atomic force microscopy, cantilever, single-cell force spectroscopy, T cells, dendritic cells, adhesion force, solid particles, live-cell fluorescence imaging
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Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Beijing,China

TITLE:

Functionalization of Atomic Force Microscope Cantilevers with Single-T Cells or Single-Particle for Immunological Single-Cell Force Spectroscopy

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KEYWORDS:

Atomic force microscopy, cantilever, single-cell force spectroscopy, T cells, dendritic cells, adhesion force, solid particles, live-cell fluorescence imaging

SUMMARY:

We present a protocol to functionalize atomic force microscope (AFM) cantilevers with a single T cell and bead particle for immunological studies. Procedures to probe single-pair T cell-dendritic cell binding by AFM and to monitor the real-time cellular response of macrophages to a single solid particle by AFM with fluorescence imaging are shown.

ABSTRACT:

Atomic force microscopy based single cell force spectroscopy (AFM-SCFS) is a powerful tool for studying biophysical properties of living cells. This technique allows for probing interaction strengths and dynamics on a live cell membrane, including those between cells, receptor and ligands, and alongside many other variations. It also works as a mechanism to deliver a physical or biochemical stimulus on single cells in a spatiotemporally controlled manner, thus allowing specific cell activation and subsequent cellular events to be monitored in real-time when combined with live-cell fluorescence imaging. The key step in those AFM-SCFS measurements is AFM-cantilever functionalization, or in other words, attaching a subject of interest to the cantilever. Here, we present methods to modify AFM cantilevers with a single T cell and a single polystyrene bead respectively for immunological studies. The former involves a biocompatible glue that couples single T cells to the tip of a flat cantilever in a solution, while the latter relies on an epoxy glue for single bead adhesion in the air environment. Two immunological applications associated with each cantilever modification are provided as well. The methods described here

can be easily adapted to different cell types and solid particles.

INTRODUCTION:

Atomic force microscopy (AFM), a versatile tool, has found many applications in cell biology research[1-5]. Apart from its high-resolution imaging capability, the native force-probing feature allows biophysical properties of living cells to be investigated directly in situ at the single-cell level[6,7]. These include the rigidities of subcellular structures or even whole cells[8-12], specific ligand/receptor binding strengths at the single-molecule level on the cell surface[13], and adhesion forces between single-pairs of solid particles and cells or between two cells[1,2,14,15]. The latter two are often categorized as single-cell force spectroscopy (SCFS)[16]. Owing to the readily available cantilevers with various spring constant, the force range accessible to AFM is rather broad from a few piconewtons (pN) to micronewtons (μN), which adequately covers the entire range of cellular events involving forces from a few tens of pN. For example, receptor-based single-molecule binding, such as phagocytic cellular events are in the range of nN[15]. This large dynamic force range makes AFM advantageous over other force-probing techniques such as optical/magnetic tweezers and a biomembrane force probe, as they are more suitable for weak-force measurements, with force typically less than 200 pN[17,18]. In addition, AFM can function as a high-precision manipulator to deliver various stimuli onto single cells in a spatiotemporally defined manner[4,19]. This is desirable for the real-time single-cell activation studies. Combined with live-cell fluorescence imaging, the subsequent cellular response to the specific stimulus can be monitored concurrently, thus making AFM-based SCFS exceedingly robust as optical imaging providing a practical tool to probe cellular signaling. For instance, AFM was used to determine the strains required to elicit calcium transients in osteoblasts[20]. In this work, calcium transients were tracked fluorescently through calcium ratiometric imaging after the application of localized forces on cultured osteoblasts with an AFM tip. Recently, AFM was employed to stretching collagen fibrils on which hepatic stellate cells (HSC) were grown and this mechano-transduced HSC activation was real-time monitored by a fluorescent Src biosensor, whose phosphorylation as represented by the fluorescence intensity of the biosensor is correlated with HSC activation[3].

In AFM-based SCFS experiments, proper functionalization of AFM cantilevers is a key step toward successful measurements. Since our research interest focuses on immune cells activation, we routinely functionalize cantilevers with particulate matters such as single solid particles that can trigger phagocytosis and/or strong immune responses[4,14,15] and single T cells that can form an immune synapse with antigen presenting cells, such as activated dendritic cells (DC)[2]. Single solid particles are normally coupled to a cantilever via an epoxy glue in the air environment, whereas single T cells, due to their non-adhesive nature, are functionalized to a cantilever via a biocompatible glue in solution. Here, we describe the methods to perform these two types of cantilever modification and give two associated applications as well. The first application is to probe T cell/DC interactions with AFM-SCFS to understand the suppressive mechanism of regulatory T cells from the cell mechanics point of view. The second one involves combining AFM with live-cell fluorescence imaging to monitor the cellular response of macrophage to a solid particle in real-time to reveal the molecular mechanism of receptor-independent phosphatidylinositol 4,5-bisphosphate (PIP2)-Moesin mediated phagocytosis. The aim of this protocol is to provide a reference framework for interested researchers to design and implement

their own experimental settings with AFM-based single-cell analysis for immunological research.

PROTOCOL:

The mouse experiment protocol follows the animal care guidelines of Tsinghua University

1. Cantilever functionalization with single T cells

1.1 Mouse spleen cells preparation

1.1.1 Sacrifice the mouse (8-16 weeks of age (either sex); e.g., C57BL/6 strain) using carbon dioxide, followed by cervical dislocation.

1.1.2 Clean the mouse with 75% ethanol and make a midline skin incision followed by splenectomy.

1.1.3 Homogenize the spleen in 4 mL of PBS containing 2% fetal bovine serum (FBS) using glass slides and remove aggregates and debris by passing the cell suspension through a 70 µm mesh nylon strainer.

1.1.4 Centrifuge the cell suspension at 500 x *g* for 5 min, discard the supernatant and resuspend cells in 2 mL of red blood cell lysis buffer (balanced at room temperature) for 5 min. Terminate the lysis reaction by adding 8 mL of PBS solution.

1.1.5 Centrifuge the cell suspension at 500 x *g* for 5 min and resuspend cells at a density of 1 x 10^8 cells/mL in PBS containing 2% FBS and 1 mM EDTA (Labelled as Solution A), typically 0.25-2 mL depending on the cell density. Transfer the resuspended cells to a 5 mL (12 x 75 mm) polystyrene round bottom tube.

1.2 Mouse CD4+ T cells preparation

1.2.1 Add 50 µL/mL rat serum (see **Table of Materials**) and 50 µL/mL CD4+ T cell isolation cocktail (see **Table of Materials**) to the cell sample obtained from step 1.1.5. Mix and incubate for 10 min at room temperature.

1.2.2 Vortex the stock streptavidin-coated magnetic particle solution (see **Table of Materials**) for 30 s or until the particles appear evenly dispersed.

1.2.3 Add 75 µL/mL streptavidin-coated magnetic particles to the cell sample. Mix and incubate for 2.5 min at room temperature.

1.2.4 Add Solution A to top up the cell sample to 2.5 mL and mix by gently pipetting up and down for 2-3 times.

1.2.5 Place the sample tube (without lid) into the magnet (see **Table of Materials**) and incubate for 5 min at room temperature. Carefully pour the enriched cell suspension into a new 5 mL polystyrene round-bottom tube.

1.2.6 Centrifuge the cell suspension at 500 x *g* for 5 min. Discard the supernatant and resuspend the enriched T cells in 500 µL of Solution A.

NOTE: The enriched CD4+ T cells contain both conventional and regulatory T cells.

1.3 Regulatory T cells separation from conventional T cells

1.3.1 Add 25 µL of FcR blocker (see **Table of Materials**) to the enriched T cell sample obtained from step 1.2.6. Mix and incubate for 5 min at room temperature.

1.3.2 Add 25 µL of regulatory T cell positive selection cocktail (see **Table of Materials**) to the T cell sample. Mix and incubate for 10 min at room temperature.

1.3.3 Add 10 µL of PE selection cocktail (see **Table of Materials**) to the T cell sample. Mix and incubate for 5 min at room temperature.

1.3.4 Vortex the stock dextran-coated magnetic particle solution (see **Table of Materials**) for 30 s or until the particles appear evenly dispersed.

1.3.5 Add 10 µL of dextran-coated magnetic particles to the T cell sample. Mix and incubate for 5 min at room temperature.

1.3.6 Add the Solution A to top up the T cell sample to 2.5 mL and mix by gently pipetting up and down for 2-3 times.

1.3.7 Place the T cell sample tube (without lid) into the magnet and incubate for 5 min at room temperature. Carefully pour the supernatant to a new tube.

NOTE: The supernatant contains the enriched conventional CD4+T cells.

1.3.8 Centrifuge the enriched conventional CD4+T cells at 500 x *g* for 5 min. Discard the supernatant and resuspend the cells in 4 mL of RPMI1640 containing 10% FBS, 0.05 mM β-Mercaptoethanol, 0.01 M HEPES and 1% penicillin/streptomycin (labeled as Medium B).

1.3.9 Remove the tube in which regulatory T cells are enriched from the magnet. Add 2.5 mL of Solution A to the tube and mix by gently pipetting up and down for 2-3 times. Put the tube back into the magnet, incubate for 5 min, and then carefully pour off and discard the supernatant. Repeat this step three more times.

1.3.10 Resuspend the enriched regulatory T cells in 2 mL of Medium B.

1.3.11 Incubate both purified conventional T cells and regulatory T cells with 100 U/mL hIL-2 overnight or for at least 4 h at 37 °C in a humidified incubator with 5% CO_2 before being used for cantilever functionalization.

1.4 Dendritic cells preparation

1.4.1 Prepare piranha solution, a mixture of 30% H_2O_2 (30%) and 70% H_2SO_4 (conc) (v/v). Slowly pour 3 mL of H_2O_2 into 7 mL of H_2SO_4 under constant stirring and cooling.

CAUTION: Piranha solution is highly corrosive, and it can burn and destroy body tissues. Therefore, it is safer to use the piranha solution under a hood and wear appropriate safety equipment, as the mixture will splash around the beaker. Neutralize the solution with NaOH to pH 7 after use.

1.4.2 Immerse the glass coverslip of 24 mm diameter into the piranha solution for 30 min and rinse thoroughly afterward with the sterile ultrapure water.

1.4.3 Dip a pair of pointed tweezers in 75% ethanol for 30 min for cold disinfection.

1.4.4 Introduce the cleaned glass coverslips into a 6-well culture plate by the tweezers.

1.4.5 Tilt a 6 cm plastic culture dish in which DC2.4 cells were pre-cultured with 4 mL of Medium B and aspirate all the medium. Add 2 mL of PBS into the culture dish to rinse DC2.4 cells and discard PBS. Repeat this rinsing step two more times.

1.4.6 Add 1 mL of 0.25% trypsin EDTA to the culture dish for 2 min. Add 1 mL of Medium B to this dish to end the enzyme digestion reaction. Transfer the digested cell suspension to a 15 mL tube.

1.4.7 Centrifuge the cell suspension at 500 x *g* for 5 min and resuspend DC2.4 cells at a density of 2 x 10^5 cells/mL in Medium B.

1.4.8 Seed DC2.4 cells on the glass coverslips prepared at step 1.4.4 and incubate the cells overnight in a humidified chamber at 37 °C with 5% CO_2.

NOTE: In order to measure interacting forces between two single cells, a relatively low concentration of DC2.4 cells (i.e., <10% confluency) is necessary to have a proper spacing among cells.

1.5 AFM cantilever preparation

NOTE: Cantilevers that are suitable for single cell force spectroscopy experiments are those with low spring constants, typically in the range of 0.01-0.06 N/m. Here, soft tip-less cantilevers are preferred for single cells and single solid particles functionalization.

1.5.1 Clean the cantilevers by Piranha treatment or plasma or UV-ozone cleaning.

1.5.2 Mount the cleaned cantilever to the AFM scanning head.

1.5.3 Prepare a clean sample chamber filled with pure water and calibrate the cantilever in water solution by first running a force curve on the glass substrate to obtain the sensitivity (the slope of the linear fit over the repulsive part of the approaching curve) and then recording a thermal noise spectrum to extract the spring constant according to the instruction manual of the AFM.

1.5.4 Remove the AFM scanning head from the solution, wash the mounted cantilever with a few drops of pure ethanol, and keep the cantilever dry on the scanning head.

1.6 Attaching single T cells to the cantilever

1.6.1 Preheat the living cell environment enclosure with 5% CO_2 at 37 °C.

1.6.2 Mount the glass coverslip with DC2.4 cells grown on it from step 1.4.8 to a sample chamber assembly, add 600 µL of Medium B to the chamber immediately, and then put the assembly onto the AFM sample stage.

1.6.3 Add hIL-2 incubated CD4+T cells (either conventional or regulatory T cells) into the sample chamber.

NOTE: The total sample volume should not exceed 1 mL.

1.6.4 Wait until the added CD4+T cells are fully settled down on the bottom of the coverslip.

NOTE: Air bubbles will cause great disturbance to the experiment, therefore, it is advisable to avoid any air bubbles in step1.6.2 and 1.6.3.

1.6.5 Add a drop of 2 µL of biocompatible glue onto the end of the mounted cantilever with a pipette as shown in **Figure 1** and then place the scanning head on the sample stage quickly, thus letting the cantilever coated with the biocompatible glue immerse in the solution.

CAUTION: Do not touch the glass block or the cantilever with the pipette tip. Since the biocompatible glue used here is prone to oxidation in air, this step should be done as quickly as possible.

[Place Figure 1 here]

1.6.6 Locate a healthy T cell beneath the tip of the cantilever coarsely under the microscope by moving the sample stage and then finely adjust the positioning by moving the scanning head.

NOTE: A healthy CD4+T cell typically has a relatively large size, smooth edges, and optically transmissive in the bright-field imaging.

1.6.7 Lower the cantilever manually with small step-sizes (2-5 µm) by controlling the stepper motors. Hold the position of the stepper motors and adjust the positioning of the scanning head for better alignment between the cantilever tip and the cell, once the cantilever makes a firm contact with the target T cell as indicated by a small displacement of the laser beam position in the photodetector corresponding to a typical force range of 0.5-1.5 nN.

NOTE: This step can also be done by running a single force measurement in which set-point (the force applied to the cell) and contact time can be well defined in the software. However, due to the non-adhesive nature of T cells, the manual approach provides more flexibility in controlling aiming, positioning, and contact time than does the automatic approaching and it works reliably for T cell adhesion. Future experimentalists should try both the manual and automatic approaching to find out which works better for their systems of interest.

1.6.8 Retract the cantilever after 30 s of contact.

NOTE: If the cell moves with the cantilever, the attachment is successful. If not, repeat step 1.6.6 but on a different T cell. The biocompatible glue is easily oxidized. Step 1.6.5-1.6.7 should be completed within 5 min. In addition, if the same cantilever fails three times for the T cell attachment, a new cantilever should be used, and the attachment procedure should begin from Step 1.5.2 again.

1.7 Force spectroscopy of single-pair T cell/ dendritic cell interaction

NOTE: To probe cell/cell interactions, an AFM with a Z-range larger than the conventional 10-15 µm is required in order to fully separate the two cells. The AFM used here has a Z-range of 100 µm, which is adequate to separate T cell from the dendritic cell after cell/cell contact.

1.7.1 Position the attached T cell above a separate DC2.4 cell by moving the sample stage and/or the scanning head (see **Figure 2**).

[Place Figure 2 here]

1.7.2 Set proper parameters and run force spectroscopy.

NOTE: The following key settings are typically used: Setpoint 0.5 nN, Pulling Length 50 µm, Z movement Constant Speed, Extend Speed 5 µm/s, Contact Time 10 s, Delay Mode Constant Force. For each T-DC pairs, 20 repeats of force curves are collected and a minimum of 14 force curves are used for further analysis.

1.7.3 Mount a new cleaned cantilever, calibrate it in pure water as in Step 1.5.3, and go back to the same T-DC cells sample to repeat Step 1.6 and 1.7 for a different T-DC pair. Probe at least 3

pairs for each condition.

2. Cantilever functionalization with single polystyrene beads

2.1 Single beads preparation

2.1.1 Dilute the stock suspension of 6 µm polystyrene beads in 100% ethanol.

NOTE: The concentration of diluted beads solution should be low enough so that when added to a glass coverslip surface, individual beads are well separated without significant clustering after solvent evaporation.

2.1.2 Clean the 24 mm diameter glass coverslip with ethanol and remove any dust by N_2 air flow.

2.1.3 Mount the cleaned glass coverslip to a sample chamber assembly and put the assembly onto the microscope.

2.1.4 Put a drop of diluted beads solution to the left side but close to the center of the coverslip (see **Figure 3**) and check the spacing among the beads after solvent evaporation in bright-field under the microscope with a 20x objective. Proceed to the next step if individual beads are well separated.

2.1.5 Dip a micropipette tip or a toothpick into a well-mixed epoxy glue and then transfer a small amount of such glue to three separate spots with successive gentle touches on the right side but close to the center of the coverslip.

NOTE: The three glue spots should be vertically aligned (see **Figure 3**). The last spot with the least amount of the glue will be used later.

[Place Figure 3 here]

2.2 AFM cantilever preparation

2.2.1 Mount a cleaned tip-less cantilever to the AFM scanning head.

2.2.2 Calibrate this cantilever in the air with a clean surface to obtain the spring constant.

2.3 Attaching single beads to the cantilever

2.3.1 Position the cantilever tip over the left boundary of the last epoxy glue spot as shown in **Figure 4.**

2.3.2 Bring the cantilever close to the glue slowly by lowering the stepper motors with small step sizes.

2.3.3 Pull the cantilever away swiftly from the glue laterally by moving the AFM scanning head backward (to the left) manually once the tip is immersed in the glue.

NOTE: Make sure that only a tiny amount of the glue adheres to the very end of the tip. If there is excessive glue on the tip, it is possible to reduce the amount of the glue by touching followed by sliding the tip on an empty surface.

2.3.4 Move the cantilever tip on top of a well isolated single bead.

2.3.5 Approach the cantilever to the single bead slowly and make a firm contact with the bead (as indicated by the displacement of the laser beam position in the photodetector corresponding to a typical force range of 2-5 nN) for about 10 s during which a fine adjustment of the tip positioning laterally will help better locate the bead at the very end of the tip. Retract the tip at the end of the contact.

NOTE: The disappearance of the very bead from the original focal plane indicates a successful adherent event.

2.3.6 Demount the bead-modified cantilever carefully and store it in a cantilever box overnight for full solidification of the glue.

2.4 Fluorescence imaging of cellular response of macrophage to a single bead delivered by AFM.

NOTE: Fluorescence imaging was performed on a home-made objective-type total internal reflection fluorescence microscope based on a commercial microscope stand. This imaging system is equipped with 4 laser sources (405 nm, 488 nm, 561 nm, 647 nm), a splitter viewer for two-color detection, and an electron multiplying charge coupled device (EMCCD) for wide-field imaging.

2.4.1 Grow RAW264.7 cells on a glass coverslip at 37 °C in a 5% CO_2 humidified chamber.

2.4.2 Transfect Moesin-EGFP and PLCδ-PH-mCherry to RAW264.7 cells using a transfection kit (see **Table of Materials**) as per the manufacturer's protocol to fluorescently visualize Moesin and phosphatidylinositol 4,5-bisphosphate (PIP2) molecules respectively.

NOTE: Moesin has an ITAM motif that can activate Syk, a key player in phagocytosis. PIP2 is known to recruit Moesin to the cell membrane.

2.4.3 Put the glass coverslip with cells onto a sample chamber assembly and mount the assembly onto the AFM sample stage.

2.4.4 Mount the bead-modified cantilever to the AFM scanning head.

2.4.5 Run a force curve in an empty area and calibrate the force with the sensitivity from this curve and the spring constant measured in step 2.2.2.

2.4.6 Find a well-isolated cell with proper fluorescence intensities in both green (Moesin-EGFP) and red (PLCδ-PH-mCherry) channels with 488/561 nm excitations.

2.4.7 Deliver the naked 6 µm polystyrene bead with AFM to the cell surface with 1 nN constant force and 500 s contact time.

2.4.8 Record fluorescence image series of the cell in contact with the bead for analysis (typically 10 frames/s).

NOTE: To reduce the photobleaching of the fluorophores, a relatively low excitation power should be used for searching the cells of interest. In addition, an intermittent excitation scheme can be employed to prolong the fluorescence time traces if the dynamics of cell responses is on a slow time scale.

REPRESENTATIVE RESULTS:

Figure 4A shows typical force-distance curves from the binding interaction between single- T cell and single-DC in one approach-retract cycle. The light red curve is the extension curve and the dark red one is the retraction curve. Since the extension curve is typically used for indentation or rigidity-analysis, here only the retraction curve is concerned for cell adhesion. The minimum value (the green circle) in the curve gives a measure of the maximum adhesion force. The area under the curve (shaded area) represents the work (energy) required to separate the T cell from DC. Before a complete separation, sharp and stepwise rupture events are often observed. This is interpreted as membrane tethers being pulled out from the cell surface due to the strong binding at the cell/cell interface and then breaking discretely under the continuous pulling. The number, the height, and the length of the steps can be used to characterize the mechanical properties of the cell membrane[21]. For T cell-DC interactions, we are interested in the binding strength, thus only the maximum adhesion force is analyzed for each curve. **Figure 4B** compares adhesion forces between conventional T cell (Tconv)/DC and regulatory T cell (Treg)/DC. Tconv recognizes peptide antigens presented by antigen-presenting cells such as DC, whereas Treg is suppressor T cells that shut down Tconv -mediated immunity toward the end of an immune reaction. Clearly, Treg shows much stronger binding with DC than Tconv does (see **Figure 4C**). This is related to differential turnover rates of LFA-1 on the T cell surface between Treg and Tconv[2]. However, the exact mechanism that governs this is still under the investigation. The strong binding of Treg to DC is consistent with long binding times between Treg and DC observed in vivo[22]. More importantly, if a DC prebinds to a Treg, this DC cannot engage another Tconv forcefully (data not shown), leading to reduced T cell priming or immune suppression[2]. Thus, the force measurements on T cell/DC pairs provide new insight into the suppressive mechanism of Treg.

[Place **Figure 4** here]

Figure 5 shows how the fluorescently labeled phagocytic RAW264.7 cell responds to a single

naked 6 μm polystyrene bead delivered by AFM[4]. The experimental scheme is shown in **Figure 5A**. PIP2 is labeled by PLCδ-PH-mCherry (red) and Moesin is labeled by EGFP (green) (See **Figure 5B**). Although they are both enriched at the bead/cell interface, the accumulation of Moesin to a great extent traced the intensity changes of PIP2 as indicated by the kymograph plots showing the intensity profiles as the function of time along the indicated white line in **Figure 5C**. Together with the fact that Moesin can bind to PIP2 via its FERM domain[23], the result here suggests that upon engaging the bead, membrane PIP2 is first sorted at the contact site, which then induces membrane recruitment of Moesin whose ITAM domain then activates Syk-PI3K pathways, resulting in a phagocytic event. Thus, a new phagocytic pathway involving PIP2-Moesin-Syk axis has been identified and most importantly, it doesn't depend on any receptors on the cell membrane.

[Place **Figure 5** here]

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic representation of adding a small drop of biocompatible glue onto the mounted cantilever. The cantilever is mounted via a clamping spring on the glass-block holder that is installed on the AFM scanning head (not drawn here). When the scanning head stands on a leveled surface, the cantilever is vertically oriented as shown in the drawing. About 2 μL biocompatible glue can be added to the tip of the cantilever with a micro-pipette.

Figure 2: Experimental configuration of force-probing between a single T cell and DC. (A) Schematic drawing of the experimental configuration in which a T cell attached to the cantilever is brought to a DC grown on the substrate for force-probing. (**B**) Bright-field image of a T cell-functionalized cantilever and a DC. Scale bar, 20 μm.

Figure 3: Schematic representation of work flow for single-beads functionalization on the cantilever. Well separated micron-sized beads are prepared on the left side of the substrate and a tiny amount of epoxy glue is transferred onto the right side of the substrate through 3 successive gentle touches, resulting in 3 glue spots. Only the last spot with the least amount of the glue (indicated by a circle) is used to coat the very end of the cantilever. Approach the cantilever into the glue from the left and then move the cantilever backward once it is immersed into the glue to confine the glue at the very end of the cantilever. Bring the target bead underneath the cantilever and align them properly before making a firm contact (typically 2-5 nN) for the bead adhesion. When the bead is successfully functionalized on the cantilever, a new cantilever can be mounted to start a new functionalization cycle.

Figure 4: Treg cells show strong adhesion to DCs. (**A**) Typical force curves between a Tconv cell and a DC2.4. The light red curve is the extension curve and the dark red curve is the retraction curve. The minimum value of the dark red curve indicated by the green circle is the maximum adhesion force. The shaded area represents the energy required for complete separation between the two cells. (**B**) Force readings for T cells of indicated types interacting with DC2.4 cells. Each T-DC pair has at least 14 force readings and 5 independent DC-T cell pairs were probed for each cell type. Gray symbols are for Tconv cells (5); Dark blue, Treg cells (5). All data points

were collected on the same day. C. Mean forces of T cells of indicated types interacting with DC2.4 cells. Error bars are SEM. ***, P < 0.001 (Student's t-test).

Figure 5: Moesin signaling is downstream of PIP2 sorting driven by the solid structure. (**A**) Schematic representation of fluorescence imaging of bead/cell contact with a bead delivered by AFM. (**B**) PH-PLCδ-mCherry and Moesin-EGFP were co-expressed in RAW264.7 cells. A polystyrene bead was used to contact the cell surface. Images were taken at a 6 s interval for 500 s. Scale bar, 5 µm. C. Localization of PIP2 and Moesin at the site of contact (indicated with "*") was examined with kymographs generated from the indicated line. This figure has been modified from[4].

DISCUSSION:

AFM-based single-cell force spectroscopy has evolved to be a powerful tool to address the biophysical properties of living cells. For those applications, the cantilever needs to be functionalized properly in order to probe specific interactions or properties on the cells of interest. Here, the methods for coupling single T cell and single micron-sized bead to the tip-less cantilever are described respectively. To attach a single T cell to the cantilever, a biocompatible glue was chosen as cell adhesive. It is a specially formulated protein solution extracted from marine mussel. Its adhesivity originates from polyphenolic residues whose hydroxyl group can form hydrogen bonding with residues exposed on the cell surface in a nonspecific manner. This method of binding does not usually interfere with cellular functions of the bound cell. For proper adhesion, the conditions of the cells to be attached are also important. In case of freshly purified mouse CD4+T cells, they have to be first treated with human IL-2 overnight before they can be used for cantilever functionalization. Otherwise, they will undergo cell death rather quickly. If the cells are not in good conditions, even if they can be attached to the cantilever, they will very likely detach from the cantilever after only several cycles of force probing due to weaker adhesion strength, leading to a high failure rate of the force measurements. The downside of this biocompatible glue is that it is prone to oxidation, therefore the coupling procedure has to be finished as fast as possible, which can be challenging sometimes for operators. To reduce the oxidation of the biocompatible glue, it is highly recommended to prepare 30 µL aliquots in cryogenic vials filled with N_2 gas from the stock solution in a N_2 air environment and store them in liquid N_2 for the best performance. Most importantly, this biocompatible glue proves to be inert to T cells unlike other protein-based adhesives such as fibronectin and lectins which can cause unintended receptor clustering in the cantilever/cell contact zone and thereby activate T cells[24-26]. Thus, the effects of glues on the cells of interest have to be experimentally checked before they can be used for cell adhesion. This also applies to other cantilever-functionalization schemes relying on specific molecular recognitions, such as antigen/antibody, biotin/streptavidin, and Concanavalin A/carbohydrated-receptors, to couple single cells to the cantilever.

To probe the T cell/DC interactions, the optimized parameters as indicated in the protocol (step 1.7.2) were chosen for force spectroscopy. Among them, the set-point value and contact time are the two key parameters. Generally, the optimization of set-point begins with small values (0.1-0.2 nN). For cell/cell interaction, a high set-point value (>2 nN) leads to large deformation of

cells and a large contact area. This normally results in a large adhesion force readout. However, the probed force originates from both specific and nonspecific interactions at the cell/cell interface due to the complicated nature of cell surfaces, which, to some extent, scales with the contact area. Therefore, the larger the set-point value, the larger the contribution of nonspecific interactions. The latter is what should be minimized in any force measurements. In addition, to learn about nonspecific interactions, control force measurements such as using specific antibody or knockout (or knockdown) cells to block specific interactions of interest need to be considered to get basal force readout at given set-point values. This will help to identify the specific interactions in the testing measurements. As to the contact time, it depends on the time scale at which the probed molecular or cellular events take place. Therefore, prior knowledge of the events of interest is important. In case of T cell/inactivated DC interactions, adhesion molecule LFA-1 on T cell and ICAM-1 on DC form a binding pair. This intermolecular bonding occurs rather fast once the two counterparts are in position, but it takes time for the molecules to diffusion to the right places on the cell membrane where the counterpart molecules are ready for bonding, a diffusion-limited process. Therefore, we set 10 s of contact time to allow multiple LFA-1/ICAM-1 bonding pairs to form according to the shear flow experiments by Bongrand group[27,28]. Although a longer time can be set, at certain point, longer time doesn't help when the bonding formation is saturated at the interface. Moreover, long contact times may result in downstream cellular responses which may be not of the interest. On the other hand, if certain cellular events are of the interest, such as the phagocytic event described in the second application where the bead was partially engulfed by the macrophage, long contact time is absolutely necessary.

To accumulate enough statistics, 20 repeats of force curves were collected for each T cell/DC pairs and at least 14 forces curves were used for maximum adhesion force analysis. At least 5 pairs were tested in each testing groups. To remove sample histories, only fresh T cell/DC pairs were probed, meaning that the selected T cells and DCs for probing had no contact histories with any other cells. Although the above procedure makes the overall experiments time-and cost-consuming, it is perhaps the proper way to perform such force measurements on T cell/DC pairs.

Attaching single beads to the cantilever is relatively easy compared to cell-functionalization on the cantilever. Since epoxy glues with various solidification times are available in the market, it is possible to choose an epoxy glue with a long solidification time, typically on the order of hours. This allows multiple bead-modified cantilevers to be prepared in one beads sample. Alternatively, a UV-curable glue can be used, which can shorten the curing time significantly, about 10 min under UV light[28]. No matter what glues are being used, the only critical step throughout the protocol is to attach a minimum amount of glue to the tip of the cantilever. The excess glue will not only bury the bead easily, making the cell engage with the glue instead of the bead during the measurement, but also change the mechanical property of the cantilever, making force-calibration inaccurate. Therefore, it is important to control the contact area between the cantilever and the glue so that only a tiny amount of the glue is confined at the very end of the cantilever. The method described here is also applicable to other micron-sized solid particles, such as monosodium urate crystal[15], alum[14], and cholesterol crystal[29].

Apart from force-probing, AFM can also deliver a physical or chemical stimulus to the cells and

subsequent cellular events can be monitored fluorescently in real-time, as demonstrated in the second application. This opens a great opportunity to address spatiotemporally more complicated biological events, such as the formation of immunological synapse[30], which cannot be characterized in real-time by conventional approaches.

ACKNOWLEDGMENTS:

This work is supported by the National Natural Science Foundation of China General Program (31370878), State Key Program (31630023) and Innovative Research Group Program (81621002).

DISCLOSURES:

The authors have nothing to disclose.

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Figure

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Name of Material/Equipment
Material
10 µl pipette tip
15 ml tube
6 cm diameter culture dish
6-well culture plate
AFM Cantilever
β-Mercaptoethanol
Biocompatible glue
CD4+ T cell isolation Cocktail
DC2.4 cell line
Dextran-coated magnetic particles
EDTA
Epoxy
Ethanol
FBS
FcR blocker
Glass coverslip
Glass slides
H_2O_2 (30%)

H_2SO_4

HEPES

Magnet

Mesh nylon strainer

Moesin-EGFP

Mouse CD25 Treg cell positive isolation kit

Mouse $CD4^+$ Tcell isolation kit

NaOH

PBS

PE selection cocktail

Penicillin-Streptomycin

PLCδ-PH-mCherry

Polystyrene microspheres 6.0μm

polystyrene round bottom tube

Rat serum

RAW264.7

Recombinant Human Interleukin-2

Red blood cell lysis buffer

Regulatory T cell positive selection cocktail
RPMI 1640
Sample chamber
Streptavidin-coated magnetic particles
Transfection kit
Trypsin 0.25% EDTA
Tweezers

Equipment

20x objective NA 0.8
Atomic force microscope
Centrifuge
Fluorescence imaging
Humidified CO_2 incubator
Inverted light microscope

Company

Thermo Fisher
Corning
NALGENE nunc
JET
NanoWorld
Sigma
BD Cell-Tak
STEMCELL
A gift from K. Rock (University of Massachusetts Medical School, Worcester, MA)
STEMCELL
GENEray
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BD Falcon

STEMCELL

ATCC

Peprotech

Beyotime

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Life
Home made
STEMCELL
Clontech
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JD

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07312-5

352054

13551

Peprotech, 200-02-1000

C3702

18782C
C11875500BT

50001
631318
25200114
N/A

420650-9901
cellHesion200
Allegra X-12R

HERACELL 150i

Observer A1 manual

	Comments/Description
tipless cantilever	
	24mm diameter, 0.17mm thinckness customized

cloned in laboratory

Component: FcR Blocker,Regulatory T cell Positive Selection Cocktail, PE Selectio

Component:CD4+T cell isolation Cocktail, Streptavidin RapidSpheres, Rat Serum

Plan-Apochromat

n Cocktail, Dextran RapidSpheres,

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Title of Article:	Functionalization of atomic force microscope cantilevers with single-cells or -solid particles for single-cell force spectroscopy
Author(s):	Jiahuan Chen, Ying Xu, Yan Shi, Tie Xia

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Date:	2018-12-24

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Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

Response: Done

2. Please rephrase the Summary section to clearly describe the protocol and its applications in complete sentences between 10-50 words: “Here, we present a protocol to …”

Response: Done

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For example: Rapidspheres, Cell-Tak glue, Cellhesion200, etc

Response: Done

4. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

Response: Done

5. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions

should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.”

Response: Done

6. The Protocol should contain only action items that direct the reader to do something.

Response: Done

7. Please write the protocol in complete sentences.

Response: Done

8. Please ensure you answer the “how” question, i.e., how is the step performed? This includes graphical user interface, button clicks, knob turns, etc if any.

Response: N/A

9. Please explain why different cells types are used in different steps.

Response: Done

10. Please provide volumes and concentrations of all solutions used. Also provide the composition. If obtained commercial mention commercially obtained and see table of materials.

Response: Done

11. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

Response: The protocol highlighted in yellow is for filming.

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Response: Done

13. Figure 2: Please remove Cell tak and use generic term instead.

Response: Done

14. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol

b) Any modifications and troubleshooting of the technique

c) Any limitations of the technique

d) The significance with respect to existing methods

e) Any future applications of the technique

15. Please expand the journal title in the reference section.

16. Please sort the table of Materials in alphabetical order.

Response: Done

Reviewers' comments:

Please note that novelty is not a requirement for publication and reviewer comments questioning the novelty of the article can be disregarded.

Please note that the reviewers raised some significant concerns regarding your method and your manuscript. Please revise the manuscript to thoroughly address these concerns. Additionally, please describe the changes that have been made or provide explanations if the comment is not addressed in a rebuttal letter. We may send the revised manuscript and the rebuttal letter back to peer review.

Reviewer #1:

Manuscript Summary:

Reviewer report on "Functionalization of atomic force microscope cantilevers with single-cells or -solid particles for single-cell force spectroscopy" by J. Chen et al.

In this manuscript the authors provide a comprehensive description of the experimental protocols needed to perform atomic force microscope (AFM) cantilevers functionalization if live cells and particles to perform AFM force spectroscopy. The protocol will be very helpful for beginners and entry level scientist that want to perform force spectroscopy with functionalized particles. The experimental context of the protocol is adequately described (however it can be improved by providing more references). The manuscript needs to be revised for typographical errors.

Broadly speaking, this is a good and timely contribution. I do recommend its publication in JoVe after addressing the following minor comments:

Minor comments:

1) There are multiple typographical errors that need to be corrected. For example, line 59 instead of board it should say "broad". Please revise thoroughly for typos.

Response: “board” has been changed to “broad”.

2) Need to provide additional references. For example, the sentence in lines 64 and 65 needs a reference. Please provide more relevant a adequate citations through the manuscript.

Response: Two references have been added here:

Lamprecht, C., Hinterdorfer, P. & Ebner, A. Applications of biosensing atomic force microscopy in monitoring drug and nanoparticle delivery. Expert Opinion on Drug Delivery. 11 (8), 1237-1253, (2014).

Mu, L. B. et al. A phosphatidylinositol 4,5-bisphosphate redistribution-based sensing mechanism initiates a phagocytosis programing. Nature Communications. 9, (2018).

Reviewer #2:

Manuscript Summary:

The authors aim to present novel methods and procedures to functionalize AFM cantilevers with a single cell or a single particle. In the eye of the reviewer, the manuscript lacks novelty and most of the approaches are already described elsewhere. Furthermore, the manuscript contains many mistakes, literature to be cited is missing and large parts of the description are hard to follow (or to perform on your own after just reading the protocol). Finally, the presented method itself does not seem to have the advantages the authors claim with respect to "conventional" cantilever functionalization with cells (using e.g. ConA or FN), or at least there is no prove.

Overall, the present study lacks novelty, is not properly designed/described, literature is missing and a future reader will probably not be able to perform the measurements according to the manuscript/protocol.

Therefore, the reviewer suggests to reject the manuscript.

Major Concerns:

Title: The title itself can be misleading because the authors only show the functionalization of the tip with one kind of cell (T-cells) and one kind of particle (PS). Showing the usability also for other cell types and particles would have been beneficial. The wording "solid

particle" should be changed to just particle. (Minor)

Response: The title has been changed accordingly.

“**Functionalization of atomic force microscope cantilevers with single-T cells or -particle for immunological single-cell force spectroscopy**”

Whole text: Essential references are missing. Many of the materials used are missing in the list. A more thorough description of the AFM that has to be used (with a bigger z-range than the conventional 15 µm) is missing.

Response: More references have been added. Missing materials have been added into the Table of Matrials. The travel distance of Z-range of the AFM has been described as a note in the protocol 1.5.

“Note: To probe cell/cell interactions, an AFM with a Z-range larger than the conventional 10-15 µm is required in order to fully separate the two cells. The AFM used here has a Z-range of 100 µm, which is adequate to separate T cell from dendritic cell after cell/cell contact. ”

The authors do not describe where the DC2.4 and the RAW264.7 cells come from, how they cultivate them or how they prepare them for the measurements.

Response: The sources of DC and Raw cells have been added in the Table of Materials.

DC2.4 cell line was a gift from K. Rock (University of Massachusetts Medical School, Worcester, MA). RAW264.7 was from ATCC.

The authors often use the term "firm contact" (e.g. line 231, 301, 386 …) and get to this point by manually approaching the respective sample with the cantilever in "small" steps of 2 to 5 µm. This is not reproducible for any reader and prone to errors. The reviewer strongly suggests to work with defined force thresholds. The same applies for section 2.3.5

Response: We have defined “firm contact” with force thresholds throughout the protocol.

Compared to other literature, the parameters chosen for force spectroscopy (described in 1.5.2) seem to be quite low (low force set-point and low contact time).

Response: We have discussed why low force and low contact time were chosen for the measurements in the discussion section.

“To probe the T cell/DC interactions, the optimized parameters as indicated in the protocol (step 1.7.2) were chosen for force spectroscopy. Among them, the set-point value and contact time are the two key parameters. Generally, the optimization of set-point begins with small values (0.1-0.2 nN). For cell/cell interaction, a high set-point value (>2nN) leads to large deformation of cells and a large contact area. This normally results in a large adhesion force readout. However, the probed force originates from both specific and nonspecific interactions at the cell/cell interface due to complicated nature of cell surfaces, which, to some extent, scales with the contact area. Therefore, the larger the set-point value, the larger the contribution of nonspecific interactions. And the latter is what should be minimized in any force measurements. In addition, to learn about nonspecific interactions, control force measurements such as using specific antibody or knockout (or

knockdown) cells to block specific interactions of interest need to be considered to get basal force readout at given set-point values. This will help to identify the specific interactions in the testing measurements. As to the contact time, it depends on the time scale at which the probed molecular or cellular events take place. Therefore, prior knowledge on the events of interest is important. In case of T cell/inactivated DC interaction, adhesion molecule LFA-1 on T cell and ICAM-1 on DC form a binding pair. This intermolecular bonding occurs rather fast once the two counterparts are in position, but it takes time for the molecules to diffusion to the right places on the cell membrane where the counterpart molecules are ready for bonding, a diffusion-limited process. Therefore, we set 10 s of contact time to allow multiple LFA-1/ICAM-1 bonding pairs to form according to the shear flow experiments by Bongrand group. Although a longer time can be set, at certain point, longer time doesn't help when the bonding formation is saturated at the interface. Moreover, long contact times may result in downstream cellular responses which may be not of the interest. On the other hand, if certain cellular events are of the interest, such as the phagocytic event described in the second application where the bead was partially engulfed by the macrophage, a long contact time is absolutely necessary."

In 1.5.3 the authors describe that after each measurement pair of T-cell to dendritic cell, a new cantilever has to be cleaned, pre-calibrated, functionalized and used. This seems to be the biggest drawback of the presented method, increasing the experimental times, using up many cantilevers to get to the aspired statistics, and also making the approach more prone to errors.

Response: Indeed, the protocol described here involves multiple cycles of tip preparations, and is thus time-consuming, not cost-effective, and very demanding for the operator. However, we have no intention to emphasize that our protocol is better than the existing methods, rather we want to share with others the method that we believe is appropriate for probing the T cell/DC interaction with AFM. We have discussed this point in the discussion section.

"To accumulate enough statistics, 20 repeats of force curves were collected for each T cell/DC pairs and at least 14 forces curves were used for maximum adhesion force analysis. At least 5 pairs were tested in each testing groups. To remove sample histories, only fresh T cell/DC pairs were probed, meaning that the selected T cells and DCs for probing had no contact histories with any other cells. Although the above procedure makes the overall experiments time-and cost-consuming, it is perhaps the proper way to perform such force measurements on T cell/DC pairs."

In 2.1.1 3 µm polystyrene beads are described while in the rest of the manuscript, 6 µm beads are used.

Response: It is a typo. We have changed "3µm" to "6µm".

The whole section 2.1 to 2.3 (Cantilever functionalization with single polystyrene beads) has already been extensively described in literature and therefore lacks novelty.

Response: Agree.

The whole section 2.4 is only possible to be performed if one has a set-up where an AFM is combined with a fluorescence microscope. This should be already mentioned earlier in the text. The description of the fluorescence microscopy performance lacks details as does the description of the force spectroscopy method.

Response: We have added two sentences regarding what the two applications are about in the Introduction section to inform readers what set-up will be used.

“The first application is about probing T cell/DC interactions with AFM-SCFS to understand the suppressive mechanism of regulatory T cells from cell mechanics point of view. The second one involves combining AFM with live-cell fluorescence imaging to monitor cellular response of a macrophage to a solid particle in real-time to reveal the molecular mechanism of receptor-independent phosphatidylinositol 4,5-bisphosphate (PIP2)-Moesin mediated phagocytosis.”

In addition, a note has been inserted in 2.4 to describe the performance of the fluorescence microscope used here.

“Note: Fluorescence imaging was performed on a home-made objective-type total internal reflection fluorescence microscope based on a commercial microscope stand. This imaging system is equipped with 4 laser sources (405nm, 488nm, 561nm, 647nm), a splitter viewer for two-color detection, and an electron multiplying charge coupled device (EMCCD) for wide-field imaging.”

The authors claim that they only use the maximum adhesion to determine the strength of

T cell - dendritic cell interaction. The force curve shown in Figure 4.A exemplifies that there are many other parameters (work of pulling, tether formations (position, size)) that could in addition be quantified to better understand the ongoing process. Furthermore, they don't describe where the conventional and regulatory T-cells come from (or how to prepare them).

Response: Indeed, the work of pulling and tether formations can add more information of the ongoing process such as the elasticity of the cell membrane. However, the maximum adhesion force is the key parameter that dominates the duration of binding between T cell and DC in vivo (Journal of Experimental Medicine. 214 (2), 327-338, (2017)). Therefore, only the maximum forces were analyzed. Tconv(CD4+CD25-) and Treg (CD4+CD25+) were isolated from murine spleen. In the protocol section 1.1, only CD4+T cell preparation is described. At this stage, CD4+T cell contains both Tconv and Treg. Therefore, further separation is needed for specific T/DC pairs probing. However, for tip-modification, the procedure is identical for both Tconv and Treg. We have added a few steps for Tconv and Treg preparation in section 1.3.

In the discussion section the authors describe the main reason for using the bio-adhesive as glue, being that fibronectin activates T-cells according to literature. The cited literature performed tests with fibronectin T-cell contact times of 60 minutes, thus not being fully comparable for the shorter contact time between fibronectin and the cells in AFM measurements.

Response: It is well known that fibronectin can activate T cells via VLA (β integrin) family

of proteins at the cell membrane, therefore we chose the bio-adhesive instead of fibronectin to avoid introducing unwanted variables (downstream events of T cell activation), making the testing system relatively “simple”. Although the cited paper used 60min for their adhesion assays, the initial signaling (ligand-receptor recognition) at the cell surface happened long before that, typically on the order of seconds, followed by downstream signaling events, which take place on the orders of minutes to hours. Again, we don’t want any secondary cellular responses to be involved in T cell/DC interaction, making data interpretation difficult. Therefore, the bio-adhesive is more suitable for T cell-tip modification than fibronectin.

Reviewer #3:

Manuscript Summary:

In this paper the authors describe two methodologies for functionalizing AFM cantilevers: how to attach a cell or a bead to an AFM-cantilever for single cell force spectroscopy, along with examples of these modified-cantilevers applications. These protocols are widely used in the study of cell-cell cell-extracellular matrix adhesion and cell stiffness by AFM, while less common is nano-injection, which represents a very interesting additional application of bead-cantilever functionalization.

In general, the procedure described in the paper leads to the final outcome, but some steps

have to better explained and details have to be added to clarify the key steps of the protocol.

Major Concerns:

- In the introduction the number of citations regarding rigidity of single structures and whole cell is quite poor. There is a wide number of papers on this topic. Same for adhesion study about cell-cell interaction by single cell force spectroscopy. This should be revised.

Response: More references regarding rigidity and cell-cell interaction have been added in the Introduction.

"Radotic, K. *et al.* Atomic force microscopy stiffness tomography on living Arabidopsis thaliana cells reveals the mechanical properties of surface and deep cell-wall layers during growth. *Biophysical Journal.* **103** (3), 386-394, (2012).
Kuznetsova, T. G., Starodubtseva, M. N., Yegorenkov, N. I., Chizhik, S. A. & Zhdanov, R. I. Atomic force microscopy probing of cell elasticity. *Micron.* **38** (8), 824-833, (2007).
Scheuring, S. & Dufrene, Y. F. Atomic force microscopy: probing the spatial organization, interactions and elasticity of microbial cell envelopes at molecular resolution. *Mol Microbiol.* **75** (6), 1327-1336, (2010).
Berdyyeva, T. K., Woodworth, C. D. & Sokolov, I. Human epithelial cells increase their rigidity with ageing in vitro: direct measurements. *Phys Med Biol.* **50** (1), 81-92 (2005).
Sokolov, I., Dokukin, M. E. & Guz, N. V. Method for quantitative measurements of the elastic modulus of biological cells in AFM indentation experiments. Methods. 60 (2), 202-213, (2013)"

- Pg 4 point 1.3.3 which is the method used for AFM-cantilever calibration? They state that used the instruction in the manual of the AFM. This is too generic description. Which AFM microscope they used (JPK; Asylum; Park...) ? …and then which is the method they used for calibrating the spring constant of the cantilever? Even the method to obtain sensitivity should be described.

Response: More detailed information regarding how to obtain the sensitivity and spring constant has been provided in 1.5.3. However, due to JoVE policy, commercial language

cannot be published in the protocol, JPK cellhesion200 has to be appeared in the list of materials.

“1.5.3 Prepare a clean sample chamber filled with pure water and calibrate the cantilever in water solution by first running a force curve on the glass substrate to obtain the sensitivity (the slope of the linear fit over the repulsive part of the approaching curve) and then recording a thermal noise spectrum to extract the spring constant according to the instruction manual of the AFM.”

- Pg.5 point 1.4.9 I don't understand why, to attach a cell to the cantilever, they lowered the cantilever manually? Once sensitivity and spring constant are calibrated it is possible to apply a controlled force to contact the cell, more gentle and less destructive than lower manually. This should be discussed

Response: T cells do not adhere to the substrate, thus prone to drift away. With manual approaching and fishing, the operator has more flexibility in controlling aiming, positioning, and contact time. This makes fishing more efficient although the force applied to the target cell is not well controlled. Nevertheless, this manual approaching works reliably for us. Alternatively, it can be done in a gentle and less destructive way as suggested by the Reviewer. It is a matter of personal preference. We have added this alternative method of fishing as a note in 1.6.9,

“Note: This step can also be done by running a single force measurement in which set-point (the force applied to the cell) and contact time can be well defined in the software.

However, due to the non-adhesive nature of T cells, we find this manual approaching provides more flexibility in controlling aiming, positioning, and contact time than does the automatic approaching and it works reliably for T cell adhesion. We recommend that the future experimentalists try both the manual and automatic approaching to find which works better for their systems of interest."

- Pg.5 point 1.5.1. here it would be better to add a movie (or images) that shows the interaction between the cell attached to the cantilever and the DC2.4 grown on glass. This is the best way to show how measurements work. Moreover the parameters for measurements are chosen according to the cell and the kind of information the operators wants to extract. "Typical parameters" is too generic, it would be more important to explain how to chose these parameters.

Response: A movie will be made and will appear in the visualization part of the protocol. "Typical parameters" have been discussed in the discussion section.

"To probe the T cell/DC interactions, the optimized parameters as indicated in the protocol (step 1.5.2) were chosen for force spectroscopy. Among them, the set-point value and contact time are the two key parameters. Generally, the optimization of set-point begins with small values (0.1-0.2 nN). For cell/cell interaction, a high set-point value (>2nN) leads to large deformation of cells and a large contact area. This normally results in a large adhesion force readout. However, the probed force originates from both specific and nonspecific interactions at the cell/cell interface due to complicated nature of cell surfaces, which, to some extent, scales with the contact area. Therefore, the larger the set-point

value, the larger the contribution of nonspecific interactions. And the latter is what should be minimized in any force measurements. In addition, to learn about nonspecific interactions, control force measurements such as using specific antibody or knockout (or knockdown) cells to block specific interactions of interest need to be considered to get basal force readout at given set-point values. This will help to identify the specific interactions in the testing measurements. As to the contact time, it depends on the time scale at which the probed molecular or cellular events take place. Therefore, prior knowledge on the events of interest is important. In case of T cell/inactivated DC interaction, adhesion molecule LFA-1 on T cell and ICAM-1 on DC form a binding pair. This intermolecular bonding occurs rather fast once the two counterparts are in position, but it takes time for the molecules to diffusion to the right places on the cell membrane where the counterpart molecules are ready for bonding, a diffusion-limited process. Therefore, we set 10 s of contact time to allow multiple bonding pairs to form according to the shear flow experiments by Bongrand group. Although a longer time can be set, at certain point, longer time doesn't help when the bonding formation is saturated at the interface. Moreover, long contact times may result in downstream cellular responses which may be not of the interest. On the other hand, if certain cellular events are of the interest, such as the phagocytic event described in the second application where the bead was partially engulfed by the macrophage, a long contact time is absolutely necessary.

To accumulate enough statistics, 20 repeats of force curves were collected for each T cell/DC pairs and at least 14 forces curves were used for maximum adhesion force analysis.

At least 5 pairs were tested in each testing groups. To remove sample histories, only fresh T cell/DC pairs were probed, meaning that the selected T cells and DCs for probing had no contact histories with any other cells. Although the above procedure makes the overall experiments time-and cost-consuming, it is perhaps the proper way to perform such force measurements on T cell/DC pairs."

- Pg. 5 2.1.2 it is better to clean all tips and support for AFM with nitrogen flux cleaner than an air flow.

Response: We have changed "air flow" to "N_2 air flow" in 2.1.2.

- Fig. 3: to describe the attachment of the bead to the cantilever I would use a movie or a sequence of images acquired during the procedure. As discussed by the authors the step, where the glue is fished by the cantilever, is a tricky step and the best way to visualize the procedure is to show real images, rather than a scheme. By using a 40x and DIC microscopy, it is possible to clearly see the bead attached to the cantilever and confirm bead binding to the cantilever. At least one image should be shown.

Response: a movie will be added in the visualization part of the protocol.

- Chamber of measurements has a control in temperature?

Response: The sample and the AFM scanning head were inside an enclosure where the temperature and CO2 concentration can be controlled. The enclosure will be filmed in the visualization part of the protocol.

- In the results (line 351) they say that clearly Treg shows much stronger binding with DC than Tconv. Why? This reason should be briefly explained.

Response: The differential binding forces with DC between Treg and T conv is related to a differential turnover rate of LFA-1 on the T cell surface between the two cell types. However, the exact mechanism that governs this is still under the investigation. We have added this short discussion in the result section.

“This is related to differential turnover rates of LFA-1 on the T cell surface between Treg and Tconv. However, the exact mechanism that governs this is still under the investigation.”

Minor Concerns:

- Fig. 2 behind the attached cell, there is a white large spot on the cantilever. What is that? is the laser spot?

Response: The white large spot on the cantilever is indeed the laser spot.

- Fig. 4A: in the y axes what does it mean vertical deflection retract? In a force-distance graph the y axes reports the cantilever deflection, while in the graph the retraction and approaching curves registered are reported. This has to be amended.

Response: We have changed “Vertical Deflection Retract” to “Vertical Deflection”.

- Fig4: which test is used to determine difference and statistical significance?

Response: Student’s t tests were used here. We have added this in Figure 4 legend.

"***, P < 0.001 (Student's t test)."

Reviewer #4:

Manuscript Summary:

This work by Chen et al. presents 2 AFM tip functionalization protocols allowing the immobilization of single T cells and single polystyrene micro beads, respectively. Such tip functionalization protocols already exist in literature (e.g. Helenius et al., 2008; Beaussart et al., 2014), but the protocols are well detailed, and will easily be reproduced by other labs. One example of application is given for each protocol. Overall the paper is interesting and worth publishing, but i think it would benefit from a few more references and an extension of the introduction and discussion of the protocols.

I recommend this paper be published with minor modifications, after the authors reply to my questions / comments below:

Major Concerns:

- L. 52: Though appropriate, these are all references from the same group. Other groups have worked on cell biology using AFM based techniques, notably Muller, Dufrene or Alsteens, and one or two references should be added. They have a few review papers out (for instance, Muller et al. 2011, Force probing surfaces of living cells to molecular

resolution, but there are others)

Response: More references have been added in the introduction section.

“Muller, D. J., Helenius, J., Alsteens, D. & Dufrene, Y. F. Force probing surfaces of living cells to molecular resolution. *Nat Chem Biol.* **5** (6), 383-390, doi:10.1038/nchembio.181, (2009)

Muller, D. J. & Dufrene, Y. F. Atomic force microscopy: a nanoscopic window on the cell surface. *Trends Cell Biol.* **21** (8), 461-469, doi:10.1016/j.tcb.2011.04.008, (2011).”

- L. 61 : needs a reference for phagocytic cellular events. What about the uN that you mention earlier?

Response: The reference has been added for phagocytic events.

“Ng, G. et al. Receptor-independent, direct membrane binding leads to cell-surface lipid sorting and Syk kinase activation in dendritic cells. Immunity. 29 (5), 807-818, (2008).”

“μN” was only used to highlight the large dynamic range of forces with AFM. To remove any confusion, the sentence has been rephrased as follows.

“Owing to readily available cantilevers with various spring constant, the force range accessible to AFM is rather broad from a few piconewtons (pN) to micronewtons (μN), which adequately covers the entire range of cellular events involving forces from a few tens of pN, such as receptor-based single-molecule binding, to nN, such as phagocytic cellular events.”

- L. 65 : needs a reference again

Response: Two references have been added.

“Mu, L. B. et al. A phosphatidylinositol 4,5-bisphosphate redistribution-based sensing mechanism initiates a phagocytosis programing. Nature Communications. 9, (2018).

Lamprecht, C., Hinterdorfer, P. & Ebner, A. Applications of biosensing atomic force microscopy in monitoring drug and nanoparticle delivery. Expert Opinion on Drug Delivery. 11 (8), 1237-1253, (2014)”

- L. 66 - 68 : The authors should provide a few examples of what is possible with AFM coupled to fluorescence microscopy.

Response: We have added two example applications of combining AFM with fluorescence microscopy to address cellular responses to mechanical stimuli.

“For instance, AFM was used to determine the strains required to elicit calcium transients in osteoblasts. In this work, calcium transients were tracked fluorescently through calcium ratiometric imaging after the application of localized indentational forces on cultured osteoblasts with an AFM tip. Recently, AFM was employed to stretching collagen fibrils on which hepatic stellate cells (HSC) were grown and this mechano-transduced HSC activation was real-time monitored by a fluorescent Src biosensor, whose phosphorylation as represented by the fluorescence intensity of the biosensor is correlated with HSC activation”

- Authors should also add that it is safer to use Pirahna under a hood, wearing appropriate safety equipment, as the mixture will "splash" around the beaker.

Response: This notion has been added in 1.4.1

- L. 152 : 3 rinses is enough? Pirahna is very thick.

Response: We have rephrased the sentence as follows.

“1.4.2 Immerse the glass coverslip of 24 mm diameter into the piranha solution for 30 minutes and rinse thoroughly afterwards with sterile ultrapure water.”

- L. 183 : When you treat the cantilevers by Pirahna, do you let them for 30 min as well? Did you have any problem of loss of coating on your cantilevers? Also, did you try UV-Ozone cleaning instead of plasma cleaning? It should be an appropriate alternative.

Response: Yes, the cantilevers stayed in Pirahna for 30min. And we did notice loss of coating on the cantilevers. However, as long as they give adequate sum signal in the photodetector, they can be used. We don’t have Ozone cleaner, but we have mentioned it in 1.5.1 as an alternative approach.

- What is the sample chamber? Is it a petri dish? Also, could you provide a little bit more information about the living cell environment enclosure you use? Is it commercially available or home-made? (JPK has a heating module, but I don't think I've seen a module for gas content control?). Is it absolutely necessary to work at controlled CO2 and temperature, or do you observe you can work for a few hours on living cells without this?

Response: The sample chamber is a home-made fluid chamber containing two stainless steel parts that sandwich the round glass substrate with proper rubber O-ring sealing.

This sample assembly and the home-made living cell environment enclosure will be filmed in the visualization part of the protocol. To keep the cells in their best conditions, the measurements were always carried out at 37°C with 5% CO2 inside the enclosure. In addition, the measurements on Tcell/DC pairs typically run more than 6 hours. Without proper environmental controls, the cells will not survive such long time and this also introduces temperature- and pH-dependent cellular processes, making data interpretation more complicated. Therefore, it is always recommended that proper environmental controls are necessary for living cell experiments.

- L. 210 : 1.4.6 : What is the proportion of tip breaking in this stage of the procedure? Wouldn't it be easier to deposit the Cell-tak on a glass slide, then use the AFM head to gently dip the tip in the glue, like you do for the other protocol? It would not be as fast, but I would think this would lead to less breaking. Or is it possible to incubate the entire tip in Cell-Tak?

Response: Tip breaking is unlikely to happen if the operator has steady hands. Putting Cell-Tak on a glass surface, dipping the tip using the head, and changing back to the cell samples will take more time, which is not good for the performance of Cell-tak. Oxidation is a key issue here. Incubating the entire tip in Cell-Tak, mounting the tip, and aligning the laser also involves too much time, again not good for Cell-Tak. The reason that we come up with the described procedure is to shorten the exposure time of Cell-Tak in air, thus increasing the success rate of cell-adhesion on the cantilever.

- 1.4.7 : Why do you calibrate the tip 2 times?

Response: In principle, the tip calibration in pure water is good enough. However, the spring which fixes the position of the cantilever on the glass block becomes loose gradually with time. This makes the cantilever prone to small displacement with respect to the glass block when the cantilever goes in and out of the sample solution multiple times. Therefore, we measure the sensitivity twice. Since the issue is hardware specific, we delete the second calibration in the protocol to make the procedure more generic.

- Figure 1 : I recognize JPK glassblock, and the pipet tip, but it will be more readable with a more complete legend (pipet tip, cantilever, glassblock or AFM tip holder).

Response: Proper labeling has been added in the figure1.

- L. 232 : Do you control the force during this step? Can you precise what you mean by a firm contact, do you lower the probe until the laser moves in the photodetector, or do you lower it until a certain setpoint is reached in the photodetector? Did you try several forces and what works best? What is the advantage of approaching manually compared to having an automated approach with controlled force?

Response: We did not control the applied force in this step and we lower the probe until the laser position moves in the photodetector. However, the force is about 0.5-1.5 nN. We have quantified “firm contact” in the protocol.The force is not the only factor that influences the successful cell-adhesion. The performance of Cell-Tak and the conditions of cells also affect the cell-adhesion.

T cells do not adhere to the substrate, thus prone to drift away. With manual approaching

and fishing, the operator has more flexibility in controlling aiming, positioning, and contact time. This makes fishing more efficient although the force applied to the target cell is not well controlled. Nevertheless, this manual approaching works reliably for us. Alternatively, it can be done in a gentle and less destructive way as suggested by the Reviewer. It is personal preference. We have added this alternative method of fishing as a note in 1.6.9,

"Note: This step can also be done by running a single force measurement in which set-point (the force applied to the cell) and contact time can be well defined in the software. However, due to the non-adhesive nature of T cells, we find this manual approaching provides more flexibility in controlling aiming, positioning, and contact time than does the automatic approaching and it works reliably for T cell adhesion. We recommend that the future experimentalists try both the manual and automatic approaching to find out which works better way for their systems of interest."

- L. 239: 1.4.10 : Can the cantilevers that do not lead to a successful immobilization be recycled in any way? Through Pirahna + UV-ozone treatment?

Response: Yes, the cantilevers can be recycled up to three times with Pirahna treatment according to our experiences.

- Fig 3 is nice!

Response: Thanks!

- L. 274 : 2.1.5 : This protocol is very similar to the one used to prepare colloidal probes for SCFS in microbiology, please reference Beaussart et al., 2014 (Quantifying the forces guiding microbial cell adhesion using single-cell force spectroscopy, Nature Protocols volume 9, pages 1049-1055) somewhere in the manuscript.

Response: UV-curable glues have been added in the discussion section together with this reference.

“Alternatively, a UV-curable glue can be used, which can shorten the curing time significantly, about 10min under UV light.”

- L. 301: 2.3.5 : again, what do you mean by firm contact?

Response: “Firm contact” means the laser position changes in the photodetector. The force is typically 2-5nN for bead adhesion. We have added the force range for “firm contact”

- L. 307: The use of a UV-curable glue would allow you to gain time (cures in ~ 10 min under UV light, compared to hours for epoxy glue) (Beaussart et al., 2014).

Response: UV-curable glues have been added in the discussion section together with this reference.

“Alternatively, a UV-curable glue can be used, which can shorten the curing time significantly, about 10min under UV light.”

- L. 329: how long do you stay in contact?

Response: Typically 8-10min. For the results shown in Fig.5, the contact time is 500s.

We have defined the contact time in the protocol.

- Fig. 4B : a bit confusing for me with circles, stars, triangles,…, could you simplify a bit? It would be easier to read with the same symbol for all Treg and another symbol for Tconv. In addition, maybe choose colors that will contrast more with black and gray?

Response: A simplified Fig 4B has been made as suggested by the Reviewer.

- L. 346: maybe add a reference for the tethers

Response: A new reference has been added.

“Sun, M. Z. et al. Multiple membrane tethers probed by atomic force microscopy. Biophys J. 89 (6), 4320-4329, (2005)”

- What is the difference between conventional T cells and regulatory ones? I'm a novice in that field, and a sentence or two should be added for comprehension.

Response: a short description about Tconv and Treg has been added.

“Tconv recognizes peptide antigens presented by antigen-presenting cells such as DC, whereas Treg is suppressor T cells that shut down Tconv -mediated immunity toward the end of an immune reaction.”

- Fig 5 C : the kymograph seems to indicate that the cell has moved over time? If so, how do you adjust for AFM tip contact? Can you keep the force constant while moving? Also, please indicate the contact site on the kymographs (I mean orientate the white line, A-B or with an arrow, on fig 5B and 5C).

Response: Yes, the cell indeed moved over time. However, the cell would try to internalize the bead instead of moving away from it, so there is no need to adjust AFM-tip-bead/cell contact. In addition, the AFM software allows a constant force to be applied even the cell moves or deforms. We have added proper labeling to indicate the orientation of the white line and the kymographs.

- How different is Cell-Tak to a simple polydopamine solution?

Response: In principle, these two are very similar in term of surface coating performance. However, it is much faster and more convenient to work with Cell-Tak for AFM-tip coating. In addition, Cell-Tak is a real polymer compared to polydopamine, which makes Cell-Tak more reliable for cell adhesion on AFM cantilevers.

- L. 436 : again, a UV-curable glue is also a good alternative, that allows to modify your cantilever as much as needed and does not have time constraints until you cure it under UV-light. The only downside is the necessity to have a UV lamp.

Response: UV-curable glues have been added in the discussion section together with this reference.

“Alternatively, a UV-curable glue can be used, which can shorten the curing time significantly, about 10min under UV light.”

- L. 449 : needs a reference

Response: A new reference has been added.

"Hosseini, B. H. *et al.* Immune synapse formation determines interaction forces between T cells and antigen-presenting cells measured by atomic force microscopy. Proceedings of the National Academy of Sciences of the United States of America. **106** (42), 17852-17857, (2009)"

- Did you try to deliver other beads to the cells in figure 5? Other diameters, or other materials? How different is the cell reply? Is it material dependant? Force dependant?

Response: We only tried polystyrene beads in Figure 5. However, we have tried different microsized materials on DC cells such as alum, MSU (monosodium urate) crystal, cholesterol crystal. The DC cells responded differently. The outcome is largely material-dependent.

Minor Concerns:

- L. 54 : even whole cells

Response: revised

- L. 59 : broad

Response: revised

- L. 68 : tool

Response: revised

- L. 77 : due to their non-adhesive nature, are immobilized on a cantilever via a biocompatible glue in solution

Response: revised

- L. 100 : g/rcf = g RCF (relative centrifugal force)

Response: revised

- L. 147 : prepare a piranha solution

Response: revised

- L. 420 : even if

Response: revised

Reviewer #5:

Manuscript Summary:

The submitted manuscript describes a method to immobilize a single living cells or a single solid particle (i.e. polystyrene bead) on a tipless AFM cantilever for subsequent single-cell force spectroscopy (SCFS) experiments.

Title, introduction section, referencing

General points: The SCFS technique is by now well-established and has been used to quantitate cell-surface or cell-cell adhesion for a number of different cells types. Likewise, bead attachment to cantilevers is a well-established technique frequently used in cell adhesion and elasticity measurements. There are a number of excellent reviews and

methods descriptions describing these SCFS applications in detail, but unfortunately, only few of them are cited. A representative selection of these overview articles could therefore be added to the introduction section to point readers to other useful descriptions of this technique. Instead, references to some of the highly specific papers could be removed from the first part of the introduction. Also, I think it would be helpful to include at least one of the original SCFS papers, for instance from the Gaub group, to establish the historical development of his technique.

Response: Addition references have been added as suggested by the Reviewer, including the original AFM-SCFS paper from Gaub group.

"Benoit, M., Gabriel, D., Gerisch, G. & Gaub, H. E. Discrete interactions in cell adhesion measured by single-molecule force spectroscopy. Nature Cell Biology. 2 (6), 313-317, (2000)."

However, SCFS has been less frequently used to study immune cell adhesion and a JOVE contribution providing an informative introduction to suitable SCFS techniques specifically applied to study immune cell adhesion could indeed be very helpful. Here I see the real impact of this contribution. Consequently, the manuscript would benefit from an even tighter focus on SCFS applications for immune cell studies, including a clearer motivation for the described experiments. For instance, the motivation for using solid beads (to study macrophage-mediated engulfment I presume?) does not really become clear throughout the manuscript. Likewise, the title should be changed to reflect the immune cell-specific content of the manuscript. I believe there are also few additional important studies that could be cited, such as papers from the Bongrand group and others.

Response: We have changed the title to highlight the immunology-oriented content of the manuscript and have clarified the motivations behind the example applications in the introduction. In addition, the work from Bongrand and other was cited as well.

Methods section

This sections contains much useful information and in principle readers should be able to perform the described experiments according to the provided instructions. However, parts are confusing, such as the separation of spring constant and sensitivity determination of the cantilevers. There may be good reasons to split up these steps, instead of performing them together in situ, but this should be motivated better.

Response: For, single T cell functionalization, we did tip calibration twice. One in pure water and the other in cell solution. In principle, the tip calibration in pure water is good enough. However, the spring which fixes the position of the cantilever on the glass block becomes loose gradually with time. This makes the cantilever prone to small displacement with respect to the glass block when the cantilever goes in and out of the sample solution multiple times. Therefore, we measure the sensitivity twice. Since the issue is hardware specific, we delete the second calibration in the protocol to make the procedure more generic.

Also, a clearer structuring of the different experimental sections using more subheaders would be helpful to prevent the reader from getting lost. Many subpoints are very short

and appear to be single sentences copied from previous publications. For example Line 144: "All experiments performed were approved by the animal research committee at the Tsinghua University". This fact is not informative to a wider audience.

Response: We have restructured the protocol as requested.

The entire Methods section should be thoroughly revised to focus less on the authors´ specific research interests, but to provide a more general description of the SCFS application for immune cells. For example, the authors provide no motivation why cells should be transfected with moesin-GFP and PLCdelta-PH-mCherry. This may have been a valuable strategy in one of the autors´ recent (and excellent) publication, but it is not generally required for SCFS, and other fluorescent markers may work as well. It is fine to describe these particular markers as an example for the power of combined fluorescence microscopy with SCFS, but a clearer distinction between essential and elective steps in SCFS should be made.

Response: More information has been added in the introduction, protocol, and representative results sections to explain the motivation of the experiment described.

Figures

The figures are overall informative and of good quality.

Discussion

The discussion contains many useful points that should be considered when using SCFS

for investigating immune cells. Additional reasons, such as the good control over cell-cell contact time and force, could be added. Also, the possible advantage of using CellTak over other immobilization approaches, such as lectin-based techniques, which may cause unintended receptor clustering in the cantilever-cell contact zone and thereby Tcell activation, could be expanded upon.

Response: Discussion on the force and contact time has been added. In addition, lectin-based cell adhesion was discussed as well.