Title

Biochemical Stimulation of Immune Cells and Measurement of Mechanical Responses Using Atomic Force Microscopy

Journal

Current Protocols in Chemical Biology, 11(2)

ISSN

2160-4762

Authors

Hu, Kenneth H
Bruce, Marc A
Liu, Jianwei
et al.

Publication Date

2019-06-01

DOI

10.1002/cpch.63

Peer reviewed

HHS Public Access

Author manuscript

Curr Protoc Chem Biol. Author manuscript; available in PMC 2020 June 01.

Published in final edited form as:

Curr Protoc Chem Biol. 2019 June ; 11(2): e63. doi:10.1002/cpch.63.

Biochemical stimulation of immune cells and measurement of mechanical responses using atomic force microscopy

Kenneth H. Hu, **Marc A. Bruce**, **Jianwei Liu**, and **Manish J. Butte***

Department of Pediatrics, Division of Immunology and Department of Microbiology, Immunology, and Molecular Genetics, UCLA, 10833 Le Conte Avenue, MDCC Building, Room 12-430, Los Angeles, CA 90095

Abstract

This manuscript details methods to ligate cell-surface receptors on live cells with precise spatiotemporal control using an atomic force microscope (AFM) to deliver ligands. This approach can be used to image cellular responses upon triggering of T cell receptor when the AFM is mounted on an optical microscope. Moreover, the AFM measures forces generated by the cell during the contact. Using AFM to trigger cellular responses adds an important capability to the field of mechanobiology. We demonstrate here the steps needed to incorporate anti-CD3 antibodies or other molecules onto an AFM cantilever and triggering T cells.

Keywords

Atomic force microscope; T-cell; mechanobiology; cantilever; mechanical cell forces

Introduction

The uses of atomic force microscopy (AFM) in biology have expanded beyond early applications in measuring the topography of cells to include force spectroscopy, measuring polymer biophysics, observing protein unfolding, measuring cellular elasticity, mapping molecular interactions and receptors, measuring adhesion of cells with one another, and delivering and measurement of forces of cells. In this protocol, we describe a method to activate cell-surface receptors on live cells using an AFM-delivered ligand. AFM offers exquisite control over the movement of the tip; A typical AFM can easily achieve precisions of a few nanometers in positioning within a timespan of a few microseconds. Using this capability allows for contact of the molecules attached to the tip with a cell-surface receptor with high spatiotemporal precision. Subsequently, different responses of the cell can be imaged using conventional optical microscopy. The mechanical responses of the cell can also be measured using the AFM tip itself – this kind of measurement can be made of spontaneously moving cells, such as cardiomyocytes (Liu et al., 2012), or of cells that mechanically respond to contact or receptor ligation (Hu et al., 2014; Hu and Butte, 2016).

*Corresponding Author: mbutte@mednet.ucla.edu, Phone: 310-825-6481.

Conflicts of Interest

The authors cite no financial conflicts of interest. US Patent US8495760B2 was issued to the authors for “Atomic force microscope manipulation of living cells” but the patent was unlicensed and abandoned.

Changing the mechanical control of the tip allows for “clamping” the forces experienced by the receptor. Taken together, this use of AFM allows for unprecedented visualization and manipulation of cells.

A variety of AFMs have the capability to perform the studies described in the protocols here, including the Asylum MFP-3D Bio (the AFM employed in this study) and instruments from other manufacturers including Bruker, JPK, and others, including home-brew instruments. The key features needed in a suitable instrument include a heated stage or other way to ensure the cells remain at physiological temperature throughout the experiment and an enclosure to ensure that the cells remain submersed in media. The AFM employed should be able to work with small forces and soft cantilevers. The forces measured and delivered by the AFM are often well below 200 pN, and the cantilevers employed usually have a spring constant well under 100 pN/nm (0.1 N/m).

The first protocol below describes linking molecules onto the cantilever tip. An alternative of the first protocol is offered that describes how to link a single molecule onto the tip. The second protocol describes the use of AFM to ligate T cell receptors (TCRs) on T cells and measuring their responses.

Basic Protocol 1: Attachment of antibodies or peptide-MHC onto the cantilever tip

This protocol describes the attachment of stimulatory molecules like antibodies or peptide-MHC onto the cantilever tip. These molecules are then used in the second protocol below to contact T cell receptors (Fig. 1). To ensure that contact with the cell is reversible, molecules can be attached through a strong link like biotin-streptavidin or through covalent chemistry. The cantilever tip is prepared and coated with a silane that allows for covalent attachment of streptavidin. Then a biotinylated molecule is attached to the streptavidin on the tip.

Materials

(3-Mercaptopropyl)-trimethoxysilane (Sigma Aldrich)

Streptavidin (Jackson ImmunoResearch)

Sulfosuccinimidyl 6-(3’-(2-pyridyldithio)propionamido)hexanoate (sulfo-LC-SPDP, ThermoFisher)

EDTA (Sigma Aldrich)

PBS (ThermoFisher), Ca2+ and Mg2+ free

Biotinylated I-A^b-OVA (323–339) (called peptide-MHC in the protocol) (NIH tetramer core facility, http://tetramer.yerkes.emory.edu/)

Biotinylated anti-CD3 (Biolegend, clone 145–2C11)

Poly-dimethylsiloxane (PDMS, Dow Corning Sylgard 184)

Acetone

Isopropanol

Distilled, deionized water Zeba Desalting spin column (0.5 mL size, Thermo Scientific)

AFM cantilever tips (we mostly use HYDRA6R-200N from AppNano)

Plasma (Harrick Plasma Cleaner PDC-001)

Vacuum oven

Desiccator

Chemical fume hood

10 cm petri dishes, polypropylene

8 mm biopsy punch (Robbins True-Cut Disposable)

streptavidin solution (reagents and solutions)

Plasma cleaner (we use the Harrick PDC-001 Expanded plasma cleaner with a quartz sample tray)

5 cm glass dish (e.g., Corning Pyrex petri dish with cover)

Plastic forceps, solvent resistant (e.g., Ted Pella Celcon tweezers)

Note: *Perform all steps involving volatile solvents in the fume hood wearing appropriate personal protective equipment including face & eye protection, gloves, and coat.*

1. Prepare petri dishes with a floor of PDMS.

   Fill the bottom of multiple, standard 10 cm petri dishes with a thin layer (2–5 mm) of PDMS (~10–20 mL of PDMS), tap the dish to mobilize and remove air bubbles, place under vacuum in desiccator for 24 hours to cure.

2. Store the PDMS treated petri dishes covered with a lid, to prevent dust accumulation.

   Before using the these treated petri dishes, remove dust from the PDMS using a piece of scotch tape. These dishes can be prepared weeks ahead of time and stored in ambient conditions. We use a biopsy punch to cut many 8 mm circles from the PDMS petri dishes. These circles are large enough to serve as a platform holding one cantilever each and allow for the various coating and wash steps below.

3. Measure the spring constant of each cantilever prior to coating with biomolecules.

This step is essential in order to achieve precise mechanical measurements and should be conducted prior to coating with biomolecules. Follow the manufacturer's directions for your AFM. We use the thermal noise method for measuring the spring constant (Butt and Jaschke, 1995). Alternatively, the geometry-based Sader method is also popular for measuring the spring constant (Sader et al., 1999).

4. Expose cantilever tips in plasma for one minute.

   A typical experiment might entail five to ten cantilevers at a time. Gently place one cantilever each onto clean circular pads of PDMS prepared above. Ensure that the tip of the cantilever is facing upwards and not down into the face of the PDMS when placed, otherwise the tip will be damaged. Place the cantilevers resting on their pads into the plasma cleaner and close the door. Engage the vacuum pump. When the pressure is suitably low (100–200 mTorr), activate the RF power. Set a timer for one minute. Open and close the vacuum slowly to prevent a "wind" of air tossing the cantilevers around. Securing the cantilevers on pads of PDMS is typically sufficiently adherent to prevent this movement. After one minute, turn off RF power and slowly vent the chamber to access the cantilevers.

5. Submerge cantilevers in acetone plus 4% (3-Mercaptopropyl)-trimethoxysilane for 2 hours.

   Fully submerge the cantilever using approximately 10 mL of solution, pipette the liquid into a small glass dish (5 cm) and then placed tips into the dish using forceps. Transfer the tip carefully to another clean PDMS pad for subsequent steps.

6. Wash cantilevers with excess 100% acetone.

7. Wash cantilevers with excess 100% isopropanol.

8. Wash cantilevers in 100% distilled, deionized water.

9. Place cantilevers in vacuum oven and cure at 110 °C under vacuum for 1 h.

10. While the cantilevers are curing, prepare the streptavidin solution that will be used to attach the biomolecule to the cantilever.

    First, prepare a 20 mM sulfo-LC-SPDP solution in ultrapure water (2 mg of sulfo-LC-SPDP in 200 μL of ultrapure water). Then, in a separate Eppendorf tube add 3.125 μL 20 mM Sulfo-LC-SPDP in water to a solution of 250 μg of streptavidin in 125 μL of PBS containing 1 mM EDTA.

11. To remove un-reacted cross-linker, buffer exchange the solution with a Zeba Desalting spin column.

    Equilibrate the desalting column with PBS-EDTA (1 mM). Transfer all the solution from step 10 onto the column, then spin down following the manufacturer's directions (1500 g x 1 min). Repeat this step two additional times

to exchange the buffer and remove the unreacted cross-linker. We typically end with the same volume at the end as at the start (125 μL).

12. Remove cantilevers from oven and return to ambient conditions and place onto a fresh PDMS pad (as prepared in step 2).

13. Pipette 25 μL of the Streptavidin-sulfo-LC-SPDP onto each silanized cantilever, allow to react overnight at 4 °C.

The reaction will be performed within the droplet that fully submerges the cantilever and rests on the pad of PDMS. The entire cantilever and chip should be contained within a droplet of solution. If portions of the chip are not submerged, the cantilever may not get coated; pipette more solution onto the chip. To reduce evaporation, we gently place the pads (holding the cantilever and the crosslinker droplet) into a polypropylene petri dish. In the same dish, we placed wadded tissue (e.g., Kimwipe) that has been wetted with water into the sides of the dish. These tissues provide humidity and reduce evaporation. Then the plastic cover is placed onto the dish and placed into the refrigerator overnight.

14. Remove unbound Streptavidin using a pipette and wash three times with PBS.

To wash the cantilever, place a 25 μL drop of PBS onto the PDMS pad. Gently pick up the cantilever with the forceps and remove from the previous drop. Try not to transfer liquid. Place the cantilever into the droplet of PBS. Ensure the entire chip is submerged within the droplet. Wait 15 min. Transfer to another fresh drop of PBS for another 15 min. Finally, transfer to another fresh drop of PBS for another 15 min.

15. Subsequently, submerge the cantilever in 25 uL of 1 % BSA in PBS to block unbound binding sites.

Follow a similar strategy as step 13 and 14 for immersing cantilevers in droplets of solution. Blocking can be done at room temperature for 4 hours or overnight at 4 °C.

16. Wash three times with PBS.

Follow the process of step 14.

17. Transfer the cantilever into a drop of 25 μL of biotinylated molecule. Concentrations of 25 μg/mL were used successfully for coating the cantilever with the anti-CD3 antibody, and 50 μg/mL were coating the cantilever with pMHC. Cover at ambient conditions for 1 h.

To avoid evaporation during the coating steps, follow the procedure in step 13 for placing the pads and cantilevers and coating solutions into a petri dish with wetted tissue. Longer times than one hour are fine but will not significantly increase coating of proteins. Do not allow the drop to dry out, even momentarily, from this point on.

18. Wash cantilever with PBS twice.

Follow the process of step 14.

19. Store the prepared cantilevers submerged in PBS at 4 °C until ready to use. We recommend preparing cantilevers the day before an experiment whenever possible.

    Cantilevers coated in the manner of this protocol and stored submerged in PBS will be suitable for experiments for up to one week after preparation.

Basic Protocol 2: AFM contact with T cells

This protocol describes the use of the AFM cantilever to make contact with a T cell and measuring the responses optically and mechanically. Because the optical microscope and the AFM are not usually designed to collect data together, major design considerations are needed to successfully integrate AFM with video data. First, with almost all commercial AFMs, the software controlling the microscope and the AFM are on two separate computers or using two different software programs. This separation makes difficult the synchronization of optical events (e.g., “movement of cell occurred on this video frame”) with mechanical events (e.g., “the cell began pushing”). A second consideration is that AFM “events” are typically captured at rates of 10,000–1,000,000 measurements per second, whereas optical events are typically recorded at a rate of 1–50 frames per second. Combining these two radically different data rates requires special software.

To address these concerns, we capture AFM data and microscope events onto a single computer using LabVIEW software and a data acquisition board. The microscope sends TTL events for each frame, which are recorded as digital events. The AFM sends analog signals for deflection and z-piezo position. The events are captured onto a single event stream and written out to the hard drive as fast as possible. Later, a MATLAB program is used to decimate and filter the events, synchronize AFM data streams with image acquisitions and perform analyses.

Materials

10 cm petri dishes, polypropylene

4 mm biopsy punch (Robbins True-Cut Disposable)

8 mm biopsy punch (Robbins True-Cut Disposable)

Poly-D-lysine (Sigma Aldrich)

PBS (ThermoFisher), Ca2+ and Mg2+ free

Nylon mesh strainer, 40 μm (BD Falcon)

Conical tube 50 mL (BD Falcon)

Syringe 3 mL, sterile, individually wrapped (BD)

Easy-Sep T cell isolation kit (Stemcell Technologies)

Media: RPMI 1640 (Life Technologies) + 10% v/v FBS (Life Technologies, sterile filtered, heat inactivated) + Pen/Strep (Thermo Fisher 10,000 U/mL, 100x)

PCI-6115 acquisition board (National Instruments)

BNC-2110 Shielded Connector Block (National Instruments)

MATLAB software (Mathworks)

Labview software (National Instruments)

Glass bottom dishes (FluoroDish 50 mm from World Precision Instruments)

AFM (Asylum MPF-3D Bio with stage mounted upon a Nikon Ti-E microscope)

1. Prepare glass-bottom FluoroDish with Poly-L-Lysine.

   The design consideration is to slow the cells from their other rapid crawling but not entirely immobilize the cells. A typical concentration we use is 0.1% diluted in water. 2 mL of Poly-L-lysine will fully coat the bottom of a glass-bottom fluorodish. Pour the poly-lysine out of the dish and wash the dish with 2 mL of water twice. Finally, place 2 mL of PBS in the glass-bottom dish.

2. Prepare T cells (typical sources include human blood or mouse splenocytes)]

   Institutional approvals and protocols should be established before using mice. Mouse T cells can be purified from the spleen following an approved euthanasia procedure. Remove the spleen from the peritoneum in sterile fashion. Place a nylon mesh strainer onto the top of an open, 50 mL conical tube. Wet the strainer with 5 mL of media. Place the spleen onto the filter. Gently but thoroughly mash the spleen with the plunger of a 3 mL syringe. Intermittently rinse the filter with media, collecting all the cells that flow through the strainer in the conical. Continue to mash with plunger until the spleen is completely dissociated. Centrifuge the suspension of cells to form a pellet (500 g x 5 min). Aspirate the supernatant and resuspend the cell pellet with antibodies as per the Easy-Sep T cell isolate kit protocol. Perform the isolation protocol as per the manufacturer's directions.

3. Label T cells with fluorophores (such as the calcium sensitive dye Fluo-4) or transduce with fluorescent proteins. These steps are out of the scope of this protocol and are detailed elsewhere (Hu and Butte, 2016).

   Briefly, the T cells are incubated with the calcium sensitive fluorophore and afterwards washed to remove fluorophores that did not get into the cell. This step is not optional. To ascertain the biological impact of ligating the T cell receptor (step 13 below), some kind of labeling of the T cells is essential.

4. Prepare the AFM by performing a inverse optical lever sensitivity (invOLS) calibration using a clean FluoroDish with temperature equilibrated to 37 °C.

The setup and calibration of the AFM is dependent on each instrument and the manufacturer's instructions. The calibration of the optical lever sensitivity (or its inverse) enables the instrument to recognize the mounting of the cantilever in its holder and how much movement of the z-piezo results in movement of the cantilever based on light reflected from the back of the cantilever.

5. Place the FluoroDish onto the temperature-controlled AFM stage and set to 37 °C. Fill the FluoroDish with 3 mL media and allow to equilibrate for 30 min. Pipette T cells into the petri dish and let settle/equilibrate for 10 min at 37 °C.

   The area of the FluroDish is large compared to the area accessible by the AFM cantilever, which is typically the central 0.5–1 cm. Thus, try to confine cells to the center of the petri dish when pipetting. We often employ this solution when trying to confine T cells to the center of the FluoroDish. Cut an annulus from PDMS (8 mm outer diameter, 4 mm inner diameter) using the biopsy punches and the cleaned PDMS prepared in Protocol 1 step 1. Place the annulus at the center of the bottom of the poly-lysine coated FluoroDish before you add any media, allow the PDMS to reversibly adhere to the glass floor of the dish. Then fill the FluoroDish with media as above and allow to fill and totally cover the annulus. Pipette the T cells into the center of the annulus and allow them to settle for 10 min. Then very gently remove the annulus. The T cells will remain in the center of the FluoroDish for subsequent interrogation by the AFM..

6. Place dish containing cells onto the stage and lower the AFM head into place. Allow for 30 minutes for the cantilever to equilibrate to the temperature of the media, as changes in temperature of media can introduce a drift in deflection.

   The AFM head starts well above the meniscus of the media and needs to be lowered in to the media and into the approximate neighborhood of the T cells. The procedure for moving the head is dependent on the instrument and may be a manual or automated process.

7. Maneuver the stage so that the cantilever tip is above the T cell of interest and the microscope si focused on the cell of interest.

   The movement of the stage may be manual or automated. Position the cantilever so that it is approximately 10 μm above the T cell. This step is critical and may take 10–30 min because the cell to be interrogated by the AFM must be positioned right under the cantilever but also within clear sight of the microscope objective below. We judge the tip height by changing the focus of the microscope to focus on the T cell and then up to the cantilever and back, noting the change of the objective position on the microscope controller.

8. Setup the force trigger to an appropriate gentle touch – we use 50 pN to ensure contact. Program the controller to dwell on the surface for 3–5 minutes. *This time frame is typically adequate for measuring the biochemical and mechanical responses of T cells upon triggering of the TCR.*

9. Close the acoustic hood, turn off lights, and remain silent for the duration of the experiment.

Talking or any loud noises can disrupt the force measurements. Ventilation systems, nearby elevators and autoclaves, and other instruments can also dramatically affect the measurement of force. Ensure that the setup of the AFM accounts for these external factors.

10. Begin recording the LabVIEW stream.

11. Begin recording video frames on the microscope.

12. Engage the AFM to begin a force-triggered contact onto the cell.

13. We typically see a wait of a few milliseconds to a few seconds before the cell becomes triggered, as evidenced by calcium flux, changes in cell morphology, or movement of fluorescently-tagged cell surface receptors. This interval could be due to movement of receptors to the region of contact of the cantilever.

    The flux of calcium and fluorescence of the calcium-sensitive dyes that had been loaded into the T cells is visible with the microscope software in a real-time fashion. If calcium flux is not observed, consider repositioning the cantilever 500 nm-1 μm away, which will be on the same T cell but in a slightly different region of the cell. The collection of AFM data in LabView and microscope frames allows for analysis afterwards.

14. After the dwell is over and the contact has ended, switch off the microscope recording and LabVIEW stream.

15. Transfer data to another computer running MATLAB for analysis of the touch.

Alternative Protocol 1

Coating a single molecule onto the cantilever tip

This alternative protocol describes how to attach a single molecule onto the AFM cantilever tip. Sparse coating of molecules can allow the AFM to act as a molecular sensor for a single-molecule biophysical measurements, such as dynamic force spectroscopy or receptor mapping. Basic protocol 1 results in large numbers of molecules coating the cantilever tip, which is suitable for many experiments but not those that study a single molecular interaction. This protocol, adapted from a previous publication (Liu and Butte, 2012), will allow for a single, or very few, molecules at the tip. As with Basic Protocol 1, the cantilever tip is prepared and coated with a silane that allows subsequent chemistry. The aminosilane employed here enables covalent attachment of streptavidin through EDC-NHS chemistry. Then a biotinylated molecule is attached to the streptavidin on the tip by soaking it in a paper that has been soaked with the molecules to be attached.

The key to this approach is touching the solution within the matrix of fibrous paper, rather than submerging the chip with solution (Fig. 2). We believe that the fibers of the paper engage the labeling solution in a competition of wicking into the paper versus release and coating onto the cantilever tip. The wicking force likely prevents a large amount of liquid from coating the tip, and thus allows for extremely sparse labeling of the very apex of the cantilever tip.

In the Basic Protocol 1, we employed a design strategy of linkage to the cantilever tip using streptavidin-biotin chemistry, but that approach would not work for single-molecule labeling because streptavidin is tetravalent. We chose instead to covalently attach free amines to the tip as a tether for subsequent covalent cross-linking using EDC chemistry. Free carboxyls on the surface of the protein of interest (can be conjugated.

The cantilever tips used were reflex-side gold-coated silicon tips (SHOCONG, AppNano), though we and others have subsequently found that gold-coating increases signal at the expense of greater noise in biological experiments (Churnside et al., 2012). Our current recommendation is to avoid the use of gold-coated tips for biological experiments requiring live-cells and optical microscopy. This same protocol will work for the HYDRA6R-200N tips described in Basic protocol 1.

Materials

Poly-dimethylsiloxane (PDMS, Dow Corning Sylgard 184)

8 mm biopsy punch (Robbins True-Cut Disposable)

3-Aminopropyltriethoxysilane (APTES) – Thermo Fisher Scientific

N-hydroxysuccinimide (NHS) – Thermo Fisher Scientific

1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) - Thermo Fisher Scientific

2-Mercaptoethanol (BME) - Sigma Aldrich

2-(N-morpholino)ethanesulfonic acid hydrate (MES) - Sigma Aldrich

Harrick Plasma Cleaner PDC-001

Reflex-side gold-coated silicon cantilever tips (SHOCONG, AppNano)

I-A^b-OVA (323–339) (called peptide-MHC in the protocol) (NIH tetramer core facility, http://tetramer.yerkes.emory.edu/)

Anti-CD3 (Biolegend, clone 145–2C11)

Square cover slips (18 mm x 18 mm, ThermoFisher)

Ethanol (200 proof)

Printer paper

Dessicator

1. Measure the spring constant for each cantilever experimentally. Clean cantilever tips in plasma for 1 min. Follow Basic Protocol 1 steps 1–4, and maintain the cantilevers on a PDMS pad to facilitate steps.

2. Coat the cantilever with APTES by vapor deposition. Pipette a droplet of 50 μL of APTES onto an 8 mm PDMS pad. Place a cantilever tip-side up onto another PDMS pad. Place the pad holding the droplet and pad(s) holding the cantilevers gently into a desiccator nearby. Pump down with house vacuum (~30–50 torr). Wait overnight at ambient temperature. Wash the cantilever tip with ethanol and water.

   For washing the cantilever while it rests on a pad of PDMS, see Step 14 of Basic Protocol 1.

3. Prepare the biomolecules to be attached to the tip with EDC and NHS. These molecules are antibodies (anti-CD3 monoclonal antibody) or peptide-MHC, depending on what experimental triggering is desired. Bring protein to a concentration of 10 mg/mL. Mix with 2 mM EDC and 5 mM NHS in pH 5.5 100 mM MES solution. The reaction is completed in 30 min. Remove the protein from the unreacted molecules with buffer exchange using a spin column (follow Step 11 of Basic Protocol 1) using 100 mM MES at pH 5.5.

   This step prepares the protein to be conjugated to the cantilever tip. A detailed discussion of the chemistry of linking to proteins to aminosilane-functionalized cantilevers is offered in other work (Ebner et al., 2008).

4. Mount the APTES-functionalized cantilever tip to the AFM cantilever base

   The specific process of mounting a cantilever to the AFM depends on the instrument.

5. Center the cantilever tip over the AFM stage. Cut a piece of common laser printer paper (Staples, white 30% recycled 20 lb paper) to 1 cm x 1 cm piece, and place onto a coverslip on the AFM sample stage below the cantilever tip.

   The cantilever (i.e., the AFM head) should be raised high enough to eliminate the chance of touching and damaging the tip.

6. Pipette ~10 μL of 100 mM MES buffer to wet the paper

7. Add 5 μL of the protein-NHS solution (concentration ~10 mg/mL) to the center of the wet region

8. Set the AFM mode to make contact with the surface at a particular force of contact and then then dwell in contact with the paper. On the Asylum software, this mode is called a “triggered force curve.” Use a trigger force of 200 pN. Remain in contact with the paper for 10 sec, then fully retract the *z*-piezo. Repeat this contact again, for a total of 10 cycles or 100 sec of contact.

   The dwell periods offer ~100 sec of contact time for the cross-linking chemistry to occur, but do not allow the tip to be drawn deeply into the fibrous matrix of the paper, which would increase labeling broadly and not just on the very tip. Typically, when labeling of the cantilever is complete we observe for an adhesion force when withdrawing the tip from the wet paper.

Commentary

Background Information—Numerous approaches exist to link molecules to the AFM cantilever tip, depending on the chemistry of the cantilever itself and the nature of the molecules being linked (Ebner et al., 2008). The protocols here depend on silane chemistry and thus require the cantilever to be made of silicon, the most common commercial material for cantilevers, though it works well for silicon nitride as well.

Critical Parameters and Troubleshooting—Contact with living cells requires a careful experimental set up. The cells should be maintained in media that has sufficient nutrition to measure responses. At the same time, media should be optically clear and free of phenol red or other colored molecules that could interfere with fluorescence microscopy.

Intense light from lasers or halogen bulbs is needed to illuminate fluorescent proteins or fluorophore-tagged antibodies during live-cell microscopy. This same light can influence the movement of the cantilever due to a combination of photon pressure and thermal changes of the cantilever's material. In fact, this effect can be exploited to drive cantilevers by cyclic photo-thermal excitation (Labuda et al., 2014). In practice, however, the light used to image cells undergoing contact by AFM will alter the measurement of forces to some degree. Using the bare minimal amount of light will be necessary to reduce this adverse effect.

The biomolecules on the tip, though covalently attached, should not be expected to survive contact with cells for more than an hour or two. The biomolecules on the cantilever tip are likely lost over time due to abrasion from the moving cell or by molecular interactions with the receptors of the cell. In addition, debris from contacted cells can potentially remain on the tip, masking or interfering with ligand binding in later experiments.

Understanding the Results—Basic protocol 1 will result in coating of a cantilever tip with plentiful molecules that, when engaged onto a cell surface, will result in ligation of cell-surface receptors. Basic protocol 2 will allow triggering of T cells from ligands tethered to the cantilever.

Time Considerations—Preparation of cantilever tips for specific chemical reactions takes time and should not be attempted the day of an AFM experiment. We suggest preparing cantilevers the day before each use. Staggering AFM experiments so that fresh cantilevers are available is not always conducive to the underlying biological experiment, for example, if primary cells are obtained from human donors or mice. There is no simple solution to balancing the reproducibility of the AFM experiments, which is paramount, to the inconvenience of provisioning the cells being probed and other considerations of the underlying biological questions being asked. We anticipate greater ease when working with cell lines, which allow greater flexibility in terms of provisioning, but at the expense of relevance.

The degree of cellular response depends on the amount of signal given to the T cell. It is known that as little as one peptide MHC contact is sufficient to trigger calcium flux in an ideal situation (Irvine et al., 2002). We find in general that calcium flux has begun and even reaches a peak within 2 minutes of contact with the AFM cantilever (Hu and Butte, 2016).

The various subsequent responses of T cells can last for minutes (e.g., movements of the T cell towards the antigen presenting cell (Thauland et al., 2017; Negulescu et al., 1996)), hours (upregulation of cell-surface proteins like CD69 and CD25), and days (e.g., proliferative responses). Most of these events would be likely measured after the AFM-delivered signal is completed. Yet, late events after T cell activation could be observed on the microscope if a suitable arrangement exists for long-term culture of cells on the microscope stage. That would include the provision of CO_2 gas and long-term temperature control.

Acknowledgements

The authors acknowledge current funding sources support for work came from the National Institutes of Health R01 GM110482 (to M.J.B.). Because the protocols developed for this paper span many years, we acknowledge seven other, completed funding sources: National Science Foundation (CBET 1264833, to M.J.B.); the Stanford Center for Probing the Nanoscale, an NSF NSEC (PHY-0830228, to M.J.B.); and the Stanford BioX Interdisciplinary Initiatives Program; the Stanford Child Health Research Institute; and the Morgridge Family Foundation; and National Institutes of Health T32 GM008294 (to K.H.H.).

Literature Cited

Butt H-J, and Jaschke M 1995 Calculation of thermal noise in atomic force microscopy. Nanotechnology 6:1–7.

Churnside AB, Sullan RMA, Nguyen DM, Case SO, Bull MS, King GM, and Perkins TT 2012 Routine and timely sub-picoNewton force stability and precision for biological applications of atomic force microscopy. Nano letters 12:3557–61. Available at: http://pubs.acs.org/doi/abs/10.1021/nl301166w. [PubMed: 22694769]

Ebner A, Wildling L, Zhu R, Rankl C, Haselgriibler T, Hinterdorfer P, Gruber HJ, Haselgrübler T, Hinterdorfer P, and Gruber HJ 2008 Functionalization of Probe Tips and Supports for Single-Molecule Recognition Force Microscopy In Topics In Current Chemistry pp. 1–48. Springer Berlin / Heidelberg.

Hu KH, Bruce MA, and Butte MJ 2014 Spatiotemporally and mechanically controlled triggering of mast cells using atomic force microscopy. Immunologic Research 58:211–217. [PubMed: 24777418]

Hu KH, and Butte MJ 2016 T cell activation requires force generation. Journal of Cell Biology 213:535–542. [PubMed: 27241914]

Irvine DJ, Purbhoo MA, Krogsgaard M, and Davis MM 2002 Direct observation of ligand recognition by T cells. Nature 419:845–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12397360. [PubMed: 12397360]

Labuda A, Cleveland J, Geisse N, Kocun M, Ohler B, Proksch R, Viani M, and Walters D 2014 Photothermal excitation for improved cantilever drive performance in tapping mode atomic force microscopy. Microscopy and Analysis 28:23–27. Available at: http://www.microscopy-analysis.com/magazine/issues/photothermal-excitation-improved-cantilever-drive-performance-tapping-mode-atomic.

Liu J, and Butte MJ 2012 Single molecule labeling of an atomic force microscope cantilever tip. Applied Physics Letters 101.

Liu J, Sun N, Bruce MA, Wu JC, and Butte MJ 2012 Atomic force mechanobiology of pluripotent stem cell-derived cardiomyocytes. Plos One 7:e37559 Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=22624048. [PubMed: 22624048]

Negulescu PA, Krasieva TB, Khan A, Kerschbaum HH, and Cahalan MD 1996 Polarity of T cell shape, motility, and sensitivity to antigen. Immunity 4:421–30. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8630728. [PubMed: 8630728]

Sader JE, Chon JWM, and Mulvaney P 1999 Calibration of rectangular atomic force microscope cantilevers. Review of Scientific Instruments 70:3967–3969.

Thauland TJ, Hu KH, Bruce MA, and Butte MJ 2017 Cytoskeletal adaptivity regulates T cell receptor signaling. Science Signaling 3737:1–11. Available at: http://stke.sciencemag.org/content/10/469/eaah3737 [Accessed June 5, 2017].

Significance Statement

The methods in this paper offer an approach to achieving precise spatiotemporal control over the ligation of cell-surface receptors on live cells using an atomic force microscope (AFM) to deliver ligands. This approach allows for imaging cellular responses and mechanical forces upon triggering of the receptors, like the T cell receptor, and adds an important capability to the field of mechanobiology. This protocol outlines the steps needed to incorporate stimulatory ligands such as anti-CD3 antibody onto an AFM cantilever and showcases its application in triggering T cells.

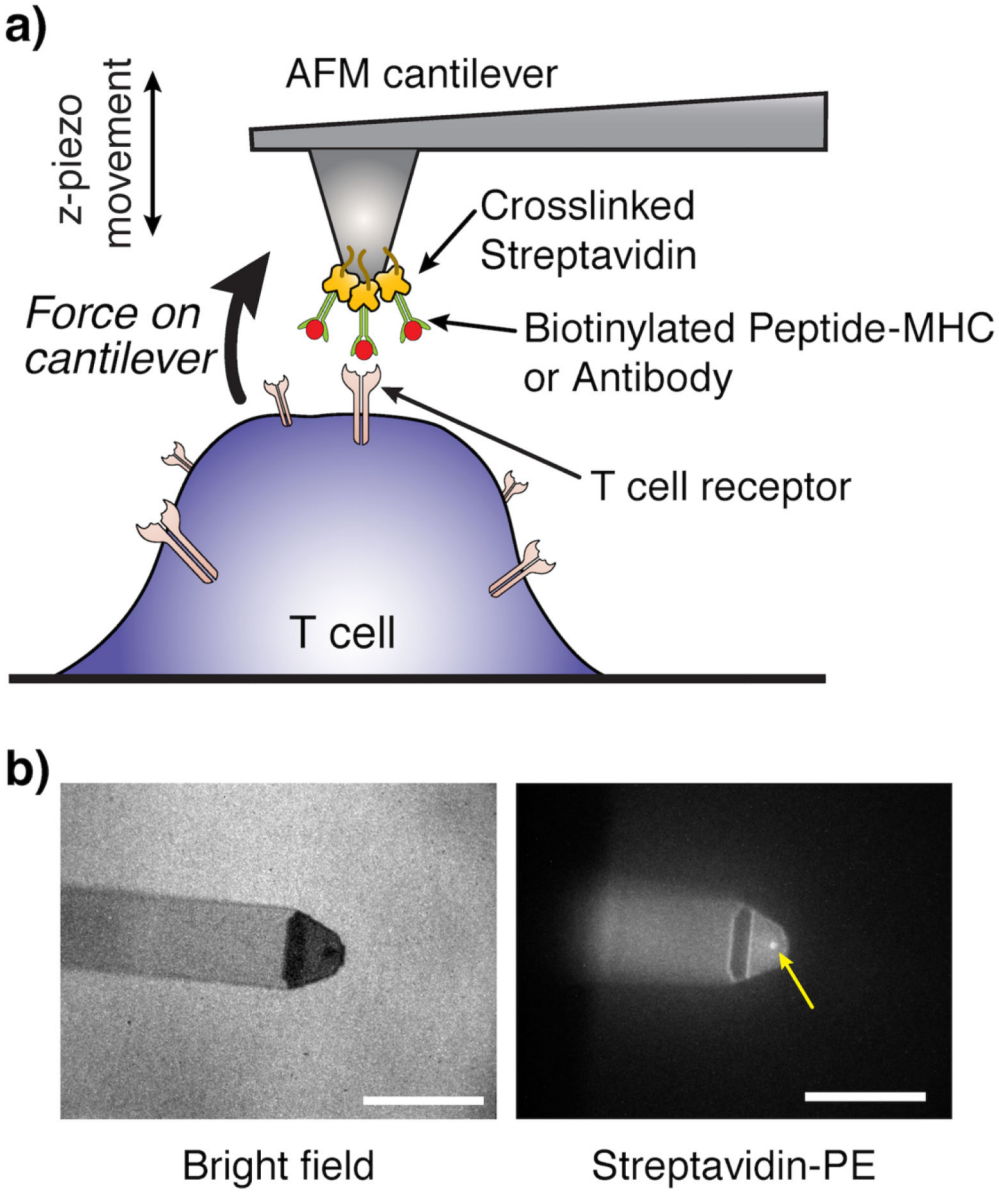

Figure 1.

(A) Schematic showing AFM cantilever for stimulation of T cells and for monitoring mechanical responses.

(B) (Left) Brightfield image of cantilever showing the dark silicon pad with the tip and the silicon nitride body of the cantilever. (Right) Fluorescence image of fluorescent Phycoerythrin-conjugated streptavidin assembled from a projection of multiple slices of spinning-disk confocal images. The cantilever tip is bright with labeled streptavidin (arrow). Scale bar, 50 μm. ©Hu KH and Butte MJ, 2016. Originally published in The Journal of Cell Biology. https://doi.org/10.1083/jcb.201511053.

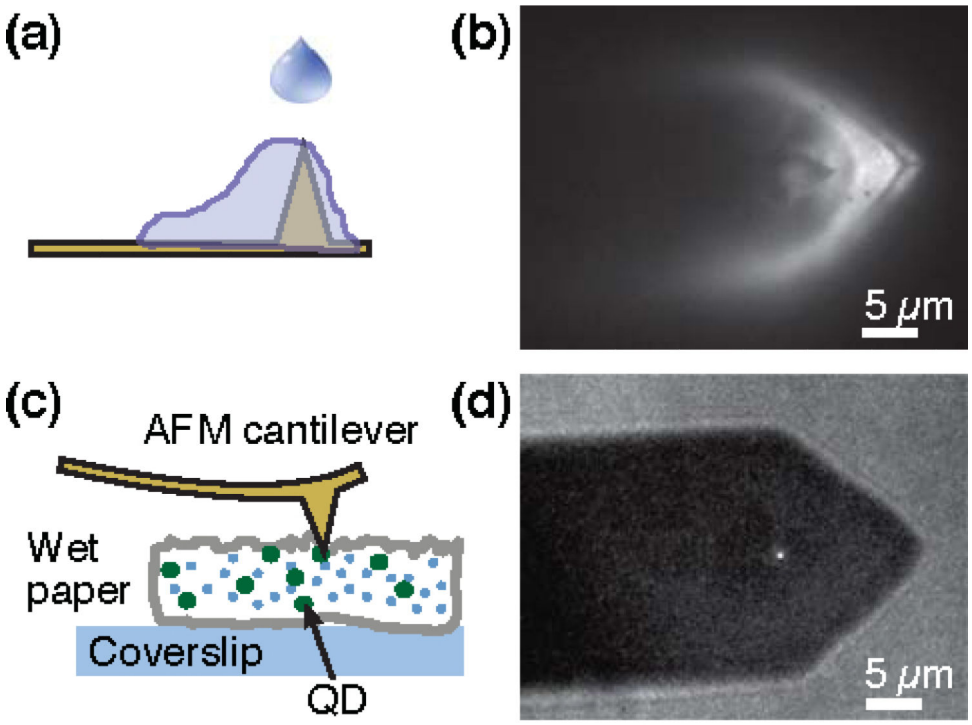

Figure 2.

(A) A conventional way to label AFM tip is to immerse the tip or to dip the tip into a small droplet.

(B) Fluorescent widefield image (ex. 405 nm, emission filter 525/50) of AFM tip labeled using conventional labeling method, shows QD525 molecules all over the tip.

(C) Alternative Protocol 1 in this paper entails touching the tip gently and repeatedly to wetted paper.

(D) Fluorescent widefield image of AFM tip labeled using our method shows QD525 molecules are confined to the very end of the tip (bright spot). Reproduced from Liu J and Butte MJ, Applied Physics Letters, Vol. 101, Page 163705, (2012), with the permission of AIP Publishing.