bio-protocol

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Characterization of the Elasticity of CD4$^+$ T Cells: An Approach Based on Peak Force Quantitative Nanomechanical Mapping

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Abstract

CD4$^+$ T cells are essential players in orchestrating the specific immune response against intracellular pathogens, and in inhibiting tumor development in an early stage. The activation of T cells is triggered by engagement of T cell receptors (TCRs). Here, CD3 and CD28 molecules are key factors, (co)stimulating signaling pathways essential for activation and proliferation of CD4$^+$ T cells. T cell activation induces the formation of a tight mechanical bond between T cell and target cell, the so-called immunological synapse (IS). Due to this, mechanical cell properties, including stiffness, play a significant role in modulating cell functions. In the past, many approaches were made to investigate mechanical properties of immune cells, including micropipette aspiration, microplate-based rheometry, techniques based on deformation during cytometry, or the use of optical tweezers. However, the stiffness of T lymphocytes at a subcellular level at the IS still remains largely elusive.

With this protocol, we introduce a method based on atomic force microscopy (AFM), to investigate the local cellular stiffness of T cells on functionalized glass/Polydimethylsiloxan (PDMS) surfaces, which mimicks focal stimulation of target cells inducing IS formation by T cells. By applying the peak force nanomechanical mapping (QNM) technique, cellular surface structures and the local stiffness are determined simultaneously, with a resolution of approximately 60 nm. This protocol can be easily adapted to investigate the mechanical impact of numerous factors influencing IS formation and T cell activation.

Keywords: CD4$^+$ T cell, AFM, Stiffness, Elasticity mapping, Peak Force QNM

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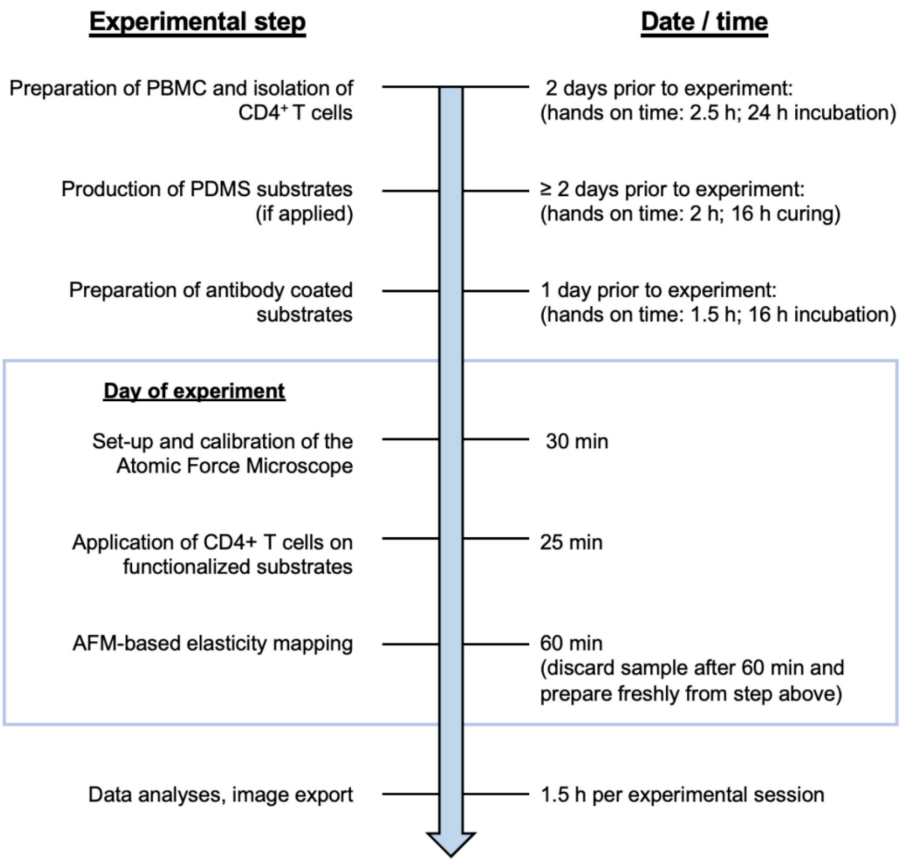
Graphical abstract:



Overview of the experimental workflow.
Individual experimental steps are shown on the left, hands on and incubation times for each step are shown right.

Background

T cells belong to the adaptive immune system and can be classified as $CD4^+$ T cells or $CD8^+$ T cells. $CD4^+$ T cells are essential for orchestrating the immune responses, and $CD8^+$ T cells, also known as cytotoxic T lymphocytes, are the key players in eliminating tumor and pathogen-infected cells (Taniuchi, 2018). T cells are activated by the engagement of T-cell receptors (TCRs) with the matching antigen. Consequently, CD3 molecules, one key component of the TCR complex, transduce the signal to activate downstream pathways, leading to the formation of a tight junction between T cells and target cells, termed the immunological synapse (IS). In addition, engagement of co-stimulatory factor CD28, which triggers the production of interleukins (*e.g.*, IL-6), initiates signaling pathways essential for sustainable activation and proliferation (Esensten *et al.*, 2016). In addition to a complex ballet of receptor-ligand bonds, a fundamental rearrangement of the cytoskeleton takes place during IS formation, including the reorientation of the microtubule-organizing center (MTOC) towards the IS, and the formation of an F-actin ring structure at the IS. In between, the adhesion molecule lymphocyte function-associated antigen 1 (LFA-1) binds its ligand on the target cells, to seal and stabilize the IS (Bromley *et al.*, 2001). Upon T cell activation, the intracellular concentration of Ca^{2+} ions is drastically enhanced via Ca^{2+} influx (Friedmann *et al.*, 2019). Ca^{2+} serves as an essential second messenger in T cells to regulate their activation, proliferation, and effector functions (Trebak and Kinet, 2019).

Mechanical properties, including stiffness and mechanical forces, play a significant role in modulation of cell functions, including those of T cells (Jansen *et al.*, 2015). A recent study has demonstrated that cytotoxic T

lymphocytes optimize their killing function by applying a mechanical force (Basu *et al.*, 2016). T cells are able to generate mechanical forces (pushing and pulling forces) in the piconewton (pN) range upon activation (Husson *et al.*, 2011; Hu and Butte, 2016). This force generation requires a sustained elevation of intracellular calcium, as well as integrity of the functional F-actin network, and phosphoinositide 3-kinases (PI3K) signaling (Hui *et al.*, 2015; Basu *et al.*, 2016). Other work shows that activation of T cells requires mechanical forces (Hu and Butte, 2016), and that T cells respond to environmental stiffness cues (Majedi *et al.*, 2020). Changes in cell stiffness can regulate force generation (Harrison *et al.*, 2019). However, the stiffness of T lymphocytes at a subcellular level at the IS still remains largely elusive.

Over the last 20 years, many different approaches were used to address the stiffness and stiffening processes in eukaryotic cells, especially immune cells. Early approaches, such as micropipette aspiration, and microplate-based rheometry, involved complex technical structures, in which the cells were inserted or incorporated and mechanically stressed (Hochmuth, 2000; Desprat *et al.*, 2005). Cytometry-related approaches are based on principles like deformation during deceleration in flow, or twisting triggered by membrane-bound metal beads in a magnetic field. Those methods are limited to a constant fluid stream to operate. In contrast, methods which are able to characterize elastic properties of cells in an environment mimicking physiological conditions, like cell monolayers and cell-cell interactions, are capable of providing much deeper insights into biomechanical processes. Optical tweezers use focused laser beams to generate attractive or repulsive forces in the pN range, and are considered a promising tool to characterize and modify biomaterials. Hence, this method is mostly used to move or sort cells, and is still not broadly applied for the characterization of local cell properties (Killian *et al.*, 2018).

Atomic force microscopy (AFM) is another biophysical method that can be used to determine cell forces in the pN range. AFM applies a mechanical probe, often made of silicon or silicon nitride with a nanometer-thin tip, that is moved along the cell surface, and interacts with each point of a defined scanning grid. Hence, it enables the examination of height profiles, or measures mechanical properties with spatial and force resolutions at the nanometer and pN ranges, respectively (Loskill *et al.*, 2014; Thewes *et al.*, 2015). In recent years, AFM has evolved as one of the most important tools in cell biology to study adhesive forces, and cell properties, such as deformation and elasticity (Scheuring and Dufrêne, 2010; Pi and Cai, 2019).

Recently, we established a peak force quantitative nanomechanical mapping-based method, for simultaneously determining the surface profile and stiffness of live T cells during IS formation at a subcellular level, with a resolution of ~60 nm (Jung *et al.*, 2021). In this method, antibodies against CD3, CD28, and LFA-1 are immobilized on substrates, mimicking the focal stimulation of target cells that would induce IS formation in T cells. This protocol can be easily adapted to investigate the mechanical impact of a broad spectrum of intracellular or extracellular factors influencing IS formation and T cell activation.

Materials and Reagents

1. Cell culture flasks 25 mL (Sarstedt, catalog number: 83.3910.502)
2. Glass slides (Carl Roth, catalog number: H872.1)
3. Glass cover slips 25 mm (VWR, catalog number: 631-1584)
4. Falcon® 24-well plates (Corning, catalog number: 353047)
5. Scalpel blades (B. Braun Melsungen, catalog number: 5518075)
6. Fluorodish Petri dish 50 mm (World Precision Instruments, catalog number: FD35-100)
7. Polypropylene tubes 15 mL (Fisher Scientific, catalog number: 11507411)
8. Polypropylene tubes 50 mL (Fisher Scientific, catalog number: 10788561)
9. MLCT AFM probes (Bruker AFM Probes, catalog number: MLCT-10)
10. Lymphocyte separation media 1077 (PromoCell GmbH, catalog number: C-44010)
11. Hanks' balanced salt solution (Sigma-Aldrich, catalog number: H6648)
12. $CD4^+$ T Cell Isolation Kit human (Miltenyi, catalog number: 130-096-533)
13. AIM V cell culture medium (Thermo Fisher Scientific, Gibco, catalog number: 31035025)
14. Fetal bovine serum (FBS) (Thermo Fisher, Gibco, catalog number: A4766801)
15. Phosphate-buffered saline (PBS) (Thermo Fisher, Gibco, catalogue number: 10010056)
16. Polyornithine (Sigma-Aldrich, Merck, catalog number: MFCD00286305)
17. anti-LFA-1 (ITGAL) antibody, mouse monoclonal (Antibodies-Online, catalog number: ABIN135680)

18. mouse anti-human CD28 antibody, mouse monoclonal (BD Pharmingen, catalog number: 555725)
19. mouse anti-human CD3 antibody, mouse monoclonal (Diaclone, catalog number: 854.010.000)
20. Sylgard 184 Silicone Elastomer Kit (Dow Europe GmbH, catalog number: 1317318)
21. For alternative cantilevers (see Notes): qp-BioT/qp-BioAC (Nanoandmore GmbH, catalog numbers: qp-BioT-20/qp-BioAC-20)

Equipment

1. CO_2 incubator (Heracell Vios 160i, Thermo Fisher Scientific, catalog number: 51030403)
2. Centrifuge (Hettich Universal 32R, with 50 mL fixed angle rotor)
3. Centrifuge (Eppendorf, model: 5424, with 2 mL fixed angle rotor)
4. AutoMACS Pro Separator (Miltenyi Biotec, catalog number: 130-092-545)
5. Cell and Particle Counter (Beckman Coulter Z2, catalog number: 8043-30-0016)
6. Electrostatic deionizer (Eltex Elektrostatik GmbH, catalog number: W0150L025U99)
7. Stove for 60°C PDMS curing (Memmert, model: 100-800)
8. Diaphragm laboratory pump (KNF, catalog number: N811KN.18)
9. Atomic Force Microscope (Bruker Corp., model: BioScope Catalyst)
10. Bruker calibration table for laser alignment (Bruker Corp., model: Easy Align)
11. Fluorescence microscope (Leica Microsystems, model: DMI 4000 B)
12. Tweezers (*e.g.*, Agar Scientific, catalog number: AG5596-TI)
13. Cantilever Holder (for measurements in fluids) (Bruker Corp., model: CAT-PCH)

Software

1. Research NanoScope, AFM operation software (Bruker Corp., version 9.1, 119071)
2. NanoScope Analysis software (Bruker Corp., version 1.8, 132257)
3. Excel 2016 (Microsoft)
4. GraphPad Prism 6 (GraphPad)

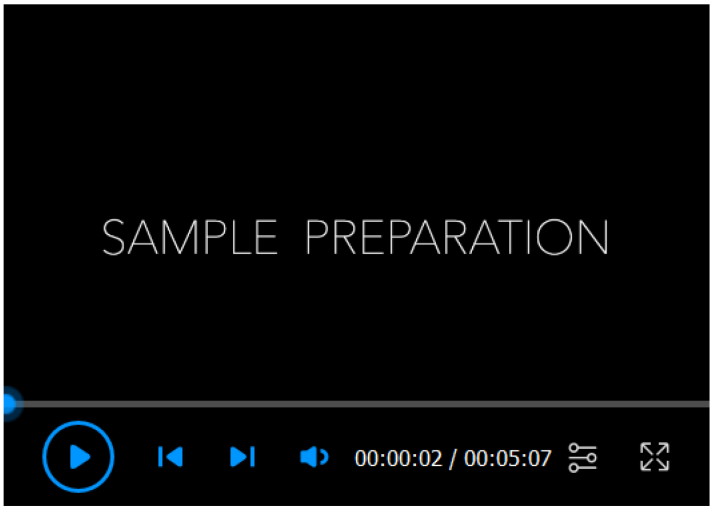
Procedure

A. Isolation and culture of $CD4^+$ T cells

1. Prepare peripheral blood mononuclear cells (PBMCs) from healthy donors, as previously described (Kummerow *et al.*, 2014). Briefly, for separation of mononuclear cells from human blood, we used lymphocyte isolation medium, according to the manufacturer's instructions, and carry out the density gradient centrifugation at 450 g (acceleration: 1, deceleration: 0) and room temperature (RT) for 30 min. PBMCs were collected, and remaining erythrocytes were lysed with 1–3 mL of erythrocyte lysis buffer (see Recipes) for 1–2 min. Estimated duration: 1.5 h.
2. Negatively isolate human $CD4^+$ T cells from PBMCs, using the AutoMACS Pro Separator with the $CD4^+$ T cell Isolation Kit human, according to manufacturer's protocols. Estimated duration: 1 h.
3. Resuspend isolated $CD4^+$ T cells at a density of 3×10^6/mL, in a 24-well plate with AIM V medium with 10% fetal bovine serum (FBS) (1 mL/well), and keep the plate at 37°C with 5% CO_2 for 24 h.
4. Prior to AFM experiments, measure the cell density with an automated cell counter, remove the media by centrifugation (1,200 rpm at RT for 5 min) and adjust the cell suspension to a density of 2×10^5–3×10^5/mL, by resuspending the cells in AIM V medium without FBS.

B. Preparation of antibody-functionalized coverslips (see Video 1)

1. Clean glass coverslips, by wiping with 70% ethanol.
2. With a permanent marker, mark a center spot with a diameter of approximately 7 mm at the backside of the glass coverslip.
3. Coat the center spot with 40 µL of Polyornithine solution (optimized concentration for T cells: 100 µg/mL, in sterilized ddH_2O) at RT for 30 min.
4. Remove the polyornithine solution with a diaphragm laboratory pump and leave the coverslip to air dry for 10 min.
5. Apply 20 µL of antibody dilution (in PBS) and incubate at 37°C for 30 min, to functionalize a spot of the glass coverslip. Store at 4°C overnight. In our experiments, the following combination of antibody concentrations showed the best results for IS formation (optimal concentrations might differ if other factors are investigated):
   - αLFA-1 (9 µg/mL)
   - αCD-3 (30 µg/mL)
   - αCD28 (90 µg/mL)



Video 1. Sample preparation.

C. Preparation of PDMS, and antibody functionalization of PDMS surfaces

1. Slowly pass all materials (*e.g.*, Petri dishes, spatulas) through the arch of an electrostatic deionizer (~2 s), to remove any static charges before bringing them into contact with Polydimethylsiloxane (PDMS) elastomer components A (methylhydrosiloxane-dimethylsolioxane) and B (vinyl-terminated polymethylsiloxane).
2. Mix component A and B in a 10:1 ratio in polypropylene tubes, to create PDMS with a stiffness of approximately 2.5 MPa (adapt according to the manufactures' protocol, if other stiffnesses are addressed)

   Note: When handling the individual PDMS components, strictly use polypropylene materials (e.g., VWR Graduated Polypropylene Beaker 100 mL, catalog number 213-3918; Sarstedt Stirring Rod 120 mm, PP, catalog number 81.970), and Kimtech G3 Sterling Nitrile gloves (e.g., VWR catalog number 112-4879).

3. Pour the mixture into 50-mm flat bottom Petri dishes (*e.g.*, Fluordish), and make sure to cover the surface with a layer of approximately 1.5 mm. Leave to settle for 1 h.

4. Place in an incubator to cure at 60°C for 16 h.
5. Prior to AFM measurements, cut 10 × 10 mm squares from the PDMS layer with a scalpel blade, turn the PDMS piece over, and continue with the bottom side.
6. Continue the functionalization process like described for glass cover slips (B), and apply cells as described below (D).

D. Application of T cells on antibody functionalized surfaces (see Video 1)

1. Pipette 100 µL of $CD4^+$ T cell suspension in AIM V medium (without FBS) into the marked center spot of the functionalized surface (Figure 1A, approximately 2×10^4–3×10^4 will be transferred).
2. Allow the cells to settle and interact with the functionalized surface in a wet chamber at 37°C and 5% CO_2 for 15 min (plastic Petri dish lined with wet paper tissue; Figures 1A–1B), to avoid drying.
3. Mount the functionalized glass cover slip onto a glass slide. Pipette 2 µL of sterilized ddH_2O between the glass layers, to make sure that the functionalized cover slip or PDMS piece is held by cohesion, and will not move during the measurements (Figures 1C–1D).
4. The sample should be used immediately. Discard the sample and freshly prepare a new one after 60 min of measurements, since the cells will die and start detaching from the surface at RT and without increased partial CO_2 pressure.

E. AFM preparation and calibration

Note: In our experiments, the NanoScope 9.1 software was used to operate a BioScope Catalyst system (Bruker, Santa Barbara, USA) mounted onto a Leica DMI 4000 B optical microscope. All methods are specifically described for these systems, but the procedures can be adapted for other software versions and AFM systems, allowing peak force tapping techniques.

1. Insert the MLCT cantilever into the Bruker Fluid Cantilever Holder, using tweezers and a preparation plate (Figure 1E).
2. Make sure to slide the Cantilever back, until it takes up the entire space of the white base plate, and is properly held by the clamp (Figure 1F).
3. Attach the Fluid Cantilever Holder to the BioScope Catalyst Scan Head, and adjust the laser spot to Cantilever B (rectangular), using the BioScope Catalyst EasyAlign table.
   Attention: Soft cantilevers are prone to strong thermal drift. Allow the cantilever to equilibrate, which can take up to 10 min. Do not continue until the laser spot has come to rest.
4. In NanoScope software version 9.1, choose PeakForce QNM in Fluid (Standard Amplitude), from the Mechanical Properties Experimental Group.
5. Determine the cantilevers spring constant, using the thermal tune technique, integrated in the NanoScope 9.1 software (Ohler, 2007).
6. Bring the BioScope Catalyst Scan Head to a vertical position on the BioScope Catalyst EasyAlign table.
7. Pipette a drop of approximately 30 µL of AIM V medium (without FBS) onto the cantilever holder. Make sure that the cantilever is fully submerged in the fluid, but is not damaged by the pipette tip during application of the medium.
8. Bring the BioScope Catalyst Scan Head to a horizontal position, and allow the cantilever to equilibrate, which can take another 10–15 min. Then, readjust the laser spot.
9. Mount an ethanol-cleaned glass slide on the measurement stage, and pipette a 50 µL drop of AIM V medium (without FBS) onto the center of the glass slide.
10. Transfer the BioScope Catalyst Scan Head to the measurement stage, and engage the cantilever to the functionalized surface (a manual pre-approach can be done, until the drop of media on the cantilever holder and the media on the glass slide merge).
11. Calculate the Deflection Sensitivity in V/nm, using the Update Sensitivity tool, which is integrated in the NanoScope 9.1 software.
12. The system is calibrated and ready to measure.

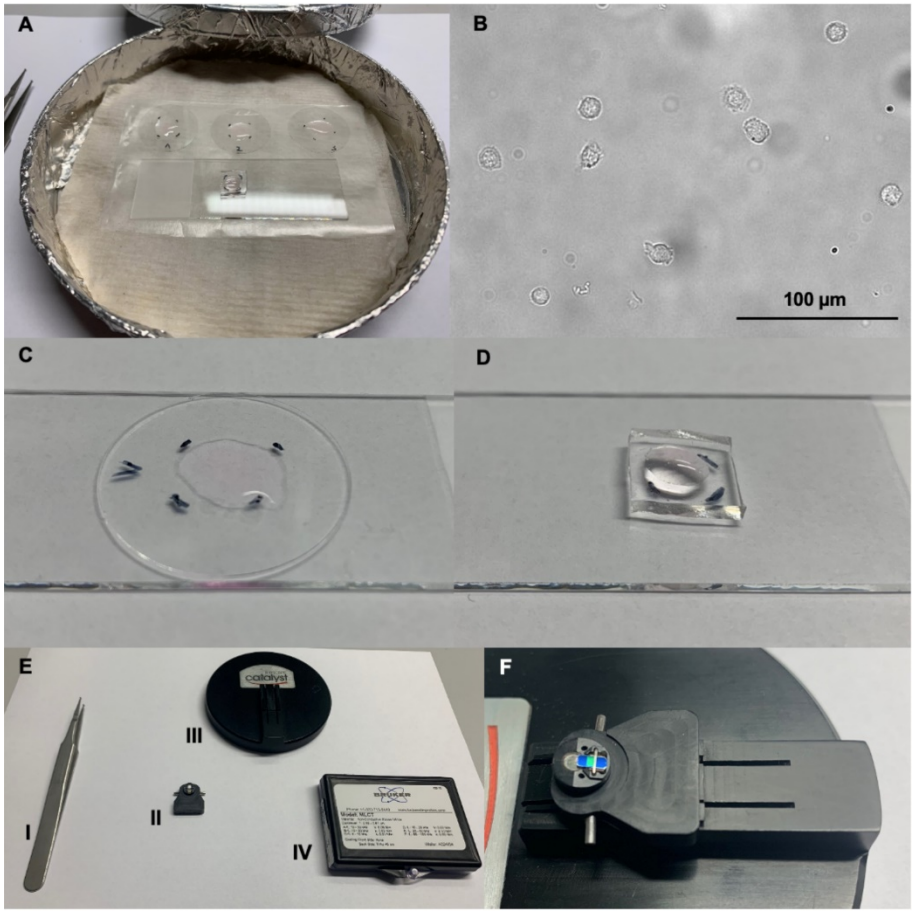


Figure 1. Experimental setup for CD4$^+$ T cell sample and AFM preparation.
A. Incubation of CD4$^+$ T cells on functionalized surfaces (glass coverslips: top; PDMS substrate on glass slide: bottom). The samples were kept in a wet chamber during a 15-min incubation step at 37°C, to avoid drying (for more details on sample preparation, refer to Procedure part B, C, D and Video 1). B. Microscopic view of CD4$^+$ T cells forming an IS on the functionalized substrate after 15 min of incubation. C. Functionalized glass coverslip mounted onto a glass slide after sample preparation. D. Functionalized piece of PDMS after sample preparation. E. Equipment used to insert a cantilever into the cantilever holder (I: tweezers, II: cantilever holder, III: preparation plate, VI: box of cantilevers). F. Detailed view of cantilever holder mounted onto the preparation plate, with adequately inserted cantilever.

F. AFM based elasticity mapping

1. Move the prepared sample (T cells attached to functionalized surfaces) to the measurement stage, and lock it in position with the magnetic Teflon clip.
2. Mount the calibrated BioScope Catalyst Scan Head onto the measurement stage, and engage.
3. Use an empty spot on the functionalized surface between the cells to set all parameters for elasticity mapping. In our experiments, deactivating the ScanAsyst Auto Control, and using the following parameters, showed the best results (if not mentioned here, the NanoScope default value was kept, or automatically set by the system):
   - Starting scan size: 10 µm
   - Line scan rate: 0.25 Hz
   - Lines: 256
   - Feedback gain factor: 0.5
   - Peak Force Setpoint: 700 pN

- Peak Force Amplitude: 100 nm

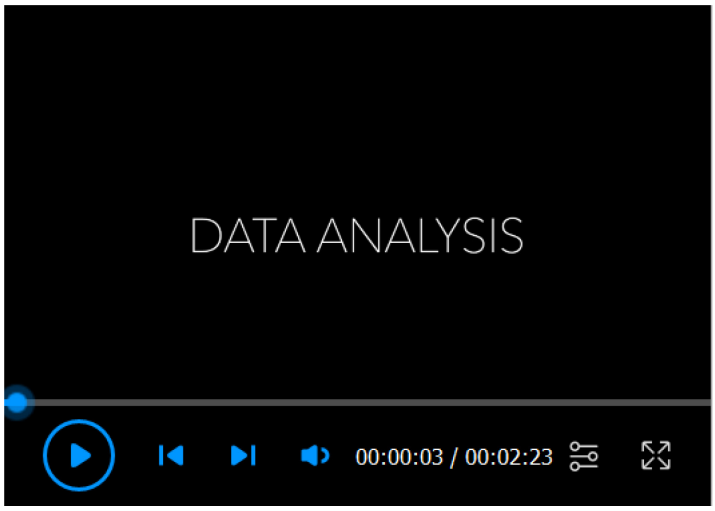
4. Several attempts are expected to establish contact between cantilever and surface. If this is difficult to achieve, increase the systems Engage Gain from factor 0.5 to factor 1.5, then withdraw and re-engage.
5. While scanning, use the optical microscope (20× objective, leading to a 200-fold total magnification) to locate a T cell in close proximity, and approach it by shifting the cantilevers' X-Y offsets, until approximately a quarter of the cell is covered by the cantilever movement (Figure 2A; see also Notes, for information regarding scanning whole cells).
   Attention: The manufacturer gives a relatively wide range of possible tip heights for MLCT cantilevers (2.5–8 µm). Therefore, collisions between the T cell and the front cantilever edges, leading to measurement artifacts, cannot be excluded. In our experiments, mostly the lower right or lower left cell quarter was approached, to make use of the cantilever tilt. However, some cells still turned out to be too large to scan, and some cantilevers turned out to have a tip too short to be used for this application (see also Notes 1, 2, Figure 2E).
6. Use the NanoScope 9.1 "Capture next" or "Capture now" tools to save the data map (Figure 2B, C).
7. If the cantilever is withdrawn from the surface before navigating to the next cell, make sure to set the cantilevers' X-Y offsets to 0 again, to allow a maximum degree of navigational space within the X-Y movement range of 100 µm for the scan heads.

For a detailed introduction of PeakForce QNM data acquisition, we recommend the manufacturer's video tutorial: https://www.youtube.com/watch?v=3I1Hl1gl3Uk.

Data analysis

A. Extraction of elasticity data from elasticity maps (see Video 2 "Data Analysis")

1. Open the NanoScope 9.1 data map in the NanoScope Analysis 1.8 software, and switch to the Derjaguin-Muller-Toporov (DMT) modulus channel (Figure 2C), which represents elasticity (Derjaguin *et al.*, 1975).
2. Switch to the NanoScope Analysis "Bearing Analysis" tool, to mark square shaped spots on the elasticity map, covering the cellular structures of interest (see Note 3).
3. Extract elasticity data from the Depth Histogram view, as XZ data in .txt format.
4. Import the .txt data to a spreadsheet analysis software (*e.g.*, Microsoft Excel), to calculate mean values and standard deviations of elasticity values.
5. Wilcoxon matched-pairs signed rank tests can be used to test for statistical significance (*e.g.*, using GraphPad Prism 6).



Video 2. Data analysis and image export.

B. Extraction of graphical images from elasticity maps (see Video 2)

1. If necessary, crop the data map to the size that needs to be displayed.
2. To extract images from the DMT modulus channel, right-click the scale and color axis next to the image, and select the "Choose Color" bar to choose a color scheme. To change the data scale, select the "Modify Scale" bar, and set the minimum and maximum values of the data scale that need to be displayed (*e.g.*, 0–1,000 kPa).
3. Use the "Export" Tool to save images from NanoScope Analysis software.
4. To create image overlays in NanoScope Analysis (*e.g.*, height profile overlayed with elasticity map), change to the height channel and to "3D Image". Choose "Skin Type" from the image input menu, and select the DMT modulus channel (Figure 2D).
5. Choose the preferred 3D image settings (*e.g.*, zoom, rotation angle, label type), and export the image.

For an overview of the complete experimental workflow and data analysis, including hands on and incubation times, see the Graphic abstract.

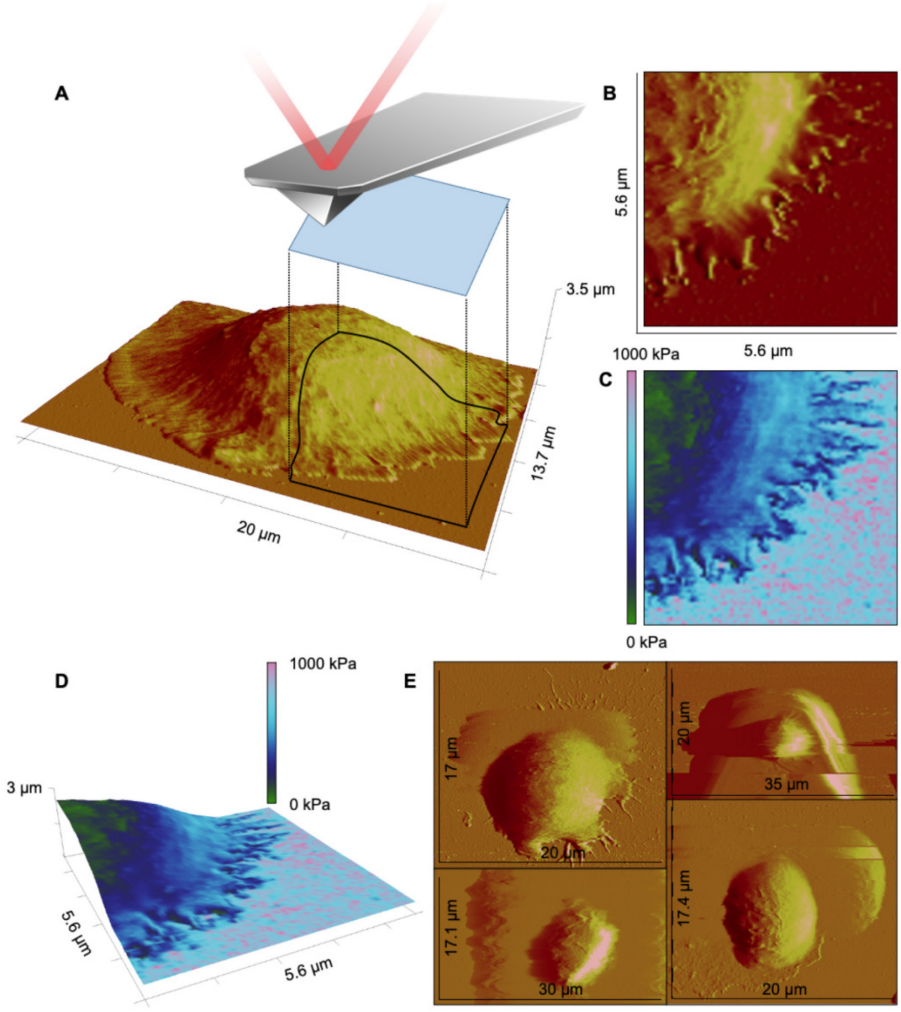


Figure 2. AFM-based elasticity mapping (PeakForce QNM) and data analysis.
A. Ideal cantilever orientation during elasticity mapping of a quarter $CD4^+$ T cell, after shifting the X- and Y-offsets. The resulting elasticity map is indicated as a blue square, and the height profile as a black frame. B, C. Height profile and according elasticity channel (DMT) of a quarter of a T cell after data capture. Shown are scan size and elasticity data scale. D. 3D overlay of height profile and elasticity map, with scan dimensions and elasticity data scale. E. Examples of data maps showing measurement artifacts; top, left: lamellipodia region

appears covered by blurry structures (single filopodia are unintentionally detached by the movement of the cantilever tip); top, right and bottom: blurry areas surrounding cell bodies (cantilever edge colliding with the cell body) (see also Notes 1, 2).

Notes

1. When initiating the study, we were not aware of problems related to insufficient tip height and collision between the cell and the cantilever edges. Since we started with the cantilever type described, we kept that type during our measurements to guarantee comparability. Users that are initiating a new series of measurements might want to consider trying an alternative cantilever type. We suggest qp-BioT or qp-BioAC (Nanosensors, Neuchatel, Switzerland).
2. Scanning whole cells is possible by increasing the scan size to 20–30 µm, since the IS forming T cells possess sizes of approximately 9–15 µm. In our experiments, we decided to focus on cell quarters, to minimize measurement artifacts due to collision between cantilever and cell (see also Note 2), and insufficient contact between the cell and the cantilever tip. These problems might be less severe with alternative cantilever types or other AFM systems.
3. We strongly recommend analyzing several individual elasticity squares of the cellular structure(s) that needs to be addressed. This protocol was adapted from our Short Report "T cell stiffness is enhanced upon formation of immunological synapse" (see Jung *et al*., 2021). One focus of this work was to distinguish between the local stiffness of lamellipodia and cell body of $CD4^+$ T cells. The data analysis strategy is given here as one possible option: for lamellipodia, three individual square-shaped surface segments of 500 × 500 nm were analyzed per cell. If very slender filopodia structures with a lateral width of less than 500 nm were seen, the segment size analyzed was reduced to 250 × 250 nm. To determine the elastic moduli of the cell bodies, one 1.5 × 1.5 µm surface segment was investigated.

Recipes

1. Erythrocyte lysis buffer
155 mM NH_4Cl
10 mM $KHCO_3$
0.1 mM EDTA
in ddH_2O, pH 7.3

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The protocol presented in this manuscript was adapted from our previous work "T cell stiffness is enhanced upon formation of immunological synapse", published in eLife 2021;10: e66643 (see Jung *et al*., 2021).

Competing interests

There are no conflicts of interest or competing interests.

Ethics

Research carried out for this study with healthy donor material (leukocyte reduction system chambers from human blood donors) is authorized by the local ethic committee [declaration from 16.4.2015 (84/15; Prof. Dr. Rettig-Stürmer].

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