Chapter 26

Measuring αβ T-Cell Receptor-Mediated Mechanosensing Using Optical Tweezers Combined with Fluorescence Imaging

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Abstract

T-cell antigen receptors (TCRs) are mechanosensors, which initiate a signaling cascade upon ligand recognition resulting in T-cell differentiation, homeostasis, effector and regulatory functions. An optical trap combined with fluorescence permits direct monitoring of T-cell triggering in response to force application at various concentrations of peptide-bound major histocompatibility complex molecules (pMHC). The technique mimics physiological shear forces applied as cells crawl across antigen-presenting surfaces during immune surveillance. True single molecule studies performed on single cells profile force-bond lifetime, typically seen as a catch bond, and conformational change at the TCR–pMHC bond on the surface of the cell upon force loading. Together, activation and single molecule single cell studies provide chemical and physical triggering thresholds as well as insight into catch bond formation and quaternary structural changes of single TCRs. The present methods detail assay design, preparation, and execution, as well as data analysis. These methods may be applied to a wide range of pMHC–TCR interactions and have potential for adaptation to other receptor-ligand systems.

Key words Optical tweezers, Mechanosensor, T cell, T-cell receptor, T-cell activation, Catch bond

1 Introduction

T cells are a critical component of the adaptive immune system responsible for attacking cancers and diseases. The T-cell activation process must be finely tuned to discriminate between self and non-self, otherwise autoimmunity or immunodeficiencies can result. In the best scenario, T cells not only fight off the initial infection but hibernate in memory where they can mount a rapid anamnestic response against the same assault many years later. Understanding the molecular mechanism behind T-cell activation opens doors for more optimized vaccine and immunotherapy opportunities.

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Using their plasma membrane arrayed T-cell antigen receptor complexes (αβTCRs), T cells recognize antigenic "foreign" peptides (i.e., derived from infectious pathogens or malignant tumor transformations) typically 8–11 amino acids long that have been intracellularly processed and displayed in the groove of major histocompatibility complex molecules (collectively termed pMHC) on the surface of antigen-presenting cells (APCs) [1]. αβTCRs are each comprised of a conformationally flexible ligand-binding disulfide linked heterodimer (TCRαβ) associated with CD3εγ, CD3εδ, and CD3ζζ dimeric subunits in a 1:1:1:1 stoichiometry. CD3 subunits function to transduce signal through noncovalent interactions with the TCR heterodimers in order to activate the T cell [2, 3]. TCRαβ heterodimers are structurally similar to antigen-binding fragments (Fabs) of antibodies, comprised of variable domains that bind pMHC, and constant domains that link to the cell membrane, but the TCRαβ-pMHC interaction (K_D in the μM range) is orders of magnitude weaker than the typical antibody-antigen interaction (K_D in the pM to nM range). Paradoxically, exquisite sensitivity and specificity is observed, and T cells can identify fewer than ten aberrant or foreign peptides in an array of ~100,000 "self" peptides on an APC surface [4, 5].

A second class of TCRs are found on γδ T cells which comprise less than 5% of peripheral T cells in humans and are distinct from the major αβ T cells. The former assume prominence in body tissues rather than blood, binding self-ligands distinct from pMHC that are displayed on stressed cells. Although γδ TCRs have a similar subunit organization and important surveillance functions [6, 7], those receptors are different in many respects from αβTCRs and are not discussed herein.

In general, T-cell activation is a remarkably complex, nonequilibrium process involving forces and cell cytoskeletal machinery. As T cells crawl and surveil their environment, lamellipodia and microvilli protrude at the leading edge to facilitate cell migration and antigen recognition [8]. At the leading edge of the T cell, several nN of force can be generated through actin polymerization [9]. As the cell scans, TCRs are clustered in the uropod, and actin retrograde flow occurs, which is caused by contractile forces from myosin motors [9–11]. After activation, an immune synapse (IS) is formed between the T cell and APC, which is driven by actin retrograde flow and microtubule-based transport [12–14]. Traction force microscopy has been used to observe internal forces associated with immune synapse formation, in which T cells interacted with micropillars and generated forces peaking at 200 pN per pillar through TCRs for murine cells and 100 pN for human cells [15]. Similar studies show that internal forces generated by cytotoxic T cells enhance target cell killing [16].

T-cell activation is typically probed at the population level. ELISAs (enzyme-linked immunosorbent assays) are frequently

used to characterize bulk T-cell activation by measuring effector cytokines in response to pMHC, however these assays are unable to provide information regarding early triggering events. Conventional assays determine if a T cell binds pMHC in a tetramer form to observe ligand associations, but tetramers artificially cluster TCRs in a non-physiological manner. Tetramer methods may mask which cell-peptide pairs are elite matches to evoke T-cell triggering and foster binding cross-reactivity. Here, hundreds of hits can arise from a tetramer screen necessitating extensive follow-up studies to identify the best pairings. To date, our understanding of the relationship between pMHC sequence and efficient T-cell activation is limited largely due to a lack of mechanism capable of elucidating such sensitive and specific recognition. Various models have been proposed to account for the robust triggering and sensitivity of T cells, including kinetic proofreading, kinetic segregation, serial engagement, TCR oligomerization, and mechanosensing [17, 18]. The mechanosensing model resolves the paradox by tying T-cell triggering to an activated process that scales exponentially with force rather than an equilibrium mechanism that scales linearly with concentration. Mechanosensing is an energetic based model that reconciles the discrepancy between low affinity TCR–pMHC interaction and highly sensitive response, by suggesting that T cells harness mechanical forces generated during immune surveillance and operate outside of thermal equilibrium [19].

Optical tweezers were the first single molecule technique used to probe the αβTCR–pMHC interaction with mechanical force, giving insight to initial triggering events [20]. Additional single molecule biophysical techniques, including atomic force microscopy (AFM), biomembrane force probe (BFP), and micropipette, have corroborated the evidence that mechanical forces underpin peptide discrimination and initiation of downstream signaling [21–24]. A micropipette was used to apply shear and normal forces to a T cell bound to CD3 ligand extending from APC, and triggering was observed only when force was applied to T cell bound via TCR [21]. BFP and AFM were used to watch T cell generated pushing and pulling upon triggering and linked the forces to actin polymerization [22, 23]. When actin polymerization was inhibited, no forces were generated and no triggering was observed due to the loss of dynamic actin network, however triggering was rescued when force was applied externally [23]. Another group used BFP to apply force to TCRs, and identified catch bond behavior, with optimum force of 10 pN [24].

The first mechanosensing model of T-cell activation was developed using optical tweezers, in which a pMHC-coated bead was trapped and an oscillating 50 pN force was applied to T cells in normal and shear directions [20]. Optimal triggering with pMHC only was observed in the shear force case, which is analogous to physiological forces that a TCR would experience during immune

surveillance. The method was refined in Feng et al. with experiments in which pMHC-coated beads are presented to single T cells to form bonds, and shear force applied to mimic T-cell crawling [14]. Single cell triggering was monitored over a period of time through increased intracellular Ca^{2+} flux, an early triggering marker, via fluorescence. A force in the range of 10–20 pN range was identified as being optimal at limiting number of interfacial pMHC molecules. Triggering events were linked to acto-myosin activation and transport stepping. The studies demonstrated that for the best TCR-pMHC pairs, triggering is digital and can be achieved with as few as two pMHC molecules. Such procedures are detailed in this paper and termed SCAR for Single Cell Activation Requirements (Fig. 1a).

While conventional bond lifetimes decrease exponentially with force [25], so called slip bonds, TCR–pMHC bonds were predicted [26] and shown to respond nonlinearly through a catch bond profile. αβT cell receptor catch bonds were first observed by the Zhu lab using a BFP [24]. Optical tweezers have also been used to measure catch bonds and corresponding quaternary structural changes of single αβTCRs in response to various forces, both isolated to a coverslip and on the surface of T cells [27]. The highest resolution force and position sensitivity measurements via optical tweezers utilized a classical tethered bead geometry where pMHC was immobilized directly on a PEG-coated coverslip surface and tethers formed through αβTCR linked bead using a ~1 μm strand of DNA as a tether. This geometry permits precise single molecule measurements of conformational changes and bond lifetimes at various forces but requires recombinant production and purification of components. T cells display ~30,000 copies of the same TCRαβ, and by immobilizing a T cell on a coverslip surface, single molecule experiments can be performed directly on the T-cell surface through pMHC tethered beads (Fig. 1b). The single molecule on isolated single cell experiment (SMSC) in particular permits analysis of the catch bond profile and even visualization of the conformational change despite additional compliance of the cell. Perhaps the greatest advantage is that these measurements are of the intact αβTCR including all transmembrane components in the context of the living cell, and thus target most native receptor possible. The protocols for SMSC are also detailed herein.

Additional studies have been conducted more recently using these optical tweezers-based techniques. The single molecule assay isolated on a coverslip was used to implicate mechanosensing in pre-TCRs during thymic selection where only the beta subunit of the heterodimer is present [28]. These protocols were also used to analyze the effects of mutations in the α subunit of the TCR to determine the role of the α subunit in triggering [29]. Single molecule and single cell studies have revealed many structural and molecular aspects of the T-cell mechanosensing mechanism

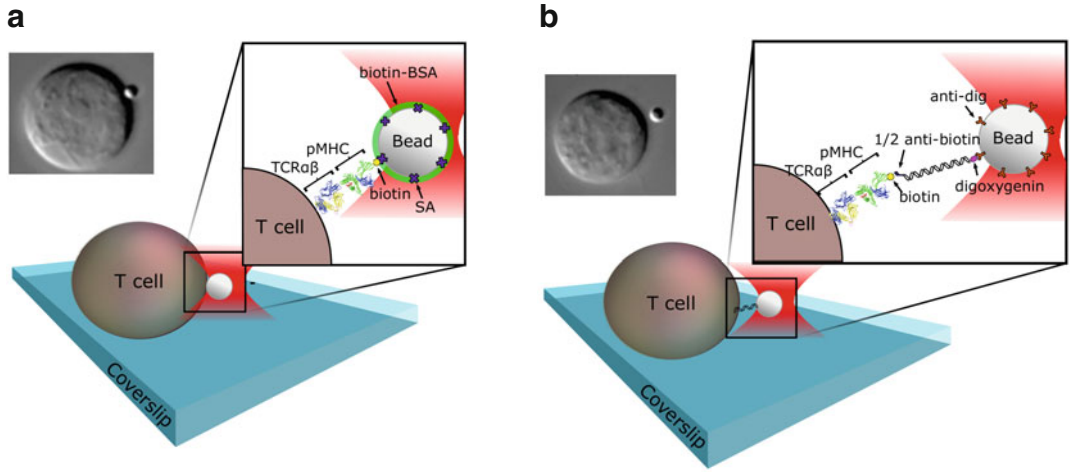

Fig. 1 (**a**) Single cell activation requirements (SCAR) assay. T cells are immobilized on the glass coverslip. A 1 μm polystyrene bead is functionalized with pMHC at varying interfacial densities via biotin-streptavidin linkage. The remaining surface of the bead is blocked from nonspecific binding with biotin-BSA. Beads are trapped and positioned nearby a cell. Beads are then calibrated for position and trap stiffness. The final TCR-pMHC interaction is facilitated by translating the cell relative to a fixed trap. Beads are typically placed at the "equator" of the coverslip bound cell. (**b**) Single molecule on isolated single cell (SMSC) assay. As in SCAR, T cells are immobilized on the glass coverslip. A 3500 bp DNA linker with a digoxygenin (dig) tag is bound to a 1 μm bead coated with anti-dig antibody. The opposite end of the DNA linker is crosslinked to a half anti-biotin antibody, which is used to bind an individual biotinylated pMHC molecule. Optical tweezers trap the bead, calibrate and facilitate tether formation. Tethers are pulled by translating the cell relative to a fixed trap with a defined force until bond breakage

through direct observation of individual T-cell triggering under various conditions. Each study guides the pursuit of molecular interactions involved in T-cell mechanosensing and further helps us uncover the complexity of this masterful immunological machine.

In this chapter, we describe protocols for both SCAR and SMSC assays. SCAR can be used to find chemical and physical triggering thresholds for a given T cell–pMHC combination, and SMSC provides detailed analysis at the individual TCRαβ-pMHC bonding interaction level. Together, these experiments provide a comprehensive understanding of αβ T-cell biophysics, and the same approach can be applied to study TCRγδ–ligand interactions in the future.

2 Materials

2.1 Generation of BW5147 Cell Lines

An overview of materials and methods pertaining to the production of the BW5147 cell lines are found below. Cells are retrovirally transfected to express TCR complex and CD8 dimers. Further details for each step can be found in Feng et al. [14], Brazin et al.

[29] and the manufacturer's instructions for the commercial reagents.

1. BW5147 cells (ATCC).
2. Cell growth medium: 500 mL DMEM-high glucose (Sigma D6546) supplemented with final concentrations of 10% heat inactivated, fetal bovine serum (FBS) (Sigma, F2442), 100 U/mL Penicillin and streptomycin (PS) (Gibco 15140-122), 4 mM L-glutamine (Gibco 25030-081), 0.4 mg/mL Hygromycin B (Invitrogen 10687010), and 0.4 mg/mL G418 Geneticin (Gibco 10131-035).
3. CD3 expression plasmid: pMIY encoding CD3δγεζ (a gift of the Vignali Lab, St. Jude Children's Research Hospital, Memphis TN).
4. TCR expression plasmid: pMIGII (Addgene) encoding the TCRαβ.
5. CD8αβ expression plasmid: pcDNA3.1 vector encoding the CD8αβ gene.
6. Retroviral supernatant: Phoenix-Eco packaging cells (ATCC, CRL-3214) transduced with expression plasmids using X-tremeGENE 9 (Roche, 06366511001) DNA transfection reagent.
7. Antibodies for FACs: anti-CD3ε-APC (145-2C11, eBioscience 17-0031-83) or anti-TCRβ-APC clone specific antibodies. Armenian hamster IgG isotype control (HTK888, BioLegend 400923).
8. FACS cell sorter.

2.2 T-Cell Activation IL-2 ELISA

A T-cell activation assay is used to determine the correlation between the T-cell triggering assays and the functional avidity of the surface-expressed TCR on the generated TCR-BW5147 cell lines. Thus, an IL-2 ELISA assay is performed to measure the secretion of IL-2 in response to graded peptide stimulation.

1. Antigen-presenting cells: R8, H-2K^b cells.
2. BW5147-CD8αβ-CD3δγεζ-TCRαβ cells.
3. Stimulatory peptide VSV8 stock, RGYVYQGL, 10 mg/mL.
4. Positive control: Phorbol-12-Myristate-13-Acetate (PMA), Santa Cruz Biotechnology (sc-3575). Ionomycin (ION), Santa Cruz Biotechnology (sc-3592).
5. 96-well plate round bottom tissue culture treated plate (Falcon 30377).
6. IL-2 ELISA kit: mouse IL-2 DuoSet and ancillary reagent kit 2 (R&D systems, DY402-05, DY008).

2.3 Media for Cell Culture and Single Cell Experimentation

1. Cell growth medium: 500 mL DMEM-high glucose (Sigma D6546) supplemented with final concentrations of 20% FBS (heat inactivated), 100 U/mL Penicillin and streptomycin (Gibco 15140-122), 2 mM L-glutamine (Gibco 25030-081), 0.5 mg/mL Hygromycin B (Invitrogen 10687010), and 0.5 mg/mL G418 Geneticin (Gibco 10131-035), 1% nonessential amino acids, 0.1% 2-mercaptoethanol (*see* **Note 1**).
2. Colorless medium: 50 mL colorless DMEM medium supplemented with final concentrations of 100 U/mL Penicillin and streptomycin (Gibco 15140-122), 2 mM L-glutamine (Gibco 25030-081), and 1% nonessential amino acids.
3. Dye loading: Colorless medium, 3% (v/v) FBS (heat inactivated), Pluronic F-127 in PBS for final concentration of 0.02% (v/v).
4. Blocking buffer: Colorless medium, 5 mg/mL BSA.

2.4 Refolding and Biotinylation of the pMHC VSV8/H-2K^b

1. Purified inclusion body preparation β2M light chain (20 mg) [30].
2. Purified inclusion body preparation H-2K^b heavy chain with C-terminal BirA tag (60 mg) [30, 31].
3. Synthetic peptide VSV8, RGYVYQGL (10 mg).
4. Refolding buffer: 8 M Urea (144 g), 20 mM Tris–Cl pH 8.0 in 300 mL DI water.
5. Dialysis Buffer 1: 2 M Urea (480 g), 20 mM Tris–HCl pH 8.2 in 4 L DI water.
6. Dialysis Buffer 2: 1 M Urea (240 g), 20 mM Tris–HCl pH 8.2 in 4 L DI water.
7. Dialysis Buffer 3: 0.5 M Urea (120 g), 20 mM Tris–HCl pH 8.2 in 4 L DI water.
8. Dialysis Buffer 4: 20 mM Tris–HCl pH 8.2 in 4 L DI water.
9. Dialysis Buffer 5: 20 mM Tris–HCl pH 8.2 in 4 L DI water.
10. Dialysis tubing (Spectra/Por) 6-8000 MWCO, regenerated cellulose. Note that 500 Da MWCO tubing had been generally used to prevent loss of peptide during dialysis, but in our hands there has been no decrease in yield in changing to this higher cutoff and more convenient tubing.
11. BirA kit (Avidity, BirA500).
12. Chromatography system with Superdex 200 (cytiva) size exclusion column and MonoQ (cytiva) anion exchange column.
13. Ultrafiltration membranes, 30 kDa MWCO for centrifugation (Amicon ultra centrifugal units, Millipore Sigma) or stirred cell concentration (Ultracel, Millipore Sigma).

2.5 Bead Decoration with pMHC Molecules

1. PBST: PBS buffer at pH 7.4 with 0.02% Tween-20 (Sigma-Aldrich).
2. Streptavidin-coated polystyrene microparticles (1.09 μm, Spherotech).
3. Biotinylated pMHC.
4. Biotin-BSA (Thermo Fisher).
5. Microcentrifuge tubes (0.7 mL, Fisher 05-408-120).
6. Cup sonicator (Sonics Vibra-Cell).

2.6 Chemical Etching of Coverslips and Flow Cell Assembly

1. Scotch permanent double-sided tape (3M cat #137DM-2).
2. Potassium hydroxide.
3. 200 proof ethanol.
4. Bath Sonicator (Branson 2510).
5. Glass coverslips (Fisher cat#12-544-C).
6. Glass microscopy slides (VWR cat#48312-068).
7. 1 L beakers.

2.7 Determination of pMHC Coating Density

1. PBST buffer pH 7.4.
2. Sodium chloride.
3. pMHC-coated beads.
4. PE anti-mouse MHC Class I ($H\text{-}2K^b$), clone AF6-88.5 (eBioscience 12-5958-80).
5. Dual channel flow cell.
6. Flow cytometer (BD LSRFortessa).

2.8 Single Cell Activation Requirement Assay Preparation

1. T Cells.
2. pMHC-coated beads.
3. Flow cell.
4. Cell media: colorless medium, dye-loading medium, blocking buffer.
5. Quest Rhod-4 Ca^{2+} indicator (AAT Bioquest).
6. Microcentrifuge tubes (1.5 and 0.7 mL, Fisher 02-682-556 and 05-408-120).

2.9 Anti-Digoxigenin Beads

1. 1 μm carboxylate polystyrene beads (Polysciences, Inc. 08226).
2. EDC crosslinker (Thermo Scientific 22980).
3. PBST buffer pH 7.4.
4. Anti-digoxigenin polyclonal antibody (Sigma-Aldrich).
5. 0.1 M MES buffer with 0.01% Tween-20 (pH 4.5): 1.95 g MES, 10 μL Tween-20, DI water up to 100 mL total volume. pH adjusted to 4.5.

6. 0.1 M borate buffer (pH 8.5): 0.618 g boric acid in 100 mL DI water. pH adjust to 8.5 using NaOH.
7. Ethanolamine (Sigma-Aldrich).
8. Bovine serum albumin (BSA).
9. Cup sonicator (Sonics Vibra-Cell).

2.10 3500 Base-Pair DNA Linker

1. M13mp18 plasmid (Bayou Biolabs #P-105).
2. Forward oligonucleotide primer with Dig tag at 5′ (5-′-Dig-AAT CCG CTT TGC TTC TGA CT-3′) (Integrated DNA Technologies custom oligo).
3. Reverse oligonucleotide primer with amino tag at 5′ (5′-NH_2-TTG AAA TAC CGA CCG TGT GA-3′) (Integrated DNA Technologies custom oligo).
4. TE buffer (Ambion #A9849).
5. dNTPs (New England Biolabs #N04475).
6. Phusion polymerase with 5× GC buffer (New England Biolabs M0530S).
7. UltraPure water (Invitrogen #10977-015).
8. UV-Vis spectrophotometer (Thermo Scientific NanoDrop 2000).
9. PCR machine (Bio-Rad).
10. QIAQuick PCR purification kit (Qiagen #28106).

2.11 Half Anti-Biotin Cleavage and Coupling to DNA Linker

1. Anti-biotin antibody (Sigma-Aldrich).
2. PBST/EDTA (1 mM EDTA).
3. 2-MEA (2-Mercaptoethylamine·HCl, Thermo Scientific 20408).
4. Bio-Rad Micro Bio-Spin (MBS6 and MBS30) size exclusion columns.
5. Sulfo-SMCC.
6. DNA linker.
7. UV-Vis spectrophotometer (Thermo Scientific NanoDrop 2000).

2.12 Single Molecule on Single Cell Assay Preparation

1. T cells.
2. Anti-dig beads.
3. DNA linker with half anti-biotin.
4. Biotinylated pMHC.
5. Flow cell.
6. Casein.
7. Colorless medium.

8. Microcentrifuge tubes (1.5 and 0.7 mL, Fisher 02-682-556 and 05-408-120).

2.13 Combined Optical Tweezers and Fluorescence Instrumentation

The optical trapping and fluorescence combined microscope is custom-built using high-end components to minimize noise and maximize stability. The system is comprised of trapping laser, detection laser, fluorescence excitation lasers, acousto-optic deflectors (AODs) for trap steering, piezo stage for sample positioning, bright-field and back thinned cameras for conventional imaging and single molecule (SM) fluorescence imaging respectively, as described in Brau et al. [32]. In order to successfully execute these assays, the system must contain trap/detection capabilities with simultaneous fluorescence acquisition.

1. Microscope: Nikon TE 2000-U, bolted to the optical table. Microscope fitted with a Nikon Oil IR, 100×, 1.40 NA objective and Nikon Oil 1.4 NA condenser.
2. Trapping laser: Coherent laser model Compass 1064-4000M. Fiber coupled to diode pump source such that acoustical noise and heat generation from the power supply does not impact the trapping room. Acousto-optic deflectors (AODs; IntraAction, model DTD-276HD6) control laser power and position.
3. Detection laser: The detection laser is a butterfly format fiber Bragg grating 975 nm, Corning Lasertron. The signal is collected on a position-sensitive device, PSD, Pacific Silicon. The signal is anti-alias filtered and amplified through a Kron-Hite filter model 3384 before collection.
4. Fluorescence: Blue Sky Research FiberTec 488 nm laser and World Star Tech 532 nm laser, with power control via IntraAction AOD model AFM-404A1 and shutter control via TTL pulses. Two fluorescence lasers are not necessary for experiments, but simply expands the list of usable fluorophores. Fluorescence images are collected with Andor iXon camera and associated Andor iXon software.
5. Automation: AODs control position of the trapping laser through computer program and permit mapping of position sensitivity. Piezo stage (Physik Instrumente p-517.3CD stage and E-710.3CD controller) enables precise controlled stage motions, various Uniblitz shutters and shutter driver box control beams and camera acquisition through TTL signals as digital outputs from LabVIEW programs. Newport picomotor actuators control positioning of detection laser relative to the trap for optimal sensing on the PSD.

3 Methods

3.1 Generation of BW5147 Cell Lines

1. Culture BW5147 cells in DMEM supplemented media in a 37 °C incubator with 5% CO_2.
2. Use the viral 2A-linked system to generate multicistronic vectors for co-transfection of the CD3 and TCR genes.
3. Sub-clone the CD8αβ gene into the pcDNA3.1 vector and transduce the plasmid into the BW5147 cells.
4. Generate the TCR and CD3 retroviral supernatant by transfection of the CD3δγεζ and TCRαβ plasmids into Phoenix-Eco packaging cells.
5. Harvest the viral supernatants and use to retrovirally transduce the BW5147-CD8αβ cells to first incorporate the CD3 genes.
6. Select for CD3-retrovirally transduced cells by positively selecting on YFP by FACs.
7. Retrovirally transduce the BW5147-CD8αβ-CD3δγεζ with the TCRαβ viral supernatant.
8. Select for TCR-retrovirally transduced cells by positively selecting on GFP by FACs.
9. Routinely FACs sort the transduced cell line to maintain equivalent surface TCR levels through selection by anti-CD3ε-APC or anti-TCRβ-APC clone specific antibody staining.
10. Use an APC Armenian hamster IgG isotype control as a negative control in the FACs samples.
11. Detect APC by FACs using a 660/20 band pass filter, excited with a 633 nM laser and sort with a 70 mM nozzle.

3.2 T-Cell Activation IL-2 ELISA

1. Irradiate the R8 cells at 3000 rads prior to use and wash cells with DMEM media containing PS, FCS, and glutamine but lacking hygromycin and geneticin.
2. Wash the BW5147-CD8αβ-CD3δγεζ-TCRαβ cells with DMEM media containing PS, FCS, and glutamine but lacking hygromycin and geneticin.
3. Set-up the activation assay in triplicate in a 96-well plate. Add prepared R8 cells at 2×10^5 cells per well.
4. Add the stimulatory peptide at the desired final concentration in each well usually within the range of 50 pg/mL to 5 mg/mL.
5. Add the prepared BW5147-CD8αβ-CD3δγεζ-TCRαβ cells at 2×10^5 cells per well.
6. Prepare positive control wells containing 2×10^5 each of R8 and BW5147-CD8αβ-CD3δγεζ-TCRαβ cells. Do not add

stimulatory peptide but instead add a mixture of 10 μg/mL PMA/1 mg/mL ION.

7. Prepare negative control wells containing 2×10^5 each of R8 and BW5147-CD8αβ-CD3δγεζ-TCRαβ cells without stimulatory peptide but replace the volume of the peptide with DMEM media.
8. Place the plate in an incubator for 16–18 h overnight in a 37 °C incubator supplemented with 5% CO_2.
9. Following incubation, harvest the cell supernatants by centrifugation of the 96-well plate and then transfer the supernatants to a new plate.
10. Carry-out an IL-2 ELISA assay using the mouse IL-2 DuoSet and ancillary reagent kit 2 following the manufacturer's instructions.
11. If necessary, during the assay set-up dilute the cell supernatants in media such that the O.D. 450 nm readings fall within the standard curve for the assay. This is frequently needed for higher stimulatory peptide concentrations.
12. Subtract the negative control values from each sample point and concentrations in pg/mL were calculated from the standard curve.
13. Plot the measured IL-2 was vs. the concentration of stimulatory peptide and fit to a 4-parameter logistic model.

3.3 Refolding and Biotinylation of the pMHC VSV8/H-2K^b

1. Add protein and peptide components to Refolding buffer. Mix well and allow to equilibrate for 1 h.
2. Place in equal aliquots in 6–8 kDa MWCO dialysis tubing and dialyze against Dialysis Buffer 1 through 5 stepwise. Keep protein in each condition at least 2 h or overnight once the sample is in Dialysis Buffer 3–5.
3. Remove from dialysis, centrifuge at high speed to remove large precipitate and filter with 0.22 mM low protein binding filter, cellulose acetate or similar.
4. Concentrate protein using stirred cell with ultrafiltration disk (Ultracel, Millipore Sigma) with 30 kDa MWCO and separate via size exclusion chromatography (Superdex 200) followed by anion exchange (MonoQ). Buffer exchange protein into phosphate buffered saline pH 7.4 using either SEC or concentration and redilution using Amicon ultra centrifugal units 30 kDa MWCO. Yields will typically be 1 mL × 300 μM complex or ~15% yield mol/mol of H-2K^b. Care should be taken to work relatively quickly once the protein is purified since proteases act to remove the BirA site.

5. Following manufacturer's directions, adjust BirA tagged pMHC concentration to 40 μM. Suggested reaction volumes are 1:1:8 of BiomixA:BiomixB:VSV8/H-2K^b-BirA (40 μM) with 1 μg of BirA enzyme/4 nmol VSV8/H-2K^b-BirA. A typical reaction is 12 μL A + 12 μL B + 100 μL VSV8/H-2K^b-BirA +1 μL BirA. BirA enzyme is stored at −80 °C and Components A and B at −20 °C.
6. Incubate at room temperature approximately 5 h or overnight. Optionally incubate 37 °C for 1 h.
7. Separate via SEC (Superdex 200) with the column equilibrated in PBS pH 7.4, expecting a single protein peak plus large small molecule peak. Collect fractions associated with the protein peak and concentrate to approximately 500 μL, adjusting volume for 1 mg/mL concentration or as convenient.
8. BirA- (i.e., before biotinylation) or Biotin-tagged pMHC can be flash-frozen and stored in aliquots at −80 °C indefinitely.
9. Conjugation efficiency can be quantified using SDS-PAGE separation to measure a shift in the migration of MHC heavy chain to a higher molecular weight when it is successfully biotinylated and can bind streptavidin [33]. Prepare a sample for separation with MHC and streptavidin (lane A): 7.5 μL (pMHC-Biotin + PBS (5 μg total)) + 5 μL of 1 mg/mL Streptavidin.
10. Prepare three samples (lanes B–D) to make a serial dilution of pMHC containing 5, 1, or 0.2 μg, i.e. a 1×, 1/5× and 1/25× sample each in 12.5 μL total volume of pMHC + PBS.
11. Wait 1 h for Streptavidin binding to occur.
12. Add 2.5 μL 4× SDS-PAGE sample loading buffer and load 15 μL/lane without boiling. SDS alone is enough to denature most proteins but will not break the streptavidin-biotin bond.
13. Any unbound MHC heavy chain migrates as an approximately 32 kDa protein. Streptavidin-bound MHC heavy chain migrates at >90 kDa. Compare MHC heavy chain band remaining in lane A with the heavy chain in lanes B–D for an estimate of unmodified MHC. Best results show a heavy chain band density equal to between that of lanes C and D, i.e., 80–96% modified.

3.4 Bead Decoration with pMHC Molecules for Single Cell Activation Assay

1. Wash 5 μL of streptavidin bead stock with 100 μL PBST—centrifuge at 2 min at 8000 × *g* and 4 °C. Resuspend in 100 μL PBST (repeat 3×). Final resuspension in 50 μL, giving 0.1% (w/v) concentration. Sonicate 2 min at 40% in cup sonicator filled with cold water.
2. Combine 5 μL of washed streptavidin beads with 5 μL biotinylated pMHC of desired concentration in 0.7 mL centrifuge

tube and rotate for 1 h at room temperature. For N15 TCR with VSV8/K^b, 5 μL of 1 μM, 100 nM, 10 nM and 2.5 nM of VSV8/K^b with 5 μL of 0.1% (w/v) streptavidin bead gives 20,000, 200, 20, and 5 VSV8/K^b molecules per bead [14] (*see* Subheading 3.7 for density determination).

3. Wash beads with 100 μL PBST (repeat 3×).
4. Block remaining surface from nonspecific binding by adding 20 μL of 5 mg/mL biotin-BSA in PBST buffer to pMHC-coated bead slurry and rotate 1 h at room temperature.
5. Wash 3× with 100 μL PBST. Final resuspension in 50 μL PBST. For single cell activation assay dilute 2 μL beads into 30 μL PBST.

3.5 Chemical Etching of Coverslips

1. Dissolve 100 g of KOH in 300 mL of ethanol in a 1 L beaker while stirring with stir bar. It will typically be a clear, dark red-brown color when complete. This takes approximately 30 min.
2. Load glass coverslips into Teflon coverslip racks.
3. Fill one beaker with 300 mL ethanol, and two beakers with 300 mL of ddH_2O. Degas all four beakers in a bath sonicator for 5 min on degas setting.
4. Submerge one rack of coverslips in KOH beaker and sonicate in the bath sonicator for 5 min on sonicate setting.
5. Dip rack of coverslips up and down in 200 proof ethanol and swirl until ethanol runs off coverslips without beading. Repeat in one beaker of water until water runs off coverslips without beading.
6. Submerge rack of coverslips in second beaker of water and sonicate for 5 min.
7. Spritz each side of the coverslips with water until water flows off coverslips smoothly. Use plenty of water.
8. Spritz each side of the coverslips with ethanol until the ethanol flows off the coverslips smoothly.
9. Dry the rack in the oven at 90 °C for at least 15 min. Store in sealed plastic Nalgene container.
10. Repeat with other racks of coverslips.

3.6 Flow Cell Construction

1. Place two pieces of double-sided tape across the short axis of a glass microscope slide with roughly 5 mm in between. Add a second layer on top of each tape to double the height of the flow cell. This gives approximate volume 40 μL.
2. Place a KOH-etched coverslip across the tape, with the long axis of the coverslip perpendicular to the long axis of the glass slide.

3. Use the bottom of an Eppendorf tube or sturdy pipette tip to press the coverslip down along the edge of the tape. Work from one side to the other, clearing any air bubbles and creating a firm seal between the tape and glass (*see* **Note 2**).

3.7 Determination of pMHC Coating Density

3.7.1 Determination of pMHC Concentration at Limiting Molecular Count

1. For low pMHC density on beads, TIRF microscopy is used to count pMHC molecules at the cell–bead interface. Create flow cell similar to Subheading 3.6, but with only one layer of tape. Cut an additional piece of tape in half lengthwise, and use it to create two separate flow channels. One will be used for control beads, and one for pMHC-coated beads.
2. Combine 5 μL of pMHC-coated beads or control unlabeled beads, 2 μL of PE anti-mouse MHC Class I (H-2Kb) (diluted 100× in PBS), and 50 μL PBST. The peak excitation of PE is 566 nm, and emission at 574 nm. There is a second excitation peak around 490 nm. We excite with a 488 nm laser.
3. Incubate on rotator for 1 h at room temperature.
4. Wash 5× with PBST for 2 min at 8000 × *g* at 4 °C, and resuspend in 50 μL high salt solution (~0.4 M NaCl in PBST) after final wash.
5. Flow control beads in one channel and pMHC-coated beads in other channel, careful not to let bead solutions mix.
6. Image using TIRF microscopy. The number of pMHC molecules at the interface can be calculated using the following equation:

$$n = \frac{I_{\mathrm{pMHC}} - I_{\mathrm{str}}}{I_{\mathrm{Ab}} - I_{\mathrm{back}}}$$

where I_{pMHC} is the fluorescence intensity of the pMHC-coated bead, I_{str} is the intensity of the control bead, I_{Ab} is the intensity of one antibody, and I_{back} is the intensity of the background (Fig. 2a).

3.7.2 Determination of pMHC Concentration at High Molecular Count

1. For high molecular count, flow cytometry can be used to determine pMHC density on the bead. Add different amounts of PE anti-mouse MHC Class I (H-2Kb) antibody to bead solution, with the maximum ~0.1 μg.
2. Rotate for 1 h at room temperature.
3. Analyze via flow cytometry. Examine mean values of fluorescence and normalize by dividing by maximum signal from the highest antibody concentration. This gives the number of pMHC molecules per bead, and it is estimated that the cell–bead interface is ~10% of the total bead surface area (*see* **Note 3**) (Fig. 2b).

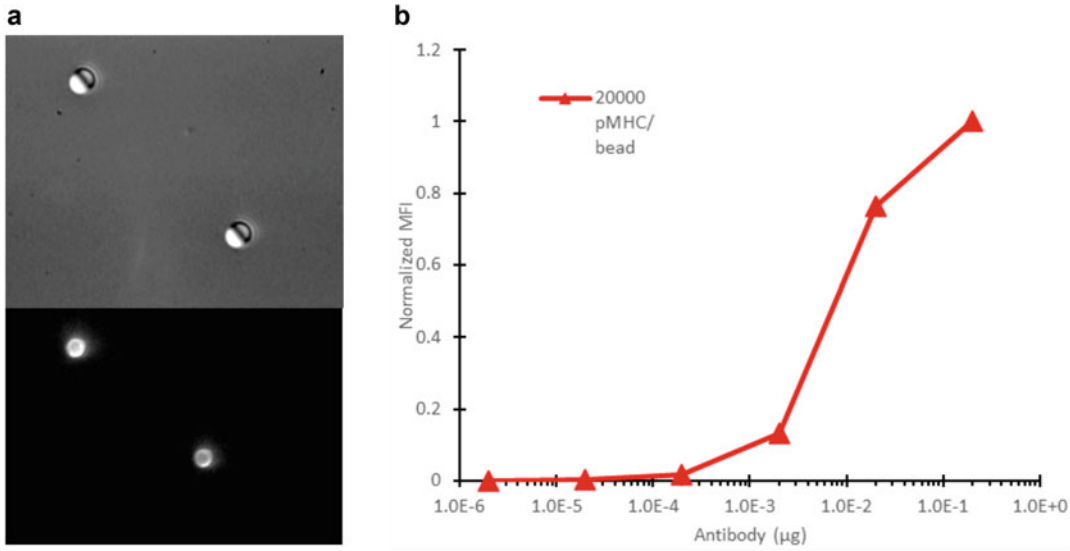

Fig. 2 Determination of pMHC density at limiting and high molecular count. (**a**) Bright-field and TIRF images of beads with 29 pMHC molecules at the interface. (**b**) Titration curves of beads with 2×10^4 pMHC molecules per bead as measured via flow cytometry. Autocorrection for flow cytometry machine variations calculated as done previously [29]

3.8 Single Cell Activation Requirement Assay Preparation

1. Wash cells for 5 min at 150 × *g* and resuspend in 100 μL at 2 million cells/mL in complete colorless dye-loading medium.
2. Add Quest Rhod-4 at 2 μM final concentration to cells and incubate for 40 min at 37 °C, gently pipette cells every 10 min for even dye distribution. Quest Rhod-4 has a peak excitation at 530 nm and emission at 555 nm. We excite with a 532 nm laser.
3. Wash cells and resuspend in 160 μL in colorless media with 3% FBS and transfer to a flow cell with KOH etched coverslip. Incubate for 1 h in incubator to allow cells to adhere to coverglass. Leave some excess medium at the edges of the flow cell to prevent evaporation from inside the channel.
4. Flow blocking buffer into flow cell and incubate for 5–10 min to block exposed coverslip surface from nonspecific binding.
5. Dilute 2 μL pMHC beads into 30 μL of blocking buffer, and flow into flow channel. Immediately load slide onto optical trap-fluorescence microscope to begin assay (Fig. 3).

3.9 Single Cell Activation Measurement

The single cell activation requirement (SCAR) assay measures single cell response to a given pMHC density and force application.

1. Warm up lasers during dye incubation, and turn on equipment for at least 30 min. For a trap stiffness of approximately 0.2 pN/nm, our instrument requires 450 mW as measured

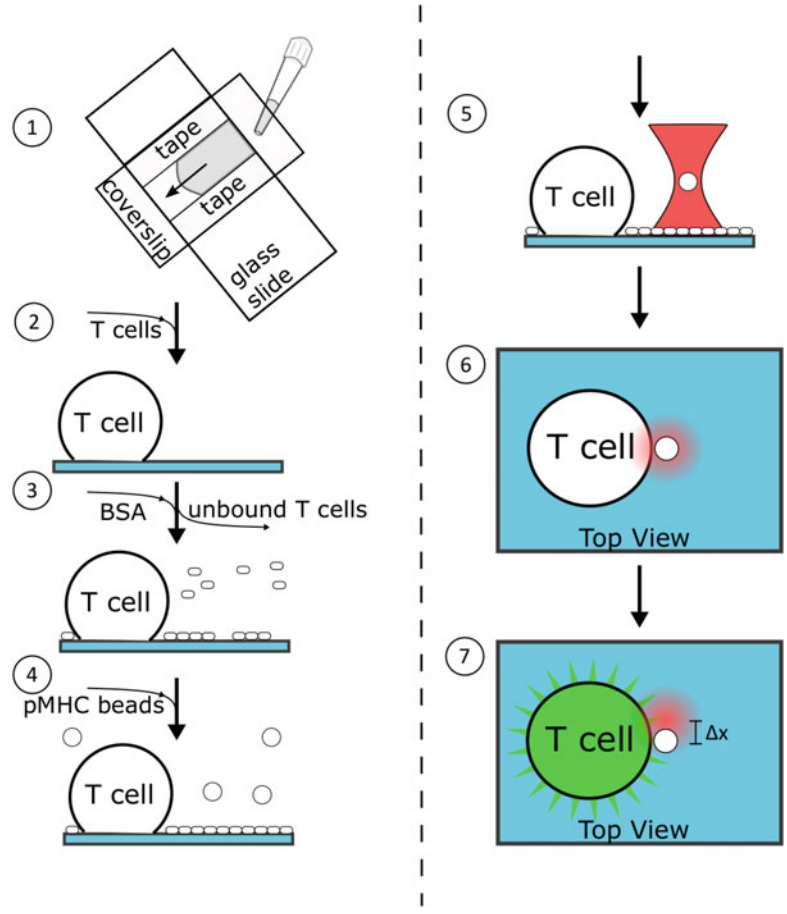

Fig. 3 Cartoon of SCAR workflow outlining flow cell preparation and measurement. A flow cell is assembled (panel 1). T cells loaded with Ca^{2+} fluorescent dye are introduced within the flow cell and incubated to adhere to the surface (panel 2). BSA in colorless medium is added to block the surface from nonspecific binding and wash out unbound T cells (panel 3). pMHC-coated beads are added to the flow cell (panel 4) and the cell is loaded on the microscope. To measure, a single pMHC-coated bead is trapped (panel 5) and the stage is translated relative to the fixed trap to facilitate bond formation between the pMHC on the bead and the TCRs on the cell surface (panel 6). A fluorescence image is obtained to establish a baseline. The stage is then translated a known distance (typically tangentially for shear direction measurements) to provide a force. Cell fluorescence is then monitored to evaluate triggering (panel 7)

before the beam enters the objective. Turn on heater to warm sample chamber to 37 °C.

2. Load slide onto microscope and focus. A single drop of type DF immersion oil (Cargille) is used between the objective and coverslip, and 3–4 drops of type A immersion oil (Nikon) between the microscope slide and condenser.

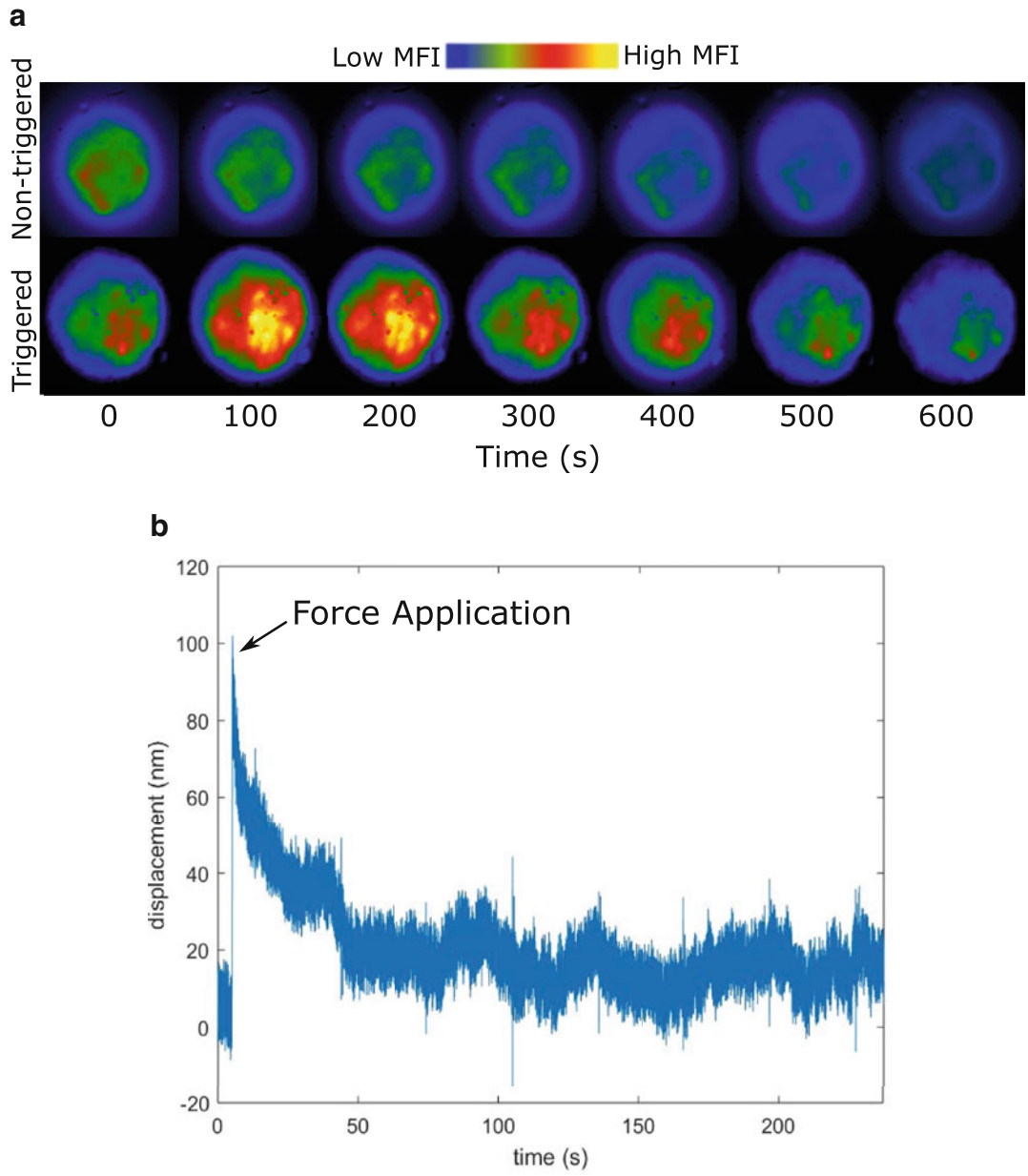

Fig. 4 Sample SCAR data (**a**) triggered vs. non-triggered fluorescence over time. The non-triggered cell, top sequence, photobleaches over time, while the triggered cell, bottom sequence, shows an increase in fluorescence before decreasing. (**b**) Sample trace at 20 pN for a triggered cell. Distance denotes bead displacement from the center of the trap. Force is applied at time zero showing a step increase in displacement. After force is applied, triggered cells transport the bead-tethered TCRs in the direction opposite of the force, resulting in relaxation back toward zero

3. Open LabVIEW programs for piezo stage control, trap position control through AODs, data acquisition through bead position voltage, fluorescence excitation shutters synchronized with camera acquisition.

4. Open Andor software to allow Andor Ixon camera to turn on and cool to −80 °C. Set exposure time to 100 ms. Increase gain. Set software to external collection, data collection frequency 0.2 Hz, and 120 total frames collected for 10-min acquisition. This allows data collection to be synchronized with fluorescence laser shutter controlled by automated LabVIEW program.
5. Find and trap a single bead near a healthy cell. A healthy cell should possess a smooth cellular surface without any blebbing. Dither the AODs while centering the detection laser to confirm proper alignment of trap and detection lasers. Run a fifth order calibration for position and calculate the trap stiffness [34] (*see* **Note 4**). Calibrate without anti-alias filtering voltage by opening the filter to 30 kHz. After calibration, reset the anti-alias filter to 1.5 kHz.
6. Carefully translate the stage relative to the trap to bring the bead near the surface of the cell. This should be done such that the tangent of the cell at the point of bead-cell contact is parallel with either the x or y stage axis. Proper bead placement will ensure that force is only applied in one axis (*see* **Note 5**). Micrometers should be used to move the stage close to this position but without actually touching the bead to the cell. The z position should be adjusted so the bead height is even with the equator of the cell resting on the slide.
7. Acquire bead position voltage at 3.0 kHz. Begin voltage acquisition storing the data to a file and visualizing the voltage on a volt vs. time chart as well as a top down *x* vs. *y* voltage plot. These visuals will be used to monitor the moment when the bead comes in contact with the cell membrane. In small (~50 nm) steps, translate the stage using the automated piezo stage control program to guide the cell toward the bead in the fixed trap. Watch the voltage acquisition to determine when the bead and cell touch. Note, there will be motion in the voltage, indicating that the cell membrane touched the bead, causing bead motion slightly out of the trap center. Ideally this results in minimal, < 5 pN, force. Using the piezo stage control routine, translate the stage slowly toward the trap, until the cell and the bead just barely touch to facilitate binding. Confirm the bead is bound to the receptor by quickly turning the trap off and on. Turn off the bright-field lamp, and quickly switch to fluorescence mode.
8. Initiate data acquisition on Andor software. Use the fluorescence shutter control routine to collect fluorescence images every 5 s. The fluorescence shutter triggers the Andor software to collect images. Note it is important to acquire at least one frame prior to applying force so that the non-triggered baseline

of fluorescence can be determined (*see* **Note 6**). After first image is collected, apply force using piezo stage control (*see* **Note 7**). Collect both fluorescence and voltage data for 10 min (Fig. 4).

3.10 Anti-Digoxigenin Beads

SCAR protocols conclude with Subheading 3.9. The remaining protocols are to be used for SMSC assay.

1. Combine 10 μL of 1 μm carboxylate polystyrene beads, 2.6% (w/v), with 100 μL MES buffer.
2. Spin down (2 min at 8000 × *g*) and resuspend in 200 μL MES buffer (repeat 5×).
3. Sonicate 2 min at 40% in cup sonicator.
4. Add 200 μL of 2% (w/v) EDC solution in MES buffer.
5. Incubate at room temperature for 3 h on rotator.
6. Spin down (4 min at 8000 × *g*) and resuspend in 1 mL borate buffer.
7. Spin down and resuspend in 400 μL borate buffer and sonicate 4 min at 40%.
8. Add 80 μL of 200 μg/mL anti-Dig antibody and rotate 1 h at room temperature, then overnight at 4 °C.
9. Stop reaction by adding 10 μL of 0.25 M ethanolamine in borate buffer.
10. Incubate 30 min on rotator at 4 °C.
11. Spin down and resuspend in filtered 200 μL 10 mg/mL BSA in PBST.
12. Sonicate 1 min at 25% with ice.

3.11 Creation of DNA Linker with Half Anti-Biotin

3.11.1 PCR Amplification of 3500 Base-Pair DNA Linker

1. Combine 25 μL of 20 μM forward primer, 25 μL of 20 μM reverse primer, 8 μL dNTPs, 5 μL of 50 ng/μL M18 plasmid, 200 μL of 5× GC buffer, 727 μL ddH_2O, 10 μL Phusion polymerase. Mix by gently pipetting up and down.
2. Separate 100 μL reaction mixture into PCR tubes.
3. Run PCR program: (1) 98 °C for 30 s, (2) 98 °C for 10 s, (3) 49 °C for 30 s, (4) 72 °C for 90 s, (5) repeat **steps 2–4** for a total of 35 times, (6) 72 °C for 10 min, (7) keep at 4 °C.
4. Purify using Qiagen PCR purification kit. Final suspension in PBS rather than elution buffer as instructed in kit.
5. Measure concentration using NanoDrop (*see* **Note 8**).

3.11.2 Antibody Splitting into Half Anti-Biotin

1. Mix 80 μL of 1 mg/mL anti-biotin antibody with 20 μL PBS/EDTA for final concentration of 0.8 mg/mL.

2. Mix 0.8 μL of MEA in PBST/EDTA (6 mg MEA in 100 μL PBST/EDTA) with 100 μL anti-biotin solution from **step 1**. Incubate at 37 °C for 90 min.
3. Purify half anti-biotin by three consecutive MBS6 column purifications. Follow buffer replacement protocol with the MBS columns using PBS.
4. Measure concentration using NanoDrop (*see* **Note 9**).

3.11.3 Coupling of Half Anti-Biotin to 3500 bp DNA Linker

1. Add 200 μL of PBS/EDTA to pre-weighed 2 mg of sulfo-SMCC and heat at 75 °C until dissolved.
2. Combine 30 μL of 3500-bp DNA linker with 6 μL of sulfo-SMCC and incubate at room temperature for 45 min (*see* **Note 10**).
3. Purify DNA-SMCC by two successive MBS6 column purifications. Follow buffer replacement protocol with the MBS columns using PBS.
4. Mix 54 μL of half anti-biotin (Subheading 3.11.2) with 60 μL of DNA-SMCC and incubate rotating overnight at 4 °C.
5. The following morning, purify half anti-biotin-DNA linker using MBS30 column. Resulting linker is 3500 bp DNA with a digoxygenin on one end, and the half-anti-biotin on the other.

3.12 Single Molecule on Single Cell Assay Preparation

1. Wash 0.5 μL of 0.25% (w/v) anti-dig beads (Subheading 3.10) with PBST for 2 min at 8000 × *g* in tabletop centrifuge (repeat 2×). Incubate with 10 μL of half anti-biotin-DNA at approximately 65 ng/μL (Subheading 3.11.3) for 1 h at room temperature. The resulting beads are functionalized with the DNA linker terminating with half anti-biotin.
2. Wash DNA-labeled beads with PBST (repeat 3×). Resuspend in 5 μL of 20 pM biotinylated pMHC (Subheading 3.3) for 1 h at room temperature. Wash with PBST (2×) and resuspend in 1 mL.
3. While DNA beads are incubating with pMHC, wash cells once in PBS at 150 × *g* for 5 min and resuspend at 2×10^6 cells/mL in colorless medium. Flow cells into flow chamber and incubate for 45 min at 37 °C.
4. Flow in 1 mg/mL casein in PBS and incubate for 10 min. Flow in 20 μL DNA-labeled beads with pMHC slurry and immediately load slide in microscope and begin experiment (Fig. 5).

3.13 Single Molecule on Isolated Single Cell Measurement

1. Warm up lasers and turn on equipment at least 30 min prior to experimentation. For a trap stiffness of approximately 0.2 pN/nm, our instrument requires 450 mW as measured before the

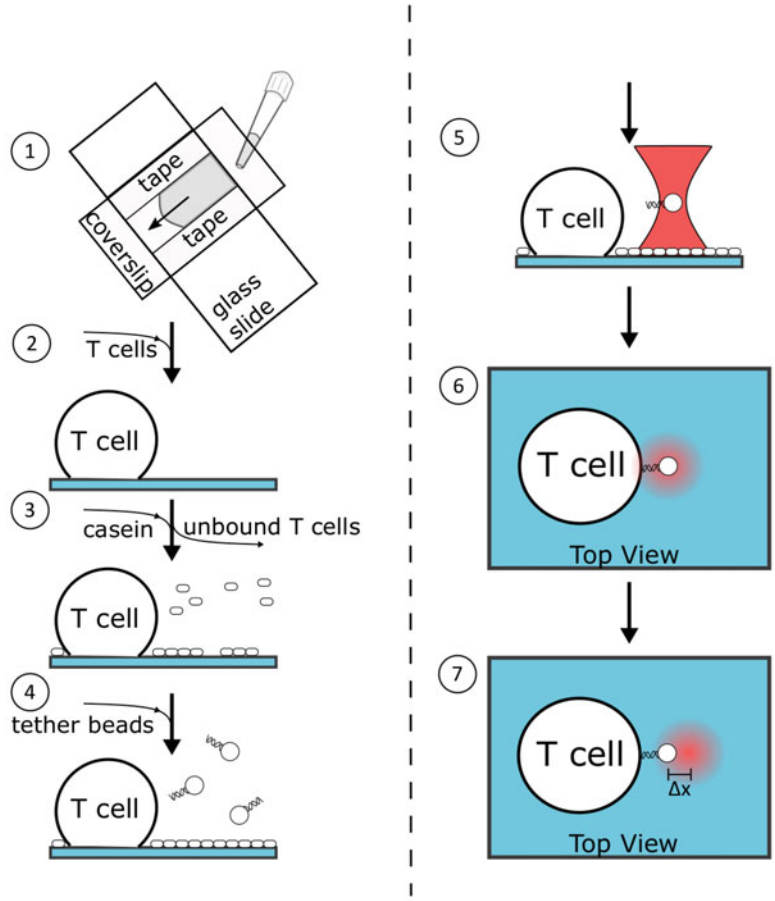

Fig. 5 Cartoon of SMSC workflow outlining flow cell preparation and measurement. A flow cell is prepared (panel 1). T cells in colorless medium are added incubated to adhere to the surface (panel 2). Casein in colorless medium is added to block the surface from nonspecific binding, and unbound T cells are wash out (panel 3). Beads containing DNA tethers bound to pMHC are added to the flow cell (panel 4) and the cell is loaded on the microscope. To measure, a single tether-functionalized bead is trapped (panel 5) and the stage is translated to position a cell near the bead. The bead is then calibrated for position and stiffness. The cell is then translated relative to the fixed trap such that a single tether forms between bead and cell surface (panel 6). The stage is translated a known distance in a direction normal to the interface to provide a force. Bead position values are collected until bond rupture (panel 7)

beam enters the objective. Turn on heater to warm sample chamber to 37 °C.

2. Load slide onto microscope and focus. A single drop of type DF immersion oil (Cargille) is used between the objective and coverslip, and 3–4 drops of type A immersion oil (Nikon) between the microscope slide and condenser.

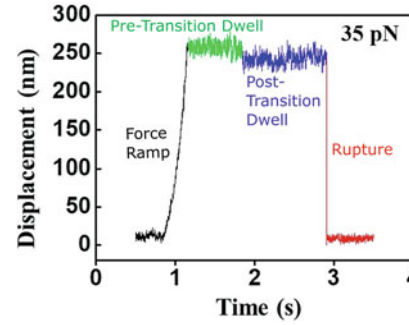

Fig. 6 Sample SMSC trace. Displacement denotes bead offset from the center of the trap. During the first phase of the experiment, the cell is translated relative to the fixed trap resulting in a force ramp to a pre-defined force value (black segment). Force is applied and held until bond rupture, a visible snap back to the center of the trap (red segment). A conformational change is noted by a slight displacement of the bead toward the trap center (green to blue transition)

3. Open LabVIEW programs for piezo stage control, trap position control through AODs, and data acquisition through bead position voltage.
4. Find and trap a single bead near a healthy cell. A healthy cell should possess a smooth cellular surface without any blebbing. Dither the AODs while centering the detection laser to confirm proper alignment of trap and detection lasers. Run a fifth order calibration for position and calculate the trap stiffness [34] (*see* **Note 4**). Calibrate without anti-alias filtering voltage by opening the filter to 30 kHz. After calibration, reset the anti-alias filter to 1.5 kHz.
5. Use fine motions of the piezo stage to bring the bead decorated with pMHC-DNA tethers close to the cell, about one bead diameter away. The z position should be adjusted such that the bead height is even with the equator of the cell.
6. The difference between a free bead and a bead bound by single tether is not visible by eye, so wait 15–30 s to allow tether to form. Begin voltage acquisition software.
7. Translate the stage relative to the fixed trap 500 nm or less to apply a certain force. Collect data until bond rupture (Fig. 6).

4 Notes

1. Minimal requirements for cell culture include: an incubator with CO_2 connection, biosafety cabinet, water or bead bath, serological pipets, and pipet aide. Cells should be cultured until they reach log phase growth before experimentation. This can be determined by counting cells daily after thawing. For best

cell growth conditions, maintain concentration ~2 × 10^5 cells/mL, and viability should remain near 90% and higher. Wash cells via centrifugation for 5 min at 150 × *g* once a week. Cultures are typically maintained for roughly 3 weeks before a new aliquot is thawed. After about a week in culture the cells can be split and frozen to build a stock. Filter media through a sterile 0.2 μm filter after mixing. Flow cytometry can be used to check TCR expression using APC anti-mouse CD3ε, clone 145-2C11 (Biolegend 100311) or PE anti-mouse TCR β chain, clone H57-597 (BD Biosciences 561081). This should be done occasionally to check TCR expression.

Sterile practices are imperative for healthy cell culture and to maintain cell functionality. Sterile practices include thoroughly spraying the biosafety cabinet with 70% ethanol and wiping down before use, spraying surfaces with 70% ethanol prior to entering the biosafety cabinet, wearing lab coat, changing gloves frequently, and keeping any bottles or flasks covered inside the biosafety cabinet as much as possible. The hood should be periodically checked for proper laminar air flow, and should be situated in the laboratory such that it is in a low traffic area away from the door to prevent hindrance to air flow patterns. Test for mycoplasma contamination occasionally to confirm clean cell culture. Mycoplasma are difficult to detect without a mycoplasma detection kit, but affect cell behavior that will can affect triggering results.

2. When exchanging solutions in the channel vacuum, filter paper wedges or kimwipes can be used to encourage flow through the channel. It is important to be gentle when using vacuum or capillary action to pull solution through so that the flow cell does not dry out. Also, it is important to keep fluid velocity gentle and low so that the laminar flow velocities will minimize fluid forces impacting cells/beads bound to the coverslip surface.
3. Fluorescence from flow cytometers is arbitrary based on user settings and machine hardware. An autocalibration to correlate mean fluorescence data to the number of fluorescent antibodies bound is described in Sarda et al. [35].
4. The AODs are scanned back and forth through a 2 MHz range (~2 μm) in one axis at a time. This is done to confirm relative overlap of trapping and detection lasers. The detection laser position can be adjusted as necessary using motorized actuators on the detector beam steering lens. The fifth order calibration routine steps a bead through the detection laser at known step sizes to calibrate bead position by mapping voltage signals to bead locations. Stiffness is calculated using equipartition as described previously [34].

5. Applying force in the shear direction mimics cell crawling through the body. αβ T cells are more responsive to shear forces than normal forces [14, 20].
6. The fluorescence shutter routine controls the shutter to the fluorescence laser and darkbox through TTL pulses. The darkbox houses the sensitive Andor camera to reduce background noise and protect it from light. Pulse sequences trigger the fluorescence and darkbox shutters to open and then trigger the Andor software to collect an image. Shutters are then closed to reduce photobleaching. Images are collected every 5 s. It is wise to check cell fluorescence before facilitating bond formation to confirm settings. Ideally, the fluorescence should be relatively high, but not saturating the signal. One must have the gain such that fluorescence is able to increase if the cell is triggered without saturation. The green laser power can be reduced via AOD power control and Andor gain can be adjusted.
7. Translating the stage 250–400 nm for a trap stiffness ~0.1 pN results in bead displacement of 100–150 nm from the center of the trap, within the linear range of trap stiffness.
8. DNA gel electrophoresis is performed to confirm length of the linker. We use an 0.8% agarose gel (VWR EM-2120) and apply 120 V for 2 h. SybrGreen at 10,000× (Molecular Probes S7563) is used to indicate DNA bands.
9. Crosslink half antibody to DNA strand in the same day. The antibody recombines if left overnight. Standard SDS-PAGE will confirm cleavage. We use Invitrogen NuPAGE Bis-Tris mini protein gels.
10. Start this step during the 90-min incubation for the anti-biotin cleavage (Subheading 3.11.2).

Acknowledgments

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