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DNA Tension Probes to Map the Transient picoNewton Receptor Forces by Immune Cells
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1 **TITLE:**

2 DNA Tension Probes to Map the Transient picoNewton Receptor Forces by Immune Cells

3

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22 **KEYWORDS:**

23 DNA tension probes, mechanobiology, receptor force, piconewton forces, immune cells,

24 mapping

25

26 **SUMMARY:**

27 This paper describes a detailed protocol for using DNA-based tension probes to image the

28 receptor forces applied by immune cells. This approach can map receptor forces >4.7pN in real-

29 time and can integrate forces over time.

30

31 **ABSTRACT:**

32 Mechanical forces transmitted at the junction between two neighboring cells and at the

33 junction between cells and the extracellular matrix are critical for regulating many processes

34 ranging from development to immunology. Therefore, developing the tools to study these

35 forces at the molecular scale is critical. Our group developed a suite of molecular tension

36 sensors to quantify and visualize the forces generated by cells and transmitted to specific

37 ligands. The most sensitive class of molecular tension sensors are comprised of nucleic acid

38 stem-loop hairpins. These sensors use fluorophore-quencher pairs to report on the mechanical

39 extension and unfolding of DNA hairpins under force. One challenge with DNA hairpin tension

40 sensors is that they are reversible with rapid hairpin refolding upon termination of the tension

41 and thus transient forces are difficult to record. In this article, we describe the protocols for

42 preparing DNA tension sensors that can be "locked" and prevented from refolding to enable

43 "storing" of mechanical information. This allows for the recording of highly transient

44 piconewton forces, which can be subsequently "erased" by the addition of complementary

45 nucleic acids that remove the lock. This ability to toggle between real-time tension mapping
46 and mechanical information storing reveals weak, short-lived, and less abundant forces, that
47 are commonly employed by T cells as part of their immune functions.
48
49 **INTRODUCTION:**
50 Immune cells defend against pathogens and cancer cells by continuously crawling and scanning
51 the surfaces of target cells for antigens, studding their surface[1,2]. Antigen recognition is initiated
52 upon binding between the T cell receptor (TCR) and the peptide-major histocompatibility
53 complex MHC (pMHC) complex expressed on the surface of target cells. Because TCR-pMHC
54 recognition occurs at the junction between two mobile cells, it has long been suspected of
55 experiencing mechanical forces. Moreover, this led to the mechanosensor model of TCR
56 activation, which suggests that TCR forces contribute to its function[3,4]. To understand when,
57 where, and how mechanical forces contribute to T cell function, it is imperative to develop tools
58 to visualize the molecular forces transmitted by T cells. Traditionally, methods such as traction
59 force microscopy (TFM) and micropillar arrays are used to investigate cellular forces[5,6].
60 However, the force sensitivity of TFM and micropillar arrays is at the nanonewton (nN) scale
61 and thus is often insufficient to study the molecular piconewton (pN) forces transmitted by cell
62 receptors[7]. To improve the force and spatial resolution for detection, our lab pioneered the
63 development of molecular tension probes, which were initially synthesized using polyethylene
64 glycol (PEG) polymers[7]. Molecular tension probes are comprised of an extendible molecular
65 "spring" (PEG, protein, DNA) flanked by a fluorophore and quencher and are anchored on a
66 surface. Forces applied to the terminus of the probe lead to its extension, separating the
67 fluorophore and quencher, and thus generating a strong fluorescence signal (**Figure 1A**)[8-10].
68
69 Over the past decade we have developed a library of different classes of molecular tension
70 probes with spring elements made from nucleic acids[11], proteins[10], and polymers[8]. Among
71 these, the DNA-based tension probes provide the highest signal to noise ratio and the greatest
72 force sensitivity, which is easily tuned from a few pN up to ~20 pN[11]. We have used these real-
73 time DNA tension probes to study the molecular forces generated by many diverse cell types,
74 including fibroblasts, cancer cells, platelets, and immune cells[11-13]. This manuscript will describe
75 protocols to synthesize and assemble DNA tension probes on a surface to map molecular
76 receptor forces with pN force resolution using a conventional fluorescence microscope. While
77 the current procedure includes chemical modifications to the nucleic acid to introduce the
78 fluorescent reporter (**Figure 1B**), it is important to note that many of the modification and
79 purification steps can be outsourced to custom DNA synthesis companies. Therefore, DNA
80 tension probes technology is facile, and accessible to the broader cell biology and
81 mechanobiology communities.
82
83 Briefly, to assemble DNA tension sensors, a DNA hairpin is hybridized to a fluorescent ligand
84 strand on one arm and a quencher anchor strand on the other arm and then immobilized on a
85 glass substrate (**Figure 1C**, real-time tension). In the absence of mechanical force, the hairpin is
86 closed, and thus the fluorescence is quenched. However, when the applied mechanical force is
87 greater than the $F_{1/2}$ (the force at equilibrium that leads to a 50% probability of unfolding), the
88 hairpin mechanically melts, and a fluorescent signal is generated.

89

90 Building on the real-time DNA tension sensor, we also describe protocols to map accumulated
91 forces, which is particularly useful for studying interactions between receptors on immune cells
92 and their natural ligand. This is because immune receptors often display short-lived bonds[3,14].
93 Accumulated forces are imaged using a "locking" strand that preferentially binds to open DNA
94 hairpins and allows for the storage of fluorescence signals associated with mechanical pulling
95 events (**Figure 1C**, locked tension). The locking strand is designed to bind a cryptic binding site
96 that is exposed upon mechanically induced melting of the hairpin and lock the hairpin in the
97 open state by blocking hairpin refolding, thus storing the tension signal, and generating an
98 accumulated tension map. Moreover, the locking strand is designed with an eight-nucleotide
99 toehold, which enables a toehold-mediated strand displacement reaction with its full
100 complement, the "unlocking" strand. With the addition of the unlocking strand, the bound
101 locking strand is stripped off the hairpin construct, erasing the stored tension signal and
102 resetting the hairpin back to the real-time state.

103

104 [place **Figure 1** here]

105

106 The main protocol consists of four major sections - oligonucleotide preparation, surface
107 preparation, imaging, and data analysis. This protocol has been successfully demonstrated by
108 our lab and others in naïve and activated OT-1 CD8^{+} T cells, OT-II CD4^{+} cells, as well as
109 hybridomas, and can be applied to interrogate different immune cell receptors including T cell
110 receptor, programmed cell death receptor (PD1), and lymphocyte function-associated antigen 1
111 (LFA-1) forces. OT-1 CD8^{+} naïve T cells are used as an example cell line in this paper.

112

113 **PROTOCOL:**
114 The OT-1 transgenic mice are housed at the Division of Animal Resources Facility at Emory
115 University. All the experiments were approved and performed under the Institutional Animal
116 Care and Use Committee (IACUC) protocol.

117

118 **1.   Oligonucleotide preparation**

119

120 1.1   Dissolve the ligand strand DNA in water (18.2 MΩ resistivity, used throughout the whole
121 protocol). Vortex and spin down the solution with a tabletop centrifuge. Tune the volume of
122 water such that the final concentration is 1 mM. Validate the concentration by using a
123 nanodrop spectrophotometer to measure the absorbance at 260 nm and determine the final
124 concentration based on the extinction coefficient of the oligonucleotide.

125

126 NOTE: The ligand strand has a modification at each terminus, 5' amine and 3' biotin, to
127 conjugate with the fluorophore and to present the biotinylated ligand. The amine group in the
128 ligand strand needs to be conjugated with a fluorophore. Cy3B dye is used for this conjugation
129 due to its high brightness and photostability, but it is not generally offered commercially and
130 requires in-house conjugation. Accordingly, the following section describes the conjugation
131 between amines and NHS ester dyes. For end users that do not have access to facilities or
132 resources for nucleic acid modification, modified nucleic acids can instead be purchased from

133 custom DNA synthesis vendors that offer bright and photostable dyes, such as the Alexa and
134 Atto family of dyes.

135

136

137 1.2   Prepare 10x PBS and 1 M NaHCO$_3$ solutions. Mix 10 µL of the 1 mM amine ligand strand
138 solution (10 nmol) with 10 µL of 10x PBS, 10 µL of 1 M NaHCO$_3$, and 60 µL of H$_2$O. Dissolve 50
139 µg of Cy3B NHS ester in 10 µL of DMSO immediately before use and add to the mixture for a
140 total reaction volume of 100 µL. Add Cy3B NHS ester last. Allow to react at room temperature
141 for 1 h or 4 ˚C overnight.

142

143 1.3   Prepare the Atto647N locking strand by conjugating the amine locking strand with
144 Atto647N NHS ester. Prepare 10x PBS and 1 M NaHCO$_3$ solution. Mix 10 µL of the 1 mM amine
145 locking strand solution (10 nmol) with 10 µL of 10x PBS, 10 µL of 1 M NaHCO$_3$, and 60 µL of H$_2$O.
146 Dissolve 50 µg of Atto647N NHS ester in 10 µL of DMSO immediately before use and add to the
147 mixture for a total reaction volume of 100 µL. Add Atto647N NHS ester last. Allow to react at
148 room temperature for 1 h or 4 ˚C overnight.

149

150 1.4   After the reactions, remove by-products, excess dye, and salts by P2 desalting gel
151 filtration. Dilute the reaction mixture with H$_2$O to a total volume of 300 µL, which is appropriate
152 for the subsequent HPLC purification step. Add 650 µL of hydrated P2 gel to a centrifugal device
153 and spin down at 18,000 x g for 1 min. Remove the liquid at the bottom of the device, add the
154 reaction mixture to the column containing P2 gel, spin down at 18,000 x g for 1 min and collect
155 the reaction mixture at the bottom of the device.

156

157 NOTE: P2 gel should be hydrated at least 4 h before use with H$_2$O.

158

159 1.5   Purify the desalted reaction mixture with HPLC using a C18 column designated for
160 oligonucleotide purification, with solvent A: 0.1 M TEAA in H$_2$O and B: ACN as the mobile phase
161 for a linear gradient elution 10-100% B over 50 min at a flow rate of 0.5 mL/min. Inject the
162 desalted reaction mixture in reverse-phase HPLC with a 500 µL injection loop for purification.
163 Collect the product that has an absorbance peak for the DNA (260 nm) and an absorbance peak
164 for the fluorophore (560 nm for Cy3B and 647 nm for Atto647N) and dry them in a vacuum
165 centrifugal concentrator overnight (see **Figure 2A**).

166

167 1.6   Reconstitute the dried oligo-dye product in 100 µL water. Determine the concentration
168 of the Cy3B ligand strand and Atto647N locking strand with the nanodrop spectrophotometer.
169 Ensure that the dye labeling ratio is close to 1:1. Correct for the 260 nm absorbance of the dye
170 if needed when determining the oligonucleotide concentration.

171

172 1.7   Validate the purified product with MALDI-TOF-MS using 3-HPA as the substrate in 50%
173 ACN/H$_2$O with 0.1% TFA and 5 mg/mL ammonium citrate using 0.5 µL of the product at 1-5 µM
174 for MALDI-TOF-MS sample preparation. An example mass spectrum can be found in **Figure 2B**.

175

176   1.8   Dissolve the hairpin strand and the quencher anchor strand in water and make sure the
177   concentration of the stock solutions is between 50 and 100 µM.

178

179   NOTE: The hairpin strand is unmodified and can be directly custom synthesized from a vendor.
180   The anchor strand has a thiol anchoring group and a quencher BHQ2 and can be directly custom
181   synthesized from a vendor.

182

183   1.9   Aliquot all of the oligonucleotides. For short-term use and storage, store these
184   oligonucleotides at 4 ˚C. For long-term storage, freeze and keep them at -20 ˚C. At this point, all
185   of the oligonucleotides are ready for the DNA tension probe assembly.

186

187   NOTE: Repeated freeze-thaw cycles are not problematic for oligonucleotides.

188

189   **2.   Surface preparation**

190

191   NOTE: The preparation of DNA hairpin tension probe substrates takes two days. The DNA
192   hairpin tension probe will be functionalized onto glass coverslips.

193

194   2.1. Day 1

195

196   2.1.1   Place the 25 mm coverslips on a polytetrafluoroethylene rack in a 50 mL beaker. Each
197   rack can hold up to 8 coverslips. Rinse the coverslips by submerging in water three times.

198

199   2.1.2   Add 40 mL of a 1:1 ratio (v:v) solution of ethanol mixed with water to the beaker
200   containing the rack and coverslips, and seal the beaker using a paraffin film.

201

202   2.1.3   Sonicate the beaker for 15 min in an ultrasonics cleaner (operating frequency 35 KHz) to
203   clean the coverslips. After sonication, discard the liquid and rinse the beaker with the rack and
204   coverslips in it with water at least 6 times to remove any remaining organic solvent.

205

206   2.1.4   Prepare fresh Piranha solution by mixing sulfuric acid and hydrogen peroxide in the ratio
207   of 3:1. To make 40 mL of Piranha solution, add 30 mL of sulfuric acid to a clean 50 mL beaker
208   first and then slowly add 10 mL of H_2O_2. The Piranha solution will rapidly heat and bubble upon
209   addition of the H_2O_2. Gently mix the piranha using the end of a glass pipette.

210

211   2.1.5   Next, transfer the rack that holds the coverslips to the beaker containing gently mixed
212   Piranha solution for etching (**Figure 3A**). Allow the Piranha solution to hydroxylate and clean
213   the coverslips for 30 min at room temperature. After Piranha etching, transfer the rack using
214   steel or polytetrafluoroethylene tweezers to a clean 50 mL beaker with water and rinse again
215   with water at least 6 times.

216

217   CAUTION: Large amounts of organic substances could react vigorously with Piranha solution
218   and may cause explosion. Be careful and always work with Piranha solution in a fume hood.

Make sure to wear a labcoat, gloves, and safety goggles. Never store fresh Piranha solution in a sealed container.

NOTE: The hydrogen peroxide to sulfuric acid ratio should be kept under 1:2 (v:v) and should never exceed 1:1. When submerging the rack with coverslips in Piranha solution, place them in the solution slowly and carefully. Do not discard the solution immediately after etching, as it is still active and hot. Leave it in the beaker overnight before pouring it in the acid waste container.

2.1.6. Immerse the rack holding the coverslips in a 50 mL beaker with 40 mL ethanol to remove water. Discard the ethanol and repeat 3 times to ensure that the water has been removed.

2.1.7. Then immerse the rack in 3% aminopropyl triethoxy silane (APTES) (v/v) in 40 mL of ethanol to react with the -OH on the coverslips for 1 h at room temperature (**Figure 3B**).

NOTE: Ethanol can be replaced by acetone.

2.1.8. Rinse surfaces 6 times by submerging them into 40 mL ethanol, then dry in oven at 80 °C for 20 min. After cooling, store the dried amine-modified coverslips at -20 °C for future use (up to 6 months).

2.1.9. Cover the bottom inner side of 10 cm diameter plastic Petri dishes with paraffin film. The paraffin film prevents the coverslips from sliding inside the Petri dish and helps in keeping the solution for the next steps of functionalization stay on the coverslips. Place the cooled-down amine-modified coverslips in the Petri dishes. The side to be functionalize should be facing up.

2.1.10. To modify the amine groups on the coverslips, add 300 μL of 0.5% w/v lipoic acid PEG NHS (LA-PEG-SC) and 2.5% w/v mPEG NHS (mPEG-SC) in 0.1 M NaHCO$_3$ onto each coverslip and incubate for 1 h at room temperature (**Figure 3C**). For each 25 mm coverslip, weigh 1.5 mg of LA-PEG-SC and 7.5 mg of mPEG-SC. Dissolve the NHS reagents immediately before adding to the surfaces, as they have a short half-life (~10 min) in aqueous solution at room temperature. After the reaction, rinse surfaces 3 times with water.

NOTE: The NHS reaction can be performed at 4 ˚C overnight. NHS reagents have longer half-life before hydrolysis at 4 ˚C, which is around 4-6 h. This will result in a three-day surface prep procedure.

2.1.11. Add 100 μL of 0.1 M NaHCO$_3$ containing 1 mg/mL of sulfo-NHS acetate to a set of "sandwich" coverslips (two coverslips facing towards each other with reaction buffer in between). Allow passivation to occur for at least 30 min. To save reagent, this step could be done with 50 μL of 1 mg/mL sulfo-NHS acetate. Rinse with water three times after passivation.

263   2.1.12. Add 0.5 mL of gold nanoparticles (AuNP, 8.8 nm, tannic acid, 0.05 mg/mL) to each
264   coverslip and incubate for 30 min at room temperature (**Figure 3D**). To save the reagent, this
265   step can be done by sandwiching two coverslips as well. Make sure no salts are present in the
266   system from previous steps to avoid aggregation of gold nanoparticles. Do not leave the
267   coverslips to dry after this step.

268

269   2.1.13. Meanwhile, pre-hybridize 4.7 pN hairpin, Cy3B ligand strand, and BHQ2 anchor strand
270   that form the DNA tension probes construct at a ratio of 1.1:1:1 in 1 M NaCl at 300 nM in a PCR
271   tube. Anneal the strands by heating the solution up to 95 ˚C for 5 min, then gradually cool down
272   by decreasing the temperature to 20 ˚C over 30 min in a thermal cycler.

273

274   2.1.14. Rinse the coverslips with water three times after 30 min of incubation with gold
275   nanoparticles. Add additional BHQ2 anchor strand (from 100 μM stock) to the annealed DNA
276   solution to make the ratio between BHQ2 anchor strand and Cy3B ligand strand 10:1. At this
277   point, the DNA solution should contain 300 nM of tension probe construct and 2.7 μM BHQ2
278   strand. Add 100 μL per two coverslips to make the "sandwich" (**Figure 3E**).

279

280   2.1.15. Carefully place a wet lab tissue ball in the Petri dish (away from coverslips) and seal the
281   dish with paraffin film to prevent the solution from drying up. Cover the dish with foil and
282   incubate at 4 ˚C overnight.

283

284   **2.2.   Day 2**

285

286   2.2.1. Wash off the excess probes from the coverslips with 1x PBS. Check for DNA tension
287   probe surface quality under an epifluorescent microscope.

288

289   2.2.2. Prepare 40 μg/mL of streptavidin in 1x PBS and incubate on coverslips for 30 min at
290   room temperature (**Figure 3F**). Usually, 100 μL is sufficient for a 25 mm coverslip. Rinse with
291   PBS 3 times after incubation to wash away the excess amount of streptavidin.

292

293   2.2.3. Prepare 40 μg/mL biotinylated antibody/ligand in 1x PBS. Add 50-100 μL per sandwich
294   and incubate for 30 min at room temperature (**Figure 3G**). Rinse with PBS three times after
295   incubation to wash away the excess amount of biotinylated antibody/ligand.

296

297   2.2.4. Assemble the clean imaging chambers with surfaces carefully. Surfaces can be easily
298   cracked when tightening the chambers. Add 0.5-1 mL of Hank's balanced salt solution (HBSS) to
299   the imaging chambers and keep them ready for imaging with cells (**Figure 3H**).

300

301   **3.   Imaging cell receptor forces**

302

303   3.1   Prepare immune cells of interest in HBSS at 1-2 x 10^6 cells/mL.

304

305   NOTE: OT-1 CD8+ naïve cells are used as an example in this paper. Purify OT-1 CD8$^+$ naïve T cells
306   a from the spleens of sacrificed mice using the MACS mouse CD8$^+$ T cell isolation kit with a

307 MACS separator following manufacturer's instruction. Isolate and enrich the CD8$^+$ T cells by
308 removing any non CD8$^+$ T cells that magnetic depleting antibody cocktail bound to. Resuspend
309 purified OT-1 CD8+ naïve T cells in HBSS at 2 x 10^6 cells/mL and keep on ice prior to use.

311 3.2   Check the quality of DNA hairpin tension probe surface under a fluorescence
312 microscope (100x objective) for quality control before adding ligands or plating cells. Image and
313 quantify the average background intensity in Cy3B channel of a DNA hairpin tension probe
314 surface from at least 5 different positions and 3 replicates. Keep the imaging acquisition
315 conditions consistent so that this value can be used as a reliable marker of surface quality and
316 probe density (**Figure 4C**).

318 NOTE: Quantify the number of DNA strands per gold nanoparticle and the number of gold
319 nanoparticles per μm^2 the first few times of surface preparation according to literature[12], which
320 can be used as another reliable marker of surface quality.

322 3.3   Plate ~4 x 10^4 - 10 x 10^4 cells onto each DNA tension probe functionalized coverslip and
323 allow them to attach and spread for ~15 min at room temperature.

325 3.4   As cells are plated onto the DNA hairpin tension probes and start to spread, image the
326 fluorescence signals that are generated in the Cy3B channel with the 100x objective (**Figure 3I**).

328 3.5   After cells start to produce real-time tension signal on the DNA hairpin tension probe
329 surface in the Cy3B channel, acquire images in both Cy3B and Atto647N channels (TIRF
330 microscopy gives better signal-to-noise ratio than epifluorescence). Subsequently, add
331 Atto647N strand to the imaging chambers at a final concentration of 200 nM for mechanically
332 selective hybridization.

334 3.6   After 10 min of incubation, quickly and gently remove the buffer containing the
335 fluorescent Atto647N locking strand and replace with fresh Hank's balanced salts. Image in
336 both Cy3B and Atto647N channels again and determine the Pearson's correlation coefficient
337 with Fiji software[15].

339 3.7   At the timepoint of interest for the investigation, introduce non-fluorescent locking
340 strand to the cells in the imaging chamber to store the tension signal. Prepare locking strand
341 stock (100 μM) and add to the cells at a final concentration of 1 μM. Gently pipette to mix. The
342 duration of locking can vary but 10 min is the recommended time.

344 3.8   Acquire time-lapse movies or end-point images in epifluorescence for both qualitative
345 tension mapping and quantitative analysis as needed (**Figure 3J** and **Figure 5**).

347 NOTE: If tension measurement at multiple time points is desired, initiate erasing of stored
348 tension signals by the addition of an unlocking strand. To avoid excess rinsing, a higher final
349 concentration of unlocking strand at 2 μM is used to initiate a toehold-mediated strand
350 displacement reaction with the locking strand for 3 min, which erases the stored signals (**Figure**

351   **3J**). Gently rinse the excess oligonucleotides off with HBSS. The DNA hairpin tension probe
352   surface and the cells are ready for another round of tension storing and mapping. Unlocking of
353   tension signals is not necessary if only one time point is of interest in the study.
354
355   **4.   Data analysis**
356
357   NOTE: Image analysis is performed using Fiji software, and the quantitative analysis is
358   performed using analysis software.
359
360   4.1   Correct any drift during image acquisition with **Correct 3D drift** command in
361   **Registration** under the **Plugins** menu.
362
363   4.2   Remove the camera background of the image with **Subtract** command under the
364   **Process** menu.
365
366   4.3   Determine the Pearson's correlation coefficient with the **Colocalization** function under
367   **Analyze** menu.
368
369   4.4   Average and subtract the fluorescence background produced by the unopened probes
370   from three different local background regions. Draw ROIs of cells on either background-
371   subtracted images or RICM (reflection interference contrast microscopy) images with Image J
372   **Freehand Selections** tool. Measure any metric of interest of the ROIs, e.g., integrated
373   fluorescence intensity and tension occupancy using **Measure** tool under **Analyze** menu (**Figure**
374   **6**).
375
376   4.5   Export the measurements for quantitative analysis with analysis software.
377
378   4.6   Plot the data with any analysis software.
379
380   **REPRESENTATIVE RESULTS:**
381   Here we show representative surface quality control images (**Figure 4**). A high-quality surface
382   should have a clean background in RICM channel (**Figure 4B**), and uniform fluorescence
383   intensity in Cy3B channel (**Figure 4C**). With the same imaging equipment and identical
384   fluorescence imaging acquisition conditions, the background fluorescence intensity should be
385   consistent and reproducible each time when conducting experiments with DNA probes. We
386   recommend using it as a marker for the surface quality control before each set of individual
387   experiments.
388
389   In this paper we show some representative data (**Figure 5**) with OT-1 naïve CD8$^+$ cells as a
390   model cell type, which specifically recognize chicken ovalbumin. The CD3ε antibody is included
391   as a positive control for the system and the cognate antigen pMHC N4 (SIINFEKL) is used as an
392   example. Cells were plated on substrates that were functionalized with 4.7 pN DNA hairpin
393   probes presenting either antiCD3ε or pMHC N4. After 30 min, cell spreading in RICM and the
394   real-time tension in Cy3B channel were captured, and the locking strand was introduced at a

395 final concentration of 1 µM. The time-lapse of tension locking showed that both TCR-antiCD3ε
396 and TCR-pMHC tension signals were amplified because of hairpin locking and signal
397 accumulation. Furthermore, TCR-pMHC N4 interaction showed weak force signal in real-time (0
398 min), likely due to its short bond lifetimes. The locking strand recorded these short-lived
399 mechanical pulling events, which facilitates quantitative analysis. Additionally, locking revealed
400 the pattern of the TCR-pMHC force as it accumulates, which was quite distinct from the
401 antiCD3ε control and resembled the "bull's-eye" pattern of immune synapses. The
402 quantification of the fluorescence signal is described in step 4.4. We typically use the tension
403 occupancy (defined as the percentage of area that has tension signal over the contact area) and
404 integrated fluorescence intensity of each cell as a metric to evaluate accumulated tension that
405 is > 4.7 pN.

406
407 **FIGURE AND TABLE LEGENDS:**

408
409 **Figure 1: Scheme of the state-of-art molecular tension probes. (A)** General design of real-time
410 molecular tension probe, **(B)** Strands for the DNA-based tension probe construct, and **(C)**
411 engineered DNA-based tension probes and their toggling between real-time state and locked
412 state.

413
414 **Figure 2: Examples of oligonucleotide preparation. (A)** HPLC chromatogram of Cy3B ligand
415 strand purification; **(B)** MALDI-TOF-MS spectra of the product; **(C)** Table of the calculated mass
416 and found m/z peaks of the starting material and the labeled oligonucleotide.

417
418 **Figure 3: Functionalization of the DNA tension probe substrates and experiment procedures.**
419 **Steps are described in the corresponding protocol.**

420
421 **Figure 4: Example of DNA-tension probe surface with good quality. (A)** atomic force
422 microscopy (AFM) characterization of the DNA tension probe surface; and raw microscopy
423 images of a DNA tension probe surface in **(B)** RICM and **(C)** Cy3B channel.

424
425 **Figure 5: Example of raw microscopy images of a successful experiment.** Images show OT-1
426 naïve CD8^{+} cells producing TCR forces against **(A)** antiCD3ε and **(B)** pMHC N4. Scale bar = 5 µm.

427
428 **Figure 6: Examples of data processing and quantitative analysis.**

429
430 **Figure 7: Examples of successful and unsuccessful surfaces and tension mapping. (A)** Cells that
431 do not spread compared to cells that spread on the pMHC N4 surfaces in RICM. **(B)** OT-1 cell
432 that spread but did not generate any tension signal on an antiCD3ε surface with low DNA
433 tension probe density. **(C)** Picture showing that the successful surface is pale pink, and the
434 unsuccessful surface is colorless. **(D)** Image in Cy3B channel showing the fluorescent aggregates
435 from an old DNA stock. **(E)** The pattern in RICM channel and Cy3B channel that is likely due to
436 either insufficient washing or accidental surface drying between steps. **(F)** Images in RICM and
437 Cy3B channels showing the effect of using Au NPs of different sizes. **(G)** RICM and Cy3B images
438 of OT-1 cells that did not exert tension signals but instead depleted DNA. Scale bar = 5 µm. Note

439 the raw data shown here was collected by different group members with different image
440 acquisition settings from 3 microscopes, thus having different fluorescence background levels.
441
442 **Table 1: Troubleshooting with the DNA-based tension probe system.**
443
444 **DISCUSSION:**
445 With the detailed procedures provided here, one can prepare DNA hairpin tension probe
446 substrates to map and quantify the receptor tension produced by immune cells. When cells are
447 plated onto the DNA hairpin tension probe substrate, they land, attach, and spread as the
448 receptors sense the ligands both chemically and mechanically, the latter of which is detected by
449 our probes. However, in some cases cells may fail to spread (**Figure 7A**) or fail to produce
450 tension signal. This is often a consequence of surface chemistry issues and requires
451 troubleshooting (**Table 1**). Low ligand density is one of the most common issues that results in a
452 failure of cell spreading. This could be a result of multiple factors, such as degraded ligand,
453 degraded DNA strands, hydrolyzed reagents like APTES and/or LA-PEG-NHS, aggregated gold
454 nanoparticles, or insufficient glass coverslip cleaning. By comparing substrate background
455 fluorescence intensity over successful experiments and failed ones (**Figure 7B**), one can quickly
456 assess the source of the problem. For example, surfaces with strong fluorescence signal from
457 the background measurements indicate that the immobilization of DNA probes was successful.
458 Low background intensity suggests issues in the preparation steps prior to the addition of the
459 streptavidin and the biotinylated ligand. If the issue is caused by the ligand, its quality can be
460 checked by coating it on a glass slide or wells in a 96-well plate at 10 μg/mL to test for cell
461 adhesion. This is a useful functional test of ligand activity for pMHC ligands and anti-CD3
462 antibodies. By observing the color change (pale pink) of the functionalized coverslips after the
463 Au NP incubation step, one can assess whether the problem is caused by DNA degradation or
464 the steps preceding incubation with DNA (**Figure 7C**). The DNA strand quality can be validated
465 with HPLC and MALDI-TOF-MS, in which a single DNA sample will show multiple peaks if
466 degraded. The quality of Au NPs can be confirmed by comparing the UV-Vis spectra and TEM
467 images with the characterization provided by manufacturer. If the quality of the ligand, DNA
468 strands, and Au NP is validated and these reagents do not cause the surface issue, we
469 recommend replacing reagents including APTES, LA-PEG-NHS, sulfuric acid, and H_2O_2 for future
470 surface preparation instead of spending time to precisely locate the cause of the surface
471 chemistry issue. Except for the low-density problem that will cause a major failure of the DNA
472 probe substrate, there are a few minor issues that could affect data quality. Since the surface
473 preparation has multiple incubation steps, buffers used during surface preparation should be
474 made fresh, filtered, and kept at 4 ˚C for storage to avoid contamination. If a surface is
475 contaminated, it will be visible in the RICM channel and may result in a strange fluorescence
476 background. Other than contamination, aggregates can form in the DNA stock solution over
477 time, which may result in bright dots in Cy3B channel (**Figure 7D**). Usually, a few aggregates will
478 not affect the data quality; however, if a cell lands on regions with such defects, data analysis is
479 less reliable. Occasionally, irregular patterns that affect tension mapping can be observed in
480 both the RICM and Cy3B channels, which could come from insufficient washing after the APTES
481 step, or accidental drying of the surface during the steps after gold particles are functionalized
482 onto the surface (**Figure 7E**). These patterns might affect the tension mapping depending on

483 how severe it is. To save relatively precious reagents such as antibodies or pMHCs, we
484 recommend always checking the surface quality (see 2.2.1 and 3.3) before functionalization of
485 DNA hairpin tension probes with antibody/ligand. Though this surface preparation protocol has
486 been robust and reliable in our hands, note that it does not always tolerate alterations at
487 certain steps very well. For example, when base and plasma etching are used instead of piranha
488 etching, it is very likely that the probe density decreases, and more surface defects are present.
489 Alterations of Au NP size and capping reagents will likely dramatically affect the surface
490 functionalization results (**Figure 7F**). For example, we have found that the 5 and 10 nm Au NPs
491 do not bind to the lipoic acid modified surfaces very well, which results in minimal DNA after
492 the surface preparation. However, 8.8 nm and 13 nm Au NPs can bind to the lipoic acid surfaces
493 at a much high density, which has been observed in RICM (roughness), and Cy3B channel
494 (fluorescence intensity).

496 Once DNA hairpin tension probe surfaces of good quality are prepared, one can conduct
497 experiments with cells of interest and acquire data with a fluorescence microscope. The real-
498 time tension can be captured with an ordinary fluorescence microscope equipped with a high
499 NA (numerical aperture) objective and EMCCD. Do note however that we have observed that
500 primary T cells occasionally display artifacts on the DNA tension probe surfaces due to that
501 particular mouse's conditions. Anecdotally, such artifacts are more likely to happen with older
502 mice. For example, we have observed negative depletion of the fluorescence instead of turn-on
503 fluorescence signals (**Figure 7G**). In this case, terminate the experiment and redo with a new
504 batch of cells. Before using non-fluorescent locking strand for quantitative analysis of receptor
505 forces, the tension signal storage and erasing need to be confirmed with a fluorescent
506 Atto647N locking strand. After initial locking for 10 min, the Atto647N signal should be strongly
507 co-localized with Cy3B signal, as it reflects tension history during this incubation. As Cy3B signal
508 reflects not only the tension history but also real-time forces after removing locking strand, it is
509 possible that a small subset of Cy3B signal is not accompanied by Atto647N signal (**Figure 3J**).
510 For a more quantitative analysis of tension, we recommend using a non-fluorescent locking
511 strand which would eliminate the potential energy transfer between the Cy3B and Atto647N, as
512 well as avoiding excess washes in between image acquisitions. Note that the addition of locking
513 strand will inevitably cause a slight fluorescence background increase, as the thermodynamics
514 will drive some minor hybridization between the hairpin and the locking strand, which can be
515 subtracted before quantification. One can quantify the integrated intensity of each cell after
516 locking procedure to study the force generated by a certain receptor. Additionally, the kinetics
517 of locking likely reflects the 2D k_{on} and k_{off} of a receptor to its ligand.

519 [place **Table 1** here]

521 The application of mechanical information storage is particularly useful for mapping receptor
522 forces generated by immune cells, which are often transient and less abundant. As determined
523 by single-molecule spectroscopy measurements, the peak bond lifetime is observed with the
524 application of ~10 pN of force with a lifetime ranging from less than 100 milliseconds to several
525 seconds long. Such short duration bonds are difficult to capture with conventional DNA hairpin
526 tension probe imaging[15]. Another use of this mechanical storage system is when the density of

the receiver of interest is relatively low on the cell surface[16], which results in a sparse signal that is often difficult to distinguish from the background with conventional DNA hairpin tension probes. Such low-density receptor forces can be revealed by accumulated tension mapping with this strategy. Note that we specifically limit the application of this strategy to immune cells, as the forces present in immune cells are known to be in the lower regime (TCRs < 19 pN) comparing to integrins in fibroblasts or platelets (up to or more than 56 pN)[12,13,15]. The reason is that the hybridization rate constant k_{hyb} is not impaired when the load is less than 20 pN according to optical tweezers measurements, which is in the range of 10^6 $M^{-1}S^{-1}$. Moreover, the k_{hyb} under forces < 20 pN is predicted to be slightly higher than at k_{hyb} at zero force, as the weak force helps aligns the strand for the complementary strand to hybridize with[17]. On the other hand, larger forces are expected to hinder the hybridization, as the k_{off} increases and creates a barrier for hybridization to happen[18]. For a lock that is 15-17 nucleotide long, we estimate that a force around 27.8 to 30.8 pN will hinder the hybridization. Given these limitations, we suggest to exclusively apply this method in investigations of immune cell receptor forces. Additionally, the presence of the locking strand is likely to tilt the energy landscape for hairpin unfolding, which might slightly decrease the effective $F_{1/2}$.

DISCLOSURES:
The authors declare no conflict of interest.

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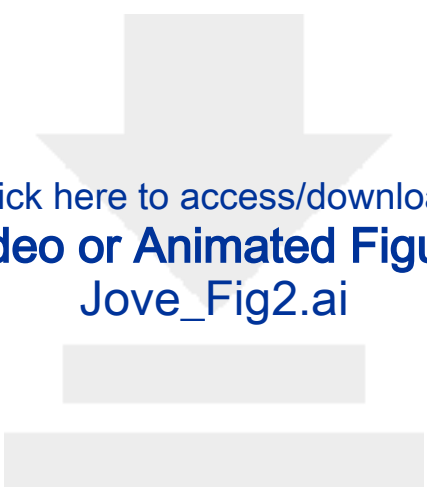591 oligonucleotide hybridization. *Nucleic Acids Research.* **45** (2), 547-555 (2016).

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594

Figure

Figures uploaded as .ai files.

Figure 1

Click here to access/download
Video or Animated Figure
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Figure 2

Figure 3

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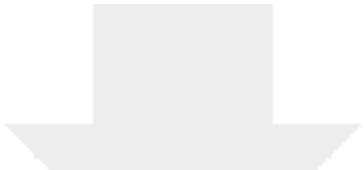**Video or Animated Figure**
JoVE_Fig3.ai

Figure 4

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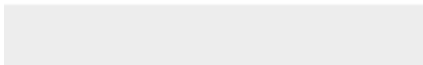**Video or Animated Figure**
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Figure 5

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Figure 6

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Figure 7

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Problem
Surfaces are not pink after AuNP incubation
Cells don't spread on the surface
Cells spread well but no tension signal or only very weak tension signal is observed even with antibody control
No locking signal is observed in Atto647N
Locking signal is anti-localized with real-time probe

Possible reason
Insufficient etching
APTES is hydrolyzed
NHS reagents are hydrolyzed
PEG-NHS reagents are hydrolyzed before added to surfaces
Au NP has aggregated
Residue water on the coverslips diluted the Au NP
Low DNA probe density
Low ligand density
Cells don't spread on on the ligand
The receptor-ligand interaction does not have force transmission
Force is short-lived or force signals are sparse
The surface is not passivated well
Oligonucleotide has degraded
The receptor force is higher than the working range of locking nucleotide

Solution
Include a base-etching step after piranha etching. Etch coverslips in 0.5 M KOH in ice-bath for 1 h with sonication.
Use new APTES
Use new PEG-NHS reagents
Work faster
Use new Au NP
Make sure there is no/little water residue before adding Au NP
If the surfaces are not as pink, troubleshoot as described above. If the Au NP functionalization is normal, synthesize and use new DNA
Use new streptavidin and ligand reagents
Check cell spreading on a ligand-coated surface, if the cells don't spread still, include adhering molecules as described in ref.12.
NA
Use locking oligonucleotide
Increase the sulfo-NHS acetate conc., up to 10 mg/mL
Check with MALDI-TOF-MS
Use 19 pN real-time hairpin probe from ref. 12 as described.

Name of Material/ Equipment	Company	Catalog Number
3-hydroxypicolinic acid (3-HPA)	Sigma	56197
mPEG-SC	Biochempeg	MF001023-2K
(3-Aminopropyl)triethoxysilane	Acros	AC430941000
10x Red blood cell lysis buffer	Biolegend	00-4333-57
8.8 nm gold nanoparticles, tannic acid	Nanocomposix	customized order
Atto647N NHS ester	Sigma	18373-1MG-F
Attofluor Cell Chamber, for microscopy	Thermo Fisher Scientific	A7816
BD Syringes only with Luer-Lok	BD bioscience	309657
biotinylated anti-mouse CD3ε	ebioscience	13-0031-82
Biotinylated pMHC ovalbumin (SIINFEKL)	NIH Tetramer Core Facility at Emory University	NA
bovine serum albumin	Sigma	735078001
Cell strainers	Biologix	15-1100
Coverslip Mini-Rack, teflon	Thermo Fisher Scientific	C14784
Cy3B NHS ester	GE Healthcare	PA63101
Dulbecco's phosphate-buffered saline (DPBS)	Corning	21-031-CM
ethanol	Sigma	459836
Hank's balanced salts (HBSS)	Sigma	H8264
hydrogen peroxide	Sigma	H1009
LA-PEG-SC	Biochempeg	HE039023-3.4K
Midi MACS (LS) startup kit	Miltenyi Biotec	130-042-301
mouse CD8+ T cell isolation kit	Miltenyi Biotec	130-104-075
Nanosep MF centrifugal devices	Pall laboratory	ODM02C35
No. 2 round glass coverslips	VWR	48382-085
NTA-SAM	Dojindo Molecular Technologies	N475-10
P2 gel	Bio-rad	1504118
sufuric acid	EMD Millipore Corporation	SX1244-6
Sulfo-NHS acetate	Thermo Fisher Scientific	26777

Comments/Description

maldi-TOF-MS matrix

surface prep

surface prep

buffer

surface prep

fluorophore, oligo prep

imaging

cells

antibody/ligand

antibody/ligand

block non-specific interactions

cells

surface prep

fluorophore, oligo prep

buffer

surface prep

buffer

surface prep

surface prep

cells

cells

oligo prep

surface prep

surface prep

oligo prep

surface prep

surface prep

Name of Equipment	Model/Cat
Agilent AdvanceBio Oligonucleotide C18 column, 4.6 x 150 mm, 2.7 µm	653950-702
Barnstead Nanopure water purifying system	Thermo Fisher
CFI Apo 100× NA 1.49 objective	Nikon
Cy5 cube	CHROMA
evolve electron multiplying charge coupled device (EMCCD)	Photometrics
High-performance liquid chromatography	Agilent 1100
Intensilight epifluorescence source	Nikon
Matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-TOF-MS)	Voyager STR
Nanodrop 2000 UV-Vis Spectrophotometer	Thermo Fisher
Nikon Eclipse Ti inverted microscope	Nikon
Nikon Perfect Focus System	Nikon
NIS Elements software	Nikon
quad band TIRF 405/488/561/647 cube	CHROMA
RICM cube	CHROMA
TIRF launcher with 488 nm (50 mW), 561 nm (50 mW), and 640 nm	Coherent
TRITC cube	CHROMA

Comments/Description

oligonucleotide preparation
water
Microscopy
Microscopy
Microscopy
oligonucleotide preparation
Microscopy
oligonucleotide preparation
oligonucleotide preparation
Microscopy
Microscopy
Microscopy
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Microscopy

oligo name	5' modification
15mer amine locking strand	no modification
15mer Atto647N locking strand	Atto647N
15mer non-fluoresccent locking strand	no modification
4.7 pN hairpin strand	no modification
amine ligand strand	/5AmMC6/
BHQ2 anchor strand	/5ThiolMC6-D/
Cy3B ligand strand	Cy3B
unlocking strand	no modification

sequence (5' to 3')	3' modification
AAA AAA CAT TTA TAC CCT ACC TA	/3AmMO/
AAA AAA CAT TTA TAC CCT ACC TA	/3AmMO/
A AAA AAC ATT TAT AC	no modification
TGAAATACCGCACAGATGCGTTTGTATAAATGTTTTTTTCATTTATACTTTAAGAGCGCCACGTAGCCCAG	no modification
CGCATCTGTGCG GTA TTT CAC TTT	/3Bio/
TTTGCTGGGCTACGTGGCGCTCTT	/3BHQ_2/
CGCATCTGTGCG GTA TTT CAC TTT	/3Bio/
TAG GTA GGG TAT AAA TGT TTT TTT C	no modification

Use

locking real-time tension signal

locking real-time tension signal

locking real-time tension signal for quantitative analysis

hairpin probe

hairpin probe

hairpin probe

hairpin probe

unlocking accumulated tension signal

<u>Respond to the review comments</u>

We thank the editors and the reviewers for their kind comments and feedback. We have revised our manuscript accordingly. Below is a detailed point to point response to the review comments.

Editorial comments:

Editorial changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. E.g. Line 354: "MALDI-TOF.." instead of "MALI-TOF.."

We apologize for this mistake. This typo has been fixed.

2. Please submit each figure individually as a vector image file to ensure high resolution throughout production: (.psd, ai, .eps.).

We have uploaded each figure individually as a vector image as requested.

3. Please define all abbreviations before use.

We have defined all abbreviations before use, except for the commonly known abbreviations like DNA and EMCCD.

4. Avoid use of personal pronouns in the protocol. E.g. "we", "our" etc.

We have revised our protocol part and avoided the use of personal pronouns as much as possible.

5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials. E.g. Nanopure, Kimwipes, Agilent AdvanceBio column, Vacfuge etc.

We have removed all commercial language from the manuscript.

6. Please ensure that the protocol consists of step-wise instructions. The text explaining the theoretical/practical aspects should be moved to the results/discussion section. E.g. Lines 119-135.

We have moved theoretical/practical aspects to results/discussion section. Some general but necessary information is kept in place as a note to readers.

7. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." E.g. Line 136: "Dissolve the DNA.." instead of "The ligand is dissolved.."etc. Lines 142-143, 273-277 can be added as a note, etc.

We have revised the protocol accordingly.

8. Please convert centrifuge speeds to centrifugal force (x g) instead of revolutions per minute (rpm).

We have revised the protocol accordingly.

9. Line 186: Dimensions of the coverslips? What is the role of slides here?
We have added the dimensions of the coverslips and described its role.

10. Line 191: Sonicator settings? Frequency used?
We have added the sonicator settings.

11. Lines 216-217: Please add this in the references.
We have removed it from our protocol.

12. Include the animal ethics statement at the start of the protocol.
We have moved the statement "The OT-1 transgenic mice are housed at the Division of Animal Resources Facility at Emory under the Institutional Animal Care and Use Committee." to the start of the relevant part in the protocol (3. Imaging cell receptor forces).

13. Please move all figure legends to after the representative results section.
We have moved all figure legends to after the representative results section.

14. Please include a Disclosures section, providing information regarding the authors' competing financial interests or other conflicts of interest. If authors have no competing financial interests, then a statement indicating no competing financial interests must be included.
We have added the disclosure section.

15. Figure 1A: Please write the Y-axis label in full including units to avoid misinterpretation. Figure 1B: Please include the X-axis label
We have changed the Y-axis label in full in Figure 1A and moved the X-axis to the center in Figure 1B.

16. Figure 3B, 4A-B, 6A,B,D-G: Please indicate the what the color code legend represents, and include the units.
These images were taken in RICM channel. They do not serve any quantitative purposes, just like bright field images.

17. Do not include "&" in the references. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).]
We have revised the reference part accordingly.

18. Please sort the Materials Table alphabetically by the name of the material.
We have sorted the table alphabetically.

_____

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
The authors describe exciting DNA based tools for mechanobiology studies which is currently one of the hot topics of scientific research. The host lab over last many years has

established a strong command on using DNA based hairpin structures and the opening-closing characteristics of those as a function of receptor changes on plasma membrane on cells. Immune cells present an perfect test beds for such tools since a lot of biology has been established where the receptors on immune cells correspond to external cues like development, growth factors, toxins, etc. How can we measure the confirmational changes on those receptors in response to external stimuli has been a long challenge and tedius methods like FCS or recently electron microscopy has been explored. DNA based hairpin loops offer a much simple solution to such problems and the best advantage is that they can be multiplexed and explored at the resolution of single receptor.

Having such a protocol or method explained via JOVE would be definitely a big plus and I am happy to see the authors come up with this report. I went through the methods and figures and the protocols and all things are in place and written in a very lucid language, easy to understand by general reader and the flow of information is absolutely smooth, with the cautions at most steps in the experiments.

I recommend this report for publication in JoVE post editorial review.

Reviewer #2:
Manuscript Summary:
The authors developed the lockable" DNA-based tension sensor and presented the step-by-step protocol in this work, where the sensor will not refold, thereby storing the transient information of tension/force generation of the cells. The manuscript was written well with essential details for the readers to succeed by following the protocol. This reviewer recommends the manuscript to be accepted for publication after the minor concerns listed below are addressed.

Major Concerns:
None

Minor Concerns:
(1) In steps 1.2 and 1.3 the order of mixing liquids should be clearly stated, or if the order does not matter, it should benefit the readers to be informed as well.
   We have added extra description of the order.

(2) What is the safest way in step 2.3 to move the Teflon coverslip rack into and out of the piranha solution? Are steel tweezers safe enough? Or Teflon tweezers are recommended?
   We have added this information in the protocol.

(3) Including recommended parameters used in image analysis (section 4) will be very helpful to reader adopting the protocol.
   We have recommended parameters for image analysis in section 4.