26, rue d'Ulm - 75248 Paris Cedex 05

Dear Editor,

Thank you very much for sending our manuscript entitled "*Traction force microscopy to study B lymphocyte activation"* to the referees for peer review.

We have modified the manuscript to conform to the all editor's and referees' requests. In particular we have modified the introduction and discussion part and we have now included a part of the protocol to describe the use of fluorescent antigen for antigen extraction quantification as requested by referee #1 (comment 11).

Please find below our response to referees. We hope we have answered to all your requests.

With our best regards,

Paolo Pierobon (on behalf of the authors)

26, rue d'Ulm - 75248 Paris Cedex 05

**Editorial Comments:**

The manuscript will benefit from thorough language revision as there are a number of grammatical errors throughout. Please thoroughly review the manuscript and edit any errors.
*We have been benefit of the help of a native English speaking collaborator. We hope that the manuscript is now more readable.*

• **Abstracts:**
1) Remove reference citations from abstract, and re-order the citations.
2) Current the abstract reads like the first paragraph of the introduction section. Please edit it so that it provides a detailed overview of the technique and a brief summary of its advantages, limitations and applications. The abstract must clearly state the goal of the protocol.
*We modified both the abstract and the introduction*

• **Protocol Language:** The JoVE protocol should be almost entirely composed of numbered short steps (2-3 related actions each) written in the imperative voice/tense (as if you are telling someone how to do the technique, i.e. "Do this", "Measure that" etc.). Any text that cannot be written in the imperative tense may be added as a brief "Note" at the end of the step (please limit notes). Please edit entire section 4 of the protocol accordingly. Descriptive sections of the protocol can be moved to Representative Results or Discussion. The JoVE protocol should be a set of instructions rather a report of a study. Any reporting should be moved into the representative results.
*We changed several points in the protocol.*

• **Protocol Detail:** Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. **Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps.** There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Some examples:
1) 1.2.1: Unclear what " becher" is. Do you mean beaker?
2) 3.2.1: Mention magnification, lens N.A., fluorescence excitation and emission settings.
*We corrected these points as long as other observations raised by the referees.*

• **Protocol Numbering:** Please adjust the numbering of your protocol section to follow JoVE's instructions for authors, 1. should be followed by 1.1. and then 1.1.1. if necessary and all steps should be lined up at the left margin with no indentations. Please add a one-line space after each protocol step.
*We corrected this point.*

• **Protocol Highlight:** Please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.

1) The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.
2) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.
3) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.
4) Notes cannot be filmed and should be excluded from highlighting.
*We highlighted what we think are the most important parts, but we are open to suggestions.*

• **Discussion:** JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.
*We modified and restructured the discussion according to these points and the ones raised by the referees.*

• **Figure/Table Legends:** Please expand the legends to adequately describe the figures/tables. Each figure or table must have an accompanying legend including a short title, followed by a short description of each panel and/or a general description.
*We modified the legends and also added a new figure according to a referee's observation.*

• **References:**
1) Use superscript citation style throughout.
2) Please make sure that your references comply with JoVE instructions for authors. Citation formatting should appear as follows: (For 6 authors or less list all authors. For more than 6 authors, list only the first author then *et al.*): [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. *Source*. **Volume** (Issue), FirstPage – LastPage, doi:DOI (YEAR).]
3) Please spell out journal names.
*In this second version we used JOVE bibliography style as implemented by the F1000 site, this should automatically conform to the editorial policy.*

• **Commercial Language:** JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are fluorodish, milliQ, Sigma cote, Chemwipes, Milteny, GlutaMax-I,etc
1) Please use MS Word's find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products

should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names.

• If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."
*We were not aware of this policy and we modified the text to avoid commercial language.*

*All figures that are not original are modified from ref 6 which is published under CC licence (figures can be used and reproduced provided we give correct citation and acknowledgement).*

---

**Comments from Peer-Reviewers:**
*We thank the referees for taking the time to evaluate the paper and for the constructive comments they provided. We hope the manuscript is not more readable and useful for the reader.*

Reviewer #1:
Comments for the authors to address:

1. Last line of the abstract: Instead of "respond specifically to certain proteins", perhaps "respond specifically to ligands for cell surface receptors". This would be more precise.
*We agree, we changed the text.*

2. First line of the Introduction: The authors state that the procedure is designed to measure forces exerted by B cells when "internalising the antigen". I'm not sure that this is completely true. Some of the force exerted by a B cell on gel-bound antigens may be used to drive the clustering and centripetal movement of the BCR-bound antigen. Antigen extraction, and subsequent internalization, would only occur if the force that the B cell exerts on the antigen exceeds the strength of the bonds holding the antigen to the gel. It is unclear what fraction of the BCR-engaged antigen is extracted from the membrane and internalized. Hence, a more correct statement might be "the procedure is designed to measure forces that B cells exert on antigens in order to cluster them and then extract them from the membrane so as to subsequently internalize them".
*The referee is right: this part is unclear; we change the introduction adding more details on the nature of the forces that are measurable by TFM in the discussion.*

3. Second paragraph of the introduction. For completeness, it would be good to state that:
B cells are often activated by APCs displaying captured antigens on their surface and that this leads to the formation of an immune synapse where the BCR exerts force on the APC-associated antigens. These forces may amplify BCR signaling but are also essential for B cells to extract and then internalize the antigen. The binding of antigen to the BCR initiates BCR signaling and that this signaling may activate force-generating mechanisms.
*Correct: we add this information in the introduction.*

4. Page 2, first paragraph. Should it be "detailed", "described" or applied in different ways" instead of "declined"? Also, the last sentence is an incomplete sentence.
*We use the verb "employed" and fuse this sentence with the following sentence.*

5. Page 2, second paragraph. This paragraph focuses on the technical aspects of the measurement. It would be good to preface this with a few lines about what one can learn from drawing a force map of the B cell immune synapse. Why is it important to quantify these forces and to elucidate their pattern?
a) Immune synapse is mentioned but not described previously. See comment #2 above.
b) The significance of the statement about 3D information is not clear. Forces perpendicular to the plane of the gel may be more directly related to antigen extraction. Is the fact that TFM is a 2D technique a significant limitation? Are there other techniques (e.g. AFM) that would allow one to assess forces perpendicular to the gel (and towards the interior of the B cell)?

*We modify this paragraph to include pros and cons of other techniques and a comment on the 3D TFM.*

6. Page 2, paragraph 3. It would be good to indicate why a soft gel with a physiological rigidity of 500 Pa is used, i.e. because this mimics the rigidity of an APC membrane.
*We added this important point (it is not the APC membrane but rather cortex).*

7. Protocol step 1 (Gel preparation): The authors should refer to figure 1 here.
a) Step 1.1. It would be good to be explicit that the Fluorodish is the gel support and that the coverslip is used just to squish and flatten the gel. This is clear in the figure but not evident from the text.
b) Step 1.2 and Figure 1. Figure 1 says that the gel support (silanized Fluorodish) is hydrophilic but silanization usually increases the hydrophobicity of a surface. Perhaps this is an error.

*Silanization is done to bind the hydrogel to the coverslip. APTMS is indeed less hydrophobic than Sigmacote but its main function here is to provide a free amine group for covalent bond (we took away this comment).*

c) Step 2.1. Why are the gel support and the coverslip silanized with different reagents?

*To conveniently remove the coverslip from the gel.*

d) Step 2.1. Should it be "beaker" instead of "becher"?

*We corrected the manuscript.*

e) Step 3.2. What type of sonicator is used -- water bath or needle probe? If the latter, what setting is used?

*We corrected this sentence (it is a common bath sonicator).*

f) Step 3.7. Does the user have to be careful to ensure that the gel sticks to the Fluorodish and isn't peeled off with the coverslip?

*This is guaranteed by the use of different silane that provide different adhesion to the gel (with the Sigmacote treatment allowing the coverslip to be peeled off more easily than the gel support).*

8. Results section. The link to the ImageJ plug-in provides a thorough description of how to analyze and quantify the microscopy data. It would be good to indicate that there is a very detailed tutorial associated with this website at: https://sites.google.com/site/qingzongtseng/piv/tuto

*We added this in the text.*

9. Results section, next to last paragraph. Is the inversion algorithm that is used to extract the local forces and generate a force map also included in the ImageJ plug-in?

*Yes, this is better specified in the text (however it needs scripting to act frame by frame).*

10. Figure 3. More information is needed in the figure legend.
a) Figure 3A, upper panel. Do the "relaxed" and "deformed" gel correspond to all of the area outside and inside, respectively, of the circle indicating the cell? Is the cell always circular or does it extend and retract membrane protrusions? Does one draw a circle that encompasses the maximal cell area or an exact outline of the cell at different time points?

*We modified the legend.*

b) Figure 3A, lower panel. It is not clear how bead displacement is depicted here. Is this an overlay of images from a time 0 point (before adding B cells) and then a later time point after adding B cells? Do the purple and white colors represent these two time points? How are the localizations of the same bead at the two time points linked so that their displacement can be measured (no tracks are shown)? A figure similar to Figure 5A in their Nature Communications paper, which shows displacements for each bead, would be much better.
*We added an inset and clarify the legend.*

c) Figures 3B and C. Again some more information about the experiment would be helpful, in particular how long the B cells were allowed to interact with the gel. How is the force (panel C) derived from

the displacement (panel B)? Are the units in panel B µm? Are the force vectors linearly related to the displacement vectors (using the value for the gel rigidity) or is there a more complex relationship?

*We added this information to the text. The classical algorithm for inversion computes the force from the displacement through linear elasticity (Boussinesq Green function), this is specified in the paragraph on inversion.*

11. Quantifying the amount of antigen internalized by the B cell directly addresses the focus of this protocol, i.e. forces that promote antigen internalization. The use of antigens whose fluorescence is quenched by the gel but reappears after internalization into the B cell is a very clever trick. Although this is depicted in Figure 7E in the Nature Communications paper, it would be nice to show an example in this protocol. I think most users would want to correlate the amount of force generated at the immune synapse with the amount of antigen that is extracted from the gel and then internalized. This would be a nice addition and enhance the utility of this protocol.

*We followed the suggestion of the referee.*

Reviewer #2:

1. The article will strongly benefit from a re-read by the senior authors and also from language editing. For example, the opening sentence of the abstract would be better written as "In order to measure force exerted by …" . The word "becher" is not standard English.

*We proofread the paper with a native english speaker scientist: we hope to have improved the style and grammar of the manuscript.*

2. A reference for "replacing beads by nano-printed patterns" is missing (between ref. 13 and 14). The analysis section also needs more references. References to previous work on force measurement of leucocytes will be welcome.

*We added this.*

3. There is a comment about confocal or spinning disc being preferable to epi-fluorescence. A explanation as to why this is so should be added. In general, a reason for choice of materials will be interesting to have. Likewise, when the choice is not critical or if multiple choices exist, it will be interesting to know about that.

*This explanation was added; we also added more comments on the choice of materials in the introduction and in the protocol.*

4. The comment about 60x objective with only 100 nm working distance is puzzling. Is this an oil immersion objective? Can air or water immersion be used too? What about the N.A?

*This is our mistake: it is obviously 100µm (and it should be half indeed): this comes from the typical working distance of a 60x objective minus the thickness of a coverslip. It is possible to use long WD objectives with smaller NA. We rewrote this part.*

5. In step 2/2 of the protocol, it is not clear whether the storage conditions refer to the Sulfo SANPAH solution or to the activated gel itself.

*We rephrased this point.*

6. Clearly a choice was made to focus on the substrate preparation and not at all on the analysis. Still, some more details to give the reader a sense of the ease or complexity of the analysis task will be nice. The authors point to a Fiji package for tracking but do not mention any package for inversion. How will a new comer go about inverting? Is (s)he expected to collaborate with experts for this? Can (s)he choose a (Fiji?) package? The authors at least need to point to literature on this.

*The extraction of the force map is perfectly possible with the Fiji package, we described the algorithm slightly better and point to the right plugins. Concerning more complex analysis: the quantities that can be extracted from the displacement and force maps (total energy, correlation lengths, local movements, etc.) depend on the biological question and indeed require the input of someone with experience in the field.*

7. The comparison analysis between two biological conditions (fig. 4B) should be presented in more detail (displacement field, force map, energy).
*We added a panel, although we refer to our paper Kumari, Pinon et al for a deeper analysis.*

8. Overall, while the protocol will be invaluable for people who are already somewhat experienced; but for a beginner of TFM, a more in depth concluding discussion about danger and pitfalls as well as advantages (for example with respect to pillars) is lacking.

*The referee is right: we modified the introduction and discussion and detailed better the pitfalls (in the discussion).*

9. Likewise, for people doing TFM on focal adhesion forming cells, a more detailed discussion comparing the two cases is important.

*We added a paragraph in the discussion about it.*

10. The final table needs to have name of the reagents. Also without the origin (name of company) the catalog number is useless!

*We assume the table was not printed correctly, as the one we sent had the name of the company and catalogue number for each product as required by the editorial policies.*