Re: Article Number JoVE58619
Title: "Measurement of force-sensitive protein dynamics in living cells using a combination of fluorescent techniques"

Response to Editor and Reviewer Comments:

We thank the editor and the reviewers for their detailed reading and thoughtful comments regarding our manuscript. We have done our best to address all comments and, as a result, the manuscript is significant improved.

Editorial comments:
Changes to be made by the Author(s) regarding the written manuscript:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

We have thoroughly proofread the manuscript and believe there are not errors.

2. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

The copyright for the figures was obtained before the first submission and not significant changes have been made.

3. Please provide an email address for each author.

This has been done.

4. Please rephrase the Short Abstract to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Here, we present a protocol to …"

The short abstract has been re-written:

Here, we present a protocol for the simultaneous use of Förster Resonance Energy Transfer-based tension sensors to measure protein load and Fluorescence Recovery After Photobleaching to measure protein dynamics enabling the measurement of force-sensitive protein dynamics within living cells.

5. Please rephrase the Long Abstract to more clearly state the goal of the protocol.

This point has been addressed through the addition of the following sentence to the Long abstract.

"Here we describe a protocol for the Förster Resonance Energy Transfer-Fluorescence Recovery After Photobleaching (FRET-FRAP) technique, which enables the measurement of force-sensitive protein dynamics within living cells."

6. Please rephrase the Introduction to include a clear statement of the overall goal of this method.

We had edited the Introduction to clearly state the overall goal of the method. Especially the following sentence in the final paragraph:

“Here we present a protocol that combines FRET-based tension sensors with FRAP-based measure of protein dynamics. We refer to this technique as FRET-FRAP. This approach enables the simultaneous measurement of protein load and protein dynamics, thus enabling assessment of the force-sensitive dynamics of proteins in living cells (Figure 1).”

7. Please define all abbreviations before use.

We believe we have properly defined all abbreviations.

8. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc.

We believe we have used SI abbreviations for all units

9. Please include a space between all numbers and their corresponding units: 15 mL, 37 °C, 60 s; etc.
We believe we have put space after all number.

10. 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 and Reagents. For example: Metamorph, MATLAB, etc.

We believe we have removed these. A search of Metamorph and MATLAB revels no occurrences.

11. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Searching for these terms, reveals no more occurrences of personal pronouns in the protocol.

12. Please revise the protocol to contain only action items that direct the reader to do something (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.” Please include all safety procedures and use of hoods, etc. Please move the discussion about the protocol to the Discussion.

We believe that the protocol only contains action items directing the reader to do something and that these items are written in the imperative tense.

13. Please add more details 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. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.

We believe we have added sufficient detail to any potentially confusing steps. We also believe we have answered the “how” question and referenced published material when necessary.

14. 1.1.1: Please describe how this is done.

We have provided references to protocols describing this procedure

15. 1.3.3: Please provide composition of the complete media.

Composition for complete media has been added.

16. 1.3.7: Please provide composition of the imaging media. Please also specify the volume of imaging media used.

The composition of the imaging media has been specified in a note as in contains a commercial production. The volume of imaging medium has been added.

17. 5.1: Please mention how CO2 is controlled.

CO2 control and more details regarding live imaging have been added.

18. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

We have highlighted less than 2.75 pages that identify the essential steps of the protocol for the video.

19. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.

The highlighted portion is written in complete sentences with actions in the imperative tense.

20. Please include all relevant details that are required to perform the step in the highlighting. 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 highlighted.

We believe that sufficient detail to preform the highlighted steps is written.

21. References: Please do not abbreviate journal titles.

References have been edited to display the full journal titles.

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
This work by Rothenberg and Hoffman describes a new methodology based on the simultaneous combination of FRET and FRAP techniques for the measurement of protein dynamics within living cells. The authors applied this methodology to measure the response of focal adhesions due to molecular load, and in particular on vinculin. This new methodology is interesting to measure the mechanotransductive properties of force-sensitive proteins, and could be applied to many other types of mechanosensitive proteins and cellular structures. Even though the described technique is highly complex and some practixe will be required to master it, in the opinion of this referee the manuscript provides interesting results based on the original publication of the same authors. However, there are minor problems that the authors need to address before being accepted for publication. See below other comments and concerns.

We thank the reviewer the detailed reading of the manuscript and positive outlook on the usefulness of this technique.

Major Concerns:
In general, more clarification and caution notes are missing below each point of the protocol. These notes should provide further information about security issues, alternative methods, or tips, among other informations. Authors are therefore encouraged to add more notes in this regard.

We agree with the reviewer and added substantial more notes. Unfortunately, the additions are too numerous to include in the letter.

For clarity, please consider adding a brief paragraph about future improvements of the described techniques at the end of the manuscript.

We feel that it will confuse readers to have a description of other possible techniques at the end of this protocol paper, and therefore have chosen not to complete this suggestion.

Minor Concerns:
INTRODUCTION SECTION

1) Page 3, Paragraphs 3-4: The mechanoresponsive properties of focal adhesions and cadherins must be described by commenting on the pioneering works from Sasha Bershadsky and Daniel Riveline. The following works need to be included in the Introduction section:
-Riveline et al, Focal contacts as mechanosensors: externally applied local mechanical force induces growth of focal contacts by an mDia1-dependent and ROCK-independent mechanism. J Cell Biol. 2001, 153(6):1175-86.
-Brevier et al, Force-extension relationship of cell-cell contacts. Phys Rev Lett. 2007, 98(26):268101.

Citations for both works have been added.

2) For clarity, a last paragraph should be included at the end of this section. It should briefly describe the protocol content and field of application. It should clearly state the cellular structure to be studied by means of the FRET-FRAP methodology (i.e. vinculin). Finally, to differentiate from the original publication, the authors must clarify that directed cell migration will not be addressed in this protocol, but only the FRET-FRAP technique.

We agree that the last paragraph of the introduction needed more refinement. It has been edited with the reviewer's comments in mind.

Unlike previous approaches in mechanobiology, the advent of FRET-based tension sensors allows direct measurement of loads experienced by specific proteins inside living cells. Here we present a protocol that combines FRET-based tension sensors with FRAP-based measure of protein dynamics. We refer to this technique as FRET-FRAP. This approach enables the simultaneous measurement of protein load and protein dynamics, thus allowing assessment of the force-sensitive dynamics of proteins in living cells (Figure 1). Already, the FRET-FRAP technique has been applied to the study of the force-sensitive dynamics of the mechanical linker protein vinculin. Tension sensors have been developed for numerous proteins that are relevant in a variety of subcellular structures. For example, sensors have been developed for vinculin and talin in FAs, cadherins and catenins in AJs, nesprin in the nuclear LINC complex, α-actinin and filamin in the cytoskeleton, and MUC-1 in the glycocalyx, among others. Similarly, FRAP is a commonly use technique has been used on mechanosensitive proteins within the focal adhesions, adherens junctions, actin cortex, and nucleus. Moving forward, the FRET-FRAP technique should be broadly applicable to any of these existing sensors or newly developed sensors, allowing for measurements of force-sensitive dynamics in a wide variety of cellular structures and contexts. Towards this end, we provide a detailed, generalized protocol for implementing the FRET-FRAP technique applicable in these different systems. Hopefully, this will enable a wide variety of experiments elucidating the roles of various mechanosensitive proteins in regulating force transmission and in mediating cell behavior.

Given the title of the manuscript, as well as alterations to the summary and introduction, we do not feel it is necessary to explicitly state the protocols regarding the study of directed cell migration are not addressed in the manuscript.

PROTOCOL
1) Page 4, point 1.1.3: Full details about the MEFs cells should be provided.

The protocol has been changed to focus on a "desired cell type". As there is no reason to suspect that FRET-FRAP will only work in one cell type, we believe the level of detailed now provided is appropriate. If researchers seek to reproduce the exact results of the Biophysical Journal paper, the details of the MEFs are provided there.

2) Page 4, point 1.1.4: This should be included as a note below point 1.1.3.

This change has been made.

3) Page 4, section 1.2.1: Hyphen in "35-mm". Consider removing to be consistent with 1.3.1.

This change has been made

4) Page 5, section 1.2.2: "invert" what? Include PBS 1x instead of just PBS (this is valid for the entire manuscript; please, correct).

The specification of the use of canonical tube has been specified in section 1.2.2. As we only discuss standard PBS, we feel the nomenclature of 1x PBS is redundant.

5) Page 5, section 1.2.3: Replace "solution" by "fibronectin solution"; Replace "hr" by "h" (the same in 1.3.7; Please, check the entire manuscript).

Both corrections have been made. We have searched and all use of h for hour have been removed.

6) Page 5, section 1.3.1. Give full details about the culturing conditions, i.e. type of media, serum, etc. Define the "complete media" in this section.

Details regarding culture conditions was added.

7) Page 6, section 2.2: Please, provide the actual experimental values used in all the subsections 2.2.x.

This section regards the calibration of the laser used the FRAP, and the pertinent value are listed. We think the reviewer is referencing Section 3 and have provided the pertinent parameters.

8) Page 8, section 4.1.4: Provide full technical details about the laser for FRAP experiments.

Details regarding the laser for FRAP experiments have been added.

9) Page 8, section 4.2.1: Provide full details about cell culture conditions: temperature (i.e. 37ºC?) and CO_2 level (5%?). Idem in section 5.1.

Pertinent details have been added to both sections. Additionally, the specific live cell imaging chamber we use is listed in associated spreadsheet.

10) Page 8, section 4.2.3. Provide references.

Appropriate references have been added. .

FIGURES

1) Figure 4A, 4D: Provide color bars next to the figures.

The requested change to the figure has been made.

2) Figure 4C and 4F: Remove fitting. The points show no trend.

We believe that indicating the lack of trend by a flat line is suitable.

REFERENCES
1) Short name in some publications is missing (e.g. see ref. 16, 23,...).

This issue has been fixed for all references.

MATERIAL/EQUIPMENT
1) For clarity, re-order the different items in alphabetical order.

Items have been alphabetized.

Reviewer #2:
Manuscript Summary:
Overall this is a timely protocol article on the FRET-FRAP technique, which was recently published by the Hoffman group. Two existing techniques FRAP and FRET (which by themselves are widely used) are being combined to understand the relationship between tensile forces and protein turnover.

We thank for the reviewer for their positive assessment of the manuscript.

Major Concerns:
No major concerns are noted.

Minor Concerns:
Some of the text is highlighted in yellow. This seems to be an obvious oversight, perhaps a draft copy was uploaded?

Highlighted text in yellow represents text that will be used for the video portion, a formatting requested by JOVE.

There is a bit of discussion on FRET efficiency vs index measurements. Could the authors link to a JOVE or other protocols paper that discusses FRET and how to apply efficiency measurements? As written it is not clear to a new user how one would go about measuring FRET and different approaches that could be taken.

We have added an extended discussion of FRET and several key citations throughout the manuscript. The key paragraph from the Discussion section is pasted below for convenience. The citations have been removed for clarity.

"FRET index and FRET efficiency measurements allow for a quantification of protein load. FRET index is a relative measure that is highly dependent on microscope settings, while FRET efficiency measurements are absolute and independent of microscope settings. We have recently shown that a previously developed method using "three-cube" imaging can be used to determine FRET efficiency from measurements of sensitized emission that are typically quantified with FRET Index when using FRET-based tension sensors. Measurements of FRET efficiency are required if measurements of the absolute forces experience by the tension sensors are to be calculated.