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800 W. Campbell
Richardson, TX 75080

March 2, 2019

Xiaoyan Cao
Review Editor
Journal of visualized experiments (JoVE)

Dear Dr. Cao,

Please find attached our revised manuscript entitled "**In Vivo two-color 2-Photon imaging of genetically-tagged reporter cells in the skin**" for consideration for publication in the Journal of visualized experiments (JoVE).

We want to thank you and the reviewers for their time and efforts reviewing our manuscript. We found there were a number of issues that several of the reviewers mirrored, like the use of LPS-FITC and a more streamlined protocol, which we have addressed. We also noticed a number of conflicting comments and have polarized to following editorial suggestions, like the improper use of trademarks. We believe that the main focus of the manuscript is to describe how we can use the genetic model to tag a subset of fibroblast and track them over time to understand their behavior after activation. We want to highlight that one could assess LPS-FITC/tdTomato overlay/interactions, morphological changes by using standard image analysis software to assess shape, among many other necessary outputs *in vivo* imaging. To our knowledge, no one has ever used this mouse line to assess these cell-types *in vivo* as we have.

The editorial production teams' comments are underlined and italicized. **Our responses are in bold.**

Editorial comments:

Changes to be made by the author(s) regarding the manuscript:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
We have thoroughly proofread the manuscript and have removed grammatical errors.

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2. Please define all abbreviations before use.
We have completed this throughout the manuscript.

3. Please remove the square brackets enclosing the reference numbers.
We have used the "JoVE" endnote style.

4. Line 70: Please ensure that references are numbered in order of appearance. References 3-7 currently appear after 8-10.
We have updated the references to appear in numerical order in the manuscript.

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 and Reagents. You may use the generic term followed by "(see Table of Materials)" to draw the readers' attention to specific commercial names. Examples of commercial sounding language in your manuscript are Olympus, MaiTai, Sigma, etc.

We have removed all commercial language from the manuscript and have provided more sufficient detail in the table of materials.

6. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.
We have added a section that includes an ethics statement: "Animal procedures were approved by The University of Texas at Dallas institutional animal care and use committee and were in accordance with National Institutes of Health Guidelines" please find page 2 at line: 110.

7. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
We have updated the protocol text and removed any personal pronouns.

8. 4.1: Please specify the age, gender, and strain of mouse.
While we have updated our protocol section that describes the characteristics of the mice used in these experiments, we have added that the animals need not be this age, sex, or strain to work in these imaging experiments. "All experiments were performed using 8-12 week old male and female mice bred in house on a C57BL/6 background. Transgenic mice were bred in house on a C57BL/6 background. Mice were group housed and given ad libitum access to food and water. Room temperature was maintained at 21 ± 2°C". Please find page 2 at line: 111.

9. When and how is lipopolysaccharide-FITC injected into the mouse? It is not mentioned in the protocol.
We have updated the protocol and it now explains we inject a 5 µg/20 µL dosing into the mouse after initial calibration using LPS-FITC: Please find page 2, line 120 and page 4, line 192: "Perform an intraplantar injection of 5

µg/20 µL LPS-FITC on the mouse, taking care to not disturb the position of the paw".

10. Please upload each Figure individually to your Editorial Manager account as a .png, .tiff, .pdf, .svg, .eps, .psd, or .avi file.
Completed.

11. Please combine all panels of one figure into a single image file; otherwise, name different labels as separate figures.
Completed.

12. Figure 1B: Please indicate the unit for the numbers on the left.
We have indicated that the figure represents base pairs.

13. Please reference all figures/panels in the manuscript.
We have now referenced Figure 1 in the protocol text.

14. JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique
We have extensively updated the discussion section.

1. 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).] For more than 6 authors, list only the first author then et al. Please do not abbreviate journal titles. See the example below:
Bedford, C.D., Harris, R.N., Howard, R.A., Goff, D.A., Koolpe, G.A. Quaternary salts of 2-[(hydroxyamino)methyl]imidazole. Journal of Medicinal Chemistry. 32 (2), 493-503 (1998).
We have updated the references using the "JoVE" endnote format.

Reviewers' comments:

Please note that the reviewers raised some significant concerns regarding your method and your manuscript. Please revise the manuscript to thoroughly address these concerns. Additionally, please describe the changes that have been made or provide explanations if the comment is not addressed in a rebuttal letter. We may send the revised manuscript and the rebuttal letter back to peer review.

Reviewer #1:

The title and abstract are appropriate for this methods article.

The authors should make the approach more general instead of focusing on their specific hardware configuration.

1. Preparation of drugs

1.1 >> How, when and for how long the drug is delivered should be delineated.
We thank the reviewer for their time. We have updated the protocol and it now explains we inject a 5 µg/20 µL dosing into the mouse after initial calibration using LPS-FITC: Please find page 2, line 120 and page 4, line 192: "Perform an intraplantar injection of 5 µg/20 µL LPS-FITC on the mouse, taking care to not disturb the position of the paw".

RED FLAG: 5 µg/20 ml solution is specified in preparation drug, but in Figure 1C 1µg/25 ml is specified
This was a typo in the protocol and we have updated this accordingly (please see above).

2. Microscope Set-up:

2.1 and 2.2 >> these are -in my view- unnecessary details.
We have deleted these steps from the protocol.

2.3 >> only applies to setup with the condenser. There are many 2-photon microscope setups that lack one.
We have deleted this step from the protocol.

2.4 >> people use different types of objectives. This is not a requirement to perform the imaging and thus one could leave out the magnification.
We appreciate the comment, however, it was brought to our attention that we must specify the magnification, type, and numerical aperture of the objective, therefore, we have added these details to the protocol: (2.1) "Set up the multiphoton system for two-color imaging. This requires the use of two separate excitation lasers (see materials table), a GFP/RFP filter cube set (see materials table), and a 25x (N.A) water-immersion objective (see materials table)". However, we have noted that many objectives and types can be used for imaging.

2.5 >> some objectives used for 2-photon imaging do not have a correction collar. This is again objective-specific.
We have deleted this detail from the protocol.

3. Turning on the Microscope and Adjusting Settings

3.1 Power on all components of the microscope...Wait until the system is equilibrated >> this does not add anything meaningful
We agree and have deleted this step from the protocol.

3.2 Turn on the associated laser >> typically lasers are first turned on since it takes longer for the lasers to be operational than the computer with acquisition software
We have deleted this step from the protocol.

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3.3 >> the lasers would already be aligned before commencing experiments
We have deleted this step from the protocol.

3.4 Wavelengths assigned to each laser? >> A laser is tuned to a specific wavelength if using a tunable laser, but not assigned.
We have rewritten this step in the protocol to make it clear: Please see page 3, line 148 (2.4) "Tune the two excitation lasers to the excitation wavelengths of GFP and RFP, 930nm and 1100nm respectively, and direct the light path of both excitation lasers to the single objective using a dichroic mirror of 690-1050 nm allowing the 930nm-tuned excitation laser to be reflected to the main scanner and the 1100nm-tuned laser to pass directly into the main scanner (Figure 1)".

4. Imaging Set-up (Animal and Equipment Prep)

4.1 >> First a piece of tape is placed on the stage and then a stereotactic apparatus is placed on top? This is just difficult to interpret. What the authors mean is that the animal has to be anesthetized during the imaging and that it is immobilized in a stereotactic apparatus. At least that is what I make out of it.
We have rewritten this step in the protocol to be clearer: (2.2) "Place a stereotaxic apparatus on the stage of the multiphoton microscope. This must be connected to an anesthesia delivery machine (see materials table) to ensure the animals are anesthetized for the duration of the experiment. Place a piece of black paper on the surface of the apparatus as a connection point for the mouse paw".

4.1 >> should be 4.2. 5% isoflurane induction is way too much! Use 3% for induction and 1.5% for maintenance (not mentioned) and specify an oxygen flow rate
We thank the reviewer for their comments. Many studies use 3-5% isoflurane induction. We use 5% but have put it is normal to use 2.5-5% based on the vaporizer and conditions. We have changed the parameters of the experiment have made these changes in the protocol in addition to specifying the oxygen flow rate: (3.1) "Place the mouse into the induction chamber of the anesthesia delivery system and use 5% Isoflurane at a 2L/min flow rate to place the mouse under deep anesthesia. Transfer the mouse to the stereotaxic apparatus and firmly affix the hind paw to the black paper with black tape on areas both proximal and distal to the area of interest (this reduces the effects of mouse respiration on image quality), making sure the plantar surface of the paw is unobstructed and facing up towards the objective. Ensure the head of the mouse is stable within the nose cone attached to the apparatus. To maintain anesthesia throughout the experiment reduce the Isoflurane to 1.5% and keep the flow rate constant. Note: Monitor the mouse for any signs of distress or dehydration throughout the experiment".

4.2 (4.3) >> what type of water-based lubricant? Liquid or gel? It is in the table but should be listed here

We have added into the protocol that this is a gel lubricant: (3.2) "Place a generous amount of water-based lubricant (gel) on the plantar surface of the paw and touch the objective to it in order to create a column of liquid between the paw and the objective".

4.3 (4.4) >> Curtains? Just state that experiments have to be performed under dark conditions without stray light
We have deleted this detail from the protocol and now state: (2.6) "Prepare the room for imaging".

5. In Vivo Imaging

5.1 >> Fluorescent light is not used to focus on something. Excitation light is focused and fluorescence is collected.
We have corrected this and have written the following in the protocol: (3.3) "Use of the FITC excitation light to focus into the dermal layer of the paw. Ensure that tdTomato-tagged fibroblasts are visualized before continuing on (this step is important in determining the correct focal plane to image)".

>> Nothing is specified about how LPS-FITC was injected into the paws.
We have re-written the protocol and it now explains when, where and how much LPS-FITC to inject into the animal: (3.5) "Perform an intraplantar injection of 5 µg/20 µL LPS-FITC on the mouse, taking care to not disturb the position of the paw".

Figures

Figures 1A, B; 2B >> are not referenced in the text.
We have altered Figure 1 and have now referenced Figure 1 in the protocol: (2.4) "Tune the two excitation lasers to the excitation wavelengths of GFP and RFP, 930nm and 1100nm respectively, and direct the light path of both excitation lasers to the single objective using a dichroic mirror of 690-1050 nm allowing the 930nm-tuned excitation laser to be reflected to the main scanner and the 1100nm-tuned laser to pass directly into the main scanner (Figure 1)".

Figure 2B was referenced in the results section mistakenly as "2C" and has now been corrected: Initially, we injected LPS-FITC into the paws of wild type mice in order to visualize the uptake of LPS-FITC in all cell types present in the dermal layer of the paw. Having observed a myriad of cells in the dermal layer of the hind paw uptake fluorescently-tagged LPS (Figure 2A, B)".

*Figure 1C >> 2 hours *post* injection*
We have expanded on figure 1C and have changed this to Figure 5. We feel as if it is useful for the reader to have a visual flow chart of the experiment and have chosen to keep it in the manuscript.

Figures 2/3 >> time points are not specified for how long post-injection imaging was performed. I would specify explicitly
We have now explicitly stated in the protocol how long to image for in

addition to adding a section to the figure legend describing how long imaging was performed for: (3.4 and 3.6) "Image and area of cells located just below the plantar surface of the hind paw with both the 930nm and 1100nm-tuned lasers and acquire a 15 minute time-lapse of about 10-15 Z slices at approximately 1 um per slice to establish a representation of the environment prior to injection with LPS-FITC. Image and area of cells located just below the plantar surface of the hind paw with both the 930nm and 1100nm-tuned lasers and acquire a 60-120 minute time-lapse of about 10-15 Z slices at approximately 1 um per slice to identify uptake of LPS-FITC by cells."

Conclusion

In my view, this write-up is not breaking any new ground, but above all is not sufficiently instructional to people wanting to apply 2-photon microscopy to study fibroblast morphology in vivo in response to LPS injection. There are a number of incorrect statements and errors. Some of the steps are unclear or very specific to the configuration the authors are using. The authors do not elaborate on how to analyze their data aside from qualitative observations. There are many published studies that already have sufficiently detailed Materials and Methods sections on how to apply two-photon microscopy to image dermal tissue and this submission does not add sufficient new details to warrant publication.

Reviewer #2:

Manuscript Summary:
The authors demonstrate the advantage of using two-photon imaging in an anesthetized, transgenic mouse for real-time detection of fibroblast changes when LPS is injected. More detailed explanations of the method and especially the figures would improve the manuscript.

Major Concerns:

1. The summary ends with the phrase "we can illustrate morphological changes that occur in dermal fibroblasts following activation in vivo". However, the authors do not explicitly show this. It would be important to track an identified fibroblast over time without LPS and then add LPS and show the change in fibroblast morphology. There is no indication that fibroblast morphology actually changes in the presence of LPS compared to changes that may occur without LPS.
We appreciate the feedback. The purpose of the manuscript was to illustrate how we use our genetically-tagged reporter lines in conjunction with 2-photon imaging. In the discussion section we have added a segment that describes potential methods of data analysis in relation to the experiment we have done. Please refer to lines 306-317.

2. There is a dearth of information about the figures. What is the total time course of the videos?
We have updated the figures legends and have provided information on what the total time course of the videos are.

Is the focal plane kept constant during the video? There is little or no description of the figures with still images.
The focal plane is kept constant throughout the z-stack, time-lapse acquisition.

Minor Concerns:
1. Line 117. The handling of LPS is important and warrants more detail. Do you use siliconized tubes to minimized sticking to the inside surface? Do you not use glass because of absorption? What additional suggestions would be useful for the reader who is not experienced with handling LPS?
We have provided additional information on how the user should handle LPS. Please refer to line 129.

2. Line 157. Is the water-based lubricant the same as that in the materials table?
We have added into the protocol that this is a gel lubricant: (3.2) "Place a generous amount of water-based lubricant (gel) on the plantar surface of the paw and touch the objective to it in order to create a column of liquid between the paw and the objective".

3. Line 168. How, where, and how much LPS is injected?
We have re-written the protocol and it now explains when, where and how much LPS-FITC to inject into the animal: (3.5) "Perform an intraplantar injection of 5 µg/20 µL LPS-FITC on the mouse, taking care to not disturb the position of the paw".

4. Lines 188-189. The sentence needs to be rewritten.
We have rewritten this section to be clearer: "To summarize our results, we show, *in vivo,* that after injection of LPS-FITC, in an FSP1cre; TLR4$^{tb/TB}$ cell-specific reactivated animal only fibroblasts interact with and uptake LPS. In contrast, whole-body knockouts of TLR4 do not bind and uptake LPS-FITC after injection".

5. Line 192. Figures 1B and 1C don't add anything to the manuscript and can be deleted.
We agree with this statement and have deleted these two figures from the manuscript.

Figure 1A should have all abbreviations defined.
Figure 1 has been changed to Figure 2 and all abbreviations have been defined.

6. Figures 2 and 3 should have text describing these figures. The videos need to be fully described.
We have updated the figure legends to more appropriately describe the videos in the figures. Please refer to lines 247-255 and 257-274.

It would help greatly to show or outline one fibroblast and show its shape changes over time (a) without and (b) with LPS. Otherwise, the videos are not informative. You don't have to quantitate this, but it should be clear that LPS

does have some effect on fibroblast morphology or movement.
We do appreciate the constructive feedback. In the discussion section, we have added text that offers suggestions on how to analyze data gathered from the experiment. Please refer to lines 340-347. Since the purpose of the protocol is to demonstrate the method used to image fluorescently-tagged fibroblasts and injecting with LPS-FITC we chose to not include image analysis…
Reviewer #3:

Manuscript Summary:
The authors study the morphological changes in immune responsive fibroblast cells following activation and promote alterations in cellular recruitments. They utilize 2-Photon imaging using their genetically engineered FSP1-cre; Tdtomato floxed-stop-floxed mouse line and fluorescently tagged lipopolysaccharide-FITC. They illustrate morphological changes that occur in dermal fibroblast following activation in vivo. The authors clearly lay out their protocol for imaging cellular alterations of cells in the dermis of the skin.

Major Concerns:

None

Minor Concerns:

On page 3 line 173 the authors mention Fig 2C but there is no figure 2C. The authors should revise this.
This was a typo and was meant to say Fig 2B. We have corrected the error in the manuscript: "Having observed a myriad of cells in the dermal layer of the hind paw uptake fluorescently-tagged LPS (Figure 2A, B)"

Reviewer #4:

Manuscript Summary:
The authors are describing a protocol for using 2-photon microscopy to image dermal fibroblast activation in vivo. In principle, this protocol could also be adapted to study other aspects of fibroblast activity in the skin in vivo.

Major Concerns:

1. The protocol is incomplete.
For example, the injection of the lipopolysaccharide marker (LPS-FITC) is mentioned in Figure 1C but is not listed and explained in the protocol. This is a critical part of the protocol and should, therefore, be explained.
We have updated the protocol and it now explains when, where and how much LPS-FITC to inject into the animal: (3.5) "Perform an intraplantar injection of 5 µg/20 µL LPS-FITC on the mouse, taking care to not disturb the position of the paw".

As a second example, there is minimal information on they performed in vivo

imaging. What power did they use? What is the appropriate range to get good SNR while not impairing cell viability?

We have added a section to the protocol that more clearly describes the parameters used in the experiment: (2.5) + (2.5.1) "Set the laser power of FITC to 5% and GFP to 20%. Note: This setup provides an optimal signal-to-noise ratio (SNR) in these experiments".

Why are the authors using those specific wavelengths? Is it based on the peak excitation wavelengths of FITC and tdTomato or did they empirically found these were the best?

We have added a section to the protocol that denotes the user may alter these wavelengths depending on their setup: (2.4.1) "Note: It is possible to alter these excitation wavelengths depending on the user's setup". We chose these because they are based on the peak excitation wavelengths for FITC and tdTomato.

What is the numerical aperture of the objective? The N.A. is a critical descriptor of the light-gathering ability of their system.

We have added a note to the beginning of the protocol that describes in more specific detail the kind of objective that we use for the experiment and have updated that in the materials table: (2.1) "Set up the multiphoton system for two-color imaging. This requires the use of two separate excitation lasers (see materials table), a GFP/RFP filter cube set (see materials table), and a 25x (N.A) water-immersion objective (see materials table)".

What type of PMT did they use? GaAsP?

We have specified the type of PMT used in the experiment. Please refer to line 168 "Note: Detect signal via multi-alkali photo-multiplier tubes (PMTs) however, GaAsP detectors may be used instead in very high-sensitivity experiments"

*How deep did they image? How deep *can* they image?*

We have added a line in the protocol that describes how deep the user should image to replicate the results seen in our experiments: (3.3 + 3.3.1) "Use the FITC excitation light to focus into the dermal layer of the paw. Ensure that tdTomato-tagged fibroblasts are visualized before continuing on (this step is important in determining the correct focal plane to image). Note: The dermal layer of the paw is about 100-150 µm into the paw". Using our setup we are able to image about 400um into un-cleared tissue.

Giving more information is critical to enable possible users to determine if they have the right equipment to repeat this protocol. Accordingly, the material lists should include more information on their imaging microscope: just listing Olympus FVMPE-RS isn't sufficient.

We have updated the Table of Materials to provide more detail on the specific setup and components of the microscope used in the experiment.

2. Problematic description of their 2-photon setup.

*The authors mentioned that they used a multiphoton equipped with "MaiTai and Sapphire" lasers. The MaiTai is a *brand* of Ti: Sapphire 2P laser, while "Sapphire" is part of the word describing the kind of laser they are probably*

*using. The authors should describe the Brand and type of *both* two-photon lasers they are using.*

We have been advised by the editor of the journal that we may not use any descriptions of branded equipment in our setup. We have updated the materials section to more accurately reflect the kind of lasers used in the experiment.

3. Missing Z-stacks

Only two are provided, but the Figure legends suggest there should be four.

We have uploaded the remainder of the z-stack videos. There are now 6 in total.

4. Incomplete figure descriptions

For example, the lanes in the gels for Figure 1B aren't labeled.

We have updated the figure and have added more detail to the figure.

5. Incomplete discussion.

The 1-paragraph discussion is not enough. I suggest that the authors also discuss the limitations of their methods

We have extensively elaborated on the discussion section of the manuscript.

*6. The paper is also difficult to read. I would suggest the authors to better explain the jargon. For example, what are FSP1+ cells? I *think* that FSP1+ are*

We have provided information on what FSP1+ cells are and where we have obtained the mice from. Please refer to line 120.

I'm also confused as to why we see many cells in Figure 3B (Z-stack) that are green but not red. I believe the authors are claiming that only FSP1+ cells, which express tdTomato, should uptake LPS-FITC. As a result, all cells which are green should also be red.

We have provided additional explanation in the manuscript describing what FSP1 is and the percentage of fibroblast that express is. Because FSP1 is only found in 72% of fibroblasts and the recombination efficiency of cre is not 100%, there will be some dermal fibroblasts that are not tomato-tagged that will uptake LPS-FITC accounting for the small number of green fibroblasts.

Minor Concerns:

Many grammatical mistakes.

We have proofread the manuscript and have removed grammatical errors.

Figure 1A uses a lot of acronyms that aren't defined.

Figure 1 has been changed to Figure 2 and all abbreviation have been defined.

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All authors have approved the final article. Thank you for considering our manuscript for publication.

Sincerely,

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