Journal of Visualized Experiments

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--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE59647R1
Full Title:	In Vivo Two-Color 2-Photon Imaging of Genetically-Tagged Reporter Cells in the Skin
Keywords:	Multi-photon Imaging; toll-like receptor 4; genetic; reporter; lipopolysaccharide; in vivo
Corresponding Author:	Michael D. Burton University of Texas at Dallas Richardson, Texas UNITED STATES
Corresponding Author's Institution:	University of Texas at Dallas
Corresponding Author E-Mail:	michael.burton@utdallas.edu
Order of Authors:	Thomas A. Szabo-Pardi
	Nilesh M. Agalave
	Ashley T. Andrew
	Michael D. Burton
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Richardson, TX, USA

TITLE:
In vivo Two-Color 2-Photon Imaging of Genetically-Tagged Reporter Cells in the Skin

AUTHORS AND AFFILIATIONS:
Thomas A. Szabo-Pardi[1], Nilesh M. Agalave[1], Ashley T. Andrew[1], Michael D. Burton[1]

[1]School of Brain and Behavioral Science, Center for Advanced Pain Studies, the University of Texas at Dallas, Richardson, Texas, USA

Thomas A. Szabo-Pardi
Thomas.Szabo-Pardi@utdallas.edu

Nilesh M. Agalave
Nilesh.Agalave@utdallas.edu

Ashley T. Andrew
Ashley.Andrew@utdallas.edu

Michael D. Burton
Michael.Burton@utdallas.edu

CORRESPONDING AUTHOR:
Michael D. Burton
Michael.Burton@utdallas.edu

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SUMMARY:
Morphological changes occur in immune responsive fibroblast cells following activation and promote alterations in cellular recruitment. Utilizing 2-photon imaging in conjunction with a genetically engineered Fibroblast-specific protein 1 (FSP1)-cre; tdTomato floxed-stop-floxed $^{(TB/TB)}$ mouse line and green fluorescently tagged lipopolysaccharide-FITC, we can illustrate highly specific uptake of lipopolysaccharide in dermal fibroblasts and morphological changes in vivo.

ABSTRACT:
Fibroblasts are mesenchymal cells that change their morphology upon activation, ultimately influencing the microenvironment of the tissue they are located in. Although traditional imaging techniques are useful in identifying protein interactions and morphology in fixed tissue, they are not able to give insight as to how quickly cells are able to bind and uptake proteins, and once activated how their morphology changes in vivo. In the present study, we ask 2 major questions: 1) what is the time-course of fibroblast activation via toll-like receptor-4 (TLR4) and lipopolysaccharide (LPS) interaction and 2) how do these cells behave once activated? Using 2-

photon imaging, we have developed a novel technique to assess the ability of LPS-FITC to bind to its cognate receptor, TLR4, expressed on peripheral fibroblasts in the genetic reporter mouse line; FSP1cre; tdTomato$^{TB/TB}$ in vivo. This unique approach allows researchers to create in-depth, time-lapse videos and/or pictures of proteins interacting with live cells that allows one to have an increased level of granularity in understanding how proteins can alter cellular behavior.

INTRODUCTION:

Lipopolysaccharide (LPS) is an endotoxin found in the outer membrane of gram-negative bacteria[1]. LPS has a high binding affinity for the toll-like receptor 4 (TLR4)/CD14/MD2 receptor complex[2]. TLR4 is a pattern recognition receptor commonly found on the outer membrane of a wide range of immune cells, mesenchymal cells, and a subset of sensory neurons[3-5]. Activation of TLR4 expressed on immune cells leads to MyD88-dependent and independent second messenger systems, ending with nuclear-factor kappa beta (NFκB) translocation to the nucleus of the cell. This causes the prototypic immune cell to produce and release pro-inflammatory cytokines such as Interleukin (IL)-1β, IL-6, and TNF-α[6]. However, how other cell-types respond to TLR4 stimulation is not as clear. Fibroblasts have been implicated in a wide range of pathologies such as cancers and cystic fibrosis and have recently been shown to play a role monocyte chemo-attraction and promoting inflammation[7-9]. Our lab is interested in the role of fibroblasts in the development of acute and chronic pain, as early evidence suggests that factors released by fibroblasts (matrix metalloproteinases (MMPs), tissue inhibitor of metalloproteinases (TIMPs), and fibroblast growth factors (FGFs)) are involved in neuropathic pain[10].

Activation states of cells can be determined by a variety of factors that include: induction of immediate-early genes, altered protein expression, cell proliferation, and morphological changes[11-13]. There are many techniques that exist to answer questions we may have about how activation of cells contributes to pathologies, but they all have their limitations. Prototypical immunohistochemistry uses fluorescently tagged antibodies to label proteins of interest in fixed tissue, which may be unspecific and often require significant troubleshooting before yielding fruitful results[14]. Western blotting is a useful technique when comparing levels of protein expression in post-mortem tissue; however, the histological component is lacking in this technique and researchers are unable to identify any changes in morphology[15]. RNA-Seq allows us to quantify the presence of messenger RNA in a sample which in many instances yields insightful data; however, the gap between transcription and translation makes it difficult to have temporal resolution after a stimulus[16]. Confocal imaging is useful in determining the expression and co-localization of proteins that exist in a cross-section of tissues[17]. Often, this is not representative of the entirety of the tissue sample. In contrast, multiphoton microscopes allow users to image roughly 1 mm deep into a sample, creating a comprehensive three-dimensional representation[18]. Therefore, we choose to focus on in vivo, 2-photon imaging preps, as data collected from these experiments are more directly relatable to the highly plastic and interconnected microenvironment of living tissue.

An advantage of studying protein-receptor interactions in vivo is that we can clearly capture how cells respond to a stimulus, real-time, in their native environment without the harmful and unpredictable influence of post-mortem tissue extraction[19]. In addition, longitudinal studies may be performed to assess cellular plasticity and priming that may occur because of activation. Using 2-photon imaging, we preserve the integrity of the microenvironment present when an external stimulus is applied. This protocol provides a way to identify uptake of molecules in fibroblasts following peripheral injection of fluorescently tagged LPS over the course of several hours in vivo and the role of TLR4 in fibroblast activation.

PROTOCOL

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. All experiments were performed using 8-12-week-old male and female mice bred in-house on a C57BL/6 background. Transgenic mice with *cre*-recombinase driven by the fibroblast-specific protein-1 promoter were purchased commercially (Jackson, 012641) $(FSP1cre)^{+/-}$ and crossed with $tdTomato^{TB/TB}$ mice, also purchased commercially (Jackson, 007914) and $(FSP1cre)^{+/-}$; $tdTomato^{TB/TB}$ and $FSP1cre^{-/-}$; $tdTomato^{TB/TB}$ mice were bred in-house on a C57BL/6 background (**Figure 1A,B,C**). Fibroblast-specific protein 1 is an endogenous protein expressed on roughly 72% of fibroblast and represents an effective cre driver in dermal tissue[20]. Mice were group housed and given ad libitum access to food and water. Room temperature was maintained at 21 ± 2 °C. While we used C57BL/6 crossed male and female mice at 8-12 weeks, we do not believe the age, sex, or genetic background are necessary requirements to run multiphoton experiments.

1. Preparation of drugs

1.1. Prepare a 5 µg/20 µL solution of lipopolysaccharide-FITC (See **Table of Materials**) in sterile 1x PBS (pH 7.4). Vortex the stock solution at medium intensity for minimally 30 seconds to ensure homogenous mixing for an equal concentration throughout the solution before pipetting (LPS is a glutinous molecule).

NOTE: Do not place LPS into a glass container, if possible, use siliconized microcentrifuge tubes. Keep on ice until use.

2. Imaging set-up

2.1. Set up the multiphoton system (see **Table of Materials**) for two-color imaging. This requires the use of two separate excitation lasers (see **Table of Materials**), a GFP/RFP filter cube set (see materials table), and a 25x (1.05 NA) water-immersion objective (see **Table of Materials**).

NOTE: Users can alter these settings to better suit alternative setups and multiphoton microscope capabilities. These are the parameters used in the experimental protocol. See **Table of Materials** for specific details.

2.2. Place a stereotaxic apparatus (see **Table of Materials**) on the stage of the multiphoton microscope. Connect this to an anesthesia delivery machine (see **Table of Materials**) to ensure the animals are anesthetized for the duration of the experiment. Place a piece of matte black paper on the surface of the apparatus as a connection point for the mouse paw.

2.3. Select the resonant scanner with a fixed scan area of 512 µm x 512 µm.

NOTE: Do not perform the experiments in this protocol using a galvanometer scanner. Due to the slower sample rate, there will be distortion in z-stack time-lapse videos due to the respiration of the animal that is unavoidable.

2.4. Tune the two excitation lasers to the excitation wavelengths of GFP and RFP, 930 nm and 1100 nm respectively, and direct the light path of both excitation lasers to the single objective using a dichroic mirror of 690-1,050 nm allowing the 930 nm-tuned excitation laser to be reflected to the main scanner and the 1,100 nm-tuned laser to pass directly into the main scanner (**Figure 2**)**.**

NOTE: It is possible to alter these excitation wavelengths depending on the user's setup.

2.5. 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. Detect signal via multi-alkali photo-multiplier tubes (PMTs); however, GaAsP detectors may be used instead in very high-sensitivity experiments.

2.6. Prepare the room for imaging under dark conditions without stray light.

3. In vivo imaging

3.1. Place the mouse into the induction chamber of the anesthesia delivery system and use 5% isoflurane (see **Table of Materials**) at a 2 L/min flow rate of oxygen to place the mouse under deep anesthesia.

3.1.1. Transfer the mouse to the stereotaxic apparatus with access to a nose cone to maintain anesthesia throughout the experiment. Reduce the isoflurane to 1.5%-2% and keep the flow rate constant.

3.1.2. **Firmly** affix the hind paw to a piece of 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 that the head of the mouse is stable within the nose cone attached to the apparatus.

NOTE: Monitor the mouse for any signs of distress or dehydration throughout the experiment and adjust isoflurane accordingly.

3.2. Place a generous amount of water-based lubricant (gel, see **Table of Materials**) 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.

3.3. 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.

3.4. Image the area of cells located just below the plantar surface of the hind paw with both the 930 nm and 1100 nm-tuned lasers and acquire a 15-minute time-lapse of about 5-10 z-slices at approximately 1 µm per slice to establish a representation of the environment prior to injection with LPS-FITC.

3.5. Perform an intraplantar injection of 5 µg/20 µL LPS-FITC on the mouse's hind paw using a 25 µL glass Hamilton syringe (see **Table of Materials**) and 30G needle (see **Table of Materials**), taking care to not disturb the position of the paw.

3.6. Image an area of cells located just below the plantar surface of the hind paw with both the 930 nm and 1100 nm-tuned lasers and acquire a 60-120 minute time-lapse of about 5-10 z-slices at approximately 1 µm per slice to identify uptake of LPS-FITC by cells.

REPRESENTATIVE RESULTS:

Initially, we injected LPS-FITC into the hind-paw 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 in a wild type mouse (**Video Figure 1, 2**), we tried to specifically target fibroblasts as they are a primary focus in our research. Before imaging the paws of animals injected with LPS-FITC we wanted to be clear that there is no inherent fluorescence of cells in the dermal layer. This is to ensure that after injection, the images we take are true interactions of cells with the fluorescently-tagged LPS and not any imaging artifacts (**Video Figure 3, 4**). After LPS-FITC injection, only $FSP1^{+}$ fibroblasts expressing TLR4 bind and uptake the injected protein, with a high level of co-localization with the tdTomato tag expressed by these cells (**Video Figure 5**). In contrast, mice that have TLR4 knocked out of the entire body ($TLR4^{KO}$) do not bind and uptake LPS after injection. As evident in the video, silhouettes of cells are visible after LPS-FITC injection which indicates that the drug is dispersing in the interstitial fluid around cells but is not actually being bound by a receptor (**Video Figure 6**).

To summarize our results, we show, in vivo, that after injection of LPS-FITC, in an FSP1cre; $tdTomato^{TB/TB}$ 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.

FIGURE AND TABLE LEGENDS:

Figure 1. tdTomato is expressed only in FSP1^{+} Fibroblasts in a Cre-Dependent Manner. A) FSP1cre transgenic mice bred on a C57BL/6 background are crossed with tdTomato mice bred on a C57BL/6 background to generate mice expressed tdTomato in FSP1^{+} fibroblast in a cre-dependent fashion. **B**) Representative PCR results depicting both positive and negative FSP1cre mice expressing tdTomato. **C**) Representative pictograph of dermal fibroblasts in extracellular space located in the mouse paw. FSP1^{+} mice express a red fluorescent protein only in fibroblasts while FSP1^{-} mice do not.

Figure 2. Light Path of Two-Color 2-Photon Microscope. The depiction of the light path set up for the 2-color 2-photon experiment set up. Laser 1 is tuned to 930 nm to excite FITC-conjugated LPS and Laser 2 is tuned to 1100 nm to excite tdTomato found in fibroblasts. Excitation light from laser 1 is reflected by the dichroic mirror (690-1050 nm) while excitation light from laser 2 passes through to the main scanner. Excitation light from both lasers is reflected by a set of mirrors to a second dichroic mirror (650 nm) allowing excitation light to pass through the objective and into the tissue to excite the fluorophores. Light is emitted from the excited fluorophores and is captured by the 25x objective and reflected by the dichroic mirror (650) to the multi-alkali photomultiplier tubes.

Figure 3. Experimental Flow Chart of 2-Photon Microscopy. Mice are anesthetized and immobilized using a low-flow anesthesia system and stereotaxic apparatus. The plantar surface of the paw faces the objective and is imaged for 15 minutes. Intraplantar injection of 5 µg/20 µL LPS-FITC is performed on the anesthetized mouse and the paw is then imaged for a duration of time necessary for the goal of the experiment.

Video Figure 1. Z-stack Videos of LPS-FITC uptake in Cells in Wild Type C57BL/6 Mice. Z-stack video of the dermal layer of the hind paw of a C57BL/6 mouse before LPS-FITC injection. The plantar aspect of a wild type mouse paw was imaged for 15 minutes prior to injection with LPS-FITC to control for autofluorescence produced in the GFP channel. There is little to none signal in the GFP channel indicating no autofluorescence.

Video Figure 2. Z-stack video of the dermal layer of the hind paw of a C57BL/6 mouse after LPS-FITC injection. The plantar aspect of a wild type mouse paw was imaged for 1.5 hours post-LPS-FITC injection to visualize uptake of LPS-FITC by all cells expressing TLR4. As evident in the video, a multitude of cells bind and uptake LPS-FITC throughout the course of the experiment.

Video Figure 3. Z-stack video of the dermal layer of the hind paw of an FSP1cre; tdTomato mouse before LPS-FITC injection. The plantar aspect of an FSP1cre; tdTomato mouse paw was imaged for 15 minutes prior to injection with LPS-FITC to control for autofluorescence produced in the GFP channel. There is little to none signal in the GFP channel indicating no autofluorescence. tdTomato-positive fibroblasts are visualized.

Video Figure 4. Z-stack video of the dermal layer of the hind paw of a TLR4^{KO} mouse before LPS-FITC injection. The plantar aspect of TLR4^{KO} mouse paw was imaged for 15 minutes prior to

injection with LPS-FITC to control for autofluorescence produced in the GFP channel. There is little to none signal in the GFP channel indicating no autofluorescence.

Video Figure 5. Z-stack video of the dermal layer of the hind paw of an FSP1cre; tdTomato mouse after LPS-FITC injection. The plantar aspect of an FSP1cre; tdTomato mouse paw was imaged for 1.5 hours post-LPS-FITC injection to visualize uptake of LPS-FITC by tdTomato-positive fibroblasts expressing TLR4. As evident in the video, highly specific uptake of LPS-FITC via TLR4 expressed on tdTomato-positive fibroblasts is seen.

Video Figure 6. Z-stack video of the dermal layer of the hind paw of a TLR4^{KO} mouse after LPS-FITC injection. The plantar aspect of a TLR4^{KO} mouse paw was imaged for 1.5 hours post-LPS-FITC injection to visualize if uptake of LPS-FITC by cells in a whole-body knockout of TLR4 is possible. As evident in the video, no uptake of LPS-FITC is seen by the cell in the dermal layer of the hind paw.

DISCUSSION:

Arguably the most important steps of in vivo 2-photon imaging are: 1) Choosing the right genetic reporter mouse and fluorescently-tagged protein for the multi-photon setup and experimental needs[21,22]; 2) imaging the correct focal plane to have an accurate representation of the population of cells in the tissue[23]; 3) minimizing movement due to an improperly immobilized animal[24]; and 4) choosing when to analyze data qualitatively vs. quantitatively[25-27]. Ensuring to address these points before beginning an experiment will provide the knowledge to collect data that is both reproducible and scientifically rigorous.

An important consideration in the protocol is properly immobilizing the region to be imaged. Respiration from the animal causes minute shifts in the focal plane during imaging and when performing z-stack and time-lapse video, this causes significant distortion in the video and can negatively impact the quality of data produced. Ensuring that the paw is properly immobilized will allow successful imaging of the paw without disruption from respiration. In addition, knowing the localization of cells in the experiment is a critical step in identifying the correct focal plane to visualize. Because we chose to focus on dermal fibroblasts, we only need to image a relatively short distance into the paw to be able to visualize our cell types of interest (~100-150 µm). In other experiments, it is important to consider the capabilities of the microscope and objective one uses because performing an experiment to image cells deep within the tissue may be challenging to impossible given the users set up. Finally, choosing how to approach data analysis is an important consideration in the final steps of the experiment. Here, we are showing that fibroblasts expressing TLR4 are able to uptake and bind fluorescently-tagged LPS which is evident by the robust co-localization of green (LPS) and red (tdTomato expressed by fibroblasts) in the videos. Although no quantitative image analysis is done in this protocol, there are a variety of ways a user could interpret data gathered from this protocol. The first being a simple co-localization analysis using open-source software to measure the intensity of two distinct colors in a given pixel[28]. This allows the user to identify if two excited fluorophores are detected on a given pixel in an acquired image or video and how much of this overlap there is in a given space. This is useful in identifying if the cells of interest

are interacting to some capacity with the injected molecule. An alternative method of quantitative analysis is fluorescence intensity[29]. The user is able to gather information on the intensity of a given signal within a cell of interest. Data gathered from these analyses may indicate how cells might uptake various amounts of a molecule in comparison to others. These methods of data analysis are an example of how the user might seek to analyze data gathered from an experiment similar to the one performed in this protocol.

Our genetic models allow us to selectively fluorescently tag (tdTomato) $FSP1^{+}$ fibroblasts in a cre/loxP-dependent manner, which allows for quick and easy visualization of cells in the dermis of the skin**.** Although this makes visualizing cells easy because of their inherent fluorescence, it is possible to conduct 2-photon imaging without genetically tagged cells if the user is experienced in determining the shift from the epidermis to the dermis. Using the robust levels of autofluorescence from the hair on the skin can be a useful indicator of where the user is focusing and the direction one needs to move in to obtain the desired focal plane. This obviously will only work if the focal plane of interest in close to the skin and will not the cell of interest is deep within the tissue.

Tracking a focal plane throughout the experiment is ideal; however, due to the respiration of a live animal, it makes it difficult to prevent frame shifts over time when incorporating z-stacks into a time-lapse experiment. In order to troubleshoot this issue, the user may consider reducing imaging quality by decreasing line-averaging when using a resonant scanner and increasing the sample rate. As mentioned previously, it is nearly impossible to use a traditional galvanometer scanner in an experiment where a z-stack and time-lapse are incorporated due to the slower sample rate of the scanner.

Changing the duration of image acquisition may allow the user to better suit the needs of their experiment. While imaging the animal for extended periods of time allows the user to gather data throughout all the steps of molecule endocytosis and metabolism, shortening the time to image only specific parts of the process will increase efficiency. It is possible to image for a shorter period of time (~1-15 minutes) to identify molecule attachment, a longer period of time (~15-30 minutes) to visualize receptor-ligand endocytosis[30], and the full duration (~30-60 minutes) to visualize cellular activation and potential metabolism of the molecule. This is highly dependent however on the molecule injected and the cells were visualized (**Figure 3**)**.**

An important note in the experimental protocol described in this manuscript is that currently, we are unable to image identical sample areas pre and post-injection. This is due to the nature of the setup and the method of drug delivery. However, we are able to track cells from a time early post-injection for several hours. While it is important to keep track of individual cells throughout the course of the experiment, regional shifts in cellular activation are equally important and can be captured using this method. Visualizing more than two fluorophores on a multiphoton microscope simultaneously at the moment is impossible given the setup, therefore, a limitation of this method is that users are only able to image two fluorophores as opposed to other imaging equipment where 4 or more fluorophores are able to be visualized simultaneously[31]. Overall, the protocol described is specific to the goals of our lab and provides

a useful tool in identifying cellular activation where the experimental possibilities outweigh the limitations of the protocol.

The methods described in this manuscript provide a number of benefits over existing methods to visualize the activation of cells. The first being that the experiments performed here are in vivo, allowing for real-time visualization of cells binding and up taking molecules which is directly indicative of activation specifically in regard to an insult. This provides advantages over other methods such as traditional live-cell imaging techniques because we are able to preserve the environment of the cells which significantly decreases the likelihood of confounding results due to hyperexcitability and ectopic activity of cells related to the trauma of dissociation[32]. In addition, the use of a multiphoton microscope in this experimental setting decreases the rate at which fluorophores photo-bleach allowing for continuous and significantly longer imaging sessions which is important if the user applies this method to studies investigating the rate of metabolism or long-term activation[33]. Lastly, if the cell types of interest reside deep within the tissue (>100 μm) using a multiphoton microscope is necessary to obtain optimal signal[34]. Overall, if the goal of a user's experiment is to study real-time uptake and activation of cells in response to an insult then using two-photon microscopy coupled with transgenic reporter lines is more suitable over other conventional imagining techniques.

This technique allows users to perform longitudinal studies on a wide variety of cell types in regard to activation, motility, and cell-to-cell interactions. The advantage of this technique is that it allows for users to use their own genetically-tagged reported animals and substitute the fluorescently-tagged molecule to suit their research interests (e.g., Tie2cre for epithelial cells). This protocol is not restricted to the specific setup shown in our experiments and can be highly modified to fit the needs of any lab utilizing genetic reporter lines and 2-photon dermal imaging in their studies. We plan to use this approach to identify activation and recruitment of immune cells to the site of injury following peripheral trauma and determine what the specific time course of activation and recruitment is so that we may better understand what the best approach is for preventive therapeutics is in regard to various forms of pain.

In conclusion, we have developed a novel technique that allows users to image uptake of fluorescently-tagged LPS by tdTomato-tagged dermal fibroblasts expressing TLR4 using 2-photon microscopy.

ACKNOWLEDGMENTS:
This work is supported by the grant NS096030 (MDB). We would also like to thank the imaging core manager Ved Prakash.

DISCLOSURES:
The authors declare that they have no competing financial interests.

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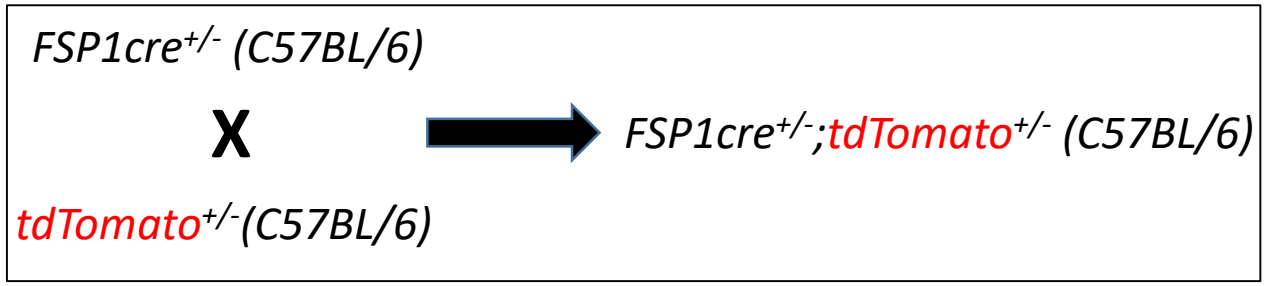
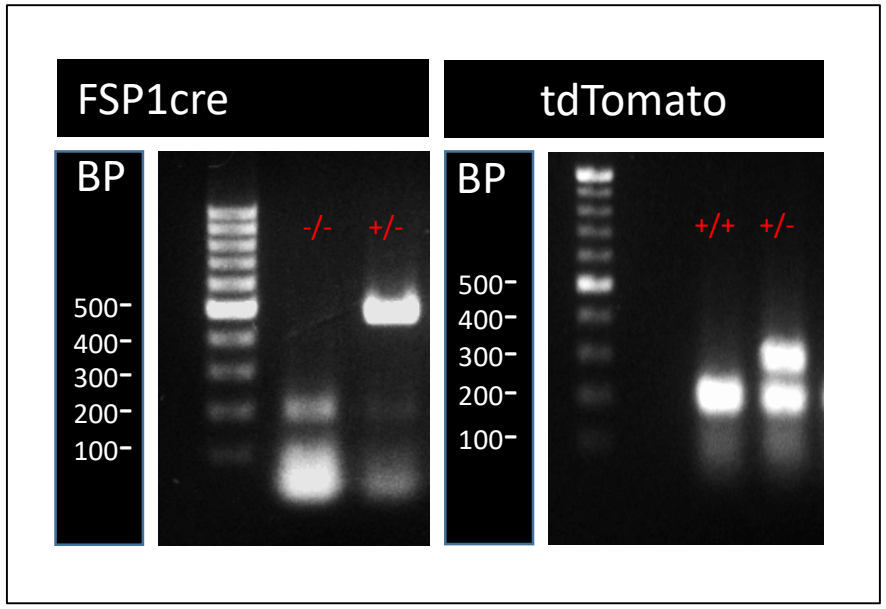
Figure 1

A)



B)



C)

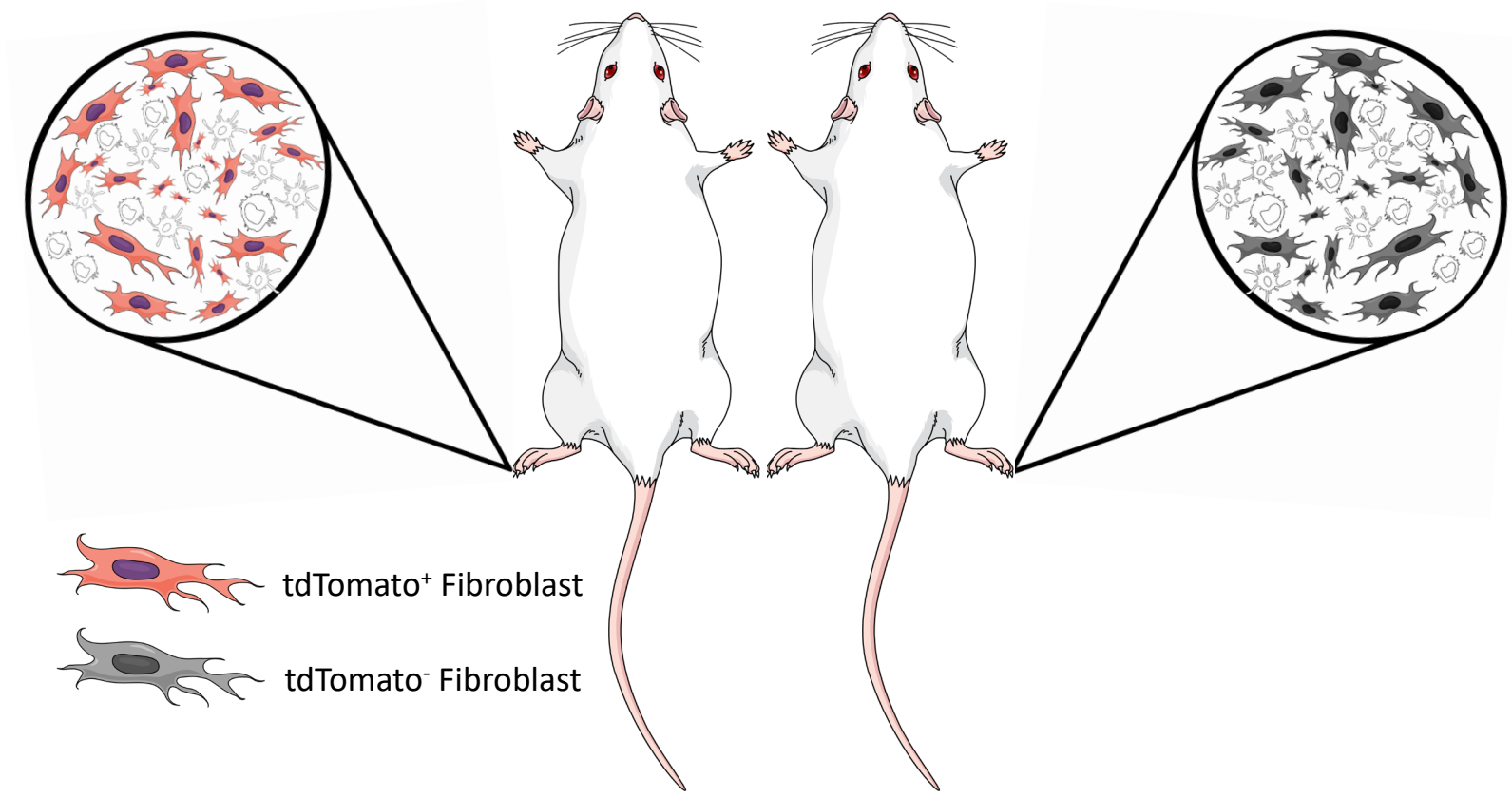


Figure 2

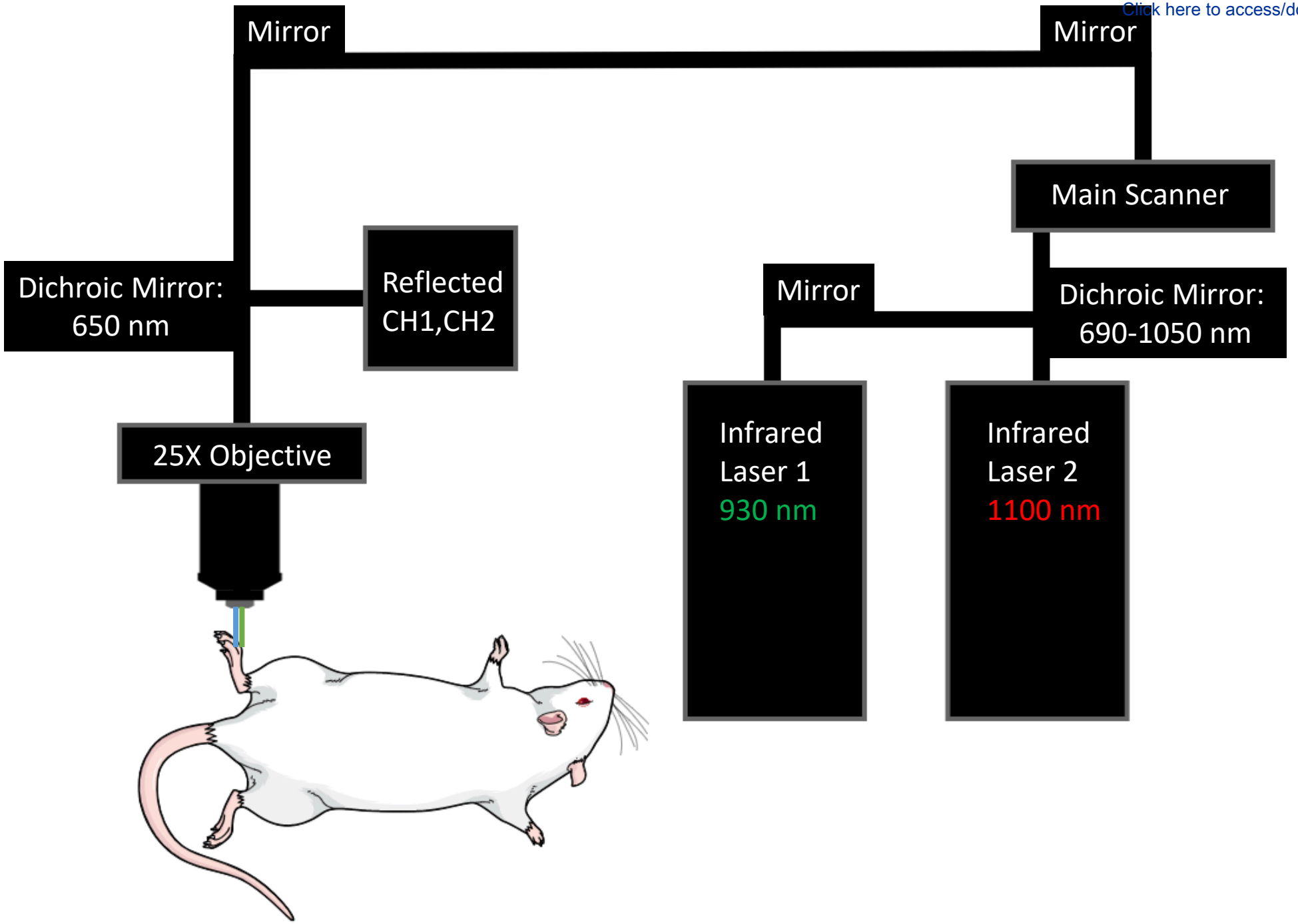


Figure 3

Anesthetize the mouse and immobilize the paw with the plantar surface facing towards the objective

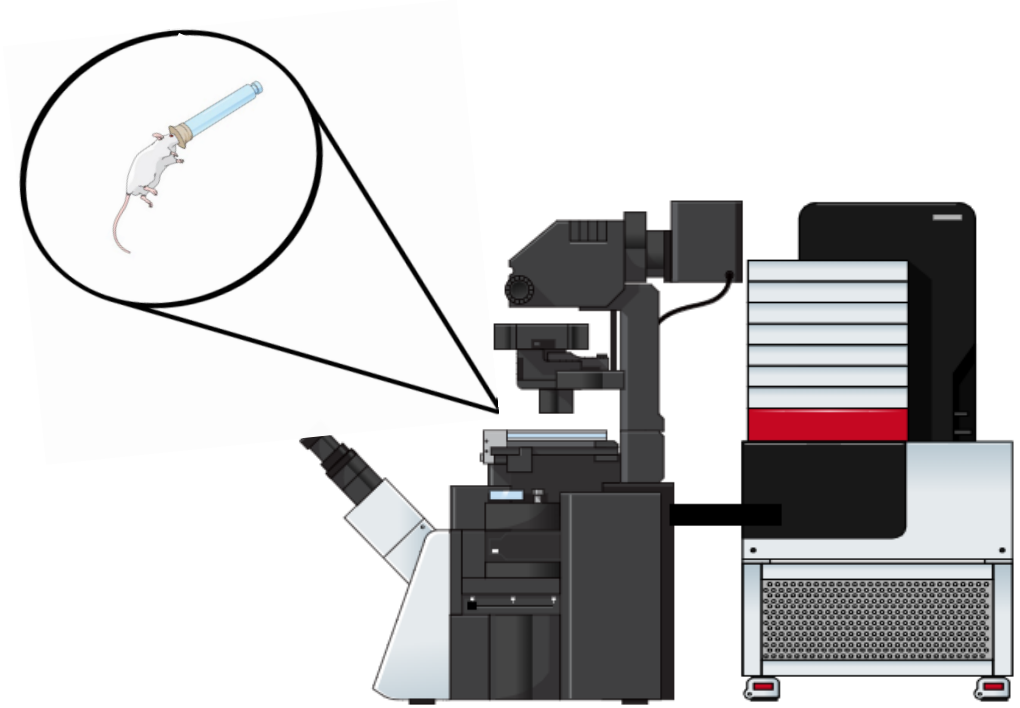
Image paw for 1-15 minutes to visualize pre-injection state of cells

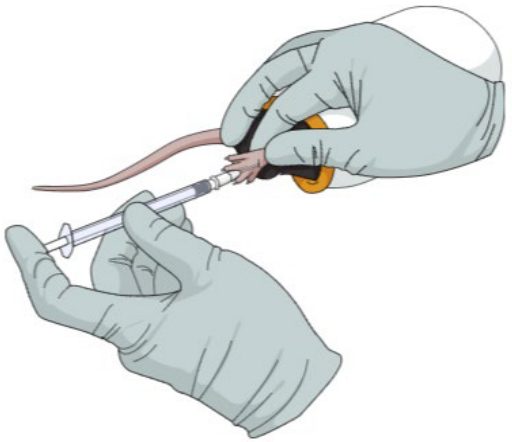
Perform intraplantar injection of 5 µg/20 µL LPS-FITC

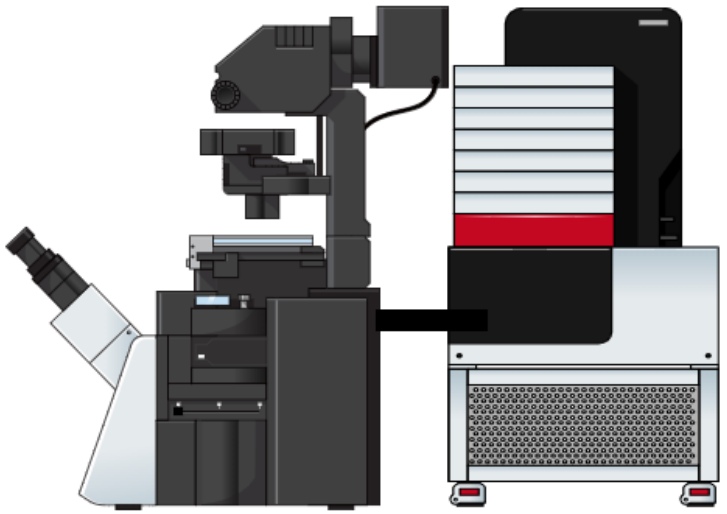
Image paw for 1-15 minutes to visualize molecule attachment

Image paw for 15-30 minutes to visualize receptor-molecule endocytosis

Image paw for 30-60 minutes to visualize clearance and metabolism







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Name
10x PBS, 4 L
700 Series MICROLITER Syringes, Hamilton, Model 705 LT Syringe
BD Precision Glide Needle 30G
Blue Pad
Filter Cube: Green/Red (BP 495-540 DM570 BP 575-645)
Isoflurane, USP 250 mL
Lipopolysaccharides from Escherichia Coli O111:B4 - FITC conjugate
Main scanner laser: Spectra Physics INSIGHT DS+ -OL pulsed IR LASER, tunable from 680 nm to 1300 nm,
Micro Centrifuge Tubes, 1.5 mL
Multiphoton Microscope: Olympus MPE-RS TWIN
Objective: Ultra 25x MPE water-immersion objective 1.05 NA, 2 mm WD
Personal Lubricant Jelly (Gel)
SGM-4 Stereotaxic Apparatus
SomnoSuite Low-Flow Anesthesia System
Stimulation laser: Olympus-specific SPECTRA PHYSICS MAI TAI HP DEEP SEE-OL pulsed IR LASER, tunable

Company	Catalog Number	Comments
Fisherbrand	BP3994	
Hamilton	80501	
VWR	305106	
Fisherbrand	1420665	
Olympus	FV30-FGR	
Vedco	50989-150-15	
Sigma-Aldrich	F3665-1MG	
Spectra Physics		
VWR	20170-333	
Olympus	MPE-RS TWIN	
Olympus	XLPLN25XWMP2	
equate	ZH727 2E F1	
Narishige	16030	
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Author(s):	Thomas A. Szabo-Pardi, Ashley T. Andrew, Nilesh M. Agalave, Michael D. Burton

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CORRESPONDING AUTHOR

Name:	Michael D. Burton
Department:	School of Behavioral and Brain Sciences
Institution:	University of Texas at Dallas
Title:	Assistant Professor

Signature: Michael Burton Date: 01/01/2019

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School of Behavioral and Brain Sciences
Cognition & Systems Neuroscience Program
Neuroimmunology and Behavior Laboratory
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.

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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".

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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.

Telephone (972) 883-7273
https://labs.utdallas.edu/niblab; Email: Michael.burton@utdallas.edu

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

Sincerely,

Michael D. Burton Ph.D.
Assistant Professor of Behavioral and Brain Sciences

Telephone (972) 883-7273
https://labs.utdallas.edu/niblab; Email: Michael.burton@utdallas.edu