Response to the editor and reviewers

Editorial Comments:

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

Yes. We have carefully proofread our revised manuscript to ensure that there are no spelling or grammatical errors.

• **Summary:** Please add a Summary (10-50 words) that clearly states the goal of the protocol.

We have added a Summary as "The goal of this protocol is to show how to use Lattice Light-Sheet Microscopy to visualize four-dimensionally surface receptor dynamics on live cell surface, shown here T cell receptor on $CD4^+$ T cells." in the revised manuscript (see page 1 in the revised manuscript).

• Please include an ethics statement before your numbered protocol steps indicating that the protocol follows the animal care guidelines of your institution.

We have included an ethics state summary "5C.C7 TCR-transgenic RAG2 knockout mice in B10.A background were used in this study according to a protocol approved by the Institutional Animal Care and Use Committee of the University of Chicago."

• **Protocol Detail:** Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. **Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps.** There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Some examples:

1) 1.3: Mention surgical steps in detail including tools used.
Detail has been added to explain the use of surgical scissors and forceps.

2) 1.4: Mention strainer mesh pore size
70 μm pore size cell strainer was used.

3) 5.2: LLSpy needs a reference
Reference has been added[1]. Detail has been added to Table of Materials.

4) 5.3: unclear how de-bleaching is done.
The Fiji pathway has been added to the manuscript (Fiji Pathway: Image>Adjust>Bleach Correction>Histogram Matching>OK).

In addition, more specific details regarding button clicks, etc. has been added.

• **Protocol Numbering:** Please adjust the numbering of your protocol section to follow JoVE's instructions for authors, 1. should be followed by 1.1. and then 1.1.1. if necessary and all steps

should be lined up at the left margin with no indentations. There must also be a one-line space between each protocol step.

We have adjusted the numbering of our protocol by following JoVE's instructions.

• **Protocol Highlight:** Please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps. Please see JoVE's instructions for authors for more clarification. Remember that the non-highlighted protocol steps will remain in the manuscript and therefore will still be available to the reader.

1) The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.
2) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.
3) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.
4) Notes cannot be filmed and should be excluded from highlighting.
5) Please bear in mind that software steps without a graphical user interface/calculations/ command line scripting cannot be filmed.

We have highlighted the text as instructed (Please find in page 3-6).

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

We have revised our discussion by following the editorial advices. Below is our discussion (Also please find the discussion in page 8-9 of the revised manuscript):

"Our protocol was optimized for the usage of CD4+ T cells isolated from 5C.C7 transgenic mice on the 3i LLSM v2, and therefore other cell systems and LLSMs may need to be optimized differently. However, this protocol shows the power of 4D imaging, as it can be used to quantify the dynamics of a surface receptor on an entire cell with the least distortion in physiological conditions. Therefore, there are many possible future applications of this technique.

A critical step is allowing the cells to settle at an appropriate concentration. If too many APCs settle on the coverslip and become too dense, it is hard to find a T cell that is interacting with only a single APC. When a T cell has multiple synapses, tracking and interpretation of data can become very complicated. Similarly, if too few APCs are present, finding a T cell forming a synapse is also difficult. In our hands, allowing 50,000 cells to settle for 10 minutes achieves an optimal density. However, this problem can be avoided if using a system with adherent cells. Cells can be grown in the incubator with the coverslips to a desired confluency.

Similarly, the number of T cells dropped into the system is dependent upon the size of the bath and the distance they can disperse. In the 3i LLSM v2 system, there is a 12 mL bath and a 2.5 mL bath, as opposed to the previous system which only had a 10mL bath available. We use the 12 mL bath for the original fluorescein imaging then switch to the 2.5mL bath for imaging the cells. This allows for less thorough washing of the bath following the beam visualization step, and

also lowers the number of T cells required for each imaging session. In turn, this would allow users to utilize cells of lower abundance.

Finding cells at the correct point of interaction is also a challenge. In our hands, T cells take about 2 minutes to settle down to the APCs on the coverslip, so it is important to begin searching the coverslip for dynamic T cells close to APCs. A major improvement of this has been the recent addition of the LED light in the finder camera. If using a home-built system, we highly recommend including this feature in your design.

Finally, fluorescent labeling strategies are another important consideration. Each fluorescent protein or dye has a different quantum yield and rate of photobleaching. Fluorescent dyes are typically brighter, but if the cells have been stably transduced with a fluorescent protein labeled molecule, the label is replenished as the cell continues to produce the molecule. Therefore, labeling strategy is an important factor to consider when designing experiments.

We would like to conclude with a discussion on future directions for the technology. LLSM is also capable of Structured Illumination Microscopy (SIM), which results in 150 nm XY resolution and 280 nm Z resolution, and is at least 10 times faster than widefield SIM2. Therefore, while LLSM provides unprecedented speed of 4D imaging, it cannot achieve the spatial resolution of current super-resolution techniques. However, this resolution could be improved if a STED LLSM could be created. Light Sheet STED and STED-SPIM (Stimulated Emission Depletion with Selective Plane Illumination Microscopy) have been utilized, but lack the temporal resolution of LLSM3–5. If STED-SPIM were adapted to incorporate a lattice, we could potentially obtain 50 nm axial resolution with far less photobleaching, and image faster than currently available techniques. Nevertheless, with current available technologies, LLSM gives us the fastest temporal resolution with high axial resolution."

• **Figure/Table Legends:** Add a title to each figure.

A title has been added to each figure and movie.

• **Commercial Language:** JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are mili-Q, Ficoll, Sigma-Aldrich, ThermoFisher, Slidebook, http://www.bitplane.com/

1) Please use MS Word's find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names.

We have removed all commercial sounding language in the manuscript. Also, all commercial products have been referenced in the table of materials/reagents as requested. (Please find the revised Table of materials)

2) Please remove the registered trademark symbols TM/R from the table of reagents/materials.

We have removed the registered trademark symbols TM/R from the table of reagents/materials.

Editorial Comments on Revised Submission:
Figure 2 has been called out before figure 1 here. Please list figures in the order of call out.

Figure order has been changed.

Age, sex, weight?

Sentence was adjusted to:
Sacrifice a 10-12-week-old 5C.C7 transgenic mouse of either sex (~ 20-25 grams) according to the approved IACUC protocol (i.e., CO_2 chamber followed by cervical dislocation).

Discard supernatant before this?
Yes. Washing means discarding supernatant. Sentences indicating "Discard supernatant" have been added.

This is a bit vague. Mention which layers provide which cells clearly. After the density gradient is first added, do you pipette up and down to mix?

Step 2.2.1 was added to include detail:
2.2.1 Add 3 mL density gradient reagent to 15mL conical tube and add cells dropwise to the edge of the tube carefully. DO NOT MIX. Centrifuge at 930 × g for 10 min at 4 °C; use acceleration/deceleration: SLOW/SLOW. Remove the middle thin layer of cells between the complete media and the density gradient reagent carefully, putting each cell type into separate conical tubes.

Something is missing before this. Which cells? T cells or APCs? Are the two cell types separated into separate tubes? Is centrifugation repeated to remove the supernatants?

Step 2.2.2. was rewritten to include more detail:
2.2.2. Wash both tubes of T cells and CH27 cells three times with 5 mL complete RPMI (300 × g for 5 min). Discard supernatant each time during wash. Resuspend each tube in 1 mL complete RPMI and count cells by a hemocytometer.

You simply mean add 2 ug to the entire cells +50 uL RPMI correct?

Yes.

What concentration?

Detail was added to indicate 30 µL fluorescein (1 mg/mL stock) was added.

What kind of bath is this? What is the size?

This is the bath on the LLSM. It is 10 mL.

Is the bead suspension diluted? What is the concentration? How are the beads mounted? Is imaging media present?

Step 3.3 was re-written to include details about the beads:

3.3. Image standard fluorescent beads in the imaging media (prepared by adhering beads to 5 mm coverslip with Poly-L-lysine, see the Table of Materials; this can be pre-prepared and re-used) for physical point spread function (PSF) in imaging media.

Please clearly mention button clicks here.

Button clicks were added wherever possible. However, many of these steps are done manually, so these steps must be shown in the video. These will also be different in home-built systems.

Add this info in the form of instructional statements in a previous step.

Step 3.3.3. was re-written to include these steps in the form of instructional statements:
3.3.3. The sample scan is how data will be collected during the experiment, so collect the sample PSF by pressing Execute in sample scan mode for deskewing and deconvolution (see section 5: Track Surface Dynamics). Change lasers to three color mode (488, 560, 647) and press Execute again.

How and when are they counted? Are they in media? Which media? Is the temperature of the LLSM maintained at a specific setting?

These are the cells from step 2.5. Counting and media have already been described.

Square or circular?

Sentence was adjusted to indicate round coverslips.

In which media?

These are the cells from step 2.5. Counting and media have already been described.

Unclear, do you mean raise the stage back by clicking **Image (Return)**?

Yes, sentence was revised to include button clicks:
Raise stage back by clicking “Image (Return)”.

Provide examples for a starting point

Sentence was added to include examples:
For example, we image 60 z-stacks at a 0.4 µm step size and input 500 time frames.

Please revise the language for grammar.

Sentence was adjusted to:
Finally, both the z+objective scan and the sample scan should show dots that look as round as possible (Figure 2E and Figure 2F, respectively).

Utilize cells at lower concentration?

Sentence was adjusted to: In turn, this would allow users to utilize fewer cells.

Comments from Peer-Reviewers:

Reviewer #1:

Manuscript Summary:
The authors present a protocol for imaging T cells using lattice light sheet microscopy. I believe this protocol could be very useful for others trying to image cellular dynamics using light sheet microscopy in general.

We thank reviewer #1 for his/her positive comments.

Major Concerns:
The paper is too based on the commercial system for lattice light sheet used by the authors. whenever possible, explanation of approaches used should be included for those not familiar with that particular system (and yet familiar with the lattice light sheet approach). In particular:

Line 134: DESKEWEING. not clear what this is to those not using a lattice light sheet system. Please add some comments on what deskweing physically represents.

Since the LLSM images on an angle (57.2°), images captured in the "sample scan" mode are collected on this angle, and are therefore "skewed". Deskewing is the process of correcting for this angle and "re-aligning" the image to a true z-stack. This detail has been incorporated into the text. See section 3.3.3.1.

Line 144: Slidebook: no idea what this is.... include reference at least

Slidesbook is the software by 3i that runs the 3i commercially available LLSM. Since we are unable to include commercial language in our text, we have replaced "Slidebook" with imaging software, and referenced it in our Table of Materials (Item 23).

Other general concerns:

Paragraph 217: I have my doubts about this paragraph. Fluorescent dyes require more power to reach photobleaching, and are, usually, brighter than fluorescent proteins. This claim is complex to prove since there are so many fluorescent proteins and dyes, all with very different quantum yield, crossections and photobleaching energies. I suggest changing the text to ensure the reader is aware that these are important factors when choosing the dyes or proteins.

We have completely re-written this paragraph as the reviewer #1 suggested (also please see page 9 in the revised manuscript).

"Finally, fluorescent labeling strategies are another important consideration. Each fluorescent protein or dye has a different quantum yield and rate of photobleaching. Fluorescent dyes are typically brighter, but if the cells have been stably transduced with a fluorescent protein labeled molecule, the label is replenished as the cell continues to produce the molecule. Therefore, labeling strategy is an important factor to consider when designing experiments."

Discussion on LLSM with STED (lines 222-228):
please include references, there are several publications doing STED light sheet (maybe not lattice light sheet, but nonetheless worth mentioning in this discussion)

We have cited and discussed relevant STED references in the discussion (see page 9 in the revised manuscript)[3, 4].

Minor Concerns:
The authors mention speed throughout the text. Please consider including a short discussion on how this approach compares to the one proposed by Huisken and Fahbrach (OL 2013 and Nat. Methods 2014). They use a tunable lens and are capable of imaging 10s of volumes per second (a more than 10x speed compared to the one proposed in this paper). Some comments on the benefits of one approach over the other would be much appreciated.

In the Huisken papers referenced here, the authors used z-steps of 1-2 µm, which is vastly larger than our 0.4 µm step size, allowing them to cover the same depth size faster. In addition, the authors are creating videos lasting only 1.5 s, which allows them to increase laser power and decrease exposure time; the authors comment on this as well: “To improve synchronization in low-frame-rate data, the duration of movies had to be increased; for example, at least 4.5 beats per movie were needed at a frame rate of only 67 fps”[6]. Therefore, the authors recognize that they must image slower to obtain longer videos. The apparent “10x speed” increase is due to settings and desired outcomes, rather than the technique used. In summary, the power of LLSM over other techniques is the decrease in photobleaching of the sample, thereby allowing for more frequent sampling, rather than the capacity of the machinery for speed. As this microscope is an adaptation of SPIM, we included these citations into our discussion on STED-SPIM.

Related to speed, in the first paragraph (line 25) you mention "immense speed". Please include a quantifiable number so that we understand the orders of magnitude discussed here. Unfortunately immense only makes sense when compared to something.

We recognize the reviewer’s concern, but unfortunately this number has not been quantified. Determining the speed with which TCRs move is a very complicated question, and they move so quickly that no one has yet been able to answer[7].

Once these changes have been introduced I believe this paper could be very useful for the light sheet community in general.

We thank Reviewer #1 for his/her feedback.

Reviewer #2:

The paper by Rosenberg et al. describes a protocol for visualising T cell receptor dynamics on the surface of an activating T-cell in response to stimulation by antigen presenting cells. The protocol describes the steps necessary to prepare the cells, and alignment and imaging procedures for the commercially available lattice light sheet microscope produced by 3i.

The authors provide a useful step-by-step procedure for isolating T-cells from transgenic mice, however this has been described in detail by others (e.g. PMID: 27842342). The alignment procedure is also welcome, but is specific for the commercially available 3i microscope, and may not be generally applicable.

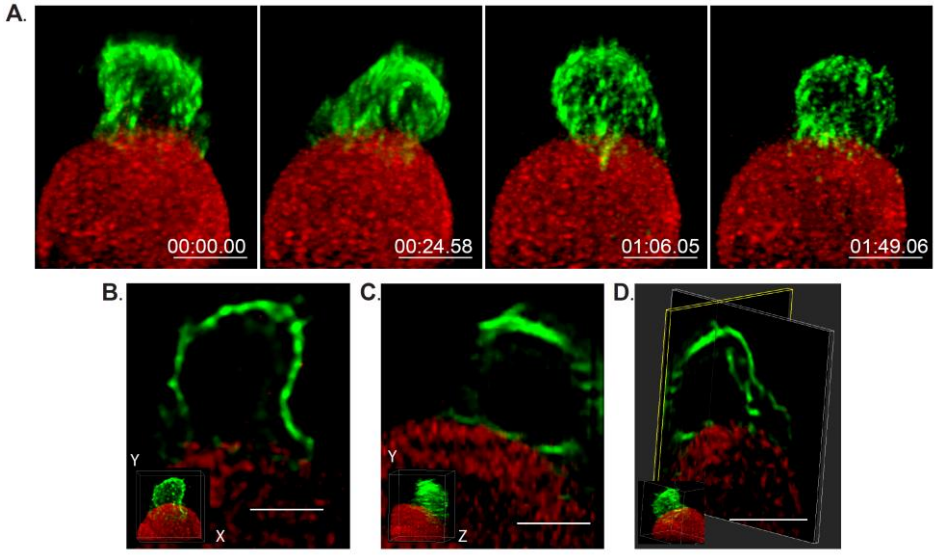
Isolating T cells from transgenic mice is not the focus of this paper, and we have simplified the T cell isolation and added a note citing relevant references: "NOTE: This part of the protocol is based on previous protocols. See citations for further detail[20, 21].". Although the alignment might be instrument dependent, the basic principle is generally applicable to other LLSM.

Major concerns:

My main concern with the manuscript is both the quality and presentation of the lattice light sheet data. We are only shown a single representative movie, and the authors claim they are able to track individual TCR clusters on the T-cell membrane. From the presented data, it is challenging to assess whether the features being tracked are indeed clusters of TCR on the membrane, rather than intracellular vesicles (caused by endocytosis of the Fab), or membrane topological features such as microvilli or ruffles. For this protocol to be widely applied, the authors should confirm that they are tracking TCR clusters. To this end, it would be beneficial to see orthogonal views of the cell, rather than simply a projection. In addition, selectively co-staining the plasma membrane, would give an indication whether or not the observed features were on the plasma membrane.

We have shown orthogonal views of the cell as well as 3D movie of the cells from different angles, to demonstrate that we indeed tracked surface TCRs, not the intracellular vesicles.

We have previously attempted to label the plasma membrane, but these cells turn over their membrane so quickly we would only see vesicles and the outer membrane was not labeled.



Minor concerns:

The authors should explain the source of their B-cells used as APCs for LLSM imaging, adding to point 2. 3. 'Resuspend 1×10^6 CH27...'

Source added[8, 9]. (see step 2.2, page 3 in the revised manuscript).

In section 5, the authors should provide further details of their approach to track features on the membrane. For example, which tracking parameters were used (feature intensity, feature size, track length). Furthermore, to display the power of the LLSM technique, the authors could show examples of quantitative data that can be extracted from such movies, for example, how rapidly the clusters are moving, how many clusters per cell etc.

Tracking details have been added in the revised manuscript as below (also please see section 5.4 on page 7 in the revised manuscript).

“NOTE: Tracking results will depend upon the tracking software used, algorithm chosen, desired output parameters selected, etc. For example, in the tracking software we utilized (see Table of Materials), we chose to allow the software to track irregular shapes, rather than assigning each feature a single spot, since TCR clusters are not organized into perfect spheres. In addition, we chose to collect 35 parameters, including speed, direction, volume, intensity, area, location, and track duration information. However, different methods or parameters will be beneficial to answer different questions.”

We thank Reviewer #2 for his/her feedback.

References:

1. Cai, E. *et al.* Visualizing dynamic microvillar search and stabilization during ligand detection by T cells. *Science (New York, N.Y.)*. **356** (6338), eaal3118, doi: 10.1126/science.aal3118 (2017).
2. Chen, B.C. *et al.* Lattice light-sheet microscopy: Imaging molecules to embryos at high spatiotemporal resolution. *Science*. doi: 10.1126/science.1257998 (2014).
3. Friedrich, M., Gan, Q., Ermolayev, V., Harms, G.S. STED-SPIM: Stimulated Emission Depletion Improves Sheet Illumination Microscopy Resolution. *Biophysical Journal*. **100** (8), L43, doi: 10.1016/J.BPJ.2010.12.3748 (2011).
4. Cella Zanacchi, F. *et al.* Live-cell 3D super-resolution imaging in thick biological samples. *Nature Methods*. **8** (12), 1047–1049, doi: 10.1038/nmeth.1744 (2011).
5. Hoyer, P. *et al.* Breaking the diffraction limit of light-sheet fluorescence microscopy by RESOLFT. *Proceedings of the National Academy of Sciences*. **113** (13), 3442–3446, doi: 10.1073/PNAS.1522292113 (2016).
6. Mickoleit, M. *et al.* High-resolution reconstruction of the beating zebrafish heart. *Nature Methods*. **11** (9), 919–922, doi: 10.1038/nmeth.3037 (2014).
7. Jansson, A. A mathematical framework for analyzing T cell receptor scanning of peptides. *Biophysical journal*. **99** (9), 2717–25, doi: 10.1016/j.bpj.2010.08.024 (2010).
8. Irvine, D.J., Purbhoo, M.A., Krogsgaard, M., Davis, M.M. Direct observation of ligand recognition by T cells. *Nature*. **419** (6909), 845–849, doi: 10.1038/nature01076 (2002).
9. Huang, J. *et al.* A Single Peptide-Major Histocompatibility Complex Ligand Triggers Digital Cytokine Secretion in CD4+ T Cells. *Immunity*. **39** (5), 846–857, doi: 10.1016/j.immuni.2013.08.036 (2013).