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Visualizing Surface T Cell Receptor Dynamics Four-Dimensionally Using Lattice Light-Sheet Microscopy

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Cover Letter

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Benjamin Werth, PhD
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Dear Dr. Werth,

On behalf of my colleagues, I would like to submit our manuscript entitled "Visualizing Surface T Cell Receptor Dynamics four-dimensionally Using Lattice Light Sheet Microscopy" for publication in JoVE.

Lattice light-sheet microscopy is a powerful technique that can image the nanostructure and dynamics of cell surface receptors, intracellular molecules and cellular interactions with unprecedented speed and resolution. In this manuscript, we used T cell receptors (TCRs) as an example to show the dynamics of immunological synapse formation in 4D (x, y, z and time) using live primary T cells. The frame rate reaches 100 frames/second, which results imaging a single cell three dimensionally in 0.85 second. This allow us to track each individual TCR molecules/microclusters with a spatiotemporal resolution that has never been reached before.

The submission is in the form of JoVE with a 151-word summary paragraph (abstract), a 1512-word main text, a 1243-word method section, 19 references, 2 figures and 2 movies.

As we discussed before, we would like to publish this JoVE method paper after the publication of our primary research paper.

We thank you for handling this paper and look forward to hearing from you.

Best regards,

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TITLE:

Visualizing Surface T-Cell Receptor Dynamics Four-Dimensionally Using Lattice Light-Sheet Microscopy

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KEYWORDS:

Lattice light-sheet microscopy, LLSM, immunology, T cell, imaging, tracking, dynamics

SUMMARY:

The goal of this protocol is to show how to use Lattice Light-Sheet Microscopy to four-dimensionally visualize surface receptor dynamics in live cells. Here T cell receptors on $CD4^+$ primary T cells are shown.

ABSTRACT:

The signaling and function of a cell are dictated by the dynamic structures and interactions of its surface receptors. To truly understand the structure-function relationship of these receptors in situ, we need to visualize and track them on the live cell surface with enough spatiotemporal resolution. Here we show how to use recently developed Lattice Light-Sheet Microscopy (LLSM) to image T-cell receptors (TCRs) four-dimensionally (4D, space and time) at the live cell membrane. T cells are one of the main effector cells of the adaptive immune system, and here we used T cells as an example to show that the signaling and function of these cells are driven by the dynamics and interactions of the TCRs. LLSM allows for 4D imaging with unprecedented spatiotemporal resolution. This microscopy technique therefore can be generally applied to a wide array of surface or intracellular molecules of different cells in biology.

INTRODUCTION:

The precise dynamics of molecules trafficking and diffusing on the three-dimensional cell surface in real time have been an enigma to solve. Microscopy has always been a balance of speed, sensitivity, and resolution; if any one or two are maximized, the third is minimized. Therefore, due to the small size and immense speed with which surface receptors move, tracking their dynamics has remained a major technological challenge to the field of cell biology. For example, many studies have been conducted using total internal reflection fluorescence (TIRF) microscopy[1–3], which has high temporal resolution, but can only image a very thin slice of the T-

cell membrane (~100 nm), and therefore misses events happening farther away in the cell. These TIRF images also only showing a two-dimensional section of the cell. By contrast, super-resolution techniques, such as stochastic optical reconstruction microscopy (STORM)[4], photoactivated localization microscopy (PALM)[5], and stimulated emission depletion microscopy (STED)[6], can overcome the Abbe diffraction limit of light. These techniques have high spatial resolution (~20 nm resolution)[4–7], but they often take many minutes to acquire a full two-dimensional (2D) or three-dimensional (3D) image, and therefore the temporal resolution is lost. In addition, techniques such as STORM and PALM that rely on blinking signals may have inaccuracies in counting[8, 9]. Electron microscopy has by far the highest resolution (up to 50 pm resolution)[10]; it can even be conducted three-dimensionally with focused ion beam scanning electron microscopy (FIB-SEM), resulting in up to 3 nm XY and 500 nm Z resolution[11]. However, any form of electron microscopy requires harsh sample preparation and can only be conducted with fixed cells or tissues, eliminating the possibility of imaging live samples over time.

Techniques to obtain the high spatiotemporal resolution required to identify the dynamics of surface and intracellular molecules in live cells in their true physiological 3D nature is only being recently developed. One of these techniques is Lattice Light-Sheet Microscopy (LLSM)[12], which utilizes a structured light sheet to drastically lower photobleaching. Developed in 2014 by Nobel Laureate Eric Betzig, the high axial resolution, low photobleaching and background noise, and ability to simultaneously image hundreds of planes per field of view make LLS microscopes superior to widefield, TIRF and confocal microscopes[12–19]. This four-dimensional (x, y, z and time) imaging technique, while still diffraction limited (~200 nm XYZ resolution), has incredible temporal resolution (we have achieved a frame rate of about 100 fps, resulting in a 3D reconstructed cell image with 0.85 seconds per frame) for 3D spatial acquisition.

LLSM can be generally used to track real-time dynamics of any molecules within any cell at the single-molecule and single-cell level, particularly those in highly motile cells such as immune cells. For example, we show here how to use LLSM to visualize T cell receptor (TCR) dynamics. T cells are the effector cells of the adaptive immune system. TCRs are responsible for recognizing peptide-MHC (pMHC) ligands displayed on the surface of antigen-presenting cells (APC), which determines the selection, development, differentiation, fate, function of a T cell. This recognition occurs at the interface of T cells and APCs, resulting in localized receptor clustering to form what is called the immunological synapse. While it is known that TCRs at the immunological synapse are imperative for T cell effector function, still unknown are the underlying mechanisms of real-time TCR trafficking to the synapse. LLSM has allowed us to visualize in real time the dynamics of TCRs before and after trafficking to the synapse with the resultant pMHC-TCR interaction (**Figure 1**). LLSM can therefore be used to solve current questions of the formative dynamics of TCRs and provide insights to understand how a cell distinguishes between self and foreign antigens.

PROTOCOL:

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.

1. Harvest and activate T cells

NOTE: This part of the protocol is based on previous protocols. See citations for further detail[20, 21].

1.1. Euthanize a 10–12-week-old 5C.C7 transgenic mouse of either sex (~ 20–25 g) according to the approved IACUC protocol (i.e., CO_2 chamber followed by cervical dislocation).

1.2. Spray mouse carcass thoroughly with 70% ethanol to soak down the fur and bring into a BSL-2 safety cabinet.

1.3. Turn mouse onto its right side and make a small incision in the body cavity with surgical scissors. Remove the spleen using surgical forceps. Cut away connective tissue as needed with surgical scissors.

1.4. Place the spleen in a 70 µm-pore mesh cell strainer and mash with the back of a 1 mL syringe plunger. Wash through the strainer thoroughly with complete RPMI (RPMI with 10% fetal bovine serum [FBS], 2 mM L-glutamine, 1% penicillin/streptomycin, 50 µM 2-mercaptoethanol).

1.5. Centrifuge the single cell suspension of splenocytes at 300 × *g* for 5 min. Discard the supernatant.

NOTE: The pellet at this point will be red.

1.6. Resuspend the splenocytes in 5 mL RBC lysis buffer. Incubate for 5 min, then quench with 5 mL of complete RPMI.

1.7. Centrifuge the single cell suspension of splenocytes at 300 × *g* for 5 min. Discard the supernatant.

NOTE: The pellet at this point should be white, not red.

1.8. Resuspend the pellet in 5 mL of complete RPMI.

1.9. Transfer to a T-25 flask and add 10 µM moth cytochrome-C (MCC, sequence ANERADLIAYLKQATK). Put the cells in a cell culture incubator at 37 °C, 5% CO_2 overnight.

1.10. On the next day, add recombinant mouse IL-2 to a final concentration of 100 U/mL.

1.11. Observe for the following days, adding fresh media as the current media turns yellow. T cells will die if left in yellow media unattended for over 48 h. Cells are ready to use 6–10 days after harvest.

2. Prepare cells

2.1. Incubate 5 mm round coverslips with 0.1% poly-L-lysine for 10 min. Aspirate off and let dry naturally.

2.2. Use a density gradient reagent (see the **Table of Materials**) to separate out dead cells and to obtain 1 × 10^6 T cells and 1 × 10^6 APCs (CH27 cells[21, 22] transduced with cytosolic mCherry) separately.

NOTE: Cells are counted using a hemocytometer.

2.2.1. Add 3 mL of density gradient reagent to a 15 mL 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 thin middle layer of cells between the complete media and the density gradient reagent carefully, putting each cell type into separate conical tubes.

NOTE: It is necessary to prepare more cells than needed for imaging, as we find that 50% is lost on average during the process. The more cells used for the process, the easier it is to harvest them from the density gradient. We use 4–8 mL of both cell types to ensure excess cells. If desired, cells can be counted before this step to ensure volume needed. Any extra cells are put back into their respective flasks.

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 the supernatant each time during the wash. Resuspend each tube in 1 mL complete RPMI and count cells by a hemocytometer.

2.3. Resuspend 1 × 10^6 APCs in 500 µL complete RPMI and add 10 µM MCC. Incubate for 3 h at 37 °C, 5% CO_2). Wash cells three times with 500 µL complete RPMI (300 × *g* for 5 min). Discard the supernatants.

2.4. Resuspend 1 × 10^6 T cells in 500 µL complete RPMI. Add 2 µg of anti-TCRβ Alexa488-labeled Fab (clone H57) to 500 µL of cells. Incubate for 30 min at 37 °C, 5% CO_2. Wash cells three times with 500 µL of complete RPMI (300 × *g* for 5 min). Discard supernatant after each wash.

NOTE: The divalent anti-TCR antibody was cut into monovalent Fab using a Fab preparation kit (see the **Table of Materials**) to avoid antibody crosslink the T cell receptors (this step is optional).

2.5. Resuspend both cell types in 500 µL of imaging media (phenol red-free Leibovitz's L-15 medium with 10% FBS, 1% penicillin/streptomycin, 2 mM L-glutamine).

3. Conducting LLSM daily alignment

NOTE: (Important) This alignment protocol is based on the LLSM instrument used (see the **Table of Materials**). Each LLSM may be different and require different alignment strategies, especially

those that are home-built. Carry out the appropriate routine alignment and continue to section 4.

3.1. Add 10 mL of water plus 30 µL fluorescein (1 mg/mL stock) to the LLSM bath (~10 mL volume), press **Image (Home)** to move the objective to image position, and look at a single Bessel laser beam pattern. Align the laser beam using the guides and pre-set region of interest (ROI) to make the beam a thin pattern balanced in all directions.

3.1.1. The beam should also appear focused in the finder camera. Use two mirror tilt adjustors, top micrometer, focus, and emission objective collar to adjust. See **Figure 2A,B** for correctly aligned beam.

3.2. Wash the bath and objectives with at least 200 mL of water to completely remove fluorescein.

3.3. Image standard fluorescent beads in the imaging media (prepared by adhering beads to a 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.

NOTE: There can only be one bead in view for later processing, so try to find a bead that is by itself in the viewer or can easily be cropped to obtain a single bead.

3.3.1. Turn on **dither** (set to 3). To do so, press **Live** to view the current field. Move along the Z direction to find the cover slip and beads. Find the center of a bead by moving along Z, press **Stop** to pause the laser. Check **3D**, press **Center** and then press **Execute**. This will collect the data.

3.3.2. Manually adjust the tilt mirror, objective collar, and focus micrometer for highest gray values, then adjust as necessary to obtain proper patterns for objective scan, z galvo, z+objective (totPSF), and sample scan (samplePSF) capture modes. See **Figure 2C–F** for properly adjusted maximum intensity projections (MIPs).

NOTE: The various captures modes (objective scan, z galvo, z+objective, and sample scan) change how the light-sheet moves through the sample. All scan modes should be used for alignment.

3.3.3. The sample scan shows how data will be collected during the experiment. Collect the sample PSF by pressing **Execute** in sample scan mode for deskewing and deconvolution (see section 5). Change lasers to three color mode (488, 560, 647) and press **Execute** again.

NOTE: Since the LLSM images at an angle (57.2°), images captured in the “sample scan” mode are collected at this angle, and are therefore “skewed”. De-skewing is the process of correcting for this angle and “re-aligning” the image to a true z-stack. These data **must** be collected in imaging media and in all channels that will be imaged during the experiment. If this is not collected properly, the data will not be properly de-skewed. Similarly, make sure media has been warmed to 37 °C (or desired experimental temperature).

4. Setting up cells with LLSM

4.1. Add 100,000 APCs (50 µL) from step 2.5 to a 5 mm-diameter circular coverslip and allow them to settle for 10 min.

4.2. Grease the sample holder then add the coverslip cell-side-up to it. Add a drop of imaging media to the back of the coverslip to avoid bubbles before placing in the bath. Screw the sample holder onto the piezo, and press **Image (Home)**.

4.3. Find an APC to image to ensure that the LLSM and imaging software (see **Table of Materials**) are functioning properly.

NOTE: We image at 0.4 µm step size with 60 z-steps and 10 ms exposure for two colors with dither set to 3, which results in 1.54 s per frame of 3D image with ~200 nm XY and 400 nm Z resolution. These settings may need to be adjusted based on cell size, desired z-resolution, and strength of signal from the fluorescent labeling technique used. Laser power usage will also vary based on fluorescent labeling technique used.

4.3.2. Press **Live** to view the current image. Move along Z to find the cover slip and cells.

4.3.3. Find the center of an APC by moving in the Z direction, then press **Stop** to pause the laser. Check **3D** and input the desired settings (see step 4.3.1), press **Center** and then press **Execute**. This will collect the data.

4.4. Lower the stage to **load** position and add 50 µL of T cells in imaging media (50,000 cells, from step 2.5) dropwise directly over the coverslip. It is best to let a drop form on the end of the pipette tip and then touch the tip to the bath liquid. Raise stage back by clicking "**Image (Return)**".

4.5. Begin imaging. Be sure to set the desired stack size and time lapse length. For example, image 60 z-stacks at a 0.4 µm step size and input 500 time frames. (Typically) stop recording before 500 frames are reached to avoid photobleaching. Use **Live** mode to search for cell pairs, and when ready and desired settings have been entered, press **Execute** to collect data. See **Movie 1** and **Figure 1** for an example.

5. Track surface dynamics

5.1. Export the data from the imaging software (see the **Table of Materials**). This will create z-stack TIF files for every time point in each color.

5.2. First deskew and deconvolve the data.

NOTE: We use the LLSpy pipeline under license by HHMI's Janelia Research Campus[18], but deskewing and deconvolution are also available within multiple imaging softwares (see **Table of Materials**).

5.3. Debleach the data.

NOTE: We use Fiji's debleach feature with histogram matching (Fiji Pathway: **Image | Adjust | Bleach Correction | Histogram Matching | OK**).

5.4. Import into the tracking software (see the **Table of Materials**). Track clusters according to software specifications. See **Movie 2** for an example.

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 the **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.

5.4.1. If tracking is not desired, use the ClearVolume plugin for Fiji for visualizing and creating movies from hyperstack data.

REPRESENTATIVE RESULTS:

Here, we describe the isolation, preparation, and imaging of primary mouse 5C.C7 T cells using a lattice light-sheet microscope. During section 3, it is imperative to align the microscope correctly, and to collect PSF daily with which to deconvolve the data after collection. In **Figure 2**, we show the correct alignment images that will be seen when aligning the microscope. **Figure 2A** and **Figure 2B** show the correct beam path and beam alignment, respectively, when imaged in fluorescein. The objective scan should show a large X shape in the XZ and YZ projections that is as symmetrical as possible; this should also be adjusted to be as small of an X as possible (**Figure 2C**). This is mainly achieved by adjusting the emission objective collar. The z galvo scan should show an oval in XZ and XY with a single dot on either side (above and below for XZ and to the left and right for YZ) (**Figure 2D**). This is mainly achieved by adjusting the galvo mirror tilt, either manually or with the motorized adjustment in the software. 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). These may have a small X, but this should be as dimished as possible. These should be well aligned if the objective scan and z galvo were set well, but if adjustments are necessary, they will be mostly conducted with the galvo mirror. It is important to note that during this alignment, any time the collar and galvo are adjusted, the focus (micrometer above the emission objective) will need to be adjusted as well.

Using this protocol, we can see the four-dimensional dynamics of the TCRs on a T-cell surface (**Figure 1**, **Movie 1**). The main advantage of this microscope lies in the ability to track the

visualized surface units of the TCRs, and to obtain quantified data from their size, motion, signal intensity, etc. (see **Table of Materials**). **Movie 2** shows an example of the tracks obtained.

FIGURE LEGENDS:

Figure 1: 4-dimensional imaging of T cell-APC Synapse. (**A**) A representative example 3D time-lapse LLSM images showing a T cell interacting with an APC. Shown are the TCR (green, labeled by anti-TCR-AF488) dynamics in recognizing antigens presented on the surface of an APC (red, cytosolic mCherry). Scale bar represents 5 µm. Also see **Movie 1**. (**B**) Orthogonal XY slice of (A). Inset is a reference frame of a whole cell. Scale bar represents 5 µm. (**C**) Orthogonal YZ slice of (A). Inset is a reference frame of a whole cell. Scale bar represents 5 µm. (**D**) Dual orthogonal slice of (A). Inset is reference frame of whole cell. Scale bar represents 5 µm. Also see **Movie 3**.

Figure 2: LLSM alignment. (**A**) Desired beam pattern for LLSM imaging experiment. (**B**) Screenshot of the beam alignment process; on the left is the focus window showing the narrowed, focused beam; at the top right is a graph showing that the beam is centered within the window; at the bottom right is the finder camera, which should also be a thin, focused beam. (**C**) Maximum intensity projections (MIPs) of a bead by objective scan. (**D**) Maximum intensity projections (MIPs) of a bead by z-galvo scan. (**E**) Maximum intensity projections (MIPs) of a bead by z+objective scan. (**F**) Maximum intensity projections (MIPs) of a bead by sample scan.

Movie 1: T cell synapse formation. Two-color volume rendering of the interaction of a T cell labeled with αTCR-AF488 (green) with a target APC expressing cytosolic mCherry (red) over 70 time points at 1.54 s interval.

Movie 2: Tracking TCR dynamic motion. Compare with **Movie 1**. Clusters corresponding to visible TCR structures were tracked in 3D over time with imaging software. Dragon tails showing cluster positions over the previous four frames are color-coded by displacement length.

Movie 3: Orthogonal slices of T cell synapse. Compare with **Movie 1** and **Figure 1B–D**. Dual orthogonal slice of **Movie 1**, showing that αTCRβ-AF488 Fab is indeed labeling the membrane of the T cell. Inset is a reference frame of whole cell. Scale bar represents 5 µm.

DISCUSSION:

The presented protocol was optimized for the usage of $CD4^+$ T cells isolated from 5C.C7 transgenic mice on the LLSM instrument used, 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 min 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 LLSM system used here, there is a 12 mL bath and a 2.5 mL bath, as opposed to the previous version of the system which only had a 10 mL bath available. We use the 12 mL bath for the original fluorescein imaging then switch to the 2.5 mL 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 fewer cells.

Finding cells at the correct point of interaction is also a challenge. In our hands, T cells take about 2 min 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 the 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 SIM[12]. Therefore, while LLSM provides unprecedented speed of 4D imaging, it cannot achieve the spatial resolution of current super-resolution techniques[4–6]. However, this resolution could be improved if a STED LLSM could be created. Light sheet STED and stimulated emission depletion with selective plane illumination microscopy (STED-SPIM) have been utilized, but lack the temporal resolution of LLSM[21–24]. 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.

DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

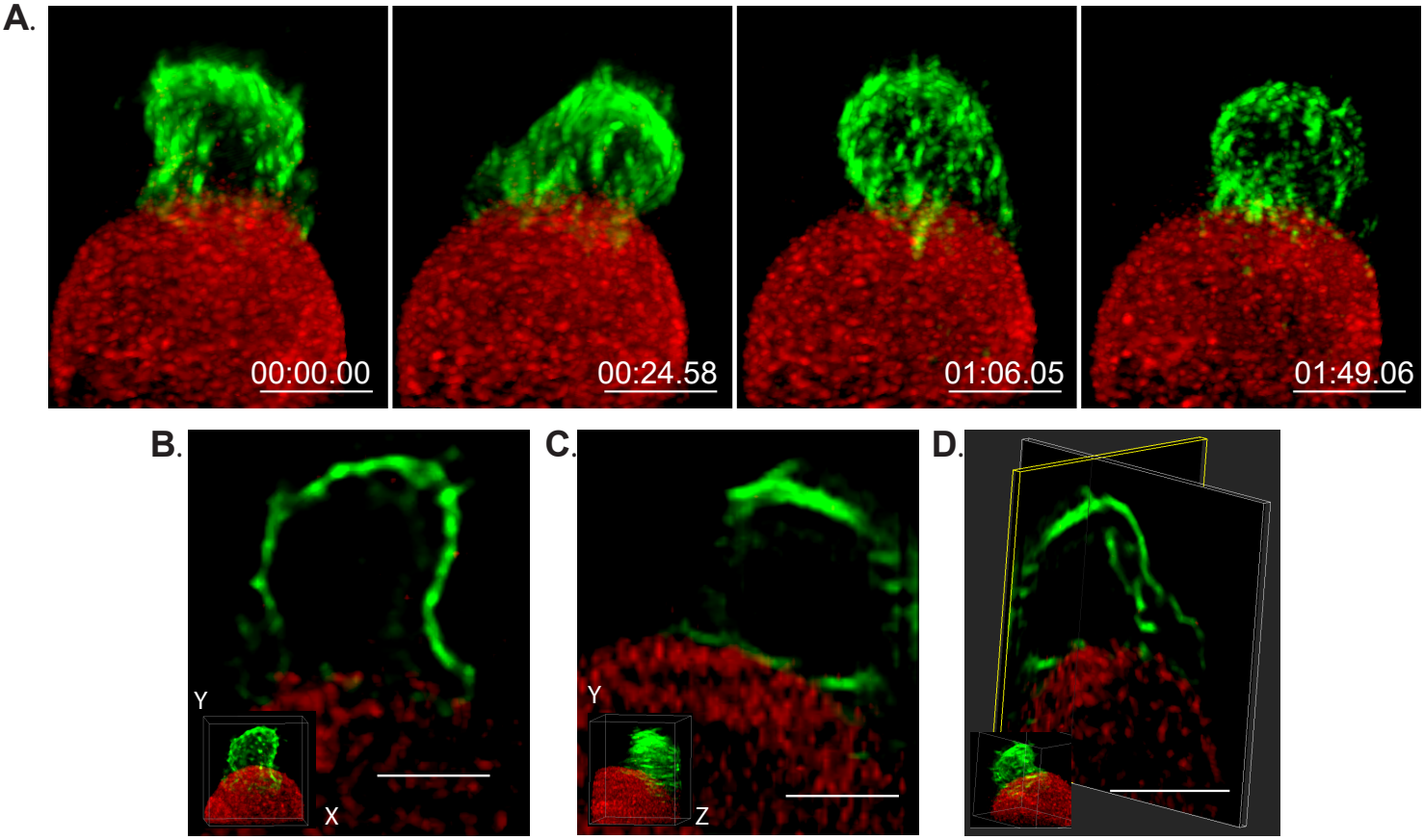

Figure 2

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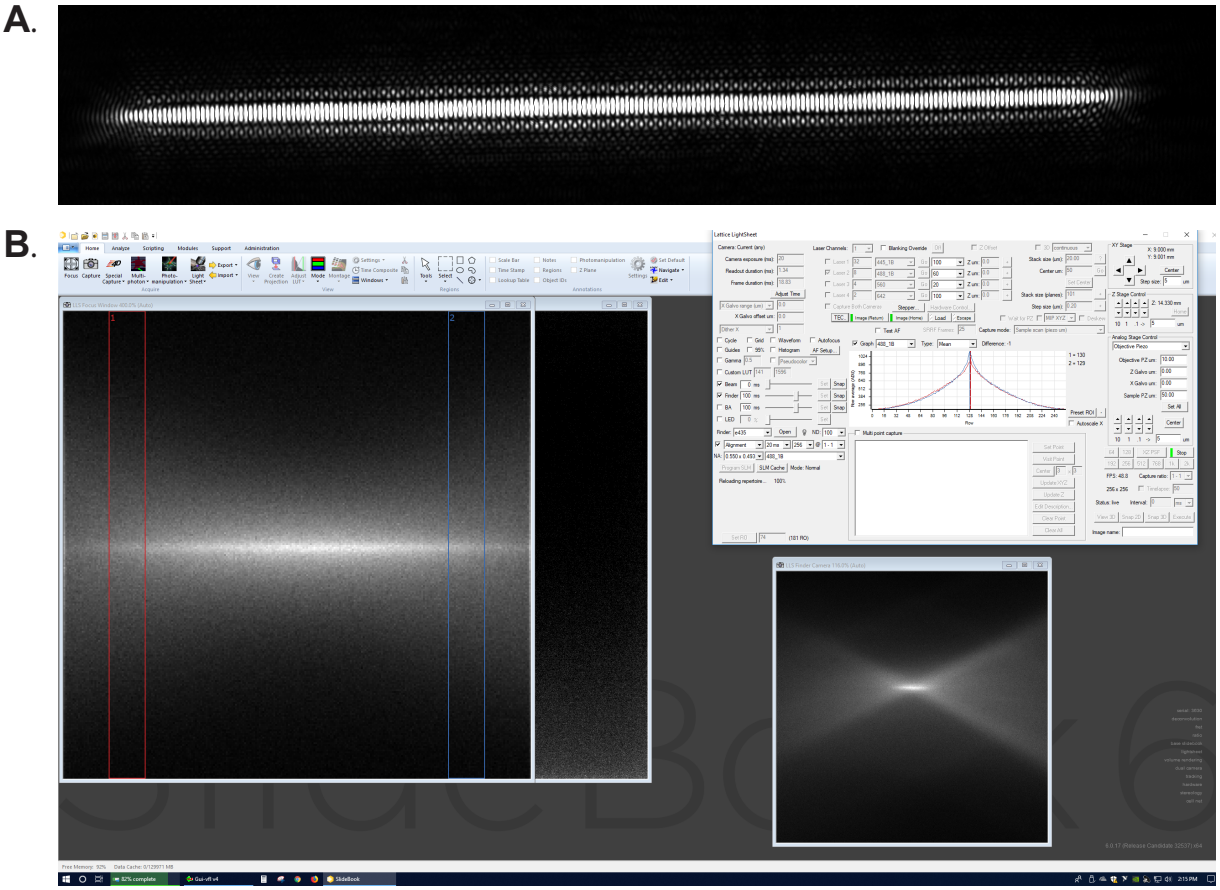

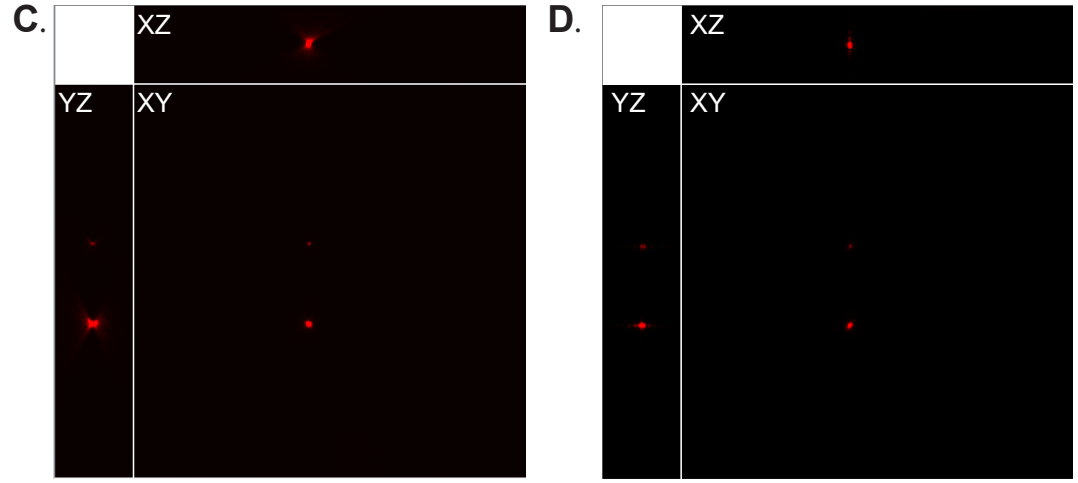

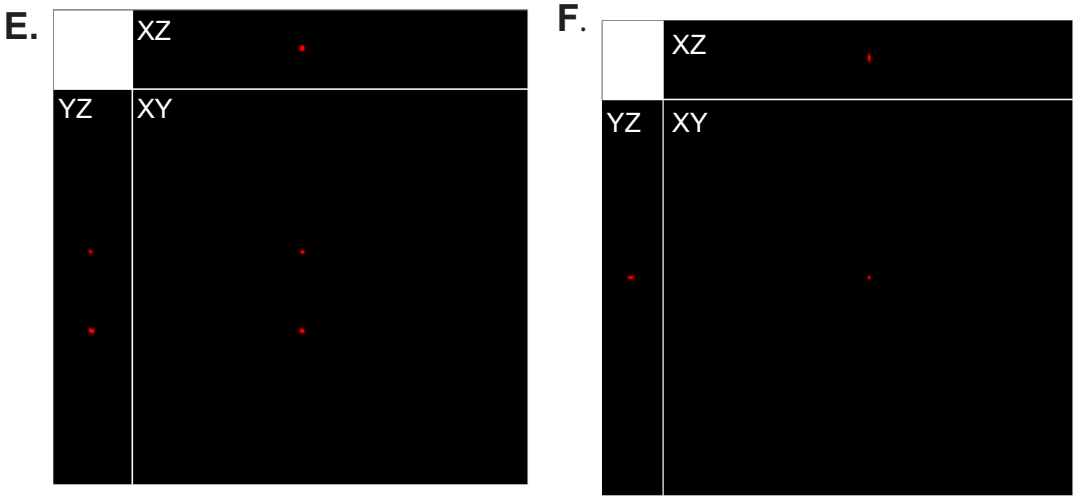

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Table of Materials

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Name	Company	Catalogue Number
1 mL Syringe	BD	309659
2-Mercaptoethanol	Sigma-Aldrich	M3148-25ML
5 mm round coverslips	World Precision Instruments	502040
70um Sterile Cell Strainer	Corning	7201431
Alexa Fluor 488 anti-mouse TCR β chain Antibody	BioLegend	109215
Fetal Bovine Serum (FBS)	X&Y Cell Culture	FBS-500
Ficoll	GE Healthcare	17-1440-02
Fluorescein sodium salt	Sigma-Aldrich	F6377
FluoSpheres Carboxylate-Modified Microspheres	Thermo Fisher Scientific	F8810
Imaris	Bitplane	N/A
Lattice Light-Sheet Microscope	3i	N/A
Leibovitz's L-15 Medium, no phenol red	Thermo Fisher Scientific	21083027
L-Glutamine	Thermo Fisher Scientific	25030-081
LLSpy	Janelia Research Campus	N/A
Moth Cytochrome C (MCC), sequence ANERADLIAYL	Elimbio	Custom Synthesis
Penacillin/Streptamycin	Life Technologies	15140122_3683884612
Poly-L-Lysine	Phenix Research Products	P8920-100ML
RBC Lysis Buffer	eBioscience	00-4300-54
Recombinant mouse IL-2	Sigma-Aldrich	I0523
RPMI 1640 Medium	Corning	MT10040CV
Slidebook	3i	N/A
Surgical Dissection Tools	Nova-Tech International	DSET10
T-25 Flasks	Eppendorf	2231710126
Thermo Scientific Pierce Fab Micro Preparation Kits	Thermo Fisher Scientific	44685

Comments

For T cell harvest
For T cell culture
For Imaging
For T cell harvest
For Imaging
For T cell culture
Denisty gradient reagent for T cell harvest
For microscope alignment
For microscope alignment
Tracking Software; Other options for tracking software include Amira or Trackmate (Fiji).
Microscope Used
For Imaging
For T cell culture
LLSpy was used under license from Howard Hughes Medical Institute, Janelia Research Campus. Contact inno
For T cell harvest
For T cell culture
For Imaging
For T cell harvest
For T cell culture
For T cell culture
LLSM imaging software
For T cell harvest
For T cell culture
For preparing Fab

vation@janelia.hhmi.org for access. Other deconvolution and deksewing methods are available in image

e processing softwares such as Fiji, Slidebook, Amira, and others. https://llspy.readthedocs.io/en/latest/

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Author(s): Jillian Rosenberg, Jun Huang

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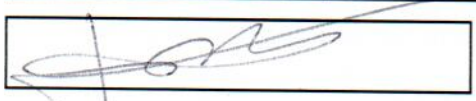
Signature:	
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Response to the editor and reviewers

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.

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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.

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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.

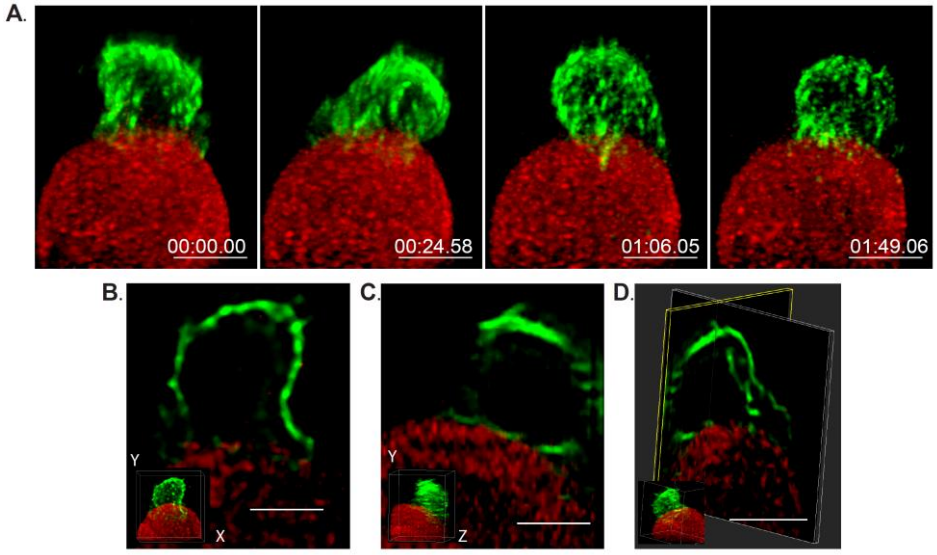
Isolating T cells from transgenic mice is not the focus of this paper, and we have simplified the T cell isolation and added a note citing relevant references: "NOTE: This part of the protocol is based on previous protocols. See citations for further detail[20, 21].". Although the alignment might be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