

Video Article

Imaging CD4 T Cell Interstitial Migration in the Inflamed Dermis

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Abstract

The ability of CD4 T cells to carry out effector functions is dependent upon the rapid and efficient migration of these cells in inflamed peripheral tissues through an as-yet undefined mechanism. The application of multiphoton microscopy to the study of the immune system provides a tool to measure the dynamics of immune responses within intact tissues. Here we present a protocol for non-invasive intravital multiphoton imaging of CD4 T cells in the inflamed mouse ear dermis. Use of a custom imaging platform and a venous catheter allows for the visualization of CD4 T cell dynamics in the dermal interstitium, with the ability to interrogate these cells in real-time via the addition of blocking antibodies to key molecular components involved in motility. This system provides advantages over both *in vitro* models and surgically invasive imaging procedures. Understanding the pathways used by CD4 T cells for motility may ultimately provide insight into the basic function of CD4 T cells as well as the pathogenesis of both autoimmune diseases and pathology from chronic infections.

Video Link

The video component of this article can be found at <https://www.jove.com/video/53585/>

Introduction

The effector function of CD4 T cells is critically dependent on their ability to rapidly enter and traverse a wide variety of peripheral tissues to survey for damage, locate foci of infection, or cause pathology from chronic infection or autoimmunity. While the processes of homing to inflamed sites¹⁻⁴ and extravasation⁵⁻⁷ from the vasculature into tissues have been well-characterized, the factors that drive and regulate the interstitial motility of T cells remain undefined. The migration of T cells in complex 3D environments has been studied *in vitro* through the use of artificial matrices⁸⁻¹⁰ or microfluidic devices^{11,12}, but these fail to recapitulate the complex and dynamic environment of an *in vivo* system. It is only recently, with the advent of high-resolution multi-color intravital imaging that it has become possible to study the dynamic behavior of immune cells *in situ*, allowing for a better understanding of intact immune responses.

Over a decade ago, several influential studies were published that first utilized multiphoton microscopy to address immunological questions. Early studies focused on the behavior of immune cells within explanted lymphoid organs¹³⁻¹⁶, which were soon followed by techniques to image exposed lymph nodes in anesthetized mice¹⁷. Imaging allowed for new fundamental observations about the stages of lymph node priming of T cells¹⁸, the mechanisms by which T cells migrate in secondary lymphoid organs¹⁹, T cell interactions with other immune cells^{20,21}, and dynamic T cell positioning within the lymph node²². Although many early studies focused on lymph node dynamics, intravital imaging has been since been utilized to image the immune response in many peripheral tissues, including the brain²³⁻²⁵, liver²⁶, lung²⁷, and skin²⁸⁻³⁰.

The mouse ear dermis is particularly well poised for imaging, due to the thinness of ear skin, a relative lack of hair, and the ease with which it can be isolated from respiratory movements³¹. Indeed, the ear dermis has been used to image the interstitial behavior of dendritic cells^{32,33}, T cells^{28,29,34,35}, and neutrophils^{36,37}, and is a well-established site for studying dermal inflammation. Increasingly, non-invasive procedures have been replacing surgical preparations of the skin, including split dermis^{38,39}, flank^{39,40}, or dorsal skin flap window^{39,41} models, that can induce changes to the local inflammatory milieu. The use of transferred, *in vitro*-primed, antigen-specific CD4 effector T cells allows for the study of a homogenous population of cells in the context of a dermal inflammatory response³⁰. Here we describe a non-invasive imaging procedure that allows for the visualization of antigen-specific effector CD4 T cells in the dermal interstitium of the inflamed mouse ear, and the ability to manipulate these cells in real-time by introducing blocking antibodies through a venous catheter. We show that this model is effective for tracking the movement of CD4 T cells in the dermis and for querying the mechanisms that govern this motility.

Protocol

All procedures involving mice were approved by the Institutional Animal Care and Use Committee of the University of Rochester, and carried out in strict accordance with the Animal Welfare Act and the Public Health Service Policy on Humane Care and Use of Laboratory Animals administered by the National Institutes of Health, Office of Laboratory Animal Welfare.

1. Preparation of Effector CD4 T Cells

NOTE: BALB/c TCR-transgenic DO11.10 mice that specifically recognize a peptide from chicken egg ovalbumin (pOVA: ISQAVHAHAHAEINEAGR). Other TCR-transgenic systems can be substituted, using the appropriate cognate peptide in place of pOVA where indicated.

1. Purify naïve CD4 T cells

- Euthanize a 6-8 week-old female DO11.10 BALB/c mouse by exposing to 2 L/min CO₂ until the mouse shows no signs of movement or breathing for 1 min, followed by cervical dislocation, or according to the guidelines of the local Institutional Animal Care and Use committee. Spray the mouse with a 70% ethanol solution and make an approximately 7 cm incision in the skin from the chin of the mouse to 2/3 of the way down the abdomen. Make 2-3 cm skin incisions from the end of the incision in the abdomen towards the hind feet. Be careful not to cut into the peritoneum.
- Carefully separate the skin from the peritoneum by gently pulling with forceps. The inguinal lymph nodes are located on the skin near the junction of the hind legs with the body. Remove by grasping and pulling with forceps and place in 8 ml of HBSS supplemented with 2% newborn calf serum (NCS).
- Harvest the axillary and brachial lymph nodes from the mouse by grasping and pulling gently with forceps. Place in the HBSS +2% NCS with the inguinal lymph nodes.
- Harvest the deep and superficial cervical lymph nodes located in the neck of the mouse with forceps and place in the HBSS + 2% NCS with the other lymph nodes.
- Gently cut into the peritoneum, taking care not to cut into the intestines. Grasp the cecum and colon with forceps and expose the mesenteric lymph nodes that are located just along the colon. Carefully remove the mesenteric lymph nodes with forceps and place with the other lymph nodes.
- Locate the spleen in the peritoneal cavity and carefully remove it, holding with forceps and cutting the connective tissue away with a pair of surgical scissors. Place the spleen with the lymph nodes.
- Prepare a single cell suspension by pouring the HBSS + 2% NCS containing the spleen and lymph nodes into a metal strainer placed in a 60 x 15 mm culture dish. Gently mash the spleen and lymph nodes through the metal strainer with the plunger from a 10 ml syringe into the HBSS + 2% NCS.
- Using a pipette, move the cell suspension into a clean 50 ml centrifuge tube. Rinse the metal strainer and culture dish with an additional 10 ml of HBSS + 2% NCS, using a pipette, and place this solution in the same 50 ml centrifuge tube as the cell suspension.
- Spin the cells at 600 x g for 5 min to pellet the cells and resuspend in 10 ml HBSS + 2% NCS by gently pipetting. Unless otherwise specified, all centrifugation should be performed at 600 x g for 5 min.
- Dilute 10 µl of the cell suspension in 90 µl of 0.1% trypan blue in PBS for a 1:10 dilution. Place 10 µl of the cells in trypan blue onto a hemocytometer by pipetting the solution into the groove at the edge of the hemocytometer. Count the white blood cells in the center grid of the hemocytometer, ignoring erythrocytes that appear as slightly smaller, round, red-hued cells and the blue dead/dying cells. Multiply the number of cells counted by 10⁴, by the dilution factor (10), and by the volume of the cells (10 ml) to determine the total number of cells collected in the 50 ml centrifuge tube.
- To enrich for CD4 T cells, centrifuge the cells to pellet, and resuspend the cells at 2x10⁷ cells/ml in a solution containing approximately 1 µg/ml anti-CD8 (clone 3.155), 1 µg/ml anti-MHC Class II (clone M5/114), and 1 µg/ml anti-CD24 (clone J11d) antibodies in HBSS + 2% NCS by gently pipetting.
NOTE: The antibodies used in this step are derived in-house from hybridoma cell lines and concentrations are approximated. The concentration and volume of these antibodies ideal for complement lysis were determined empirically and will vary between labs. Alternatively, several commercially available kits are available for purification of naïve CD4 T cells and can be substituted for the complement lysis and CD62L+ purification process represented here. If using another method to purify naïve CD4 T cells, this protocol can be continued from step 1.2.
- Incubate on ice for 30 min. At the end of this incubation, rapidly thaw guinea pig complement by placing in a 37 °C water bath for approximately 2 min. Add 100 µl of complement for every 1 ml of cells in the antibody solution by pipetting the complement directly into the cell suspension. Incubate cells in a 37 °C water bath for 30 min.
- Add HBSS + 2% NCS to bring the cells to a total volume of 20 ml in the centrifuge tube. Layer in 8 ml of RT density centrifugation media (density 1.086 g/ml) underneath the cells. Immediately centrifuge the cells at 1400 x g at RT for 15 min with the centrifuge brake off.
- Collect cells at the interface using a serological pipette and transfer cells to a new 50 ml centrifuge tube. Wash the cells by bringing the volume in the centrifuge tube to 50 ml with HBSS + 2% NCS and centrifuging.
- Resuspend the cells in MACS buffer (PBS supplemented with 2% NCS and 2 mM EDTA) by gently pipetting and count as previously, in step 1.1.10.
- To enrich for naïve CD4 T cells, resuspend the cells at 2x10⁷ cells/ml in a solution of 2.5 µg/ml biotin-conjugated anti-CD62L antibody in MACS buffer, based on the number counted in step 1.1.16. Incubate for 30 min on ice.
- Wash the cells by adding 10 ml MACS buffer, then centrifuging the cells. Resuspend the cells at 10⁷ cells/100 µl in MACS buffer and add streptavidin-conjugated magnetic separation beads at a 1:10 dilution directly to the cells. Incubate on ice for 20 min.
- Wash the cells as before, in step 1.1.17, and resuspend the cells at 2x10⁸ cells/ml in MACS buffer, in a minimum volume of 500 µl.
- Place a magnetic separation column in the magnet holder and wash the column by letting 3 ml MACS buffer flow through. Apply the cells to the column with a pipette.

20. Wash the column to remove cells not bound by the magnetic column by pipetting 3 ml MACS buffer onto the column and allowing the MACS buffer to flow through, and repeat 3 times. Discard the flow through fraction.
 21. Remove the column from the magnetic stand and hold over a new 50 ml centrifuge tube. Pipet 5 ml of MACS buffer onto the column and use the plunger enclosed with the column to push the MACS buffer through the column to release the column bound cells and collect the flow through that contains the enriched naïve CD4 T cells.
 22. Centrifuge the cells and resuspend in 10 ml RPMI supplemented with 10% Fetal calf serum (FCS), 100 I.U./ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, and 50 µM 2-mercaptoethanol (RPMI-10). Count the cells as before, in step 1.1.10.
 23. Adjust the final concentration of the naïve CD4 T cells to 6×10^5 /ml in RPMI-10.
2. Purify T cell-depleted splenic APCs
1. Euthanize a 6-8 week-old female wild-type BALB/c mouse as in step 1.1.1.
 2. Spray the mouse with a 70% ethanol solution and expose the spleen by making an approximately 3 cm incision on the left side of the mouse's abdomen. Cut open the peritoneal layer and remove the spleen by gently grasping it with forceps and cutting it away from the underlying connecting tissue with a surgical scissors.
 3. Place the spleen into 8 ml of HBSS + 2% NCS.
 4. Prepare a single cell suspension by mashing the spleen, wash, and count the cells, as before, in steps 1.1.7-1.1.10
 5. Deplete T cells by spinning the cells at $600 \times g$ for 5 min to pellet, and resuspend the cells at 2×10^7 in a solution of approximately 1 µg/ml anti-Thy1.2 antibody (J1J clone) by gently pipetting. Incubate the cells on ice for 30 min.
NOTE: This antibody was derived in-house from a hybridoma cell line and the concentration is approximated. The concentration and volume of this antibody ideal for complement lysis was determined empirically and will vary between labs. Other methods for purifying APCs from splenocytes can be substituted if desired. Naïve T cells and APCs should be prepared in culture from step 1.3.
 6. Add guinea pig complement, incubate, and separate the cells on a density centrifugation media gradient as before, in steps 1.1.13-1.1.14.
 7. Resuspend the cells in 10ml of RPMI-10 and irradiate the APCs by placing the cells in a 50 ml centrifuge tube into a gamma irradiator for a length of time that will expose the cells to 25 Gy radiation. This length of time will vary based on the irradiator and will need to be calculated every time cells are irradiated.
 8. Count the cells on a hemocytometer as before, in step 1.1.10, and adjust the APC cells to a final concentration of 2.4×10^6 cells/ml in RPMI-10.
3. Stimulate cells and differentiate CD4 T cells to a Th1 phenotype in a 5-day culture.
- NOTE: Although we present here a protocol for preparing and imaging Th1 effectors generated from naïve CD4 T cells, this protocol can be adjusted to differentiate the naïve cells to a different phenotype, or to use other cell types, such as CD8 T cells. Conditions for priming and differentiation will have to be determined empirically.
1. In each well of a 24-well culture dish, combine 500 µl of the naïve CD4 T cells and 500 µl of the irradiated APCs in each well, for a total of 3×10^5 T cells and 1.2×10^6 APCs per well.
 2. Prepare a solution in RPMI-10 containing 2 µM cognate OVA peptide, 20 U/ml recombinant IL-2, 80 µg/ml anti-IL-4 (clone 11B11) and 40 ng/ml recombinant IL-12 and filter using a 0.2 µm syringe filter. Add 1ml of this solution to each well of cells, for a total volume of 2 ml in each well.
 3. Incubate the cells in a 37 °C incubator at 5% CO₂ for 3 days.
 4. On day 3, split the cultures by gently pipetting to resuspend cells and moving 1 ml from each well into a new culture well. Bring each well to a final volume of 2 ml by adding 1 ml/well of RPMI-10 + 20 U/ml recombinant IL-2.
 5. Return the plates to the incubator and allow the cells to expand until day 5.

2. Transfer of Cells and Induction of Inflammation

NOTE: For optimal cell numbers for imaging, 5×10^6 fluorescently labeled Th1 cells should be transferred to each mouse in a total volume of 200 µl PBS. Cells here are labeled with the green dye CFSE or the near-red dye CMTMR, although other cell tracker dyes can be used. CFSE and CMTMR-labeled cells can be co-transferred to allow for tracking of two distinct effector CD4 populations.

1. Label effector T cells with CFSE
 1. Harvest the cells from the culture dishes by vigorously pipetting the cells in each well and transferring to a sterile 50ml centrifuge tube with a pipet. Count the cells as in step 1.1.10, then centrifuge and resuspend the cells at 10^7 cells/ml in PBS + 5% NCS.
 2. Dilute 5 mM stock CFSE solution 1:100 in PBS. Add 110 µl CFSE solution for each 1 ml of cells by placing a drop of CFSE solution on the side of the tube and then rapidly rocking the tube back and forth to thoroughly mix.
 3. Incubate the cells for 5 min at RT, then quench the CFSE by adding 5 ml of fetal calf serum (FCS) directly to the tube of cells. Bring the volume to 50 ml with PBS + 5% NCS.
 4. Centrifuge the cells and resuspend the pellet in 20 ml PBS + 5% NCS. Repeat this process two more times to wash the cells 3 times total. Count the cells on a hemocytometer, as before, in step 1.1.10, and resuspend the cells in sterile PBS at 2.5×10^7 cells/ml for transfer to mice.
2. Label effector T cells with CMTMR
 1. Harvest the cells as above in step 2.1.1, then count, centrifuge and resuspend the cells at 10^7 cells/ml in RPMI-10.
 2. Add 10 mM CMTMR stock solution directly to the cells for a final concentration of 10 µM. Quickly pipet the cells to thoroughly mix in the CMTMR and prevent precipitation of the dye.
 3. Incubate 30 min in a 37 °C water bath. Wash cells 3x in PBS + 5% NCS as before, in step 2.1.3-2.1.4. Count and resuspend the cells in sterile PBS at 2.5×10^7 cells/ml for transfer to mice.
3. Transfer effector T cells to naïve 6-8 week old female BALB/c mice.

1. Draw the cell suspension (step 2.1.4 or 2.2.3) into a syringe, taking care to remove all bubbles by holding the syringe needle side-up, gently flicking the side of the syringe and moving the plunger up and down, if necessary.
2. Place mice in a clean cage and heat gently under a heat lamp until the tail vein appears vasodilated. Place the mouse in a restraining device and wipe the tail with an alcohol swab. Slowly inject 200 μ l of the cell suspension into the lateral tail vein. There should be no resistance to injection or visible bubbling under the skin.
4. Induce dermal inflammation by immunizing with Complete Freund's Adjuvant (CFA)

NOTE: In this protocol, inflammation is induced by intradermal injection of CFA emulsified with either cognate or non-cognate antigen. Other inflammatory models can be substituted, although the number of cells required for transfer and the timing of imaging will need to be adjusted empirically.

 1. Prepare a 200 μ M solution of OVA peptide in sterile PBS in a microcentrifuge tube. Pipet an equivalent volume of CFA on top of the peptide solution.
 2. Emulsify the solution by drawing it into a 28 G1/2 insulin syringe and then plunging the solution back into the microcentrifuge tube, then repeating this action approximately 20 times. When fully emulsified, the CFA and peptide solution will form a thick and opaque mixture. NOTE: it is essential to use a fixed-needle insulin syringe to form the emulsion, as the emulsion will be lost in the dead space in a non-fixed needle syringe.
 3. Test the emulsion by dropping a small amount onto water placed in a petri dish. The emulsion should remain intact and not disperse into the water.
 4. Draw the emulsion into a 300 μ l 28 G1/2 insulin syringe and press down sharply on the plunger to remove large air bubbles.
 5. Immediately after the transfer of fluorescently labeled effector T cells, anesthetize the mice by *i.p.* injection of 2,2,2-tribromoethanol at 240 mg/kg. Assess anesthesia by a gentle toe pinch, administering more 2,2,2-tribromoethanol in 1 mg increments, if necessary. NOTE: other approved anesthetics may be substituted for the 2,2,2-tribromoethanol.
 6. Place a thumb on the left index finger and carefully grasp the mouse's ear between the left thumb and index finger with the ventral ear facing up. Make sure not to exert excessive pressure on the ear, which can cause mechanical damage to the skin.
 7. Slide the needle containing the CFA emulsion into the dermis, bevel side facing up, and slowly inject 10 μ l of the emulsion into the ear. Placement of the injection should be in the outer 1/3 of the pinna, slightly off-center to allow for optimal imaging.
 8. Monitor mice until the anesthesia has worn off and mice are able to right themselves and are ambulatory. Image mice 3 days following immunization, providing time for inflammation to develop and the transferred T cells to traffic to the ear dermis. NOTE: Mice may not be left unattended at any time while under anesthesia.

3. Preparing the Mouse for Imaging

1. Prepare a catheter
 1. Carefully remove the metal needle from a 30 G1/2 Tuberculin (TB) syringe needle with pliers and clean off any excess glue, using a dissecting scope to visualize that the glue is completely removed.
 2. Cut the tip off of another 30 G1/2 TB syringe needle, ensuring that the remaining needle is still patent by visual inspection. Slip an 18 cm piece of PE-10 medical tubing onto the trimmed needle, and carefully place the bare metal needle approximately 5 mm into the other end of the tubing, creating a catheter.
2. Flush the catheter by filling a 1 ml TB syringe with sterile PBS, removing any air bubbles. Gently place the catheter on the tip of the syringe and push PBS gently through the catheter to remove any bubbles in the tubing. NOTE: when pushing fluid through the catheter, it is essential to hold the catheter on the syringe to prevent fluid pressure from pushing the catheter off of the syringe.
3. Place mice in a clean cage and heat gently under a heat lamp until the tail vein appears vasodilated. Anesthetize the mouse with a mixture of room air and isoflurane (5% induction, 1-2% maintenance, at 2 L/min flow rate), delivered through the nosecone assembly that has been attached to the isoflurane vaporizer. Ensure that the mouse is anesthetized with a gentle toe pinch. Incrementally increase the isoflurane flow rate by 0.1% if movement is observed. Cover the eyes with ophthalmic ointment to prevent drying and injury while the mouse is anesthetized. NOTE: It is essential that mice never be left unattended while anesthetized. Mice should be frequently monitored for sufficient anesthesia by gentle toe pinch.
4. Immobilize the tail at the base with a pair of forceps, and then wipe the tail with an alcohol swab. Carefully slide the catheter into the lateral tail vein and check patency by gently pushing on the plunger of the syringe. There should be no resistance to movement and no visible bubbling of PBS under the skin if it is appropriately placed. Affix the catheter to the tail by applying 1-2 drops of cyanoacrylate tissue adhesive to the injection site and allow to dry, approximately 30 sec.
5. Carefully trim the hair from the back and sides of the ear with a pair of scissors, taking care not to damage the skin. Trim the whiskers as well. Using a cotton swab, moisten the inner surface of the ear with PBS.
6. Rotate the mouse and ear onto a 24 x 50 mm No. 1.5 glass cover slip. Using forceps, gently flatten the ear onto the cover slip, moving the mouse if necessary to ensure that the ear is flush with the glass. Remove excess PBS from the ear by blotting gently with a tissue wipe. NOTE: Make sure not to press too firmly on the ear, as the thin skin can be easily damaged with excessive pressure.
7. Using two pairs of curved forceps, grasp an approximately 20 mm long piece of fabric tape lengthwise at the top corners. Place the bottom of the tape onto the coverslip at the top of the mouse's ear and roll the tape over the rest of the ear, pushing excess hair out of the way with the forceps if necessary to affix the ear to the coverslip. Gently press on the tape around the ear with a dry cotton swab to ensure a tight seal, taking care not to press on the ear itself.
8. Attach the imaging platform to a 37 °C heating block with adhesive tape. Apply vacuum grease on either side of the ear area of the imaging platform. Rotate the mouse to place the coverslip onto the imaging platform, taking care to align the ear in the center of the felt. NOTE: Avoid getting vacuum grease on the tape, as this will cause it to come detached from the glass cover slip.
9. Snap the isoflurane nosecone into the holder on the imaging platform, ensuring that the nosecone is secure and completely covering the mouse's nose. Spread the vacuum grease under the coverslip by firmly but carefully pressing the coverslip onto the imaging platform with a clean, dry cotton swab.

10. Affix the coverslip to the platform with two 20 mm pieces of tape and two longer pieces of tape wrapped around the upper portion of the platform. If any air bubbles are present between the ear and the coverslip, they can be gently removed by pressing on the ear from below with a piece of folded paper.
NOTE: It is essential not to use paper that is too thick or to press firmly, as this can cause tissue blanching and damage, complicating results.
11. Move the mouse to the microscope stage and secure the platform with adhesive tape. Pipe a double layer of vacuum grease onto the coverslip around the ear to serve as a reservoir for the water immersion objective.
12. Wrap a water-filled heating blanket around the mouse through the imaging platform. Fill the reservoir with 37 °C distilled water. Ensure that everything is secured to the stage with adhesive tape and that the syringe of the catheter is easily accessible.

4. In Vivo Time-lapse Imaging and Intravenous Antibody Administration

NOTE: This protocol requires the use of a multiphoton microscope equipped with a Ti:Sa laser system. The objective used is a 25x magnification lens with 1.05 N.A., affixed with an objective heater set to 40 °C. The optimal temperature for this heater was determined empirically to be the appropriate temperature to maintain the ear dermis at 37 °C, and may need to be adjusted for use in other imaging systems. The acquisition software used may vary between instruments and adjustments to the protocol may have to be made to work on differently configured systems. Ensure that images can be saved in a format that is compatible with any desired analysis software.

1. Locate the dermis through the eyepieces by positioning the objective over the center of the ear and lowering the objective just until it contacts the surface of the water in the reservoir. Using an external light source, look through the eyepieces and continue to slowly lower the objective until the surface of the ear comes into focus. Lower the inner and outer curtains around the microscope stage.
2. Determine microscope settings and locate an area for imaging
 1. Before imaging, configure the laser for maximal excitation and detection of the desired fluorophores by adjusting the laser wavelength to 900 nm in the MP Laser Controller window, the laser power in the Acquisition Setting: Laser window, and the PMT voltages in the Image Acquisition Control window.
NOTE: This procedure uses an excitation wavelength of 900 nm with filters to detect second harmonic generation (420-460 nm), CFSE (495-540 nm), and CMTMR (575-630 nm). Other configurations can be used to detect different fluorophores as desired.
 2. Set the microscope to 512 x 512 pixel resolution, with a 2 µsec/pixel dwell time in the Acquisition Setting: Size and Mode windows. Activate a live imaging mode to allow scanning through the tissue for an area to image by clicking "XY repeat". Ideally, the area should be relatively uniform, without air bubbles, and avoiding areas of dense hair follicles.
NOTE: The CFA emulsion is brightly autofluorescent in the green and near-red channels and should be avoided. An optimal field can usually be found approximately 3 mm from the edge of the emulsion. Approximately 10 - 100 cells can be tracked in a single image. If too many cells are present, tracking software will generally encounter errors in attempting to separate closely located cells, leading to shortened and/or inaccurate cell tracks.
 3. Once an appropriate imaging field has been located, determine the Z region that will be imaged by locating the cell "highest" in the dermis, setting the Z-position to 0 by clicking the "Set 0" button, and scrolling down in the Z direction to measure extent of cell depth. An imaging depth of 35-75 µm is typical. Set the starting and end positions in the Acquisition Setting: Microscope window. Adjust the instrument PMT voltages and laser power in the "bright Z" window to optimize visualization of the cells throughout the depth of the imaging field.
NOTE: Avoid raising the laser power above 25 mW at the sample, as high power levels can cause heat damage and sterile injury to the dermis. The appropriate maximum power level for each microscope will vary and will have to be measured. It should be noted that increasing laser power or PMT voltages with tissue depth does not allow for quantitative comparison of image brightness at different depths in post-acquisition analysis.
 4. Check the "Depth" and "Time" buttons underneath the "Scan" button in the Image Acquisition Control window. Set a Kalman filter to scan the image 3 times per line in the Image Acquisition Control: Filter Mode window and adjust the Z-slice depth in the Image Acquisition: Microscope window so that it takes approximately 1 min to capture a complete stack, as noted in the TimeView window.
NOTE: The interval between stacks should not take longer than approximately 1 min, as longer intervals prevent the tracking of rapidly migrating cells. Depending on the type of cells being imaged, this interval may need to be adjusted to allow for proper cell tracking by analysis software. Z-slices should be no more than 5 µm. Generally 15-18 Z-slices between 2-5 µm thick are appropriate for a 1 min imaging interval.
3. Capture a pre-antibody time-lapse image
 1. Capture a 5 min time-lapse image of the area to assess the stability of the tissue by setting the number of repeats to "5" in the Acquisition Setting: TimeScan window and clicking the "Scan" button.
NOTE: Tissue "drift" can be caused by not allowing sufficient time for the ear to reach thermal equilibrium, poor ear preparation, or can be due to local drift in one region of the ear. If the 5 min image is not stable, image a new region in the dermis. If a second image in a new region remains unstable, it is best to carefully repeat the ear preparation and imaging from step 3.6 with a fresh coverslip.
 2. If the tissue is stable in the 5 min image, collect a 30-45 min time-lapse image by setting the number of repeats to between 30 and 45 in the Acquisition Setting: TimeScan window, monitoring for any minor tissue drift as the image is collected. Save the file in a format that is compatible with the analysis software to be used.
NOTE: At this point in the protocol, steps 4.2.2 - 4.3.2 can be repeated to image multiple locations in the same ear. A single mouse should not be kept anesthetized for longer than 4 hr, as death is more likely to occur.
4. Inject blocking antibodies and capture a post-antibody image.
 1. Draw the antibody mixture into a 1 ml TB syringe and remove the needle, making sure to remove all air bubbles. Press on the plunger so that the antibody solution forms a "droplet" at the end of the syringe.
 2. Lift the curtains around the stage and locate the catheter. Remove the original PBS-containing syringe from the catheter. Without setting the catheter down, carefully attach the new syringe containing the antibodies. Hold the end of the catheter onto the syringe and slowly inject the antibody mixture into the catheter. Ensure that there is no resistance to injection.

3. Set the catheter down and lower the curtains surrounding the microscope stage. Note the time of injection and immediately start collecting a new 20-40 min imaging sequence in the same location using the same instrument settings as the pre-antibody image. Save the file in an appropriate format for the analysis software to be used.
NOTE: Between time-lapse images, observe the mouse for sufficient anesthesia and breathing. It is not recommended to image more than one field following administration of antibodies, as there can be variable rates of clearance of the antibody.
5. Inject fluorescently-labeled dextran to locate blood vessels, assess catheter patency, and measure blood vessel permeability
 1. Prepare a 2 mg/ml solution of fluorescently-conjugated dextran (70,000 MW) in 100 μ l sterile PBS and draw into a syringe, removing any air bubbles.
 2. Using the same technique as in step 4.4.1-4.4.2, place the syringe onto the catheter and inject the dextran solution intravenously.
 3. Capture a single stack at 1024 x 1024 pixel resolution by changing the resolution in the Acquisition Setting: Mode window, with the same PMT and laser settings as for the time-lapse images. To collect the image, uncheck the "Time" button underneath the "Scan" button, and then clicking the "Scan" button. This 3D image will allow for the exclusion T cells located in the vasculature and show that the catheter was patent and properly inserted. The diffusion of fluorescent dextran out of the vessels can be also used to assess local vascular permeability.
NOTE: This high-resolution (1024 x 1024) static image is used for presentation purposes and can be additionally be used for analysis of the tissue architecture in the same area as the time-lapse images. Alternatively, a 512 x 512 image can be taken, which can be merged with the time-lapse images using image analysis software. Time lapse imaging of the dextran administration may also be desired, depending on the research needs of individual labs. In this case, the image should be set up as in steps 4.2.3-4.3.2.
6. After imaging is completed, carefully remove the mouse from under the objective and unwrap the water blanket. Remove the coverslip from the imaging platform by cutting the tape and gently lifting the glass until it detaches. While the mouse is still anesthetized, detach the ear from the coverslip. If this is to be a terminal procedure, euthanize the mouse by cervical dislocation. If saving this mouse for repeated imaging, return it to a clean cage separate from other mice and observe until the mouse can right itself and is ambulatory. Once recovered from anesthesia, the mouse can be returned to a cage with other animals.
7. Import the imaging files into an image analysis program and perform any desired corrections. Avoidance of non-linear enhancements and automated smoothing or noise reduction programs and algorithms is recommended. Typically, images need only a minor background correction by manually increasing the black point of the image before analysis. Analyze the imaging data by using an automated cell-tracking program with manual correction, as in Overstreet, Gaylo *et al*³⁰.
NOTE: There are many image analysis suites available, both proprietary and open source, that can be used to quantify cell dynamics from the multiphoton images derived from this protocol. The exact method of image analysis and the optimal software packages to be used will depend on the research needs of individual laboratories.

Representative Results

The ability to study immune responses *in situ* without altering the immune environment is essential in studying real-time interactions of T effector cells with an inflamed tissue. Imaging of the intact ear dermis by this protocol, outlined in **Figure 1A** and **B**, allows for the visualization of transferred fluorescently labeled T effector cells in the dermal interstitium. This permits both high-resolution (**Figure 1C**) and time-lapse (**Figure 1D**, **Movie 1**) images of effector T cell dynamics in the inflamed dermis.

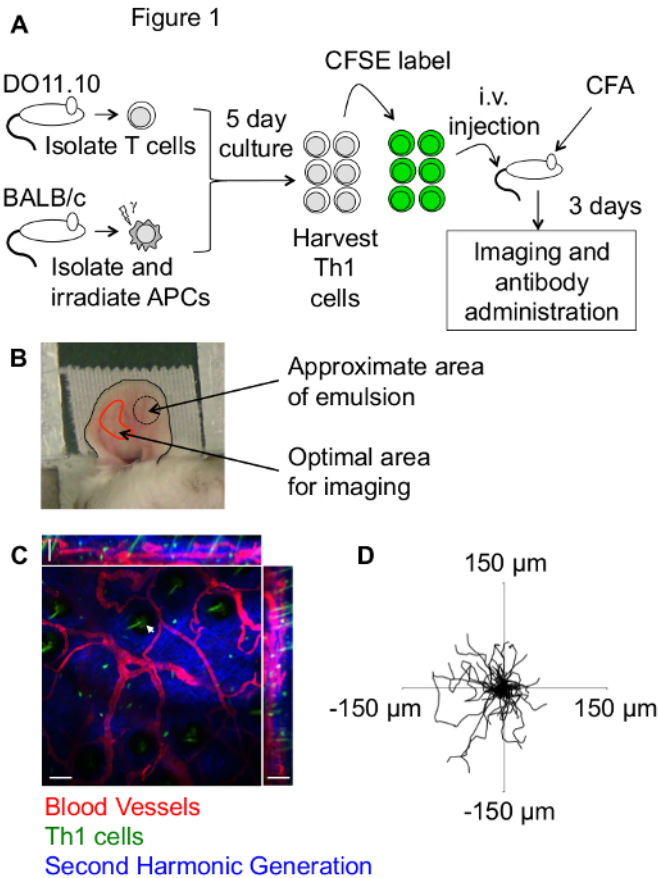


Figure 1. High-resolution and 4D imaging of T effector cells in the intact dermal interstitium. (A) Experimental outline. (B) Photograph of an ear prepared for imaging with the site of the emulsion (black dashed line) and optimal area for imaging (red line) indicated. (C) Maximal 3D projection of a high-resolution stack showing CFSE-labeled Th1 cells (green), second harmonic signal from fibrillar collagen (blue) and Texas Red-Dextran labeled vasculature (red). The white arrow indicates one of several autofluorescent hair follicles. Scale bars represent 50 μ m (D) Migratory paths of Th1 cells tracked for 30 min in the CFA-inflamed dermis. [Please click here to view a larger version of this figure.](#)

This imaging protocol requires the use of a specialized imaging platform that was constructed in-house, as well as an adapted nosecone for the delivery of inhaled anesthesia while imaging. The imaging platform (**Figure 2A-B**) consists of an aluminum base plate with a raised central section. This raised section has an inset portion lined with acrylic felt to provide support to the ear without compressing and potentially damaging the thin tissue. The geometry of our setup required the construction of a flexible and adjustable nosecone to deliver inhaled anesthesia during imaging. This nosecone, consisting of a modified microcentrifuge tube connected to a 50 mm section of flexible tubing (**Figure 2C**), is secured in place with a holder composed of modified microcentrifuge tubes (**Figure 2D**) and affixed to the imaging platform via hook and loop fastener. The use of hook and loop fastener allows for the repositioning of the nosecone assembly so that either ear of a mouse can be imaged, and can be optimally positioned for individual mice.

Figure 2

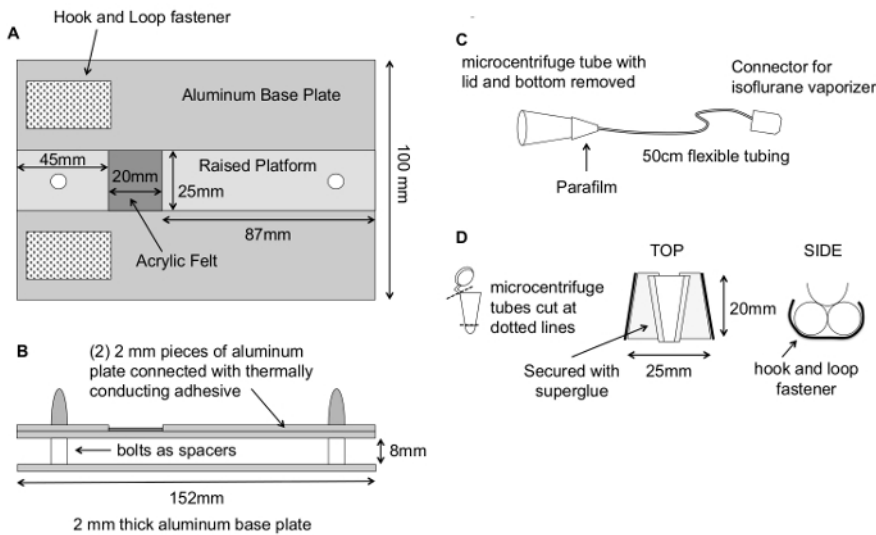


Figure 2. Equipment for intravital imaging. Custom-built imaging platform in top (A) and side (B) views. Nosecone for isoflurane administration (C) and nosecone holder (D). [Please click here to view a larger version of this figure.](#)

Catheterization of the tail vein allows for continuous access to the circulation to administer antibodies and other small molecules that can diffuse out of the vasculature, or larger fluorescent molecules such as high molecular weight dextran to label blood vessels. After the administration of 100 μg of anti- β_1 and anti- β_3 integrin blocking antibodies through the catheter, previously motile cells arrest within the dermis (**Movie 2**). These cells have a decreased average velocity after antibody administration (**Figure 3A**), as well as a significant decrease in meandering index, the ratio of the total displacement to the total track length (**Figure 3B**).

Figure 3

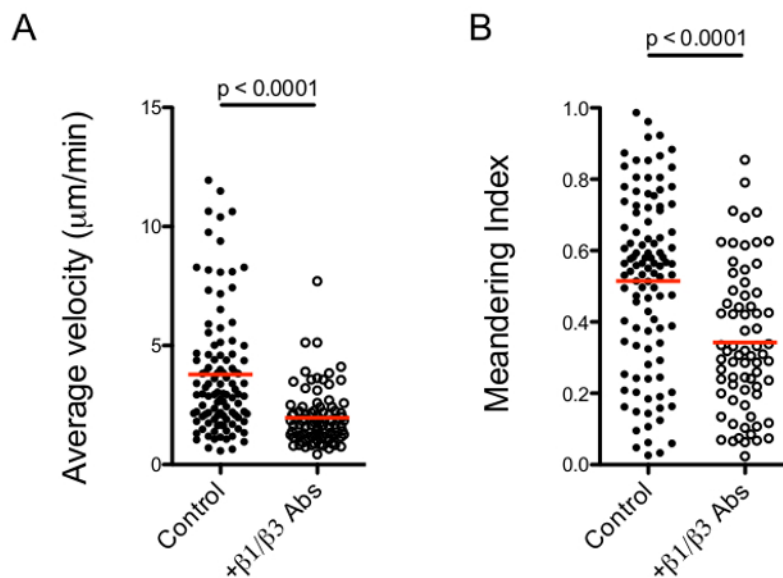


Figure 3. Administration of anti- β_1 and anti- β_3 antibodies inhibits Th1 cells migration in CFA-inflamed skin. (A) Average velocity of Th1 cells before and after administration of 100 μg anti- β_1 and anti- β_3 integrin blocking antibodies. (B) Meandering index of Th1 cells before and after antibody blockade. Approximately 100 tracked cells are from images before and after antibody blockade from a single mouse in one representative experiment. Statistics by Mann Whitney. [Please click here to view a larger version of this figure.](#)

Because hair is not removed from the surface of the ear, imaging artifacts from the hair are common. Autofluorescence from hair follicles (**Figure 4A**) and shadowing and autofluorescence from overlying hair (**Figure 4B**) should be avoided where possible as they can obscure T cells and interfere with automated image analysis software. Similarly, air bubbles trapped between the ear surface and the coverslip can lead to imaging artifacts (**Figure 4C**). Proper preparation of the ear should minimize the number and size of any remaining bubbles.

Figure 4

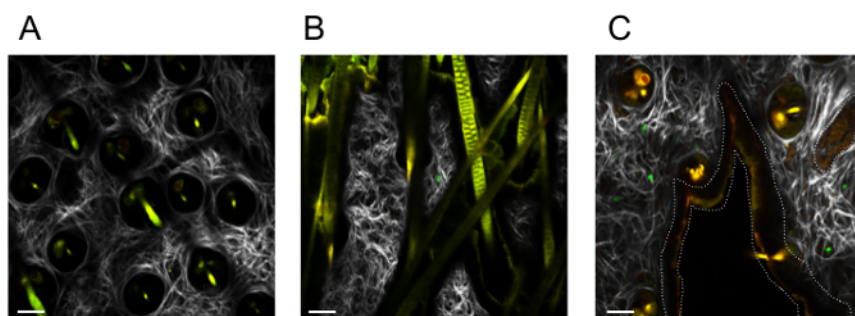
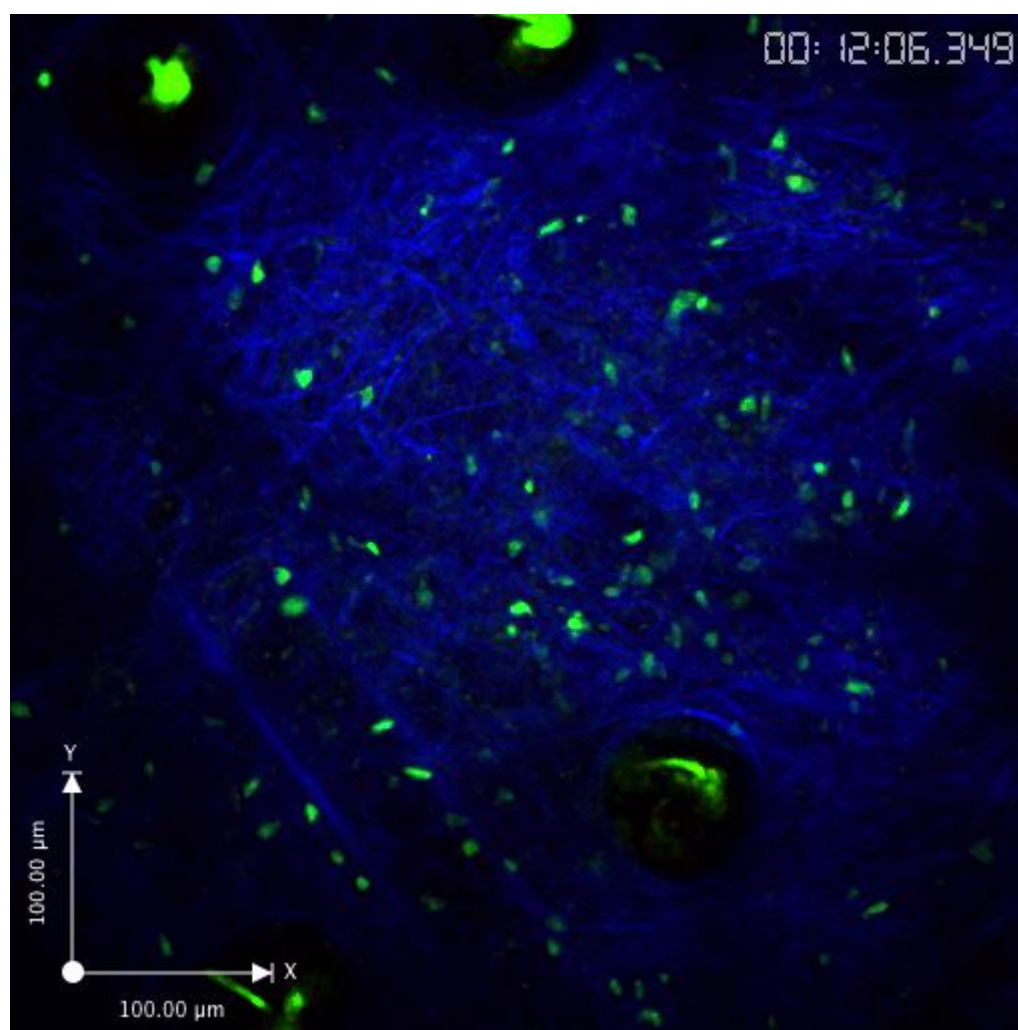
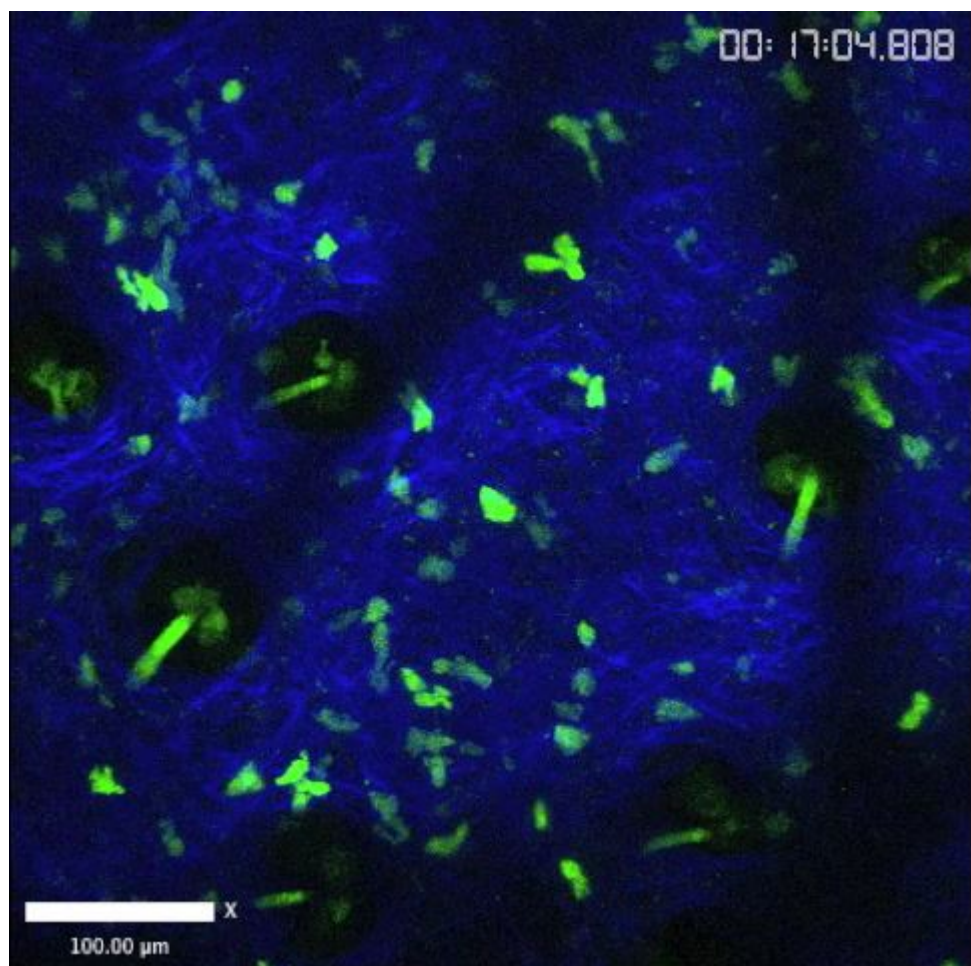


Figure 4. Common artifacts from hair autofluorescence and poor ear preparation. (A) Autofluorescent hair follicles. (B) Autofluorescent hair and hair follicles (green) and collagen (white) with overlying hair shadows, causing dark lines in the image. (C) Artifact from an air bubble, showing the edge of the displaced, autofluorescent keratinized epidermis (dotted line). Scale bars represent 50 μm . [Please click here to view a larger version of this figure.](#)

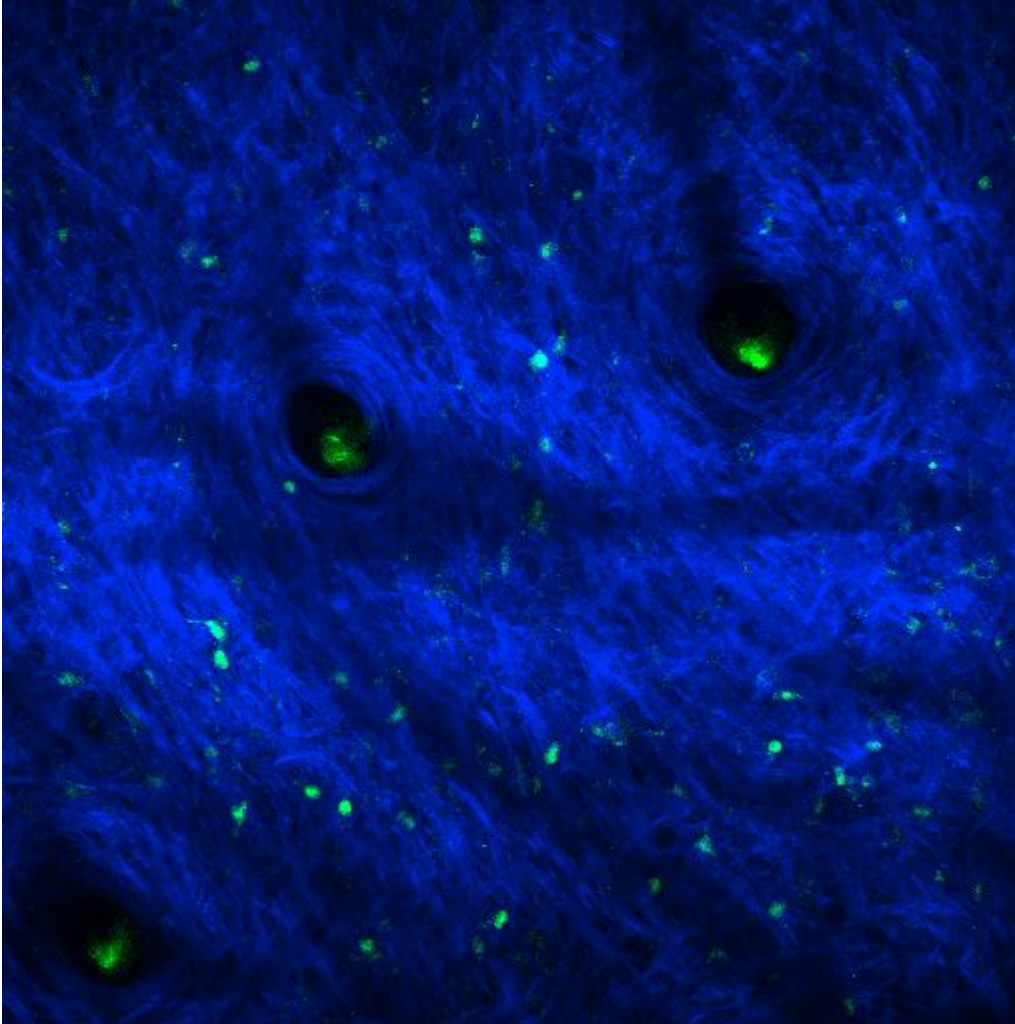
Additionally, the use of multiple sources of heat can lead to issues with stability of the tissue due to thermal expansion and contraction. Incorrect thermostat settings, for instance, can lead to large oscillations during imaging that can make interpretation of results difficult (**Movie 3**). It is essential to determine the optimal settings for any system that provides a constant temperature and maximizes stability. Ultimately, a temperature controlled imaging chamber is the best way to remove variability from changes in temperature.



Movie 1. Th1 cells migrating in the CFA-inflamed dermis ([Right click to download](#)). 30 min time-lapse image showing Th1 cells (green) migrating in the dermal collagen network (second harmonic generation, blue).



Movie 2. Th1 cells arrest upon blockade of β_1 and β_3 integrins (Right click to download). Cells (green) were imaged for 30 min before the administration of blocking antibodies, and then imaged for another 20 min in the same location.



Movie 3. Oscillations from a poorly controlled heating plate (Right click to download). 30 min time-lapse image of Th1 cells (green) and second harmonic generation (blue). After this image was collected, the heating plate used was found to have a faulty thermostat, causing oscillations in the temperature of the imaging platform and subsequent thermal expansion and contraction.

Discussion

Significance

Here we present a complete protocol for the 4D visualization of transferred, antigen-specific effector Th1 cells in the intact mouse ear dermis. This method provides advantages over some current imaging modalities for several reasons. By imaging the ventral ear dermis, we are able to forego hair removal that is required for imaging protocols involving other skin sites. Although depilatories are generally mild, they have been shown to cause disruption to the skin barrier⁴², a process that can stimulate an immune response^{43,44}. By also avoiding invasive surgical procedures to expose the dermis or hypodermis, this protocol prevents damage-induced inflammation and the rapid recruitment of neutrophils³⁷ and other immune cells into the dermis. The use of a venous catheter in this system to deliver blocking antibodies against key molecules allows for real time interrogation of the dynamic behavior of CD4 T cells. The use of this imaging protocol has revealed critical requirements for CD4 T cell interstitial motility³⁰ that were not detected in *in vitro* systems⁸.

Critical steps in the procedure

An essential step in any imaging protocol is ensuring a stable tissue preparation for imaging. It is important to ensure that there is enough PBS between the ear and the coverslip that there are no air bubbles and the ear is in contact with the glass, but not so much as to cause the tape to become de-adhered from the glass. Similarly, avoiding contact between the tape holding the coverslip to the platform and any vacuum grease will prevent the tape from loosening over time. Temperature must also be kept constant to avoid oscillations and drift from thermal contraction or expansion of the platform materials.

Limitations and Modifications

Imaging by this non-invasive protocol is limited to skin areas that are thin enough to allow for effective visualization of fluorescence through multiphoton excitation. The ear is advantageous due to ease of preparation and ability to be isolated from respiratory movements, and has

been used as a model for intravital time-lapse imaging since the 1980s⁴⁵. However, the resident immune population of the ear is distinct from skin on the flank or footpad⁴⁶, and the mouse ear has distinct vascular properties when compared to other sites⁴⁷. Thus, for some applications, comparison of ear imaging data to other skin sites may be difficult.

This protocol also requires the effective migration of transferred T effector cells out of the blood stream and into the dermis. This limits the ability to use cells that have defects in homing or extravasation as they will not be able to enter the interstitial space. Extravasation can be bypassed by injecting cells directly into the ear dermis^{37,48}, although this will cause some mechanical damage and delivery of cells in this way may not recapitulate the localization or behavior of cells that undergo *in vivo* extravasation.

It is also critical to consider using non-pigmented recipient mice for imaging experiments, such as the BALB/c strain used here or Albino C57BL/6-*Tyr^{c-2J}* mice. The melanin in pigmented mice, in addition to being highly autofluorescent, heats up under even relatively low-power excitation from a multiphoton laser³⁷. This can cause thermal damage to the skin and subsequent inflammation³⁶ or fluorescent speckling³¹, complicating results. This may limit fluorophores that can be used in a multi-parameter imaging experiment. However, some very bright or highly expressed fluorescent molecules can be effectively excited at low laser power, allowing for effective visualization in pigmented mice.

Future applications

Because this is a non-invasive procedure, it could be easily adapted for longitudinal studies on mice with sequential imaging over extended time periods. While fluorescent labeling as described here would fade over time periods greater than 3–4 days, use of endogenously fluorescent cells or fluorescent reporter cells eliminates this problem. Indeed, we have previously used this protocol to track *in vivo*-generated antigen-specific effectors bearing cytokine reporters, including the IFN γ reporter Yeti^{30,49} and IL-4 reporter 4get⁵⁰. We have additionally visualized endogenous CD4 cells in CD4-Cre ROSA26-stop-floxed eYFP fluorescent reporter mice³⁰. As the fluorescent properties of eYFP and other fluorescent proteins differ from chemical dyes such as CFSE, modifications to the imaging parameters may be needed for efficient visualization. Changes such as increasing the pixel dwell time can enhance fluorescent signal of some dim fluorophores, and decreasing the length of time lapse images can mitigate any photobleaching that may be observed. At 900 nm excitation, we have not previously observed significant photobleaching of CFSE, CMTMR, or eYFP over short imaging intervals.

Although this procedure focuses on measuring the dynamics of CD4 effector T cell motility, it is not limited to this application. Work is currently ongoing to measure the dynamic interactions of effector T cells with antigen presenting cells through the use of fluorescent reporter mice and injection of fluorescently conjugated antibodies into the dermis to label cells or tissue structures prior to imaging^{51,52}. Additionally, while this protocol demonstrates the use of the catheter to deliver blocking antibodies while imaging, other compounds, including small molecule inhibitors, can be administered. Ultimately, this protocol provides a flexible platform to measure immune dynamics over time, *in vivo*, in a non-invasive manner.

Disclosures

The authors have nothing to disclose.

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