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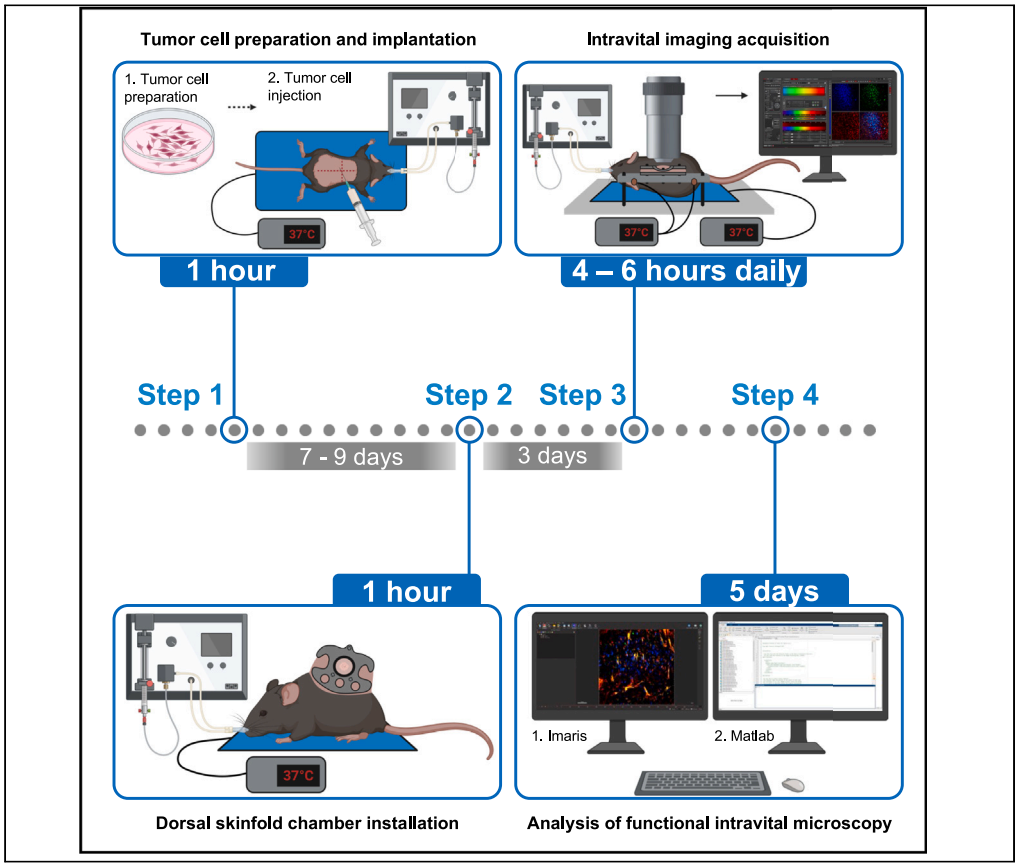
Protocol to quantify the activation dynamics of tumor-associated T cells in mice by functional intravital microscopy



Tumor-associated T cells orchestrate cancer rejection after checkpoint blockade immunotherapy. T cell function depends on dynamic antigen recognition through the T cell receptor (TCR) resulting in T cell activation. Here, we present an approach to quantify the dynamics and magnitude of tumor-associated T cell activation at multiple time points in living mice using the genetically encoded calcium reporter Salsa6f and functional intravital microscopy (F-IVM). Our protocol allows researchers to measure the activation dynamics of various immune cells *in vivo*.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Longitudinal 2-photon intravital imaging protocol for T cell dynamics in a skin tumor

Quantification of TCR signaling using the ratiometric fluorescent sensor Salsa6f

Steps include surgery, image acquisition, and analysis using Imaris and MATLAB

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Protocol to quantify the activation dynamics of tumor-associated T cells in mice by functional intravital microscopy

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SUMMARY

Tumor-associated T cells orchestrate cancer rejection after checkpoint blockade immunotherapy. T cell function depends on dynamic antigen recognition through the T cell receptor (TCR) resulting in T cell activation. Here, we present an approach to quantify the dynamics and magnitude of tumor-associated T cell activation at multiple time points in living mice using the genetically encoded calcium reporter Salsa6f and functional intravital microscopy (F-IVM). Our protocol allows researchers to measure the activation dynamics of various immune cells *in vivo*.

For complete details on the use and execution of this protocol, please refer to Geels et al.[1]

BEFORE YOU BEGIN

We present a highly efficient protocol to read out the dynamics of TCR-mediated activation *in vivo* using the genetically encoded calcium indicator Salsa6f. Salsa6f is a fusion between the Ca^{2+} sensor GCaMP6f, in which calmodulin and the M13 peptide allow GFP fluorescence only in the presence of Ca^{2+} ions,[2] and constitutive tdTomato, which allows ratiometric measurements.[3] Moreover, Salsa6f has a higher dynamic range and faster kinetics[2,3] compared to other probes (e.g., Twitch indicators[4]) used to read out TCR-mediated calcium signaling and currently is the only one implemented in a transgenic mouse. The present protocol describes the tumor implant, the surgical preparation of the mouse to ensure optical access to the tumor, and movie acquisition. We also present our current analysis pipeline, which quantifies Ca^{2+} signaling inside and outside the tumor. Our protocol has several advantages over other approaches to quantify TCR-mediated activation by F-IVM.[5–8] The expression of Salsa6f is stable, allowing imaging of the same mouse at multiple time points. Moreover, Salsa6f is expressed on all the cells in the desired subtype, offering a better alternative to *in vitro* transduction and adoptive transfer, which often results in a low abundance of labeled cells. The quantification of Ca^{2+} signaling through Salsa6f is straightforward, as it is based on peaks of GFP fluorescence or the ratio between green and red fluorescence signals. Importantly, Salsa6f has been used to visualize Ca^{2+} signaling dynamics in various immune[3,9,10] and non-immune[11,12] cell types (Table 1). Although we present the specific case of F-IVM of tumor-associated $CD4^{+}Foxp3^{+}$ T regulatory cells (Tregs),[1] our protocol can be applied to study cell motility and activation in a wide array of tumor-associated cells.

Table 1. Mouse models expressing Salsa6f, compatible with the approach described here

Mouse model	Cells labeled	Tested for	Reference
Cd4^{Cre} x *Rosa26*$^{LSL\text{-}Salsa6f}$	All T cells	Functional dynamic imaging in explanted lymph nodes	Dong et al.[3]
E8I^{Cre} x *Rosa26*$^{LSL\text{-}Salsa6f}$	CD8$^+$ T cells	F-IVM through DSFC	This paper and unpublished
Foxp3^{CreERT2} x *Rosa26*$^{LSL\text{-}Salsa6f}$	CD4$^+$Foxp3$^+$ Tregs	F-IVM through DSFC	Geels et al.[1]
GFAP^{Cre} x *Rosa26*$^{LSL\text{-}Salsa6f}$	Astrocytes	Functional dynamic imaging *in vitro*	Wakida et al.[11]
Il17^{Cre} x *Rosa26*$^{LSL\text{-}Salsa6f}$	Inflammatory CD4$^+$ T cells	Functional dynamic imaging in explanted spinal cord	Othy et al.[9]
Myhc6^{MerCreMer} x *Rosa26*$^{LSL\text{-}Salsa6f}$	Cardiomyocytes	Functional dynamic imaging in explanted whole heart	Marquez-Nogueras et al.[12]
Vav1^{Cre} x *Rosa26*$^{LSL\text{-}Salsa6f}$	All hematopoietic cells	Functional dynamic imaging *in vitro* (on bone marrow derived macrophages)	Atcha et al.[10]

Institutional permissions

All animal experiments described in this protocol, including survival surgery, were approved by the Institutional Animal Care and Use Committee of the University of California Irvine before the execution of the experiments (Protocol AUP-22-092). Investigators seeking to apply the protocol described here must obtain similar authorizations from their Institutions.

To use ketamine, we obtained a Controlled Substance User Authorization from the University of California Irvine (CSUA 2021–328).

Construction of the mouse restraint system

Timing: 1 h

The mouse restraint system (Figures 1A and 1B) allows for the immobilization of the anesthetized mouse during the acquisition of intravital images. It is comprised of a plastic base with two protruding screws that support an aluminum bar, where the dorsal skinfold chamber (DSFC) is secured. Two Caddock resistors fixed to the aluminum bar allow heating through a TC324B automatic temperature controller (Warner Instruments).

1. Drill two 1/4″ holes through the polycarbonate plastic base, as per Figure 1C. Note that the distance between holes is 5″.

   Optional: To make space for the screw head, two blind 1/2″ holes can be drilled on the reverse side of the polycarbonate plastic base. Make sure that the blind holes and the through holes have the same center.

2. Fit two $^1/_4$–20 3″-long screws through the drilled holes and immobilize them using $^1/_4$–20 hexagonal nuts.
3. Prepare the aluminum bar as per Figure 1D.
   a. Commercial aluminum bars are at least 2′ long. Cut the bar to a length of 6″.
   b. Using the drilled polycarbonate base as a template, mark the position of the two $^1/_4$″ holes that will fit the support screws. Drill the holes.
   c. Drill two 1/8″ holes 1 $^1/_2$″ from the left and the right side of the bar, and $^1/_4$″ above the bar bottom.
   d. Secure the Caddock resistors to the bar using the 1/8″ holes, 3/16″ 4–40 socket head screws, and 4–40 hexagonal nuts.
   e. Drill three 3/32″ holes in the exact position of the screws that will protrude from the DSFC. The positions of a DSFC from the vendor APJ Trading are shown in Figure 1D.

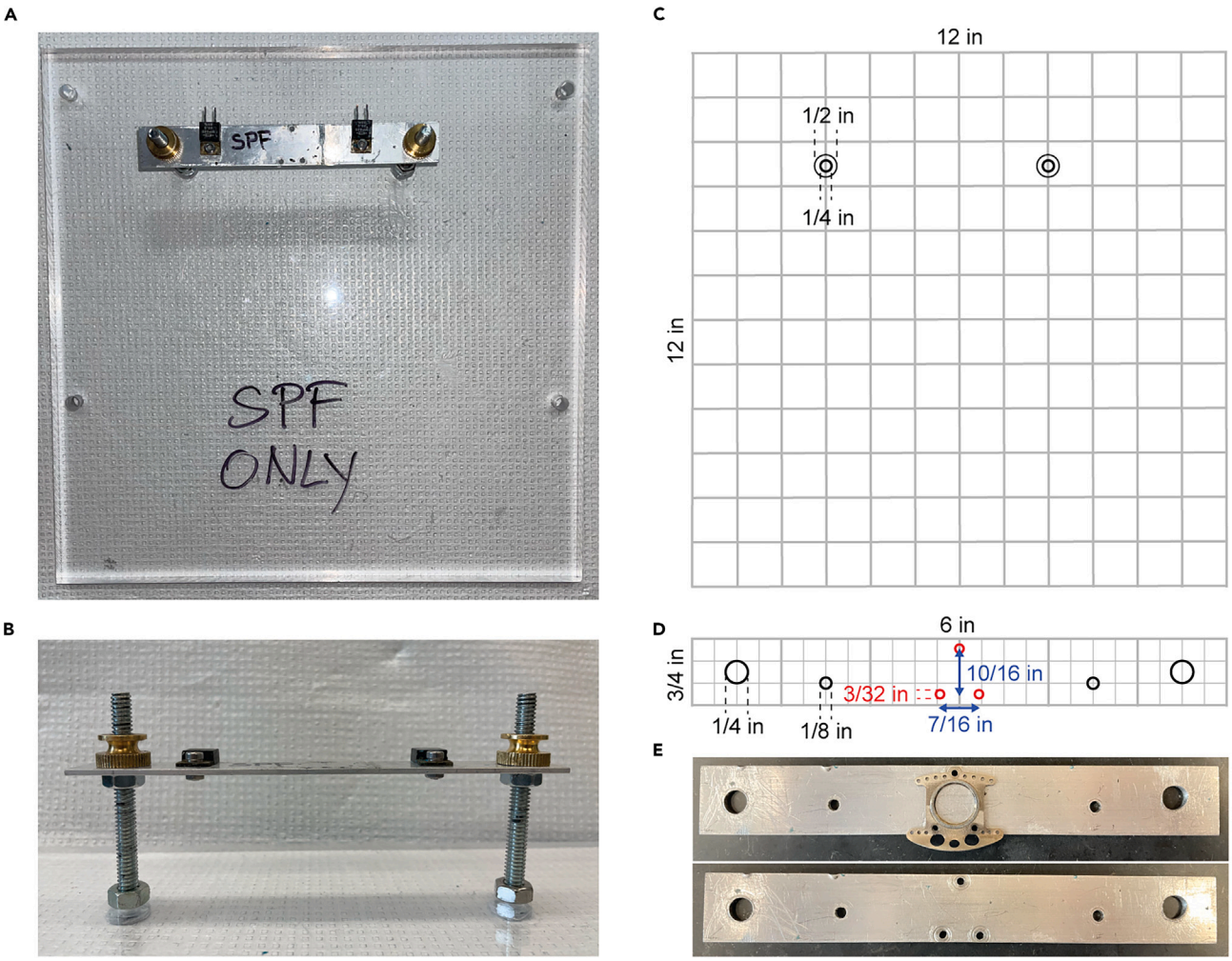


Figure 1. Construction of the mouse restraint system

(A and B) Top (A) and side view (B) of the completed mouse restraint system.

(C) Scheme to prepare the polycarbonate plastic base. The side of each square is one inch.

(D) Scheme to prepare the aluminum bar. The side of each thin-line square is $^{1}/_{4}$ inch.

(E) We highly recommend that the exact position of the holes that fit the DSFC screws are marked using a skinfold chamber as a template.

⚠ CRITICAL: The precise positions of the screws that protrude from the DSFC depend on the chamber manufacturer. It is highly recommended to use the top of a skinfold chamber to mark the position of the holes on the aluminum bar (see Figure 1E).

4. Fit two $^{1}/_{4}$–20 hexagonal nuts approximately 1″ below the top of the 3″ screws. These nuts will support the aluminum bar prepared in Step 3.
5. Insert the aluminum bar through the 3″ screws. Ensure that the bar lies flat using a small slant level.

Note: The height of the hexagonal nuts (and consequently of the aluminum bar) can be adjusted to fit the distance between the microscope stage and the objective.

6. Block the aluminum bar using two $^{1}/_{4}$–20 flanged knurled-head thumb nuts.
7. Drill holes to secure the mouse restraint system base to the microscope stage. The dimensions and positions of the holes depend on the microscope stage manufacturer.

In vivo induction of Salsa6f expression

Timing: at least 7 days

The induction of Salsa6f expression is necessary only for mouse systems based on CreERT2, such as the Foxp3^{creERT2} x Rosa26$^{LSL\text{-}Salsa6f}$.[1] This step is superfluous in mouse models expressing constitutively active Cre (e.g., CD4^{Cre} x Rosa26$^{LSL\text{-}Salsa6f}$ [3]).

Note: For this protocol, we use males homozygous for Rosa26$^{LSL\text{-}Salsa6f}$ and hemizygous for Foxp3^{CreERT2}. We used male mice to prevent the immune recognition of male-derived D4M tumor cells,[13] which would occur in female hosts. If the tumor cell line of choice was developed in female mice, male or female Foxp3^{CreERT2} x Rosa26$^{LSL\text{-}Salsa6f}$ mice can be used interchangeably. We recommend mice anywhere from 7–26 weeks of age, weighing at least 20 grams.

8. Prepare tamoxifen solution as detailed in the "materials and equipment" section. Since the solution can separate over time, thoroughly vortex it before using it to gavage.
9. Load tamoxifen solution in a 5 mL Luer-Lok syringe with a curved gavage needle.
10. Scruff the mouse and insert the gavage needle at a 45° angle into the mouth of the mouse.
    a. As the needle reaches the back of the throat, point the needle tip down toward the stomach and proceed until the whole needle is inside the mouse.
    b. Gently deliver 500 µL of tamoxifen solution.
    c. Slowly pull the needle out, carefully following the same path as before (straight up, then tilt as the needle exits the throat).

⚠ CRITICAL: When the gavage needle is placed correctly in the esophagus and stomach, there should be very little resistance. Never force the needle to avoid harming the mouse or delivering the solution to the trachea.

11. Rinse the gavage needle and store it in 100% ethanol.
12. To induce Salsa6f expression in CreERT2-expressing mice, administer up to three 10 mg tamoxifen gavages spaced two days apart.

Note: The amount and frequency of tamoxifen dosing should be optimized on the specific CreERT2-expressing strain. For Foxp3^{creERT2} x Rosa26$^{LSL\text{-}Salsa6f}$ mice, we have found success administering tamoxifen through gavage only or one 15 mg gavage followed by four 2 mg intraperitoneal injections daily.[1]

13. The same tamoxifen solution can be used for the first and second gavage but should be remade for the third to ensure the best results.

⚠ CRITICAL: In our hands, full induction of Salsa6f expression occurs as soon as three days after the last tamoxifen gavage. The duration of Salsa6f expression likely depends on the turnover of the labeled cells; for Tregs, we used mice 17 days after the first tamoxifen administration.[1] However, we detected Salsa6f-expressing blood Tregs up to 80 days after induction (not shown).

Tumor cell culture

Timing: 3–6 days

This step reports our current protocol for tumor cell culture. We routinely prepare D4M H2B-Cerulean (D4M),[14] D4M-SIINFEKL H2B-Cerulean (D4M-S),[14] and MC38 H2B Cerulean (MC38)[6] tumor

cells, but the workflow can be easily applied to other cell lines. The H2B-Cerulean construct allows for visualization of blue-fluorescent tumor nuclei in F-IVM experiments.[5,6]

14. Thaw frozen tumor cells. In our laboratory, cells are frozen in 1 mL of 90% FCS with 10% DMSO.
    a. Thaw quickly in a 37°C water bath.
    b. Warm up the cell culture media and PBS.
    c. Pipette cells into 9 mL of DMEM + 10% FCS (henceforth, cell culture medium).
15. Centrifuge cells at 500 *g* for 3 min. Gently aspirate supernatant.
16. Resuspend cells in 1 mL of cell culture medium.
17. Add cells to a 150 × 25 mm sterile cell culture-treated plate containing 19 mL of cell culture medium.
    a. Place into a 37°C incubator with 5% CO_2.
    b. Check cell confluency daily. Continue with protocol when cells reach 80% confluency.

Note: For D4M, D4M-S, and MC38 cells, one 80% confluent 150 × 25 mm culture plate should contain 6 × 10^6 - 8 × 10^6 cells. If more cells are required for experiments, proceed further in this section.

18. Work on plates at 80% confluency or less. Gently aspirate the cell culture medium in the plate. Tip the plate at an angle so the media flows to one side, and aspirate the media without disturbing the adherent cells.
19. Add 10 mL of PBS to wash the cells. Move the plate forward and backward 2–3 times and side to side to cover the plate.
20. Aspirate PBS from the plate. Keep the plate at an angle to avoid disturbing adherent cells.
21. Add 3 mL of trypsin-EDTA to the plate.
    a. Tip the plate back and forth and side-to-side to cover the entire surface.
    b. Place the plate in an incubator at 37°C with 5% CO_2 for 3 min.
22. Collect detached cells into a conical tube.
    a. Using a 5 mL serological pipette, pipette trypsin-EDTA back and forth to detach all cells. Check successful cell detachment using a microscope.
    b. Collect cells into a conical tube.
    c. Collect residual cells on the plate.
        i. Add 7 mL of cell culture medium to the plate.
        ii. Swirl the plate to wash it effectively.
        iii. Aspirate the cell culture medium with a pipette and add it to the conical tube containing the cells in trypsin-EDTA.
23. Centrifuge cells at 500 *g* for 3 min. Aspirate supernatant.
24. Resuspend cell pellet. Add 1 mL of cell culture medium and gently pipette up and down 10 times to achieve a single-cell suspension.
25. Count cells with a hemocytometer.
26. Add cell culture medium to a concentration of 2 × 10^6 cells/mL.
27. Add 1 mL of cell suspension onto a 150 × 25 mm cell culture-treated plate containing 19 mL of cell culture medium. Create as many plates as needed for tumor injection into mice ("step-by-step method details" section, steps 1–7).
    a. Place the plates into a 37°C incubator with 5% CO_2 for 48–72 h.
    b. Check cell confluency daily. Cells should not exceed 80% confluency.

⚠ CRITICAL: If a batch of cells needs to be frozen, ensure they are negative for mycoplasma.

Sterilize the DSFC and the surgical tools

Timing: 2 h

28. Sterilize all tools and chamber parts by autoclaving.
    a. Pack the chamber parts in aluminum foil, and close the packet with autoclave tape.

    Note: The pack contains one chamber half with support for the cover glass (hereafter, "DSFC front"), one chamber half without the support for the cover glass (hereafter, "DSFC back"), three 0–80 3/8″ Phillips screws, three threaded spacers, three 0–80 hexagonal nuts, and one 12 mm snap ring.

    Optional: The DSFC vendor may sell threaded spacers. If not, we found a good alternative in McMaster Carr Cat# 91780a018. The sides of such threaded spacers may need to be filed to fit the DSFC perfectly.

    b. Put surgical tools (one regular scissor, two tweezers, and one Vannas scissor) in an autoclaving box.
    c. Autoclave 30 min at 121°C.

Choose the anesthesia method

We present two alternative strategies, both routinely applied in our lab.

29. Gas anesthesia through isoflurane inhalation.
    a. Place mouse in the induction chamber and provide 3% isoflurane.
    b. Once asleep, move the mouse from the induction chamber to the nose cone, providing 2% isoflurane.
    c. Check the mouse is deeply anesthetized by pinching the hind paw.

    Note: If anesthesia is deep, no withdrawal reflex would be present. Also note that during deep anesthesia, the respiratory rate decreases.

    d. Place the mouse on a warming pad, maintaining a temperature of 37°C.
    e. As the mouse is asleep, you may need to gradually lower the isoflurane percentage from 2% to 1.5%. We usually decrease the amount of isoflurane by 0.1% per hour.
    f. To wake up the mouse, disconnect it from the isoflurane machine. Wake-up time is around 5 min.

    Note: Appropriate flow rates for anesthesia induction and maintenance depend on mouse weight and the isoflurane machine. Please consult the anesthesia machine manual before starting.

    ⚠ CRITICAL: If you observe gasping (a sudden inhalation event followed by a long breathing pause), prevent death by immediately disconnecting the mouse from the isoflurane vaporizer. Once the breathing rhythm has resumed, you can reconnect the mouse to the vaporizer, taking care of decreasing the isoflurane by at least 0.2%.

30. Anesthesia using ketamine/xylazine.
    a. Inject a solution containing ketamine (100 mg/kg) and xylazine (10 mg/kg) intraperitoneally. Such anesthesia lasts about 30 min.
    b. Check the mouse is deeply anesthetized by pinching the hind paw.

    Note: If anesthesia is deep, no withdrawal reflex would be present. Also note that during deep anesthesia, the respiratory rate decreases.

    c. Place the mouse on a warming pad, maintaining a temperature of 37°C.

d. If redosing is necessary, we recommend injecting half a dose of the original ketamine administration without xylazine upon the reappearance of the paw withdrawal reflex.[15]
e. The wake-up time varies from 10 min to 1 h, depending on how much total ketamine/xylazine was administered. Keep the mouse on the warming pad until awake to prevent hypothermia. Your institution may require monitoring of the mouse until it is fully awake.

⚠ CRITICAL: Ketamine is a controlled substance. It is mandatory to obtain institutional permissions before use.

Note: Advantages of isoflurane administration include easier maintenance of surgical anesthesia over time, quicker recovery, the fact that isoflurane is not a controlled substance, and, in our experience, a low death rate. Ketamine/xylazine injection is cheaper.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
αPD-1 (clone: 29F.1A12) 200 μg/mouse	Bio X Cell	Cat# BE0273; RRID:AB_2687796
Rat IgG 200 μg/mouse	Sigma	Cat# I8015-100MG; RRID:AB_1163629
Chemicals, peptides, and recombinant proteins		
Carprofen (Rimadyl)	MWI	Cat# 26357
Isoflurane (Fluriso)	VetOne	Cat# N/A
Tamoxifen	Sigma	Cat# T5648
Deposited data		
Practice dataset containing unprocessed and processed Imaris files and the MATLAB analyses	This paper	Mendeley Data: https://doi.org/10.17632/t7tr42ztt5.2
Experimental models: Cell lines		
Mouse: D4M.3A H2B-Cerulean (D4M)	Thorsten Mempel (Massachusetts General Hospital and Harvard Medical School, Boston, MA), Di Pilato et al.[14]	Cat# N/A
Mouse: D4M.3A H2B SIINFEKL Cerulean (D4M-S)	Thorsten Mempel (Massachusetts General Hospital and Harvard Medical School, Boston, MA), Di Pilato et al.[14]	Cat# N/A
Mouse: MC38 H2B-Cerulean (MC38)	Thorsten Mempel (Massachusetts General Hospital and Harvard Medical School, Boston, MA), Marangoni et al.[6]	Cat# N/A
Experimental models: Organisms/strains		
Mouse: B6.Cg-Gt(ROSA)26Sortm14 (CAG-tdTomato)Hze/J (*Rosa26*$^{LSL\text{-}Tomato}$)	Jackson Laboratory	JAX: 007914; RRID:IMSR_JAX:007914
Mouse: Foxp3tm9(EGFP/cre/ERT2)Ayr/J (*Foxp3*^{CreERT2})	Jackson Laboratory	JAX: 016961; RRID:IMSR_JAX:016961
Mouse: C57BL/6-Tg(Cd8a-cre)1Itan/J (E8I^{Cre})	Jackson Laboratory	JAX: 008766 RRID:IMSR_JAX:008766
Software and algorithms		
Imaris 9.7.2	Bitplane	http://www.bitplane.com
MATLAB R2021b	MathWorks	https://www.mathworks.com/products/matlab.html
Prism 10	GraphPad	https://www.graphpad.com
MATLAB cell motility analysis scripts	Geels et al.[1]	Zenodo: https://doi.org/10.5281/zenodo.11122613
Other		
Dorsal skinfold chamber	APJ Trading	Cat# SM100
Scaffold (small C-clamp)	APJ Trading	Cat# C-100
Nylon 4-0 black 1 × 18" P-3 sutures	Corza Medical	Cat# A699N
12 mm 1.5 circular coverglass	Electron Microscopy Sciences	Cat# 72230-01

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Vannas scissors	Fine Science Tools	Cat# 91501-09
Needle holder	Fine Science Tools	Cat# 91201-13
Cauterizer	Fine Science Tools	Cat# 18010-00
Pliers for snap ring placement	WLXY Tools	Cat# 359B
Gavage needles	Fisher	Cat# NC1197790
SomnoSuite isoflurane machine with RightTemp	Kent Scientific	Cat# SS-01
EZ4 stereoscope	Leica Microsystems	Cat# 10447198
Threaded spacers for chambers	McMaster-Carr	Cat# 91780a018
1 polycarbonate sheet for base (12″ × 12″ × 1/2″)	McMaster-Carr	Cat# 8574K32
Depilatory cream	Nair	Cat# N/A
2 screws (1/4-20 length 3″)	McMaster-Carr	Cat# 96640A250
4 hexagonal nuts (1/4–20)	McMaster-Carr	Cat# 95462A029
2 flanged knurled head thumb nuts (1/4–20)	McMaster-Carr	Cat# 92741A160
1 aluminum bar (24″ × 3/4″, 1/16″ thick)	McMaster-Carr	Cat# 8975K192
Socket head screws (4–40 thread, 3/16″ length)	McMaster-Carr	Cat# 91251A105
Hexagonal nuts (4–40)	McMaster-Carr	Cat# 91841A005
Hexagonal nuts (0–80)	McMaster-Carr	Cat# 91841A115
Phillips screws, 0–80 thread, 3/8″ length	McMaster-Carr	Cat# 91772A057
Silicone O-rings	McMaster-Carr	Cat# 1182N013
2 Caddock resistor MP820 20.0	Mouser electronics	Cat# 684-MP820-20
Sterilizer	Various	Cat# N/A
Sonicator	Various	Cat# N/A
#0 Phillips screwdriver	Various	Cat# N/A
Nut driver 5/32 × 60 mm	Various	Cat# N/A
Temperature controller for stage	Warner Instruments	Cat# TC324B
SP8 multiphoton microscope	Leica Microsystems	Cat# N/A
Mai Tai multiphoton laser	Spectra-Physics	Cat# N/A
InSight X3 multiphoton laser	Spectra-Physics	Cat# N/A

MATERIALS AND EQUIPMENT

Alternatives: This protocol leverages a Leica SP8 multiphoton microscope with tunable fluorescence detectors. However, our protocol has also been used on more commonly available microscopes equipped with detectors coupled to fixed-wavelength dichroic/bandpass filters. For instance, Othy and coworkers[9] used 495 nm and 538 nm dichroic filters to detect the green component of Salsa6f between 495 and 538 nm and tdTomato at 538 nm and above. Bandpass filters were not used in this case.

Tamoxifen solution for induction of Salsa6f expression

Reagent	Final concentration	Amount
Tamoxifen	20 mg/mL	10 mg per gavage per mouse
100% ethanol	10%	50 μL per gavage per mouse
Corn oil	90%	450 μL per gavage per mouse
Total	**N/A**	**500 μL per gavage per mouse**

Store at 4°C for up to 3 days. It is also possible to store aliquots long-term at −20°C. However, frozen aliquots should be used immediately after thawing and then discarded because thawed tamoxifen rapidly loses activity.

Note: This tamoxifen dosage is suitable for healthy adult mice, either male or female. We recommend determining the appropriate tamoxifen dose in case of administration to pregnant, overweight, or diseased mice.

⚠ CRITICAL: Tamoxifen powder may cause cancer, damage fertility or the unborn child, and harm the environment. For these reasons, it must be handled under a fume hood, and any leftovers must be disposed of according to the regulations in force at each Institution.

⚠ CRITICAL: Tamoxifen dissolution in ethanol takes time. Keep heating the solution in a 56°C water bath and vortex periodically until all tamoxifen is dissolved.

Alternatives: Tamoxifen can also be administered via intraperitoneal injections of 1–2 mg per day for five consecutive days (ref. 6 and 16 and data not shown). We do not favor tamoxifen chow, as it can be challenging to habituate the mice to its taste.

STEP-BY-STEP METHOD DETAILS

Preparation of tumor cells for injection

Timing: 30 min

Here, we describe how to prepare a tumor cell suspension for injection into Salsa6f-expressing mice.

⚠ CRITICAL: Do not use cells past 80% confluency. Using overgrown cells may impact their growth *in vivo*.

1. Collect cells from the plate as detailed in steps 18–23 of the before you begin section.
2. Wash cells in PBS.
   a. Resuspend cells with 1 mL of PBS. Gently pipette up and down 10 times to break up the cell pellet.
   b. Add 49 mL of PBS.
   c. Centrifuge cells at 500 *g* for 3 min.
   d. Aspirate supernatant.
3. Resuspend cell pellet. Add 1 mL of PBS. Gently pipette up and down 10 times to break up the cell pellet. Add additional PBS, as needed, to dilute cells for counting.
4. Count cells using trypan blue and a hemocytometer.
5. Add PBS to a total volume of 50 mL.
6. Centrifuge cells at 500 *g* for 3 min. Aspirate supernatant.

Note: The two 50 mL washing steps remove all FCS. FCS might adjuvant an immune response against the tumor.

7. Resuspend tumor cells in PBS at an appropriate concentration for injection. For MC38 and D4M cells, this is 5×10^7/mL, and for D4M-S, it is 7.5×10^7/mL. Place on ice until ready to inject.

⚠ CRITICAL: To account for the volume of the cells, suspend them in a lower PBS volume than calculated. Measure the total volume using a pipette and top-up PBS to achieve the desired final volume.

Tumor implant in Salsa6f-expressing mice

Timing: 30 min

Through this step, tumor cells are injected into the correct anatomical location for the subsequent implant of a DSFC, which allows optical access to the tumor environment.

8. Anesthetize the mouse and place it on a warming pad at 37°C (Figure 2A).

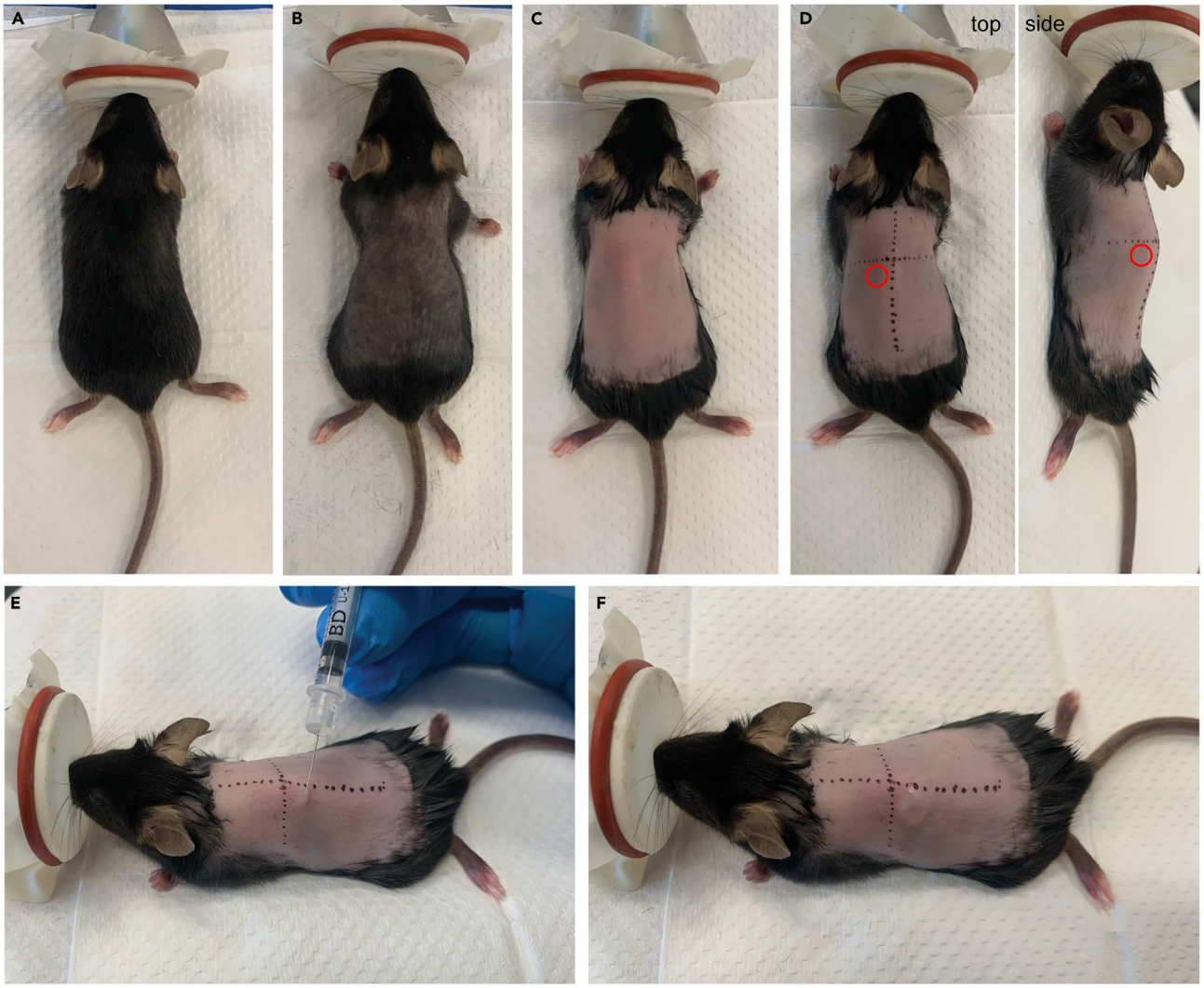


Figure 2. Tumor injection in the DSFC position

(A) Anesthetize the mouse. In this example, we used isoflurane inhalation. Place the mouse on a heated mat set at 37°C.

(B) Remove hair using a clipper.

(C) Epilate the mouse by applying Nair for 30–45 s and accurately removing it.

(D) Using a marker, draw two lines: one on the spine and one perpendicular to it, across the spine hump. Red circles highlight the location of the tumor cell injection.

(E) Tumor cell injection. Note that the needle is slightly pulled up to ensure the injection is subcutaneous.

(F) End result.

9. Shave the dorsal side of the mouse with hair clippers, approximately from the neck to the hind legs (Figure 2B). Troubleshooting problem 1.
10. Apply depilatory cream (e.g., Nair) using cotton swabs to remove the remaining fur.
    a. Cover the dorsal side of the mouse with Nair. Leave Nair on for approximately 30–45 s.
    b. Wipe off Nair. The back is now completely epilated (Figure 2C).
        i. Remove Nair with a dry piece of gauze or kimwipe.
        ii. Use a piece of gauze or kimwipe wet with water to remove the remaining Nair.
        iii. Use a piece of gauze or kimwipe to dry the mouse and remove any remaining fur.

⚠ CRITICAL: Remove Nair completely. Insufficient removal will induce an inflammatory response and may damage skin integrity. Be careful to leave Nair on for at most one minute.

11. Draw sections on the mouse dorsum for accurate tumor injection (Figure 2D).
    a. Using a marker, draw a dotted line onto the mouse spine.
    b. Draw a perpendicular line across the hump of the spine that naturally forms when the mouse lies in the prone position.
12. Inject 10 μL of tumor cells subcutaneously into the lower left quadrant (Figure 2E).

Note: The amount of injected cells is 7.5×10^5 for D4M-S and 5×10^5 for D4M and MC38 cell lines.

⚠ CRITICAL: Pull the needle up to ensure the injection is subcutaneous. If the needle cannot be pulled up without effort, it is stuck in deeper tissues. Remove needle and try again.

13. Figure 2F shows the result of the tumor injection. Place the mouse back in its original cage.
14. Allow the tumor to grow for 7–9 days, then install the DSFC.

Installation of the DSFC

Timing: 1 h per mouse

This surgery allows for longitudinal imaging of immune cells within the tumor environment. Proper installation of the DSFC is critical to successfully imaging living and functional cells later in the procedure and minimizing discomfort for the mouse.

Note: Do not perform the surgery on a small mouse (less than 20 g body weight), as this can pull the skin around the chest too tight and cause trouble breathing.

15. Anesthetize the mouse. In this example, we used ketamine/xylazine (Figure 3A).
    a. Place the mouse on a warming pad, maintaining a temperature of 37°C.
    b. Check the mouse is deeply anesthetized by pinching the hind paw. If anesthesia is deep, no withdrawal reflex would be present.
16. Gently apply Vaseline or eye ointment to protect the eyes during the procedure.

Optional: If the hair has regrown, or some hair falls into the space occupied by the DSFC, Nair can be applied again, as described in step 10.

17. Mark the back of the mouse to assist in chamber placement.
    a. With a marker, dot a line parallel to the spine and approximately 8 mm distant from the tumor.

    Note: This is used as a guide to keep the chamber straight along the midline of the mouse as you position it over the tumor (Figure 3B).

    b. Pinch the skin and pull up (as it would look once the chamber is on).
        i. Hold the DSFC front against the skin with the tumor in the middle. Troubleshooting problem 2.
        ii. Mark the position of the top screw hole of the DSFC.

    Note: This will help in knowing where to attach the scaffold.

18. Attach the scaffold to the skin. This scaffold pulls the skin tight for easier DSFC installation and is removed before the end.
    a. Fold the dorsal skin along the dotted line, and align the scaffold with the middle notch in correspondence with the chamber's top screw hole marked in step 17b.

Figure 3. Installation of the DSFC

(A) Anesthetize the mouse. In this example, we used the ketamine/xylazine strategy. The red arrow highlights the tumor.
(B) Mark a dotted line parallel to the spine, approximately 8 mm above the tumor.
(C) Scaffold placement.
(D) Using the two halves of a DSFC and keeping the tumor in the center of the frame, pierce the skin to make room for the screws. We provide views from the side and the back of the mouse.
(E) Assembly of the DSFC front. Fore and rear views are provided.
(F) Positioning of the DSFC front through the mouse skin.
(G) Place the DSFC back and secure it to the skin using surgical knots. Remove the scaffold.
(H) Dissection of the epidermis.
(I) Dissection of the dermis.
(J) Dissection of the transparent collagen layer below the dermis.
(K) DSFC appearance once the cover glass and the snap ring have been placed.
(L) Place some putty behind the DSFC to facilitate the adherence of the tumor to the cover glass.

i. Insert the suture needle into the skin along the dotted line and immediately to the right of the marked top screw hole.
ii. Once the needle is through the skin, thread it through the first hole of the scaffold to the right of the middle notch.

b. Place a suture with three alternating knots to secure the scaffold to the skin. Trim the excess thread.

⚠ CRITICAL: To maintain the correct skin tension, make sure to place the knots not perpendicularly to the skin but at an angle pointing towards the center of the DSFC.

c. Place a second suture in the first hole to the left of the scaffold's middle notch.
d. Place a third suture in the third hole to the right of the scaffold's middle notch and a fourth suture in the third hole to the left of the middle notch (Figure 3C).

Note: The scaffold should curve away from the mouse when facing the correct way.

19. Pierce the skin for the screws.
    a. Place the DSFC front on the skin with the tumor centered.
        i. Hold the DSFC back on the opposite side of the skin so that the screw holes are aligned.
        ii. Use three 16G needles to pierce through the skin in each screw hole.

    Note: Be sure to lightly pinch the skin between the chamber so that no extra tissue on the back or shoulders is pierced (Figure 3D).

    b. To widen the holes, pull each needle until it is almost removed, and then push it back in. Repeat this three times, then remove the needles and the chamber halves.

    Note: While the DSFC front will not be installed on the side with the tumor, it is helpful to use it as the guide when piercing the skin to ensure the tumor will be within the imaging window.

20. Assemble the DSFC front.
    a. Place the screws into the DSFC front. When holding the DSFC front so that it curves away from the mouse, the screws should be pointing toward the mouse.
    b. Place threaded spacers on each screw and tighten with screwdriver (Figure 3E). Troubleshooting problem 3.
21. Complete the DSFC installation.
    a. Insert the screws through each hole in the skin, making sure the DSFC front is on the side opposite the tumor.
    b. Using forceps, gently stretch the skin around each spacer to fit the spacers through the skin (Figure 3F).
    c. Once the skin is around each spacer, slide the DSFC back through the screw holes and secure it with 0–80 hexagonal nuts.
22. Secure the DSFC to the skin using sutures.
    a. Work on top holes. Begin with one suture entering the first hole to the right of the chamber center, coming out the same hole on the opposite side, then threading back through the second hole to the right of the chamber center, and tie the suture.
    b. Make the next suture the same way, going in the third hole to the right of the chamber center and back through the fourth hole.
    c. Repeat these two sutures on the opposite side of the chamber for four sutures total.
    d. Remove the scaffold by cutting the sutures that attach it to the skin (Figure 3G).
23. Prepare mouse for skin removal.
    a. Move the mouse under a stereoscope and push DSFC screws into a lump of putty to stabilize the mouse. The DSFC front should be facing up.
    b. Swab the surgical area with 70% ethanol to remove any debris on the skin. Remove loose hair by looking under the scope and grabbing it with forceps.
24. Remove the skin in the imaging window.
    a. Grip the skin with forceps and use Vannas scissors to nick the epidermis, without cutting the dermis. Extend the nick to cut the epidermis circularly around the inside of the chamber, leaving 1–2 mm of border (Figure 3H).

    Note: If performed correctly, cutting the epidermis will not result in bleeding since the epidermis is devoid of blood vessels. Bleeding may occur if the dermis is accidentally cut. In that case, cauterize immediately.

    b. Following the path of cut skin, cauterize all visible blood vessels in the dermis by lightly touching the tip of each vessel with an electric cauterizer.

c. Cut through the dermis in the same path around the chamber, carefully cutting downstream of cauterized vessels (Figure 3I). Troubleshooting problem 4.
d. Remove the extremely thin transparent collagen layer without disturbing this layer on the opposite side (Figure 3J). This layer may stick to the dermis and may have already been removed. Troubleshooting problem 5.

25. Place cover glass to seal the chamber.
    a. Clean cover glass with 70% ethanol and a kimwipe to ensure the removal of fibers or debris that may compromise imaging.
    b. Place the cover glass in the DSFC front groove and install the snap ring using pliers. If secured correctly, the glass should not be able to move up and down. Orient the snap ring so that the round holes do not obscure the tumor (Figure 3K).
    c. Check that the cover glass is intact by dragging forceps across to feel for cracks. If damaged, remove and replace.
26. Press the skin opposite to the glass to create a seal, and place putty against the skin around the tumor (Figure 3L).

Note: While there should be enough putty to force the skin against the glass, make sure not to stop the blood flow. To verify adequate circulation, observe the blood vessels within the DSFC while applying putty.

27. For pain management, administer 5 mg/kg of carprofen subcutaneously immediately outside of the chamber. Repeat injections daily.
28. Singly house mice without a wire rack to prevent the chamber from snagging on any cage components.
    a. Place food on the floor for easy access.
    b. If removing the water bottle is necessary, provide jellified water on the bottom of the cage.

Note: This protocol was written assuming the tumor was injected on the mouse's left side, but it can be mirrored if injected on the right. Throughout the procedure, always be aware of the mouse's temperature and breath rate.

Acquisition of intravital microscopy footage

Timing: 4–6 h per mouse

This section describes the longitudinal dynamic acquisition of intratumoral T cells infiltrating a specific tridimensional area of the tumor environment. Imaging the exact location before and after administration of immunomodulatory drugs (e.g., αPD-1[1]) ensures that differences in cell motility and activation dynamics reflect the action of the applied immune intervention, rather than local differences in tissue architecture.

Starting three days after the surgery to allow the surgery-related inflammation to subside, the tumor can be observed under the multiphoton microscope. This procedure necessitates anesthetizing the mouse, installing it under the microscope with the DSFC centered and fixed under the objective, and acquiring movies that record intratumoral T cells in the area of interest. The immunomodulatory drug under study is typically injected at the end of the first imaging session.

29. Turn on the microscope controlling software, the microscope, and the multiphoton laser(s).

Note: Allow adequate time to warm up the multiphoton laser(s) to achieve mode-locking.

Note: While ketamine/xylazine anesthesia can be used, we find it impractical for intravital imaging because the frequent need to re-dose will interfere with the acquisition of movies longer

than 30 min. Thus, isoflurane inhalation is our anesthesia method of choice for F-IVM footage acquisition.

30. Inspect the DSFC. Troubleshooting problem 6.
31. Anesthetize the mouse and place it under the microscope.
    a. Place the mouse on a warming pad, maintaining a temperature of 37°C.
    b. Check the mouse is deeply anesthetized by pinching the hind paw. If anesthesia is deep, no withdrawal reflex would be present.
    c. Check the putty on the back of the DSFC. Ensure that:
        i. The putty creates sufficient pressure to bring the back skin and the tumor in contact with the cover glass.
        ii. The putty does not prevent the blood flow.
        iii. No space remains between the glass and the back skin.
        iv. Ensure that the putty does not protrude beyond the hexagonal nuts on the back of the DSFC. If it does, the fixation of the DSFC to the aluminum bar of the mouse restraint system may create excessive pressure in the DSFC.
    d. Secure the DSFC to the aluminum bar using three 0–80 hexagonal nuts (Figure 4A). Troubleshooting problem 7.
    e. Attach the aluminum bar to the mouse restraint system (Figure 4B).
    f. Create a waterproof ring around the DSFC cover glass. This procedure will facilitate water retention between the objective and the DSFC.
        i. Add two layers of vacuum grease around the glass using a syringe and a blunt 16G needle. (Figure 4C).
        ii. Place a silicone O-ring on top of the vacuum grease circle (Figure 4D).
    g. Clean the DSFC glass with a cotton swab and 70% ethanol.
    h. Add a few drops of water inside the waterproof ring (Figure 4E). Troubleshooting problem 8.
    i. Adjust the set point value of the temperature control device to 37°C. Then, plug the heater controller cables into the Caddock resistors and attach the feedback thermistor to the aluminum bar (Figure 4F).
    j. Secure the mouse restraint system to the microscope stage, centering the DSFC under the objective (Figure 4G).
    k. Looking through the microscope's oculars, adjust the objective's Z position so that the tumor environment is in focus. Localization of blood vessels or immune cell fluorescence will aid in achieving this goal.

⚠ CRITICAL: Take great care not to go beyond the focal position, as the objective lens may come in contact with the DSFC cover glass. This accident can cause the DSFC cover glass to break and damage the microscope objective.

32. Mapping of the tumor environment.
    a. Install appropriate dichroic/bandpass filters to detect GFP (e.g., 525/50) and tdTomato (e.g., 590/50).[5,6] If the microscope is equipped with tunable detectors, set one to 490–545 nm and another one to 560–600 nm.[1]
    b. Tune the multiphoton laser to 950 nm. This allows for the excitation of the GCaMP6f and the tdTomato components of Salsa6f, and the generation of the second harmonic signal at 475 nm.

Note: If the microscope system has two multiphoton lasers, the other one can excite fluorescent proteins beyond the green/orange zone of the spectrum. For instance, we injected cerulean-expressing tumor cells, which were detected using an 850 nm multiphoton laser beam.[5,6] If using multiple lasers, make sure their alignment is correct.

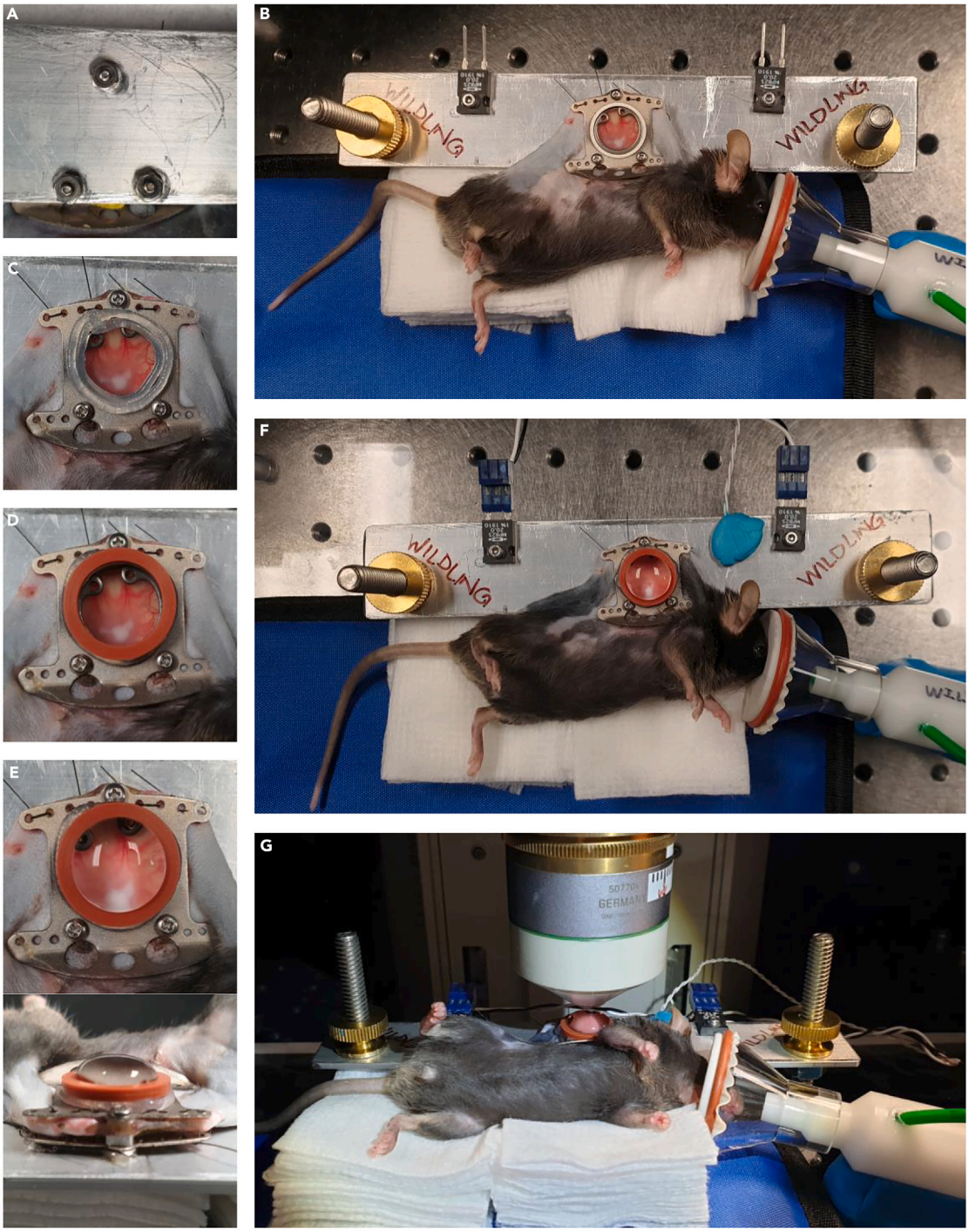


Figure 4. Installation of the mouse under the microscope

Anesthetize the mouse. In this example, we use the isoflurane strategy.

(A) Secure the three screws of the DSFC through the holes of the aluminum bar using three 0–80 hexagonal nuts.

(B Attach the aluminum bar to the stage.

(C and D) Create a waterproof ring around the DSFC by adding two layers of vacuum grease around the glass (C) and placing a silicone O-ring on top of the vacuum grease circle (D).

(E) Add a few drops of water inside the waterproof ring. Front and side views are shown.

(F) Plug the heater controller cables into the Caddock resistors and attach the feedback thermistor to the aluminum bar.

(G) Secure the mouse restraint system to the microscope stage and center the DSFC under the objective.

c. Set the laser power to the minimum that allows for visualization of fluorescence. In our system, this is typically 15%–25% of the maximum laser power (2.3–2.4 W).

⚠ CRITICAL: Be conservative with laser power, as too much of it will cause photobleaching and tissue burns. Troubleshooting problem 9.

d. Set the microscope parameters to maximize acquisition speed while retaining high image quality.
   i. While variable among microscopes, our system allows for bidirectional scanning in X and Z dimensions, and speed adjustment to 600 Hz corresponding to a pixel dwell time of 1.21 μs.
   ii. Ensure a high image quality by selecting a resolution of 512 × 512 pixels and a zoom factor resulting in a field of view of approximately 600 × 600 μm (on our Leica SP8 system, it is 0.75).

e. Acquire a merged tile-scan image of the whole tumor area. Depending on the microscope system, different strategies can be used to achieve this goal. Troubleshooting problem 10.
   i. Most systems allow for the acquisition of a tile image of the entire area after setting up the percentage of overlap between individual tiles.
   ii. In a Leica SP8 multiphoton system, go to "LAS X Navigator" and acquire a spiral image of the tumor area.

33. Acquisition of F-IVM footage.
   a. Using the tumor map acquired in step 32, identify the region to be imaged based on T cell abundance and location. We recommend saving a screenshot of the location of this area relative to the acquired montage image.
   b. Adjust Z-step size to 5 μm.
   c. Set up the time interval between Z-stack acquisitions.
      i. Set the time interval between Z-stacks to 5 s or less to capture transient Ca^{2+} signaling events (green fluorescence of Salsa6f).
      ii. Set up the duration of the movie. We recommend that the total duration of the acquisition should be at most 30 min to minimize the risk of photobleaching. Troubleshooting problem 9.
   d. Adjust the starting and ending Z-coordinates to include the tridimensional region of interest. Take note of the Z coordinates of the first and last layers of the selected region.
   e. Set the other parameters as per step 32.
   f. Start the recording.
   g. After approximately 2 min of recording, make sure T cells are moving as expected. If yes, let the microscope pursue the recording. If not, go back to step 33a and select another tridimensional region of interest to be imaged. Troubleshooting problem 11, 12, and 13.

⚠ CRITICAL: This quality control step is a crucial time-saver that allows aborting low-quality movies early on during acquisition.

Note: Microscope control software usually allows one to play a movie while it is being acquired. If your system does not support this function, take a screenshot of the movie at the beginning of the acquisition and after two minutes. Compare the two pictures to spot cell movement.

   h. When all areas of interest are recorded, save the files and turn off the microscope and the lasers.

⚠ CRITICAL: It is important to record the imaging parameters (lasers wavelength and power, range of the tunable detectors, PMT gains, resolution, zoom factor, dwell time/pixel and time interval) used in the initial session and apply them in the subsequent

session. This consistency ensures comparability of movies acquired at different time points.

34. Remove the mouse from the stage.
    a. Move the objective back to the escape position, then remove the mouse restraint system.

    Note: If possible, do not move the microscope's stage until the following acquisition of the same mouse. This precaution would facilitate going back to the same imaging area.

    b. Remove the silicone O-ring on the mouse's DSFC and clean the vacuum grease with 70% ethanol.
    c. Unplug the temperature control cables.
    d. Detach the mouse from the aluminum bar.
    e. Place the mouse back into its cage and monitor its recovery.
    f. Turn the temperature control devices off.
35. Inject the drug of interest for the next imaging session.
36. Longitudinal footage acquisition.

    Note: In Geels et al.,[1] mice are imaged again 24 h after αPD-1 administration. However, the time to re-imaging depends on the individual experiment. For instance, we have imaged the effects of CTLA-4 blockade on tumor-Treg cells 2, 4, 18, and 44 h after antibody injection.[6]

    a. Repeat steps 29, 30, and 31, and map the tumor as detailed in step 32.
    b. Find the same tridimensional region of interest as the preceding day.
        i. Adjust the objective to the recorded coordinates of the movie acquired on the previous day.
        ii. Acquire a new mosaic image of the tumor and use the previously recorded tumor's mosaic screenshot to accurately identify the area matching the one imaged the day before.
    c. Acquire F-IVM movies as per step 33. While the Z-coordinates annotated the previous day (see step 33d) can be used as a rough guide, the new coordinates will likely differ. Troubleshooting problem 14.
    d. At the end of the session, repeat steps 33 h and 34.

Analysis of F-IVM imaging data using Imaris

Timing: One to five days, depending on the manual refinement of T cell tracks

The quantitative analysis of F-IVM data is an essential step, allowing us to measure various parameters associated with cell motility relative to the tumor and the dynamics of T cell activation. However, the movie analysis protocol is not standardized, as the analysis pipeline is highly tailored to the specific focus of the experiment.[1,6,9,17] While many pieces of software to track cells are available, we chose to quantify the motility and activation of tumor-associated T cells using Imaris due to its advanced functions.

37. Data opening and verification in Imaris (Figure 5A).
    a. If necessary, use ImarisFileConverter to convert the output file from the microscope to a format readable by the Imaris software.
    b. Open the Imaris file by dragging it on the Imaris software environment.
    c. Adjust fluorescence display (ctrl-D) to visualize all fluorescence channels satisfactorily. Start by resetting all channels, as the automatic channel rendition is often unsatisfactory. Then, adjust the right-hand side of the slider to enhance the fluorescence display, and minimally tweak the left-hand slider to remove a little background. Do not change the gamma parameter (Figure 5B).

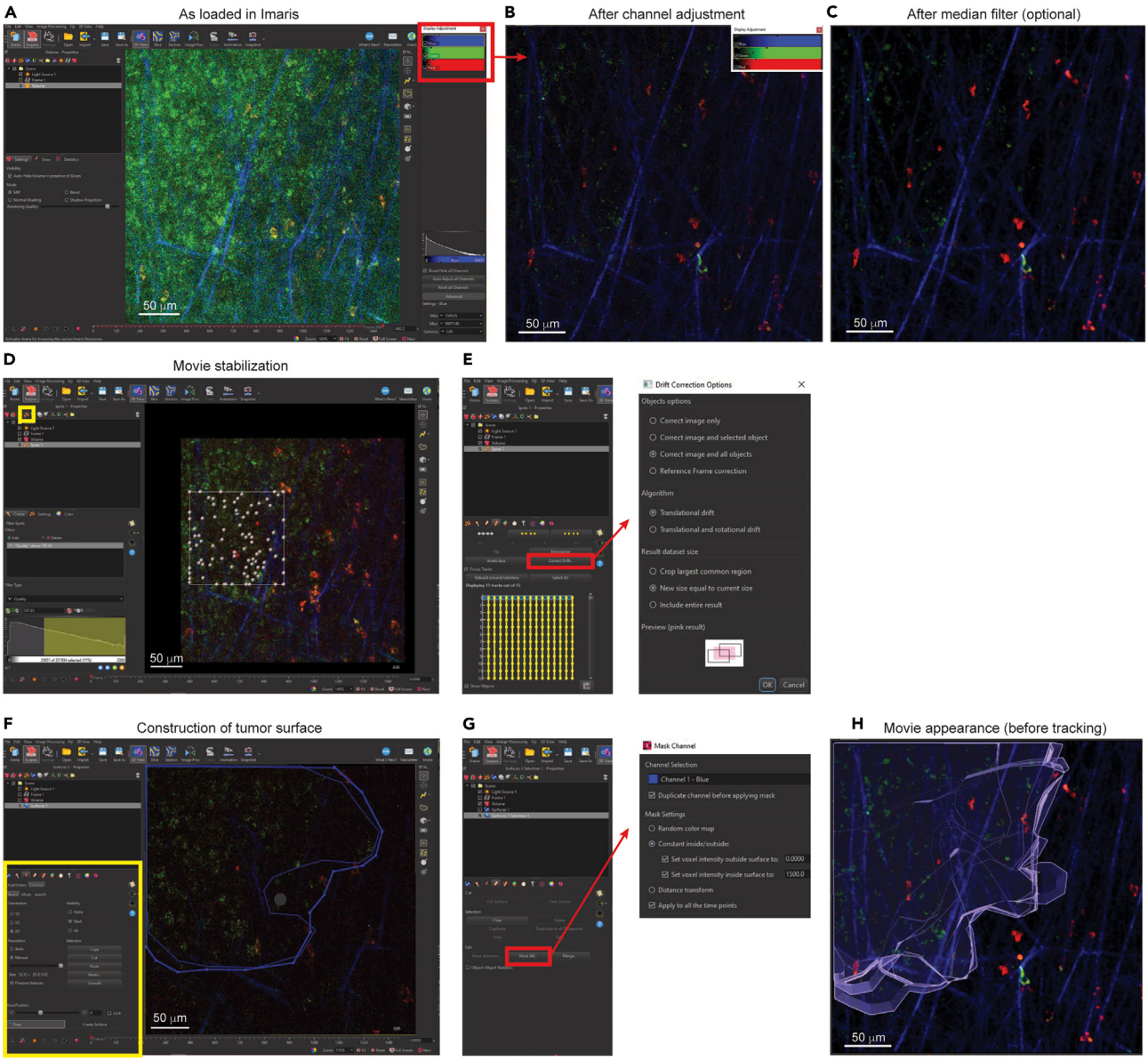


Figure 5. Movie visualization, stabilization, and tumor surface construction in Imaris

(A) Open the movie in Imaris and adjust fluorescence visualization by operating on the Display Adjustment pane (red box). Scale bar 50 μm.

(B and C) Movie appearance after a correct Display Adjustment (B) and median filtering (C). Scale bars 50 μm.

(D and E) Drift stabilization. (D) Create a new "Spot" collection (yellow box) to detect and track static objects (in this case, autofluorescent tumor cells). Scale bar 50 μm. (E) Click on the "Edit" menu, select the tracks that span the whole movie, press the Correct Drift button (red box), and insert the Drift Correction Options.

(F–H) Create a new channel corresponding to the tumor. (F) Manually create a new "Surface" object and draw the surface using the Board pane (yellow box). Scale bar 50 μm. (G) Once a surface spanning all time points has been created, press the Mask All button (red box) in the "Edit Track" pane and insert the desired options in the Mask Channel dialog box. (H) Final appearance of a correctly drawn tumor surface. Scale bar 50 μm. See also Video S1.

Optional: The movie's appearance can be improved by applying a conservative median filter, which can be accessed by clicking the "Image Proc" button (Figure 5C).

d. Open the Image Properties pane (ctrl-I) and verify the metadata has been correctly imported.

38. Stabilize the movie (Figures 5D and 5E). This step eliminates the translational drift of the movie, due to thermal artifacts or the suboptimal elimination of breathing movements and muscular spasms. Not all movies need to be stabilized, as translational drift can be acceptable or absent if the DSFC has been firmly fixed to the stage.
    a. Track static objects, such as tumor cells, collagen fibers, or blood vessels, using the Imaris Spot function applied to a specific region of interest. In Figure 5D, we tracked the green autofluorescence from tumor cells.
    b. Select only the tracks lasting for the entire movie.
    c. Select the Edit Tracks menu, and press the "Correct Drift" button. We select the options "Correct image and all objects", "Translational drift", and "New size equal to current size" (Figure 5E).
    d. Delete the tracks used for drift correction.
39. Define the position of the tumor (Figures 5F–5H). This step enables a separate analysis of T cells inside the tumor parenchyma or outside of it in the tumor stroma.[5]
    a. Select the Surface tool and press the "Skip automatic creation, edit manually" button.
        i. In the "Board" pane, select XY orientation, visibility: "Next", max resolution with preserved features (Figure 5F).
        ii. Press the button "Draw" and draw the tumor contour for the first slice. Move to the next slice and repeat.

    Note: The "Next" option selected in point i above allows to visualize the contour in the slice below the current one to facilitate the drawing of consistent contours.

        iii. Press the "Create Surface" button.
    b. Go to the Edit menu, select the surface just drawn, and press the "Duplicate to all Timepoints" button. This action will create a second set of surfaces, named "Surfaces 1 selection", whereby the original surface has been copied to all time points.
    c. In the Edit menu, press the "Mask All" button (Figure 5G). The "Mask Channel" dialog box will appear.
        i. Select any channel (in this example, we selected channel 1) and enable the "Duplicate channel before applying mask" option.
        ii. Set "Set voxel intensity outside surface" to 0, and "Set voxel intensity inside surface" to any positive nonzero value (we usually use 1500) (Figure 5G).

    Note: These operations will create a new virtual fluorescence channel representing the tumor (in this example, it is channel 4 "Tumor"). In subsequent analyses, cells with a nonzero fluorescence value in this channel are inside the tumor, while cells with 0 fluorescence are outside. A correctly drawn tumor surface is shown in Figure 5H.

    ⚠ CRITICAL: Make sure to select the option "Duplicate channel before applying mask". Failing to do so will remove the original channel, which contains actual fluorescence data.

    d. The surfaces just generated can now be deleted, as they demand significant computational power, create huge files, slow down analysis, and increase the risk of software crashes.
40. Automatically track T cells (Figures 6A and 6B).
    a. Use the "Spot" tool and select the options "Segment only a region of interest", "Process entire image finally", and "Track spots (over time)" in the wizard (Figure 6A).
    b. In the following step of the wizard, select a region of interest containing some T cells to be tracked. This region should contain bright cells and cells with lower fluorescence to ensure both are tracked.
    c. In the next step, select the source channel for tracking. In our example, we selected the red channel representing the fluorescence of tdTomato, which is constitutively expressed by Salsa6f-positive cells. Also, estimate the cell diameter. For tumor-associated lymphocytes, it is 11–16 μm.

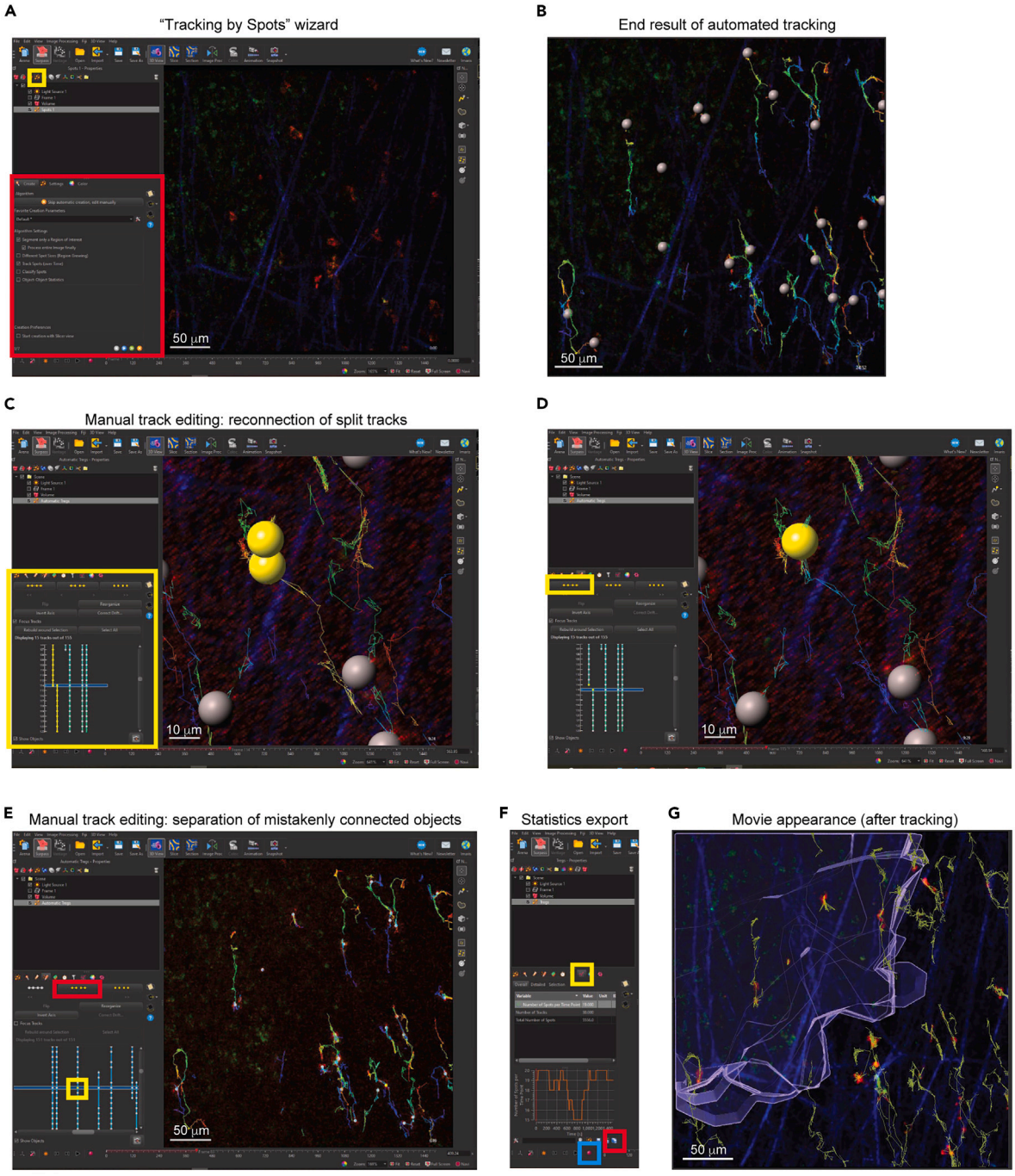


Figure 6. Tracking and statistics export from Imaris

(A and B) Automatic T cell tracking. (A) Press the Spots button (yellow box) and follow the instructions in the wizard (red box). Scale bar 50 μm. (B) Result of automatic tracking. Scale bar 50 μm.

(C) Visualization of an object duplication that led to track splitting. Note that one track ends in correspondence with the beginning of the other track in the Edit Track pane (yellow box). Scale bar 10 μm.

(D) Delete the duplicate object. The two tracks can now be joined by pressing the Join Tracks button (yellow box). Scale bar 10 μm.

(E) Separation of two track segments belonging to different cells. The yellow box highlights the object at which the tracks should be split. To separate the tracks, hit the "Split Track" button (red box). Scale bar 50 μm.

(F) To export statistics, select the "Statistics" pane (yellow box) and hit Export All Statistics (red box). Click on the record button (blue box) to export the movie.

(G) Final appearance of a correctly processed movie. Scale bar 50 μm. See also Video S1.

d. In the following step of the wizard, select values of "Quality" that ensure constant tracking of T cells at all movie time points. This goal can be easily achieved by playing the movie. If the diameter of the sphere is too big or too small compared to the cells, go back to the previous step and adjust the cell diameter.
e. In the subsequent step, maintain the "Autoregressive Motion" tracking algorithm. Initially, keep the suggested "Max Distance" and "Max Gap Size" parameters.
f. In the following step of the wizard, cell tracks appear in the region of interest. Review them carefully by playing the movie. If the cell trajectory is disconnected in several tracks, go back one step and adjust "Max Distance," which is the distance beyond which two subsequent spots will not be connected. Also, tweak the "Max Gap Size," which represents how many time points without a detected spot will be tolerated before another track is generated.
g. You may select tracks with specific characteristics in the following step of the wizard. However, we prefer not to do so to generate unbiased results.
h. In the following step of the wizard, extend the parameters to the whole movie by pressing on the green double arrow. The result is shown in Figure 6B.

Note: In our experience, automatic T cell tracking is never perfect. Aim for a reasonable level of precision to minimize subsequent manual editing of tracks.

41. Manually refine T cell tracks (Figures 6C–6E). This step is paramount because accurate cell tracking is critical for any downstream analysis of dynamic parameters. It is also a major determinant of the overall time needed for movie analysis.
    a. Track elimination. If a track does not belong to a T cell but to an autofluorescent element of the microenvironment, delete it by enabling the track selector (press "t"), clicking on the track to be erased, and hitting the "delete" key on the keyboard.

    Note: One may select multiple tracks by ctrl + click.

    b. Reconnection of split tracks. A track is split when the cell elongates beyond the estimated cell diameter set in step 40c, and the "Max Distance" value set in steps 40e and f. When this occurs, Imaris fits two spots within the same cell and generates a new track (Figure 6C). To fix this problem:
        i. Identify at which time point duplication occurred by selecting the two tracks. In the "Edit track" pane, the two tracks will appear yellow, and duplications become easily visible upon scanning time points (Figure 6C).
        ii. Activate the object selector (press "o") and select the duplicated object. Then, press the delete button on the keyboard to remove the wrong object.
        iii. Select the two objects at the extremity of the tracks you want to join and press the "connect objects" button (Figure 6D).

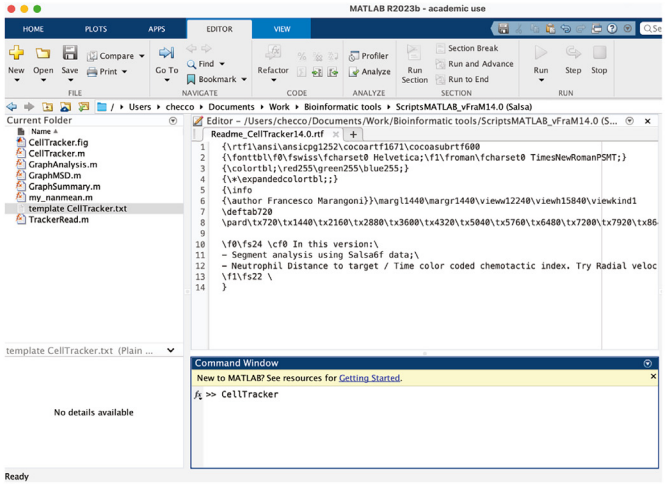
    Optional: Press the "Reorganize" button to display the newly connected track as a straight line.

    c. Separation of mistakenly connected objects (Figure 6E). This procedure is needed when part of the track belongs to the correct cell, and another part belongs to a different cell or an autofluorescent element of the environment.
        i. Identify at which time point the track becomes incorrect by playing the movie with tracks superimposed.
        ii. Activate the object selector (press "o") and select the first object that is incorrectly tracked.
        iii. Press the "Disconnect selected objects at current time point" (Figure 6E).
        iv. If the disconnected part of the track belongs to another cell, join it with the other relevant track segments. Otherwise, erase it.

A

	A	B	C	D	E	F	G	H	I	J	K
1	NAME	TrackID	Time	Position X	Position Y	Position Z	Salsa6f	Target 1 distance	Target 2 distance	Qualitative interaction Tumor	Qualitative interaction 2
2	1	1000000001	1	11607.9	11672.6	6669.3	2085.39	0	0	0	0
3	11105	1000000001	2	11609.5	11673.1	6667.48	2233.19	0	0	0	0
4	11125	1000000001	3	11609.4	11673.3	6669.42	1930.97	0	0	0	0

B



C

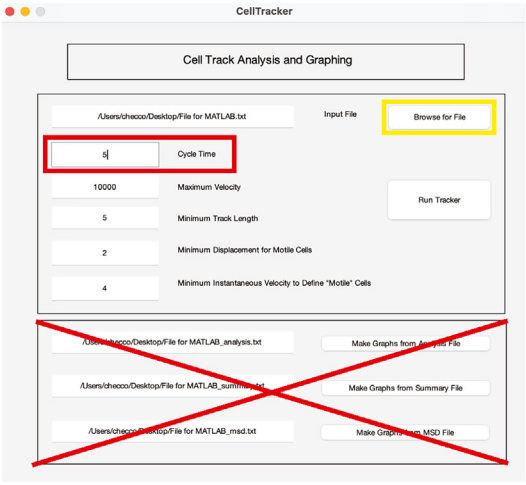


Figure 7. Parameter extraction using the Matlab "CellTracker" script

(A) Template of the Excel file for Matlab.

(B) From the Matlab environment, navigate to the folder containing the CellTracker scripts. Type "CellTracker" in the Matlab Command Window.

(C) In the CellTracker GUI, navigate to the file to analyze (yellow box) and set the Cycle Time, in this case 5 s (red box). Do not use the crossed-out options; these are deprecated options to produce graphs, which are no longer working. We customarily produce graphs of the desired parameters using Prism (GraphPad). See also Tables 2 and 3.

42. Export all parameters (Figure 6F).
    a. Select the "Statistics" pane.
    b. Press the "Export all Statistics to File" button.
43. Press the record button to export the movie (Figure 6F). The end result of a correctly tracked movie is shown in Figure 6G and Video S1.

Note: The short time interval necessary to capture Ca^{2+} dynamics leads to an overestimation of the instantaneous 3D velocity of the cells. This is because even a slight (e.g., 2 μm) imprecision in the positioning of the cell centroid every 5 seconds results in an apparent speed of 24 μm /min, much higher than the speed of T cells during interstitial motility (~10 μm/min). To solve this problem, 3D velocity should be calculated at one-minute intervals.

Quantification of motility and activation dynamics using MATLAB

Timing: Half an hour per movie

Here, we describe the Matlab script to facilitate the extraction of numerical data.[1]

44. Assemble the input file for Matlab analysis in Excel. Follow the template presented in Figure 7A and also deposited at Zenodo: https://doi.org/10.5281/zenodo.11122613.
    a. Go to the "Positions" tab in the Excel file containing all the exported statistics.
        i. Sort columns "TrackID", and "Time" in ascending order.
        ii. Copy the "ID" column of the statistics file to the "Name" column of the Excel file for Matlab.
        iii. Copy the "TrackID", "Time", "Position X", "Position Y", and "Position Z" columns of the statistics Excel file to the respective columns of the Excel file for Matlab.

b. Go to the tab "Intensity Mean Ch = 2 Img = 1".
   i. Sort columns "TrackID", and "Time" in ascending order.
   ii. Copy the content of the "Intensity mean" column into the "Salsa6f" column of the Excel file for Matlab.

Note: If photobleaching is present, we prefer to export the unprocessed green fluorescence values and calculate a Ca^{2+} signal baseline for each cell track.[1] If photobleaching is absent, one can calculate the ratio of green and red fluorescence (G/R ratio[3,9]) and report it in the "Salsa6f" column of the Excel file for Matlab. To calculate the G/R ratio, use the information in the tabs "Intensity Mean Ch = 2 Img = 1" and "Intensity Mean Ch = 3 Img = 1" of the statistics Excel file.

c. Fill the columns "Target 1 distance" and "Target 2 distance" with zeros. These columns may contain the distance (in μm) of the tracked cells to two distinct targets, e.g., antigen-presenting and control APCs,[5] if measured.
d. Go to the tab "Intensity Max Ch = 4 Img = 1".
   i. Sort columns "TrackID", and "Time" in ascending order.
   ii. Copy the content of the "Intensity max" column into the "Qualitative interaction Tumor" column of the Excel file for Matlab.
e. Fill the column "Qualitative interaction 2" with zeros. Analogously to the "Qualitative interaction Tumor" column, this column may contain discreet numerical values representing the interaction or not with a structure of the tumor environment (e.g., blood vessels).
f. Save the file as tab-delimited text.

⚠ CRITICAL: It is paramount to first sort for "TrackID" and "Time" and then copy the information on the Excel file for Matlab, because the object order may not be the same in distinct Excel tabs.

45. Run the CellTracker Matlab script. Troubleshooting problem 15.
    a. Open Matlab and navigate to the folder containing the scripts.
    b. In the Matlab command window, type "CellTracker" (Figure 7B). The CellTracker GUI appears.
    c. Set the correct parameters in the CellTracker GUI (Figure 7C).
       i. Click "Browse for File" and select the tab-delimited text for Matlab, created in step 44.
       ii. Set the "Cycle Time" to the interval between Z-stacks acquisition, expressed in seconds (in this example, it is 5 s).
       iii. Maintain the other parameters to the default value.

Note: "Maximum Velocity" splits a track in two if one step has a velocity higher than the one indicated. The "Minimum Track Length" parameter eliminates the tracks shorter than the stated value (in time points). "Minimum Displacement for Motile cells" is the displacement threshold (in μm) above which a cell is considered motile for calculating the Motile angle change. Finally, the "Minimum Instantaneous Velocity to Define Motile Cells" parameter is the velocity threshold (in μm/min) to calculate the track arrest coefficient (i.e., the percentage of time a cell is arrested).

    d. A prompt in the Matlab command window asks to insert the time (in minutes) to calculate the n-minute displacement. The n-minute displacement is a metric of cell confinement: it is 0 if the cell is completely arrested, and a higher value if it moves away from its initial position. In our recent characterization of tumor-Treg motility and activation dynamics,[6] we calculated a 10-min displacement.
    e. Hit the "Run Tracker" button. This action will generate the output files "[InputFileName]_Analysis", "[InputFileName]_Summary", and "[InputFileName]_MSD" in the same folder as the input file for Matlab created in step 44.

Table 2. List of parameters calculated in the Matlab output file "Analysis"

Parameter	Unit of measure	Column	Meaning
Track #	dimensionless	A	Unique identifier of a cell track.
Step #	dimensionless	B	Sequential integer (starting at 1) representing the step number within a track.
Centroid X-Z	μm	C-E	Position of each object in X, Y and Z.
Delta 3D distance Delta X-Z	μm	F G-I	Distance of the current object from the previous, in 3D and the individual dimensions.
Inst. 3D vel Inst. vel. X, Y, Z	μm/min	J K-M	Velocity between the current step and the previous, in 3D and the individual dimensions.
3D disp. Disp X, Y, Z	μm	N O-Q	Distance of the current object from the previous, in 3D and the individual dimensions.
Angle change	degrees	R	Angle between the current direction, and the direction at the previous step.
Inst. Vel. 2D	μm/min	S	Velocity between the current step and the previous, in 2D (XY).
Motile angle change	degrees	T	Angle between the current direction, and the direction at the previous step, if cells are considered motile. The displacement value to consider a cell motile is set in the CellTracker GUI ("Minimum Displacement for Motile Cells").
Time point	dimensionless	U	Absolute time point within the movie.
Delta Time	dimensionless	V	Distance, in time points, from the current to the previous object.
Signaling Index	Fluorescent units or dimensionless	W	Value of green fluorescence, or of the G/R ratio.
Target 1 (2) Distance	μm	X, Y	Distance of the object from a target.
Qualitative interaction Tumor	Fluorescence units	Z	A value of 0 indicates a cell outside the tumor, any value greater than 0 represents a cell inside the tumor.
Qualitative interaction 2	Fluorescence units	AA	Identical to "Qualitative interaction Tumor", but indicating if a cell is in contact or not with another structure in the tumor environment (e.g., blood vessels).
n-point displacement	μm	AB	Distance of the current object with the object detected after n minutes.
Segment ID	dimensionless	AC	Identifier of a detected signaling segment. If NaN, no segment is detected.[a]
Segment Duration	minutes	AD	Duration of a detected segment.[b]
Segment AUC	dimensionless	AE	Area under the curve of the detected segment.[b]
Peak Max Magnitude	Fluorescent units or dimensionless	AF	Highest value of fluorescence or G/R ratio within the peak.[b]
Peak Average Magnitude	Fluorescent units or dimensionless	AG	Mean value of fluorescence or G/R ratio within the peak.[b]

[a]To correctly visualize the segment ID, allow for the visualization of two decimal digits.
[b]Note that these values are associated with the first object of a detected segment only.

46. Interpret and use the output files.
    a. The "Analysis" file is a table that can be opened using Excel. Lines contain information about T cells at each time point, organized by track (column A) and the step number within the track (column B). At the end of a track, the symbol "—" separates it from the following track. Columns report the value of each parameter measured (see Table 2).
    b. The "Summary" file is a table containing parameters calculated on the entirety of a track. Each row is a different track, while the values of each parameter are reported in columns (see Table 3).
    c. The "MSD" file reports, for every track, the values of step size (in seconds), the mean displacement for the indicated time intervals D(t), and the mean squared displacement (MSD), which is the squared value of D(t). Plotting the MSD vs. time -or, analogously, D(t) vs. the square root of time- gives information on the cell's degree of confinement.[18,19]
    d. The desired parameters can be plotted using Prism (GraphPad).

EXPECTED OUTCOMES

Using the pipeline described above, we regularly and efficiently generate high-quality F-IVM footage that can be used to quantify hundreds of motility and activation parameters. Characteristics of a good movie include clarity, stability, minimal photobleaching, and movement of at least some

Table 3. List of parameters calculated in the Matlab output file "Summary"

Parameter	Unit of measure	Column	Meaning
Track #	dimensionless	A	Unique identifier of a cell track.
Total Dist. Total X, Y, Z	μm	B C-E	Measurement of the whole cell trajectory, in 3D and the individual dimensions.
Mean Vel. 3D Avg. Vel. X, Y, Z	μm / min	F G-I	Mean track speed in 3D and the individual dimensions.
Total disp. Total disp X, Y, Z	μm	J K-M	Distance from the last and the first object of the track, in 3D and the individual dimensions.
Avg. Angle change	degrees	N	Average turning angle within the track.
Confinement Ratio	dimensionless	O	Ratio between the distance and the displacement. It varies between 0 (if a cell comes back to its original position), and 1 (if a cell moves on a straight line).
Avg. Vel. 2D	μm / min	P	Mean track speed in 2D (XY).
Track Duration	minutes	Q	Track duration in minutes.
Arrest Coefficient	percent	R	Percentage of track duration a cell is arrested. The applied threshold to define a cell as motile is the one set in the CellTracker GUI ("Minimum Instantaneous Velocity to Define Motile Cells").
Avg. Signaling Index	Fluorescent units or dimensionless	S	Mean of the values of green fluorescence, or of the G/R ratio, within the track.
Track position	N/A	T	Gives a rough idea of the track trajectory relative to the tumor. "str" indicates the stroma (i.e., outside the tumor) and "par" the tumor parenchyma. For instance, "par-str" designates a track that starts in the tumor parenchyma but ends outside of it.
Percent time signaling	percent	U	Percentage of track duration a cell is activated.
Exceeded Max Allowed Velocity	N/A	V	An * indicates that the track has been split at a time point with instantaneous velocity higher than the "Maximum Velocity" limit set in the CellTracker GUI.

tumor-associated T cells at the speed they migrate within tissues, 5–10 μm/min (Video S1, Figures 6G and 8A). We also present a low-quality movie characterized by much autofluorescent debris, photobleaching, and minimal T cell movement (Video S2). The latter video is not suitable for quantification. Another key feature of our protocol is allowing repeated imaging of the same region of interest. An example of a correct identification of the same area on two subsequent imaging days is depicted in Figure 8B. We used our methodology to analyze TCR signaling in tumor-associated $CD8^+$ T cells (not shown) and Tregs[1] after administering checkpoint immunotherapy. Given the complexity of our protocol, we provide a sample dataset (correspondent to Video S1), including unprocessed and processed Imaris files and the complete Matlab analysis at Mendeley Data: https://doi.org/10.17632/t7tr42ztt5.2.

LIMITATIONS

A crucial benefit of the DSFC is the opportunity for longitudinal acquisition over several days, thus enabling the study of the impact of immunomodulatory drugs on the same area of the tumor environment. However, many factors can prevent the success of our protocol, including the skin being in the anagen phase, tumors growing too big, failure of the DSFC surgery, fuzziness within the chamber, lack of cell movement, and mice dying of anesthesia. Moreover, the quality of a perfectly placed DSFC decays over time, making it difficult to image a DSFC more than ten days after installation. For all these reasons, we always start the experiment with twice as many mice as we need to image. This structure allows us to select the best mice at each protocol step and compensate for mouse loss.

We acknowledge that the installation of the DSFC causes inflammation. For this reason, it is paramount that each dataset is accurately controlled: mice treated with immunomodulatory drugs should always be imaged together with identically prepared mice treated with an ineffective agent. Additionally, we do not image the DSFC immediately after installation but instead wait three days for inflammation to subside. We also cross-validate the results obtained with

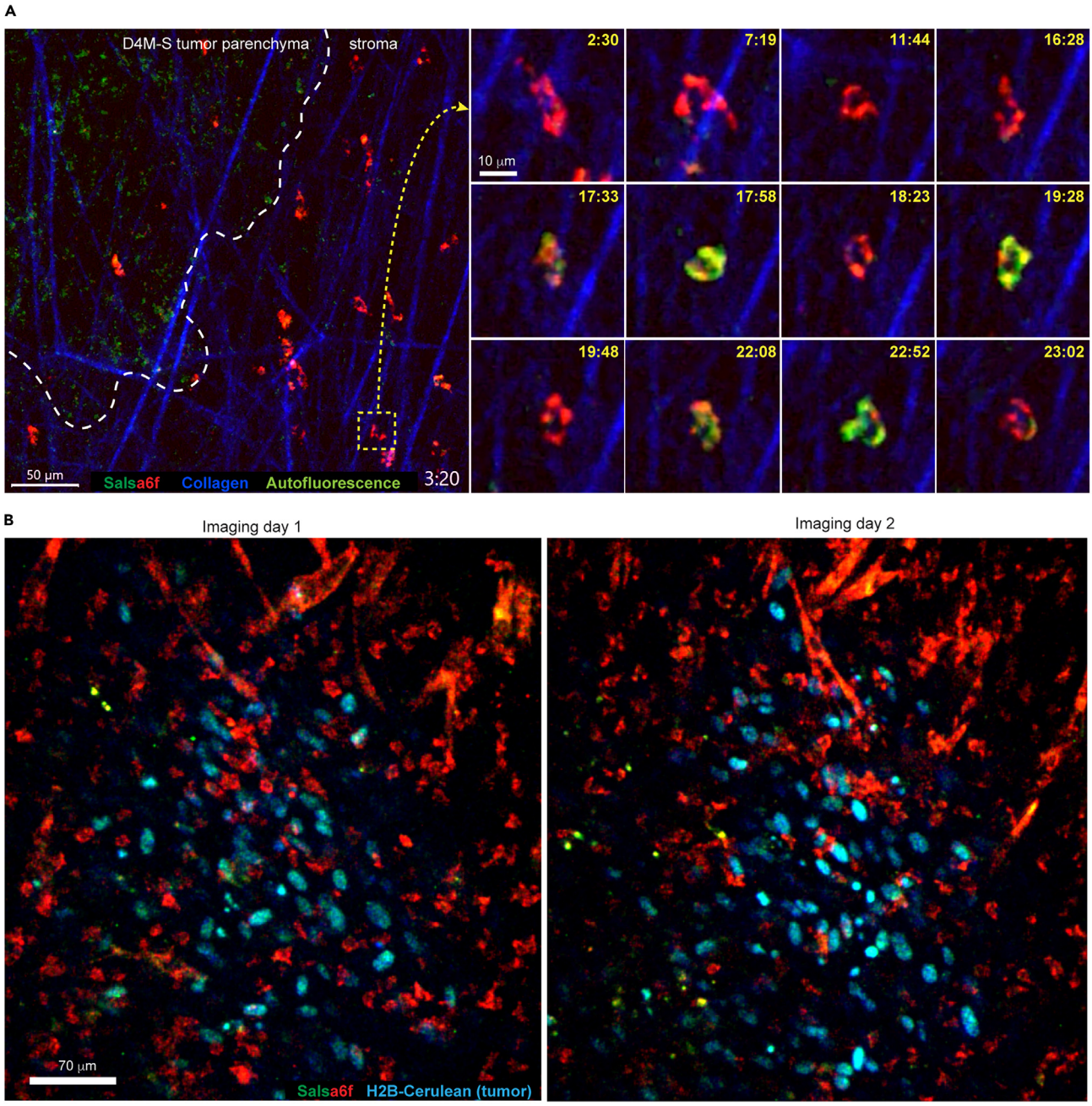


Figure 8. Expected results

(A) Micrograph of a D4M-S melanoma tumor growing in a tamoxifen-treated Foxp3^{CreERT2} x Rosa26$^{LSL\text{-}Salsa6f}$ mouse, and image sequence of a tumor-associated Treg undergoing several instances of activation. Time in min:sec. Scale bars 50 μm (full view) and 10 μm (image sequence). See also Video S1.

(B) Micrographs of an E8I^{Cre} x Rosa26$^{LSL\text{-}Salsa6f}$ mouse bearing a D4M H2B-Cerulean tumor, taken in two distinct imaging sessions one day apart. Note that we were able to locate the same region of interest. Scale bar 70 μm.

F-IVM using other technologies, including flow cytometry.[1] Another important consideration is that TCR triggering is the principal, but not the only inducer of Ca^{2+} signaling in T cells. Several other biological processes, including mechanosensation, elicit calcium transients. To specifically study the effects of drugs targeting TCR-mediated activation, it is crucial to subtract Ca^{2+} signaling in control mice from the one recorded in mice treated with the immunomodulatory drug being studied.

Lastly, the DSFC only gives access to tumors in the skin. For different organs, there are several other surgical preparations that allow for long-term imaging such as cranial, mammary, abdominal, lymph node, and kidney windows, each requiring specific expertise to install.[20–23]

TROUBLESHOOTING

Problem 1

Upon shaving, the skin appears thick and black (related to Step 9).

Potential solution

The mouse hair follicles are in the anagen phase, and the mouse is not suitable for imaging. Substitute with a mouse between 7 and 12 weeks of age, which is likely to be in the telogen hair follicle phase.[24]

Problem 2

The tumor occupies most of the DSFC (related to Step 17b i).

Potential solution

Tumors that are too big will impact the ability to install the dorsal skin fold chamber for imaging. Also, visualization of the interface between the tumor parenchyma and the surrounding stroma will become problematic. As a rule of thumb, do not use mice bearing a tumor whose largest diameter is more than half the diameter of the DSFC. In our case, the DSFC diameter is 12 mm, so we accept tumors up to 6 mm large.

Problem 3

When fixing the 0–80 screws and the spacers to the DSFC front, the spacers cannot be screwed all the way down because they run into the back side of the cover glass support (related to Step 20b).

Potential solution

File the spacers to make them fit the DSFC perfectly.

Problem 4

Excessive bleeding during skin removal (related to Step 24c).

Potential solution

When cutting through the dermis, try to cut as shallowly as possible to prevent blood vessels from rupturing. It may help to follow the vessels as they move to the outside of the chamber and cauterize there in addition to where the epidermis was cut. Gelatin-based hemostatic sponges can also be used.

Problem 5

The identification of the collagen layer to be removed is challenging (related to Step 24d).

Potential solution

Locate the white vessels that run perpendicularly to the spine using a stereoscope. Gently prod the tissue and look for these vessels moving in one layer together or in two layers separately. If there are two layers, then remove the topmost one.

Problem 6

The DSFC looks blurry or swollen upon visual inspection (related to Step 30).

Potential solution

- Minor fluid buildup: At times, some yellowish fluid can accumulate within the chamber. The fluid can be removed by gently squeezing or by removing the cover glass and cleaning the tissue. Make sure to wait 24–48 h before further imaging.

- Significant fluid buildup: The spacers may be too short, pinching the skin too tightly between the two halves of the chamber and resulting in severe tissue swelling. In this case, discard the mouse and use taller spacers in the next DSFC installations.
- Appearance of white fibrotic tissue. We observe this issue seven or more days after chamber installation. If the tissue prohibits observing the tumor environment, discard the mouse.
- Infection: Discard the mouse and sterilize the DSFC and the surgical instruments again. A heat sterilizer or an ethanol bath can be used during surgery to sterilize surgical tools.

Problem 7

The tissue within the DSFC becomes white upon securing the mouse to the aluminum bar (related to Step 31d).

Potential solution

The blood flow is interrupted because the putty is compressed between the DSFC and the aluminum bar. Remove the mouse from the aluminum bar and eliminate any excess putty.

Problem 8

The water leaks out from the vacuum grease/silicone O-ring (related to Step 31 h).

Potential solution

Press on the silicone O-ring with a wet cotton swab to remove small holes. If unsuccessful, reposition the vacuum grease and the silicone O-ring entirely, ensuring the DSFC surface is dry before applying the vacuum grease.

Problem 9

Photobleaching (related to Steps 32c and 33c ii).

Potential solution

We especially observe photobleaching of tdTomato, more rarely of the GFP. To minimize photobleaching, decrease the optical zoom factor to the minimum, and the percentage of applied laser power as much as possible to observe fluorescence.

Problem 10

The observation area is blurry (related to Step 32e).

Potential solution

- The tissue is distant from the cover glass: Replace the putty, gently increasing the pressure.
- Minor fluid buildup: The fluid can be removed by gently squeezing or by removing the cover glass and cleaning the tissue. Make sure to wait 24–48 h before further imaging.
- The cover glass is dirty: Clean the cover glass thoroughly with ethanol.
- The tumor is too deep into the dermis: Discard the mouse.

Problem 11

Cells do not move (related to Step 33g).

Potential solution

- The putty causes too much pressure: The putty's volume, as well as the tumor's volume, must not be higher than the hexagonal nuts on the back of the DSFC; otherwise, fixing the aluminum bar to the DSFC will create an overwhelming pressure in the chamber. The bloodstream can be used as a control: once the mouse is attached to the aluminum bar, the vascular system around the tumor must be visible to the naked eye.

- That specific tumor area is poorly vascularized: Select a different location. The morphology of T cells can be used as a proxy for movement: if all cells in an area appear round, they will probably be immotile. On the other hand, drop-shaped cells are likely motile.

Problem 12

The tissue drifts during acquisition (related to Step 33g).

Potential solution

- The mouse is loosely secured to the restraint system: Tighten all screws and nuts. Once fixed to the mouse restraint system, the DSFC should not move at all.
- The tumor environment is not adjacent to the cover glass: Reposition the putty, gently increasing the pressure.
- Thermal drift: Wait 20 min until the temperature of the aluminum bar, the tissue within the DSFC, and the objective equilibrate.

Problem 13

After being able to acquire part of a movie, the signal disappears (related to Step 33g).

Potential solution

Make sure water is not leaking from the vacuum grease/silicone O-ring on the DSFC. If necessary, replenish the water or reposition the vacuum grease and silicone O-ring.

Problem 14

The imaging areas from the first and second imaging sessions do not look alike (related to Step 36c).

Potential solution

- Return to the region of interest from the previous day using the recorded XYZ coordinates of the first or last layer.
- Some local tissue structures, such as blood vessels or collagen patterns, can also be used as an internal reference. Blood vessels can be visualized by the shadows they create among the fluorescent cells, or through the intravenous injection of a dye specifically designed for vascular structure visualization. We prefer not to use vessel counter coloring to not interfere with the Salsa6f but options include:
  - BSA-conjugated Evans blue.[25]
  - QDot counterstains such as QDot 655, Qdot 705 and QDot 800 from Thermo Fisher scientific (respectively #Q21021MP, #Q21061MP and #Q21071MP).

  Note: When using QDot 655, precautions must be taken to avoid fluorescence overlap with the tdTomato component of Salsa6f.

- During the first imaging session, it is possible to increase the laser power just enough to bleach a specific area, thereby creating a landmark for relocating to the same region over time. However, this technique is only applicable with fluorescent markers expressed by non-motile cells and doesn't apply to imaging Salsa6f. In addition, the second imaging session must occur before the cells generate new fluorescent molecules.
- Another method to locate the same area over multiple imaging sessions is to create sterile laser-induced damage. Please be aware that this process will cause some inflammation. The procedure is identical to setting up a video recording, yet with the following parameters:
  - Set the zoom factor to the maximum (in our microscope, 48X).
  - Turn off all PMTs. If your system does not allow acquisitions if all PMTs are off, set one to minimum gain (in our microscope, it is 0.5).
  - Adjust the Z step size to 4 μm.

- Adjust the begin and end Z-Positions in order to select 4 or more tissue layers.
- Minimize the time interval between Z-stacks.
- Set up acquisition of 3–4 stacks.
- Increase the laser power to 100% (we use a Mai Tai multiphoton laser at 2.3–2.4 W).
- Start acquisition.

Problem 15

The Matlab CellTracker script returns an error or empty output files (related to Step 45).

Potential solution

- The input file for Matlab needs to be correctly formatted.
- The input file for Matlab is saved in a format different than tab-delimited text.
- The TrackID and Time columns of the input file for Matlab are not sorted in ascending order.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Francesco Marangoni (f.marangoni@uci.edu).

Technical contact

Technical questions on executing this protocol should be directed to and will be answered by the technical contact, Claire Murat (cmurat@uci.edu).

Materials availability

The study did not generate new unique reagents.

Data and code availability

The imaging dataset used as an example in this paper is available at Mendeley Data: https://doi.org/10.17632/t7tr42ztt5.2. The Matlab code described in this study was also used in Geels et al. 2024[1] and is available at Zenodo: https://doi.org/10.5281/zenodo.11122613.

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AUTHOR CONTRIBUTIONS

S.N.G., C.M., and A.M. performed experiments and analyzed data; S.O. performed experiments and supervised movie analysis; F.M. conceived the project, performed experiments, and analyzed data. All authors wrote the paper.

DECLARATION OF INTERESTS

The authors declare no competing interests.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2024.103310.

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