Chapter 4

Intravital Microscopy Analysis of Hepatic T Cell Dynamics

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Abstract

T cells play critical roles in controlling hepatotropic viral infections and liver tumors. The protective capacity of these cells is mediated by antigen-experienced effector cells and depends on their ability to migrate to and traffic within the liver, recognize pathogen- or tumor-derived antigens, get activated and deploy effector functions.

While some of the rules that characterize T cell behavior in the healthy and cancerous antigen-expressing liver have been characterized at the population level, we have only limited knowledge of the precise dynamics of T cell interactions with different kinds of liver cells at the single-cell level. Here, we describe in detail an intravital microscopy technique that allows the analysis of T cell dynamic behavior in the liver of anesthetized mice at high spatial and temporal resolution. A detailed understanding of the spatiotemporal dynamics of T cells within the liver is important for the rational design of targeted immunotherapeutic approaches for chronic liver infections and tumors.

Key words Multiphoton intravital microscopy, In vivo imaging, Liver, CD8+ T cells, Lymphocyte motility

1 Introduction

Intravital microscopy allows for the direct visualization of lymphocyte behavior in their physiological tissue environment at cellular and subcellular resolution. This technique has been utilized in the last several years to understand the dynamic events leading to the activation, expansion and effector differentiation of naïve T cells within secondary lymphoid organs [1, 2]. By contrast, less is known on the spatiotemporal aspects that govern T cell migration and function at peripheral sites, such as the liver.

Here we detail an intravital microscopy method to study T cell dynamics within the liver at the single-cell level [3–5]. The protocol begins with T cell isolation, purification, and eventual differentiation into effector cells, T cell labeling with fluorescent dyes and subsequent intravenous injection into antigen (Ag)-expressing recipient mice. It then provides a step-by-step surgical procedure to expose the liver for intravital microscopy using an inverted

microscope setup, accompanied by protocols validated by our group to image several hepatic cells utilizing commercially available reagents. Finally, it describes the basic analyses of intravital microscopy movies to compute several motility parameters.

2 Materials

*2.1 Preparation and Injection of T Lymphocytes to be Tracked In Vivo*

1. Spleen and lymph nodes from donor wild type or TCR transgenic mice (e.g., P14 [6], OT-I [7], BC10 [3, 8]).
2. Immunomagnetic cell purification kit for $CD4^+$ or $CD8^+$ T cells (e.g., mouse CD8+ T cell negative selection kit from Miltenyi Biotec).
3. RPMI 1640 medium.
4. Recombinant IL-2 or EL-4 supernatant [9].
5. 5-chloromethylfluorescein diacetate (CMFDA, CellTracker™ Green; Thermo Fisher Scientific).
6. CellTracker™ Red (CMTPX; Thermo Fisher Scientific).
7. 5-(and-6)-(((4-chloromethyl)benzoyl)amino)tetramethylrhodamine (CMTMR, CellTracker™ Orange; Thermo Fisher Scientific).
8. CellTracker™ Deep Red (Thermo Fisher Scientific).
9. 6-((((4,4-difluoro-5-(2-thienyl)-4-bora-3a,4a-diaza-s-indacene-3-yl)styryloxy)acetyl)aminohexanoic acid (BODIPY 630/650-X) (Thermo Fisher Scientific).
10. Phosphate buffered saline (PBS).
11. Complete RPMI 1640: 10% FBS, 2 mM l-glutamine, 50 μM 2-mercaptoethanol, 10 mM HEPES, 100 μM nonessential amino acids, penicillin plus streptomycin.

2.2 Surgical Anesthesia

1. Isoflurane (Iso-Vet, Piramal Healthcare) (*see* **Note 1**).
2. Anesthetic vaporizers (G.A.S. General Anesthetic Services Ltd).
3. Oxygen supply connected to the isoflurane vaporizer.

2.3 Surgical Preparation of the Mouse Liver for Intravital Microscopy Using an Inverted Microscope Setup (Fig. 1)

1. Custom-built stage (specifics and dimensions are provided in Fig. 1a).
2. Mouse restrainer (e.g., Tailveiner Restrainer from Braintree Scientific).
3. Small animal clipper (e.g., Pocket Pro from Wahl).
4. Medical adhesive tape (e.g., Durapore™ 3M).
5. Hair removal cream (e.g., Veet).
6. 15 cm cotton tipped applicators (e.g., Heinz Herenz).
7. Gauze sponges (e.g., Heinz Herenz).
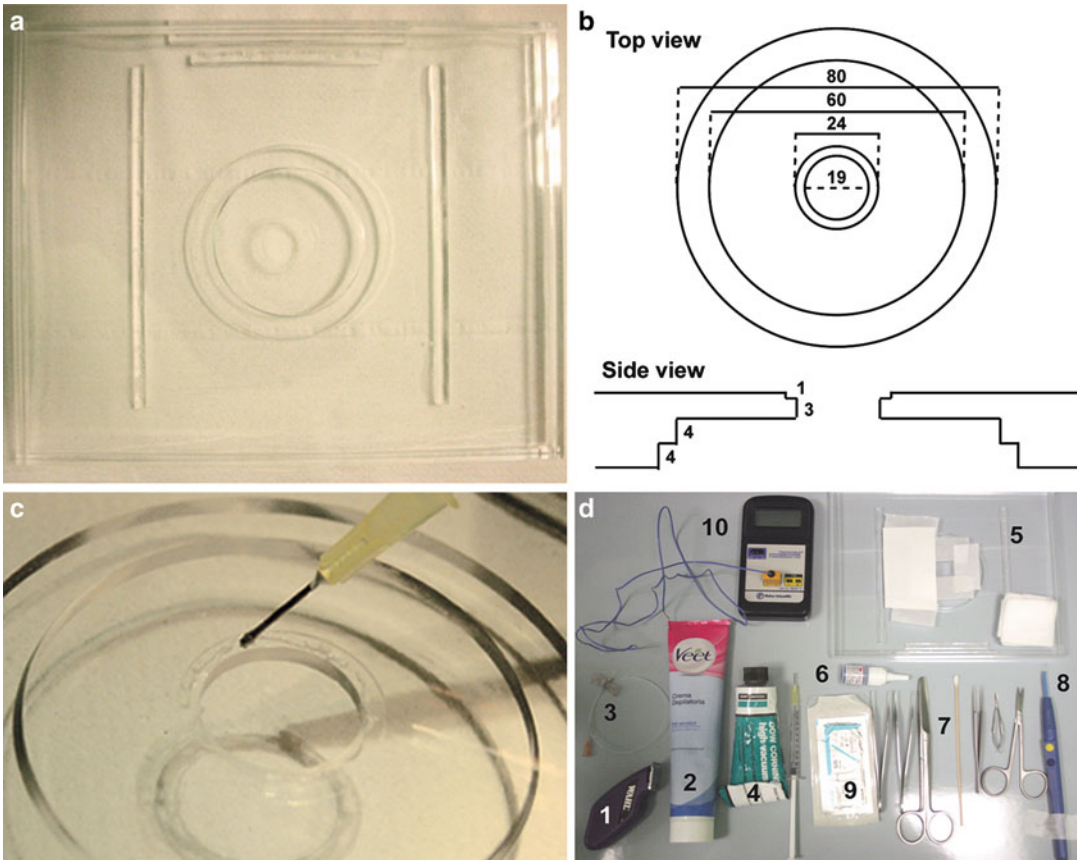
Fig. 1 Equipment. (**a**) Custom-built microscope stage. (**b**) Schematic view of the microscope stage with exact dimensions (in cm). (**c**) Application of a thin layer of grease on the perimeter of the coverslip holder. (**d**) *1*: Clipper; *2*: Hair removal cream; *3*: Tail vein catheter set; *4*: Vacuum grease; *5*: Final stage with coverslip and a rectangle (4 × 10 cm) of blotting paper; *6*: Tissue glue; *7*: Surgical instruments; *8*: Electrocautery device; *9*: Suture thread; *10*: Digital thermometer with probe

8. Precision Wipes (11 × 21 cm, Kimtech Science, Kimberly-Clark Professional).

9. Spring scissors (Cohan-Vannas 6 mm blade).

10. Extra thin iris scissors (10.5 cm).

11. Dumont #2 laminectomy forceps Inox.

12. Adson forceps (12 cm).

13. Standard pattern scissors (large loops, sharp/blunt 14.5 cm).

14. Suture thread: 5-0 braided silk (suture size), C-1 (needle type), 13.1 mm (needle length), 3/8c (needle shape) (Johnson-Johnson Intl.).

15. Tissue glue (e.g., Vetbond™ from 3M).

16. Round glass coverslips (24 mm in diameter, 0.17 mm thickness, Ted Pella Inc).

17. High-viscosity vacuum grease (e.g., Dow Corning).

18. Digital thermometer (e.g., Traceable expanded-range thermometer).

19. Miniature K type thermocouples (e.g., 5SC-TT-K-40-72 from Omega).

20. Electrocautery device (Surtron).

21. Tail vein catheter (e.g., Vevo MicroMarker™ from Fujifilm VisualSonic).

22. Blotting paper.

23. Plastic wrap.

24. 0.9% saline solution.

2.4 Multiphoton Intravital Microscopy of the Mouse Liver Using an Inverted Microscope Setup

1. Inverted multiphoton microscope (*see* **Notes 2** and **3**) equipped with: (a) at least one (preferably two) tunable femtosecond (fs)-pulsed Ti:Sa lasers (e.g., 680–1080 nm, 120 fs pulse-width, 80 MHz repetition rate, Ultra II, Coherent); (b) an optional Optical Parametric Oscillator (e.g., 1000–1600 nm, 200 fs pulse-width, 80 MHz repetition rate, Chameleon Compact OPO, Coherent); (c) at least two (ideally four or five) non-descanned photomultiplier tubes (e.g., Hamamatsu H7422-40 GaAsP High Sensitivity PMTs and Hamamatsu H7422-50 GaAsP High Sensitivity red-extended PMT from Hamamatsu Photonics K.K.); (d) a high numerical aperture, water-immersion multiphoton objective (preferably Olympus ref: XLPLN25XWMP2, 25×, 1.05 NA, 2 mm working distance).

2. Mercury arc lamp (e.g., HG Precentered Fiber Illuminator—Intensilight from Nikon).

3. Custom-made thermostatic chamber that surrounds the entire microscope with the exclusion of the scanhead (e.g., The Box, Life Imaging Services).

4. Digital thermometer (Traceable expanded-range thermometer, Fisher Scientific).

5. Fluorescently labeled anti-F4/80 antibody (clone BM8).

6. Fluorescently labeled anti-CD49 antibody (clone HMα2).

7. Hoescht 33342 (Thermo Fisher Scientific).

8. Qtracker vascular label (Thermo Fisher Scientific).

9. SYTOX dye (Thermo Fisher Scientific).

2.5 Image Acquisition, Processing and Analysis

1. Computer workstation with high graphics processing capability (e.g., Apple Inc Mac Pro computer model: Processor: 2 × 2.4 GHz Quad-core Intel Xeon or higher, Memory: 32GB 1066 MHz DDR3 ECC, Graphics: ATI Radeon HD 5870 1024 MB, System: MAC OS × Lion 10.7 or higher).

2. Image analysis software (e.g., Imaris; Volocity, Improvision; ImageJ, National Institute of Health).

3. Custom-made scripts (Matlab, Mathworks, or Python) for more complicated analyses.

3 Methods

3.1 Preparation and Injection of T Cells to Be Tracked In Vivo

1. Purify CD8^{+} T cells from lymph nodes and spleens of wild-type or T cell receptor (TCR) transgenic mice by negative immunomagnetic selection, according to the manufacturer's instructions.

2. (Optional) To generate effector CD8^{+} T cells [3, 10], incubate purified T cells from TCR transgenic mice with 10 μg/ml of cognate peptide at 37 °C for 1 h, washed, and cultured in complete RPMI 1640 medium. Two days later, replace with fresh medium supplemented with 20 ng/ml of recombinant IL-2 or with 2.5 % (vol/vol) EL-4 supernatant. Replace medium supplemented with cytokines every 2 days. After 7–9 days of culture, test the expression of CD8, CD69, CD25, CD44, CD62L, CCR7, IFN-γ, and granzyme B by flow cytometry prior to subsequent use, as described [3].

3. Wash naïve or effector CD8^{+} T cells on pre-warmed serum-free RPMI to remove residual FBS.

4. Incubate cells at 1×10^7 cells/ml and stained with 2.5 μM CMFDA, 7.5 μM CMTPX, 10 μM CMTMR, CellTracker™ Deep Red or 2.5 μM BODIPY 630/650-X for 20 min in the dark at 37 °C in plain pre-warmed RPMI 1640. Stop the reaction by adding 2 mL of FBS, which binds residual protein-binding dyes.

5. Wash cells twice with pre-warmed RPMI and resuspend in PBS at the desired concentration for injection (typically between 5×10^6 and 2×10^7 per cell population). As an alternative to cell staining with fluorescent organic dyes, T cells from mice expressing a fluorescent protein (e.g., green fluorescent protein or its variants) under a ubiquitous or T cell-specific promoter can be used.

6. Inject fluorescently labeled naïve or effector CD8^{+} T cells intravenously into recipient mice through the tail vein. Alternatively, inject cells during the imaging session through a tail vein catheter (*see* later at Subheading 3.2, **step 3** for the detailed procedure). Recipient mice typically would express the relevant cognate antigen(s) in the liver because they are transgenic mice (e.g., HBV replication-competent transgenic mice [3, 11]), because they have been transduced with hepatotropic viral vectors (e.g., adenoviruses or adeno-associated viruses [3, 12]) or because they have been infected with hepatotropic viruses (e.g., LCMV [3]).

*3.2 Microscope Setup and Presurgery Preparations*

1. Turn the system on (set the thermal chamber at 37 °C, turn on the computer, lasers, mercury arc lamp, open the oxygen supply connected to the isoflurane vaporizer system and check the isoflurane volume).

2. Stage preparation. Apply a thin layer of vacuum grease on the perimeter of the coverslip holder and gently place the coverslip on top of it (Fig. 1c). Secure the coverslip on each side with tape (Fig. 1d). Finally place on the left side of the coverslip holder a rectangle (~4 × 10 cm) of blotting paper (Fig. 1d). This paper will ultimately be positioned underneath the mouse to absorb any fluid leakage that could interfere with imaging.

3. Mouse shaving (*see* **Note 4**). The abdomen, back, and both flanks of the mouse are shaved using the clipper and the hair removal cream. Thoroughly wipe off the cream using moist gauzes. The goal is to remove hair entirely as they are very autofluorescent and therefore can interfere with imaging.

4. Tail vein catheterization (Fig. 2a). Fill the polyethylene catheter with saline solution, making sure to avoid bubble formation. Place the mouse into the restrainer and dilate the tail veins either by dipping the tail in warm water (~35–40 °C) for ~30 s or by using a heat lamp. Introduce the needle with the bevel uppermost into either tail vein and check the correct placement by saline injection (there should be almost no resistance when the syringe plunger is depressed and the vein should be flushed clear of blood as the injection is made). A drop of tissue glue on and around the needle is used to secure the intravenous line. Additional tape can be applied to ensure proper stabilization.

3.3 Surgical Anesthesia

Anesthetize the mouse with 5 % isoflurane through a nose cone also delivering oxygen at 1 L min^{-1} (*see* **Note 1**). Adequate anesthesia is achieved if the mouse does not react to firm pinching of the footpad. Perform follow-up surgery and liver intravital imaging with lower concentrations of isoflurane (between 0.8 and 1 %). Adjust anesthesia based on breathing rate (~55–65 breaths per minute).

3.4 Surgical Preparation for Liver Intravital Microscopy

1. Position the mouse on the stage by using adhesive tape to secure the nose cone and upper legs.

2. Open the skin by performing a midline incision and carefully detach peritoneal adherences (Fig. 2b). From this moment onwards, keep the surgical area moist by applying small quantities of saline solution (0.9 % NaCl).

3. Cauterize the major subcutaneous vessels prior to performing left and right subcostal incisions to expose a large peritoneal surface (Fig. 2c).

4. Perform midline and left subcostal incisions in the peritoneum through a high-temperature cautery (Fig. 2d). The left subcostal incision should be carried out along the ribcage all the way down to the spleen.
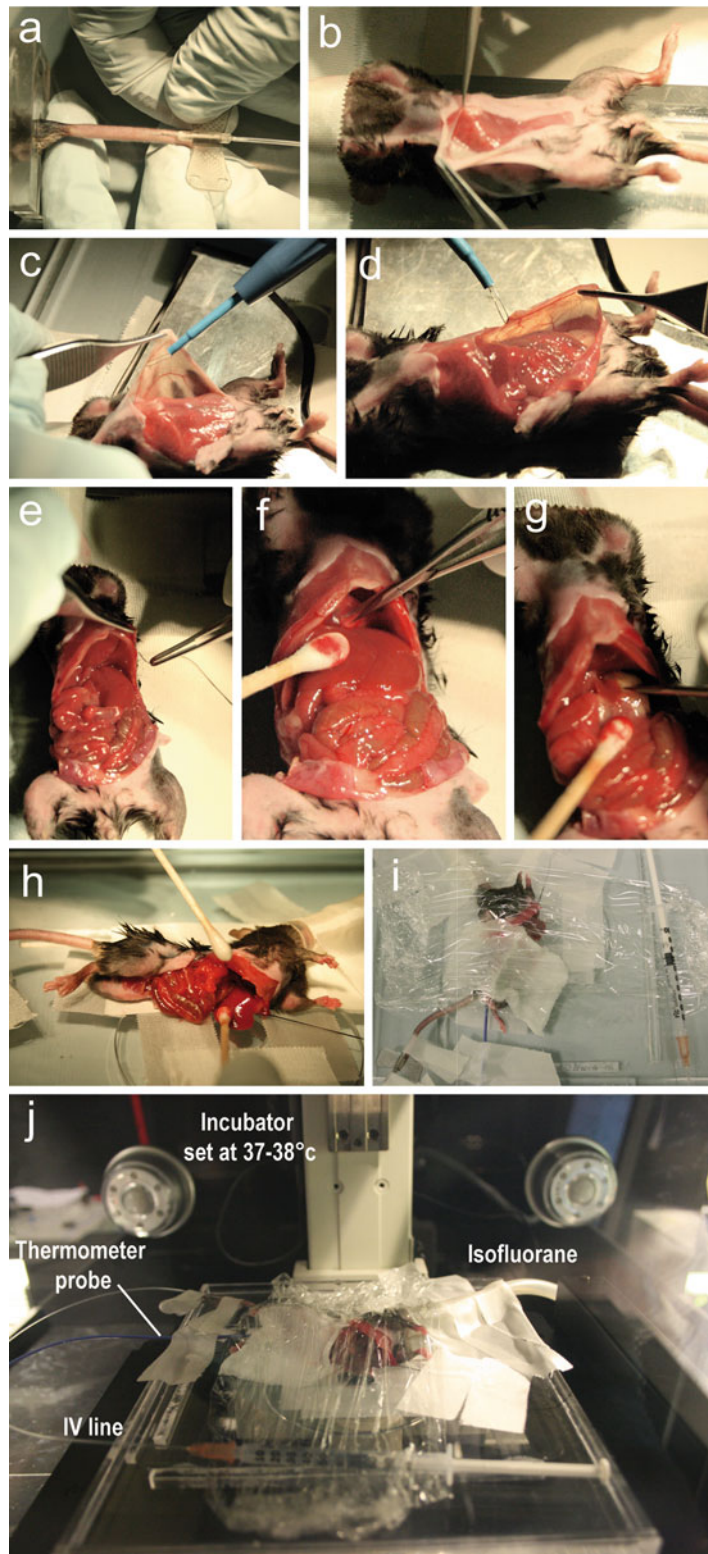
Fig. 2 Surgical procedure. (**a**) Insertion of the tail vein catheter. (**b**) Removal of subcutaneous adhesions. (**c**) Cauterization of the major subcutaneous vessels prior to performing left and right subcostal incisions. (**d**) Left subcostal incisions in the peritoneum through a high-temperature cautery. (**e**) Application of a suture thread on the left side of the peritoneum. (**f**) Resection of the falciform ligament. (**g**) Resection of the hepatogastric ligament. (**h**) Positioning of the liver lobe on the coverslip. (**i**) The preparation is covered by a plastic film to minimize heat loss. (**j**) Final positioning in the intravital microscope

5. By gently pulling on the xiphoid process, expose and cut the falciform ligament with small spring scissors, making sure to preserve the diaphragm integrity (Fig. 2f).

6. Cut the hepatogastric ligament between the stomach and the liver (Fig. 2g).

7. Place a suture thread on the left side of the peritoneum (approximately half way between the xyphoid process and the spleen, Fig. 2e).

8. Place the mouse in a left lateral position and gently exteriorize the left liver lobe onto the coverslip by simultaneously gently pulling on the suture thread (Fig. 2h) to separate the diaphragm from the left liver lobe, thus preventing transmission of breathing movements (*see* **Note 5**).

9. The suture thread is secured with the adhesive tape.

10. The liver lobe is held in place and stabilized by a small piece of plastic wrap (small enough to avoid contact with the rib cage, thereby limiting the transmission of breathing movements).

11. Internal organs are gently separated from the liver and kept moist using wet gauzes previously soaked with warm PBS.

12. Lubricate the temperature probe with vacuum grease prior to inserting into the mouse rectum.

13. Seal the whole preparation with plastic wrap to avoid heat loss and dehydration (Fig. 2i).

14. Transfer the stage to the intravital microscope into the 37 °C pre-warmed thermal chamber (Fig. 2j).

3.5 Image Acquisition

1. Check laser beam alignment according to the manufacturer's instructions. Adjust wavelengths of the laser beams according to the fluorescent dyes used in the experiment (two photon excitation spectra of most commonly used fluorescent probes can be found for example at http://goo.gl/4qYhWx). Typically, we tune the two Ti:Sa lasers at 800 nm and 900–930 nm, respectively, and the OPO at 1200 nm. This setup allows us to excite most fluorescent molecules whose emission range from ~450 to ~700 nm. Spectral separation is achieved by dichromatic mirrors and bandpass filters in front of each PMTs. Again, selection of filters should be guided by the fluorescent molecules used in the experiment. Our setup includes the following filters: 455/50 nm (to detect e.g., second harmonic, CFP, CMAC, Hoechst), 525/50 nm (to detect e.g., eGFP, CFSE, CMFDA), 590/50 nm (to detect e.g., CMTMR, dsRed), and 665/50 nm (to detect e.g., BODIPY, qDots 655).

2. As lymphocyte migration is highly temperature dependent, keep the thermostatic chamber at 37–38 °C and continuously monitor the mouse body temperature through a rectal probe to ensure that a narrow range of 37–38 °C is maintained at all time.

3. Using the ocular and the mercury lamp illumination, scan the overall preparation and choose an adequately perfused area with minimal tissue perturbation.

4. Before starting image acquisition, adjust laser power, PMT gain, offset, etc. in order to get the best possible signal-to-noise ratio. We suggest to acquire a test short time-lapse recording, in order to check stability of the preparation (with particular attention to z-drift), blood flow and cell motility.

5. Once an area of interest is found, begin actual recording. Typically, we found that stacks of 7–9 square x y sections (512×512 pixel) sampled with 4 μm z spacing acquired every 5–10 s for a period of ~60 min provide a sufficient amount of cell tracks for meaningful statistical analyses. Our experimental setup allows for continuous recording for at least 4–5 h.

6. Depending on the experimental question, liver sinusoids, several hepatic resident and infiltrating leukocytes and cell death can be visualized thanks to the intravenous injection of commercially available antibodies and probes (Fig. 3) (*see* **Note 6**). The hepatic vascular structure is directly visualized thanks to the injection of 10 μL of fluorescently labeled non-targeted quantum dots (e.g., Qtracker 655). Kupffer cells can be visualized thanks to the injection of 1 μg of fluorescently labeled (e.g., PE) anti-F4/80 antibody. Platelets can be visualized thanks to the injection of 3 μg of fluorescently labeled (e.g., FITC) anti-CD49b antibody. Cell nuclei can be visualized thanks to the injection of 10 μL of Hoechst 3342. Hepatocytes can be easily distinguished from other nonparenchymal cells or infiltrating leukocytes based on size and ploidy of the nuclei. Cell death can be visualized thanks to the injection of 4 mL of fluorescent Sytox (e.g., Sytox green).

3.6 Image Processing and Data Analysis

Initially, the sequences of image stacks are transformed into volume-rendered 4D time-lapse movies with image analysis software (e.g., Imaris Bitplane or Fiji ImageJ) (Fig. 4a). The 3D positions of the cell centroids are segmented by semi-automated cell tracking algorithm from Imaris or using the TrackMate Fiji plugin.

3.7 Qualitative Analysis

Examine migratory behavior of the cell population by plotting the trajectories of the tracked cell centroids over time in two dimensions. Shift the first position of each individual cell to the same starting point in space while maintaining its orientation (Fig. 4b), allowing to assess whether cells are traveling in a preferred direction. If all possible directions are equally covered, this indicates that motion is random on the timescale of the duration of the plotted tracks (plotting tracks only give qualitative information).

3.8 Quantitative Analysis

Several different motility parameters can be quantified, including:
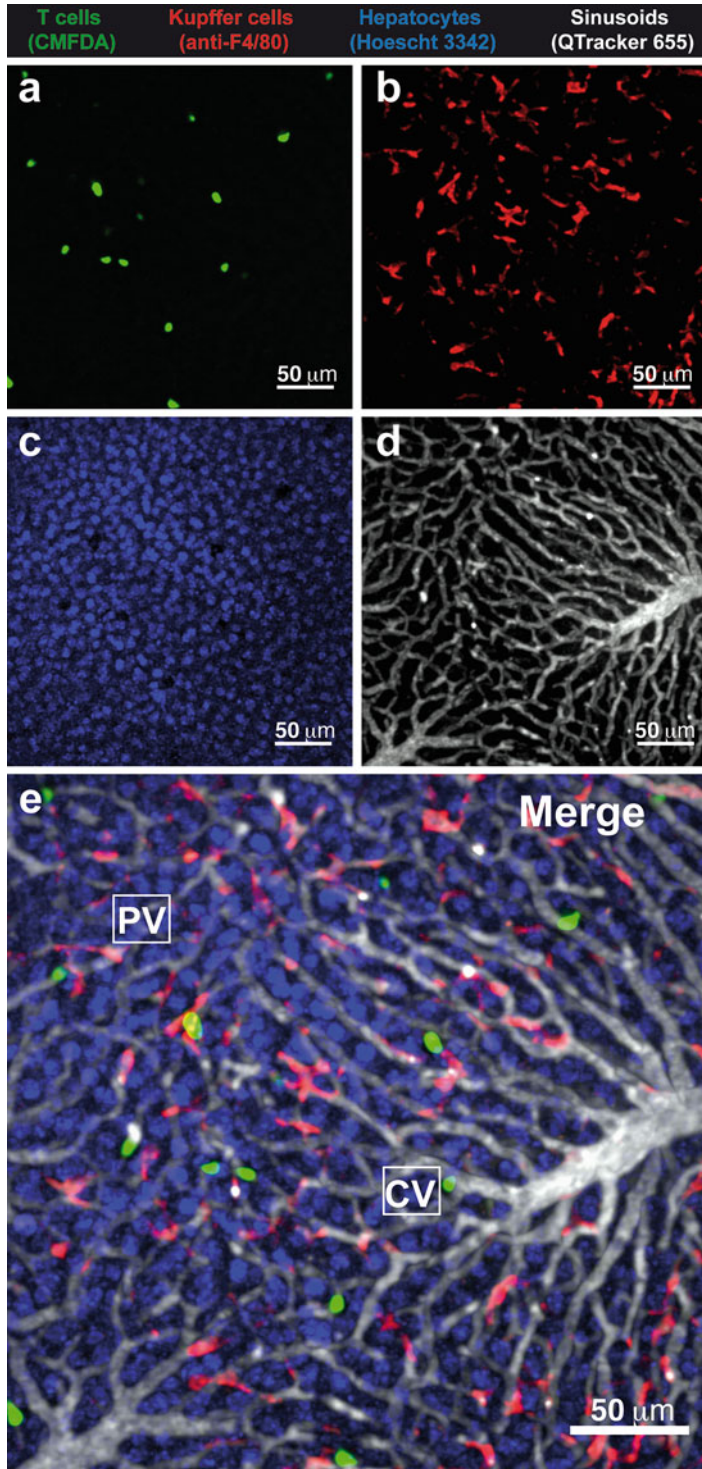
Fig. 3 Intravital multiphoton microscopy of the mouse liver. (**a**–**d**) Intravital single-channel micrographs of the liver of a mouse injected with T cells. (**a**) CMFDA-labeled T cells; (**b**) Kupffer cells (stained by the injection of PE-labeled anti-F4/80 antibody); (**c**) Hepatocyte nuclei (stained by the injection of Hoechst 3342); (**d**) Liver blood circulation (visualized thanks to the injection of Qtracker 655). (**e**) Merged image. *CV* central vein; *PV* Portal vein. Scale bars represent 50 μm

1. Mean speed (Fig. 4c): mean velocity of a cell over several time steps (usually the entire imaging period, in μm/min).

2. Displacement (Fig. 4d): distance between the first and the last imaging point (in μm).

3. Straightness (Fig. 4e): The ratio between the displacement and the total track length. It is a measure of the straightness or confinement of cell tracks. It can vary between 0 (if the cell returns to the exact position where it started) and 1 (in case of a perfectly straight cell track).

4. Instantaneous speed (Fig. 4f): the ratio between the displacement and the interval time between frames (in μm/min).

5. Contact time: the duration of physical association between two or more tracked particles (in min).

3.9 Morphological Analysis

It is also possible to analyze dynamically the morphological features of a cell. The images can be analyzed with software like Fiji to quantify several parameters that give information about a cell shape:

1. Perimeter: the length of the path that surrounds the cell shape.

2. Surface area: the total area encompassing the perimeter of the outer cell layer.

3. Circularity: $(4\pi \times \text{area})/\text{perimeter}^2$. A value of 1.0 indicates a perfect circle. As the value approaches 0, it indicates an increasingly elongated shape.

4. Shape index: ratio between the long and the short axis of the cell. It gives information about cell polarization.

Pseudopods of migrating cells can be identified with skeletonization, a technique that reduces a binary shape into a series of connected lines (the skeleton) (Fig. 4g). The temporal evolution of the skeleton highlights the dynamic nature of the cell protrusions. The software Fiji provides a plugin (AnalyzeSkeleton or Skeletonize3D) that tags all pixels in a skeleton image and calculates the total length of the skeleton and the number and length of each junction and branch (Fig. 4g) for every image in the temporal stack.

4 Notes

1. Although injectable anesthetics can be used in place of inhalational anesthetics, we favor the latter ones because they can be adjusted quickly as needed during the experiment, they allow for longer imaging sessions and for more physiological heart rate, breathing rate, and hemodynamic parameters (which are particularly critical when studying intravascular events).

2. Although we have also devised a liver intravital microscopy model suitable for an upright microscope setting [13], we favor
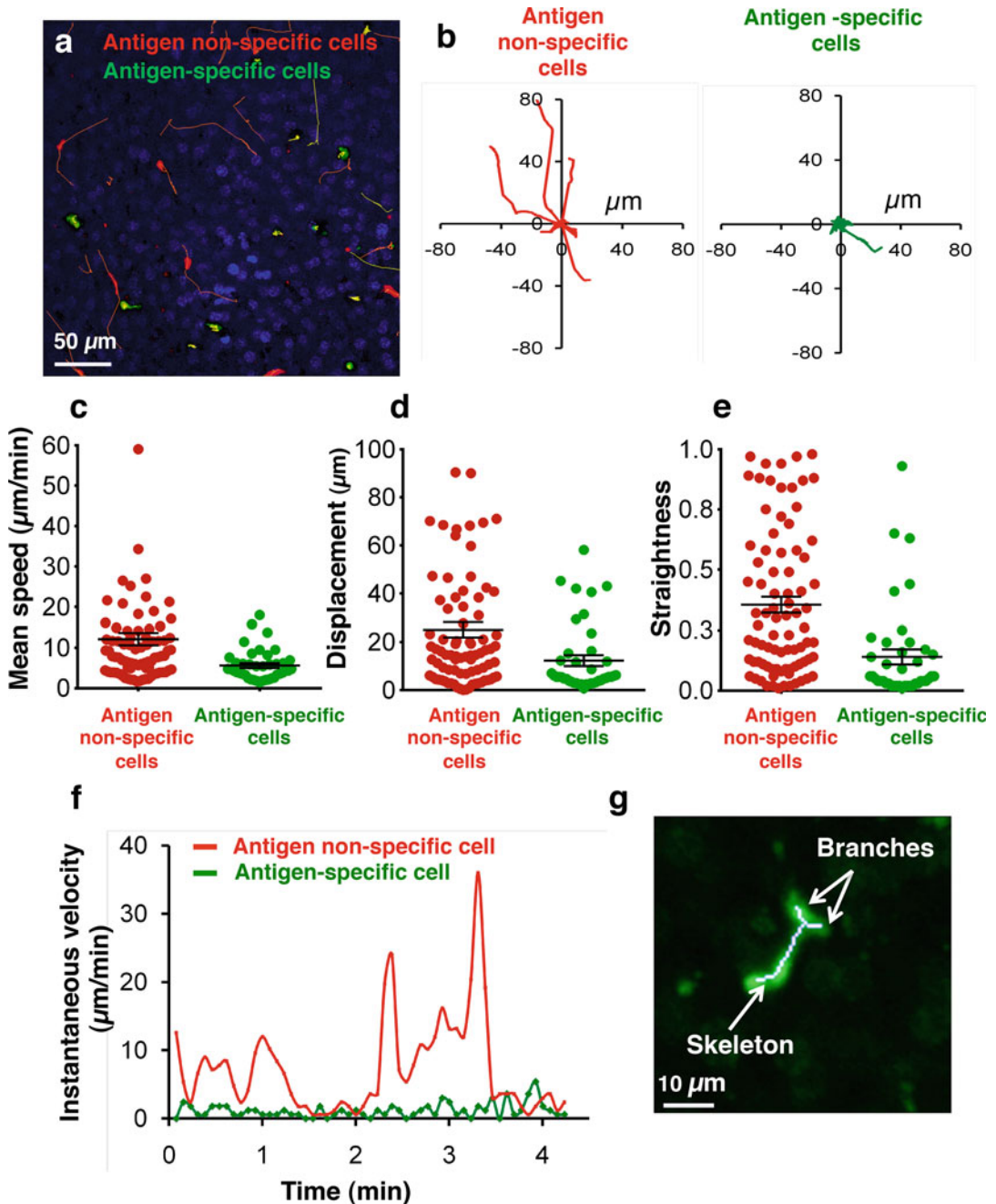
Fig. 4 Image analyses. (**a**) Cell tracks in an intravital multiphoton microscopy movie in the liver of an hepatitis B virus (HBV) replication competent transgenic mouse transferred with antigen-specific (*green*) or antigen nonspecific (*red*) T cells. Hepatocyte nuclei are depicted in *blue*. Scale bar represents 50 μm. (**b**) Track plots starting from the same origin of antigen non-specific (*red*, *left panel*) and antigen-specific (*green*, *right panel*) T cells. (**c**–**e**) Mean speed (**c**), displacement (**d**), and straightness (**e**) of individual antigen non-specific (*red*) and antigen-specific (*green*) T cells. (**f**) Instantaneous speed for individual antigen non-specific (*red*) and antigen-specific (*green*) T cells. (**g**) Example of the skeletonization of a T cell

the procedure detailed in this review as it is easier to perform, it generally offers a more stable preparation and it enables imaging over a greater liver surface area.

3. A spinning disk confocal system can be used in place of a multiphoton microscope.

4. As mouse shaving requires the use of isoflurane, we usually perform it a day before the imaging session (in order to limit isoflurane exposure right before the surgery). It is important to thoroughly rinse off the hair removal cream and hair fragments with PBS as they are both autofluorescent and can interfere with imaging. Moreover, long exposure to hair removal cream can induce undesirable skin inflammation.

5. Placement of the liver lobe on the center of the coverslip is a critical step that requires coordinated movements of the suture thread and the mouse body in order to ensure that the diaphragm is separated from the left liver lobe. We found that gently tilting the mouse head backwards (Fig. 2h) helps correct positioning of the left liver lobe.

6. The injection through the intravenous catheter during the imaging session has to be performed as slow as possible to avoid acute heart failure.

References

1. Germain RN, Robey EA, Cahalan MD (2012) A decade of imaging cellular motility and interaction dynamics in the immune system. Science 336(6089):1676–1681. doi:10.1126/science.1221063

2. Murooka TT, Mempel TR (2012) Multiphoton intravital microscopy to study lymphocyte motility in lymph nodes. Methods Mol Biol 757:247–257. doi:10.1007/978-1-61779-166-6_16

3. Guidotti LG, Inverso D, Sironi L et al (2015) Immunosurveillance of the liver by intravascular effector CD8(+) T cells. Cell 161(3):486–500. doi:10.1016/j.cell.2015.03.005

4. Iannacone M (2015) Hepatic effector CD8(+) T-cell dynamics. Cell Mol Immunol 12(3):269–272. doi:10.1038/cmi.2014.78

5. Inverso D, Iannacone M (2015) Spatiotemporal dynamics of effector CD8+ T cell responses within the liver. J Leukoc Biol 99(1):51–55. doi:10.1189/jlb.4MR0415-150R

6. Pircher H, Bürki K, Lang R et al (1989) Tolerance induction in double specific T-cell receptor transgenic mice varies with antigen. Nature 342(6249):559–561. doi:10.1038/342559a0

7. Hogquist KA, Jameson SC, Heath WR et al (1994) T cell receptor antagonist peptides induce positive selection. Cell 76(1):17–27. doi:10.1016/0092-8674(94)90169-4

8. Isogawa M, Chung J, Murata Y et al (2013) CD40 activation rescues antiviral CD8+ T cells from PD-1-mediated exhaustion. PLoS Pathog 9(7):e1003490. doi:10.1371/journal.ppat.1003490

9. Iannacone M, Sitia G, Isogawa M et al (2005) Platelets mediate cytotoxic T lymphocyte-induced liver damage. Nat Med 11(11):1167–1169. doi:10.1038/nm1317

10. Manjunath N, Shankar P, Wan J et al (2001) Effector differentiation is not prerequisite for generation of memory cytotoxic T lymphocytes. J Clin Invest 108(6):871–878. doi:10.1172/JCI13296

11. Guidotti LG, Matzke B, Schaller H, Chisari FV (1995) High-level hepatitis B virus replication in transgenic mice. J Virol 69(10):6158–6169

12. Iannacone M, Sitia G, Narvaiza I et al (2007) Antiplatelet drug therapy moderates immune-mediated liver disease and inhibits viral clearance in mice infected with a replication-deficient adenovirus. Clin Vaccine Immunol 14(11):1532–1535. doi:10.1128/CVI.00298-07

13. Sitia G, Iannacone M, Aiolfi R et al (2011) Kupffer cells hasten resolution of liver immunopathology in mouse models of viral hepatitis. PLoS Pathog 7(6):e1002061. doi:10.1371/journal.ppat.1002061