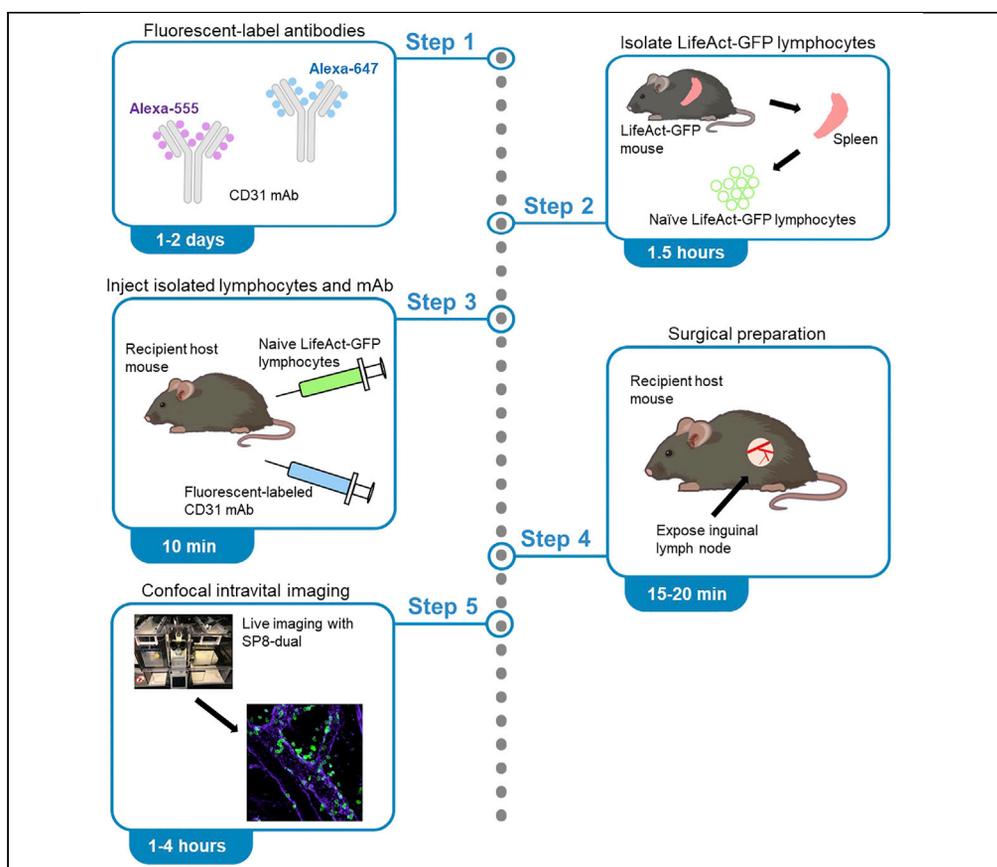


## Protocol

# An optimized confocal intravital microscopy protocol for long-term live imaging of murine F-actin organization during naïve lymphocyte migration



Serena L.S. Yan,  
John H. Kehrl

serena.yan@nih.gov  
(S.L.S.Y.)  
jkehrl@niaid.nih.gov  
(J.H.K.)

### Highlights

Protocol for high-resolution confocal intravital imaging of naïve lymphocytes *in vivo*

Protocol for LifeAct-GFP lymphocyte isolation and injection

Protocol for surgical preparation of murine inguinal lymph node for live imaging

Description of expected F-actin dynamics during naïve lymphocyte migration

Actin plays a crucial role during cell motility, but the organization of F-actin filaments during lymphocyte migration has not been visualized *in vivo*. Here, we present a 4D imaging platform using high-resolution confocal intravital microscopy to precisely determine the F-actin filament profile during lymphocyte transendothelial migration and interstitial migration. This protocol allows prolonged live imaging by laser scanning microscopy with advanced spatial resolution compared with the traditional multi-photon intravital microscopy techniques.

Yan & Kehrl, STAR Protocols 2,  
100498  
September 17, 2021  
<https://doi.org/10.1016/j.xpro.2021.100498>



## Protocol

## An optimized confocal intravital microscopy protocol for long-term live imaging of murine F-actin organization during naïve lymphocyte migration

Serena L.S. Yan<sup>1,2,\*</sup> and John H. Kehrl<sup>1,3,\*</sup><sup>1</sup>B-cell Molecular Immunology Section, Laboratory of Immunoregulation, National Institutes of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, 20892, USA<sup>2</sup>Technical contact<sup>3</sup>Lead contact\*Correspondence: [serena.yan@nih.gov](mailto:serena.yan@nih.gov) (S.L.S.Y.), [jkehr1@niaid.nih.gov](mailto:jkehr1@niaid.nih.gov) (J.H.K.)  
<https://doi.org/10.1016/j.xpro.2021.100498>

## SUMMARY

Actin plays a crucial role during cell motility, but the organization of F-actin filaments during lymphocyte migration has not been visualized *in vivo*. Here, we present a 4D imaging platform using high-resolution confocal intravital microscopy to precisely determine the F-actin filament profile during lymphocyte transendothelial migration and interstitial migration. This protocol allows prolonged live imaging by laser scanning microscopy with advanced spatial resolution compared with the traditional multi-photon intravital microscopy techniques.

For complete details on the use and execution of this protocol, please refer to Yan et al. (2019).

## BEFORE YOU BEGIN

## Preparation of fluorescent-labeled antibodies

⌚ Timing: 1–2 day

1. Prepare anti-mouse CD31 (PECAM-1) antibody fluorescent labeling with Alexa Fluor 555 or Alexa Fluor 647 Protein Labeling Kit. Prepare according to manufacturer's instructions ([https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0019835\\_AlexaFluor\\_ProteinLabelingKits\\_UG.pdf](https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0019835_AlexaFluor_ProteinLabelingKits_UG.pdf)) a few days before imaging experiments (Phan et al., 2009).

**Note:** Pre-conjugated anti-mouse CD31 antibodies such as CD31-APC or CD31-PE can also be used, however, Alexa Fluor fluorophores tend to have less photobleaching compared to the pre-conjugated antibodies.

⚠ **CRITICAL:** If the user chooses to use pre-conjugated anti-mouse CD31 antibodies, make sure to rid of sodium azide by dialysis before *in vivo* injections.

## Preparation of lymphocytes for adoptive transfer

⌚ Timing: 1.5 h

2. Sacrifice LifeAct-GFP mouse (Riedl et al., 2010) by anesthesia with Avertin (300 mg/kg) first, then followed by cervical dislocation.



- a. Use 1 donor mouse for 1 recipient host mouse.
3. Extract spleen.
  - a. Sterilize the skin of the mouse with ethanol.
  - b. Use forceps and scissors to cut through the skin and the abdomen tissue on the ventral side.
  - c. Identify and extract the spleen by using scissors and forceps.
  - d. Place the spleen in a Petri dish containing PBS.
4. Isolating naïve lymphocytes.
  - a. Place the extracted spleen on a cell strainer.
  - b. Put strainer into 100 mm plate.
  - c. Put ~1 mL PBS into strainer and mash spleen by using the plunger of a syringe.
  - d. Repeat 3–4 times and collect cellular flow-through into 50 mL conical tube.
  - e. Collect all cellular suspension with splenocytes that has gone through the strainer and finalize total volume to 30 mL with additional PBS.
  - f. Centrifuge the cells at 500 g for 5 min at ~20°C, and discard the supernatants.
  - g. Lyse red blood cells by suspending the pellet with 1 mL ACK lysing buffer for 1 min at ~20°C.
  - h. Bring total volume to 30 mL with additional PBS and centrifuge at 500 g for 5 min at ~20°C, and discard the supernatant.
  - i. Suspend the pellet with 1500 µL PBS and count cells.

**Note:** If Avertin is not an approved anesthesia for the user's institute, other anesthesia alternatives before cervical dislocation such as isoflurane or CO<sub>2</sub> asphyxia could be used instead.

### Fluorescent labeling and inhibitor treatment of isolated lymphocytes

⌚ Timing: 20–25 min

5. Obtain ~ 10 million cells from 4i and aliquot into a 1.5 mL Eppendorf tube.
6. Bring total volume to 1500 µL with PBS then add 1 µL CellTracker Orange CMTMR Dye (final concentration 0.5 µM) (Catron et al., 2010) and/or inhibitor of choice to lymphocyte suspension to obtain appropriate final concentration.
7. Incubate the cells for 15 min at 37°C, protected from light.
8. Centrifuge at 250 g for 5 min at ~20°C.
9. Discard the supernatant and re-suspend the pellet with 150 µL PBS.

⚠ **CRITICAL:** All experiments involving animals must be carried out according to institutional and national guidelines.

### KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Antibodies</b>		
Anti-mouse CD31, purified (clone 390)	BioLegend	Cat#102402
<b>Chemicals, peptides, and recombinant proteins</b>		
Avertin	Sigma-Aldrich	Cat#152463
1× PBS	Thermo Fisher Scientific	Cat#10010023
ACK lysing buffer	Lonza	Cat#10-548E
CellTracker orange CMTMR dye	Thermo Fisher Scientific	Cat#C2927
<b>Critical commercial assays</b>		
Alexa Fluor 555 protein labeling kit	Thermo Fisher Scientific	Cat#A20174
Alexa Fluor 647 protein labeling kit	Thermo Fisher Scientific	Cat#A20173

(Continued on next page)

<b>Continued</b>		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental models: organisms/strains		
Mouse, C57BL/6J	The Jackson Laboratory	JAX000664
Mouse, LifeAct-eGFP	Riedl et al., 2010	N/A
Software and algorithms		
Imaris v9.3.1	Bitplane	<a href="http://www.bitplane.com/imaris">http://www.bitplane.com/imaris</a>
Huygens	SVI	<a href="https://svi.nl/HomePage">https://svi.nl/HomePage</a>
Other		
Surgical microscope	Leica	N/A
Inverted confocal microscope (SP8 DIVE) with temperature control chamber set-up	Leica	N/A
Custom stage inserts	Leica	N/A
Corning cover glass (No. 1, 24×60 mm)	Millipore Sigma	Cat#CLS2975246
Durapore surgical tape	3M	Cat#DH888843199
Insulin syringes with 30G needle	Exel	Cat#26015
Insulin syringes with 28G needle	Exel	Cat#26027
Electric trimmer	Wahl	Cat#09990-502
Surgical scissors	Braintree Scientific	Cat#SC-M126
Micro scissors	Braintree Scientific	Cat#SC-M1150
Tissue forceps (strong)	Braintree Scientific	Cat#FC-5047
Tissue forceps (fine)	Braintree Scientific	Cat#FC-5005
Bovie Fine Tip Cautery	Tiger Medical	Cat#TM28139

## STEP-BY-STEP METHOD DETAILS

### Injection of isolated LifeAct-GFP lymphocytes

⌚ Timing: 10 min

Cells from LifeAct-GFP donor are injected into a recipient host mouse through surgically exposed femoral vein.

1. Anesthetize the recipient host C57BL/6J mouse by intraperitoneal injection of Avertin (300 mg/kg).
2. Wet with ethanol, cut away skin and gently expose the femoral vein (Figures 1i and 1ii).
3. ( $\sim 10 \times 10^6$  cells) Inject the fluorescent-labeled lymphocytes into the femoral vein using insulin syringe with 30G needle, under the dissecting microscope (Nakanishi et al., 2016).
4. Seal the needle wound with a cautery pen or apply pressure on the wound with Q-tip to stop bleeding.
5. Cover the open skin area with a strip of surgical tape.
6. Return the anesthetized mouse to a heated incubator ( $\sim 30^\circ\text{C}$ – $37^\circ\text{C}$  to maintain physiological temperature before lymph node surgery).
  - a. Ensure the animal is fully sedated by checking every 10–20 min before surgery.
  - b. Reinforce with half the dose of initial Avertin injection if necessary.

**Note:** If Avertin is not an approved anesthesia for the user's institute, other injectable anesthesia alternatives such as pentobarbital and ketamine-xylazine could be used instead.

**Note:** Tail vein injection could also be used for injecting fluorescent-labeled lymphocytes into the recipient host mouse.

**⚠ CRITICAL:** It is important to inject these cells  $\sim 20$  min before lymph node surgery. This allows the lymphocytes to fully circulate and arrive at the peripheral lymph nodes.



**Figure 1. Surgical preparation of mouse**

- (i and ii) Exposing right femoral vein for i.v. injection.
- (iii) Retro-orbital injection.
- (iv and v) Exposing left inguinal lymph node for imaging.
- (vi) Custom imaging insert with cover glass.
- (vii) Mounting of mouse on imaging insert.

### Mouse preparation and inguinal lymph node surgery

⌚ Timing: 15–20 min

Inguinal lymph node of recipient host mouse is surgically exposed and cleared of connective tissue.

7. Remove the anesthetized mouse from the incubator.
  - a. Determine if the mouse is fully sedated by lack of response to toe and/or tail pinches.
  - b. Inject half dose of initial Avertin injection if necessary.
8. At this point, inject fluorescently labeled antibodies (i.e., CD31-Alexa Fluor 647) via retro-orbital injection (Figure 1iii).
9. Use an electric trimmer to remove hair on the abdominal area of the mouse. Remove remaining loose hair by alcohol swab and gently patting the area with tape.
10. Place the mouse on a clear surgical board in a supine position and stabilize the mouse to the board with taping each footpad to the board. Body temperature should be maintained by an external heat lamp or a heating pad.

11. Place the mouse under the dissecting microscope. Using surgical scissors, make a midline incision along the ventral surface of the abdominal cavity and gently retract the skin away from the flesh (Figure 1iv).

△ **CRITICAL:** During the entire surgical procedure, ensure the exposed tissue is always kept moist with gauze dampened with PBS.

12. Once the skin is retracted so that the inguinal lymph node is visible, pull the skin away gently and stabilize the edges with surgical tape.

13. Carefully remove the thin layer of connective tissue covering the area around the lymph node (Figure 1v).

△ **CRITICAL:** Avoid contact with the vasculature of interest with surgical tools or injuring the vessels by placing too much force on them by manipulating adipose tissue and connective tissue covering the lymph node.

14. Using fine forceps and micro-scissors, clear the adipose tissue covering and surrounding the lymph node. The main arterial segment lays adjacent to the lymph node and is often covered by venule (von Andrian, 1996).

### Confocal intravital imaging

⌚ **Timing:** 1–4 h

Mouse is placed under the confocal microscope and 4D data collected.

15. Obtain optimal imaging insert best suited for the microscope stage and cover with Corning cover glass (Figure 1vi).

16. Once the lymph node and vessel segments are exposed under the dissecting microscope, gently flip the mouse up-side down, then secure the preparation onto an imaging stage (Figure 1vii).

17. The skin flap (with the attached lymph node) is maneuvered to where the lymph node is at the middle of the slide. Afterwards, secure the optimal position with surgical tape firmly adhering to the skin flap.

**Note:** All corners of the skin flap should be taped firmly to the cover glass. Since the skin flap (along with the attached lymph node) is being pulled away from the mouse body, and quite separated from the mouse body cavity, away from lung, heart, intestinal movement, there shouldn't be much motion artifacts.

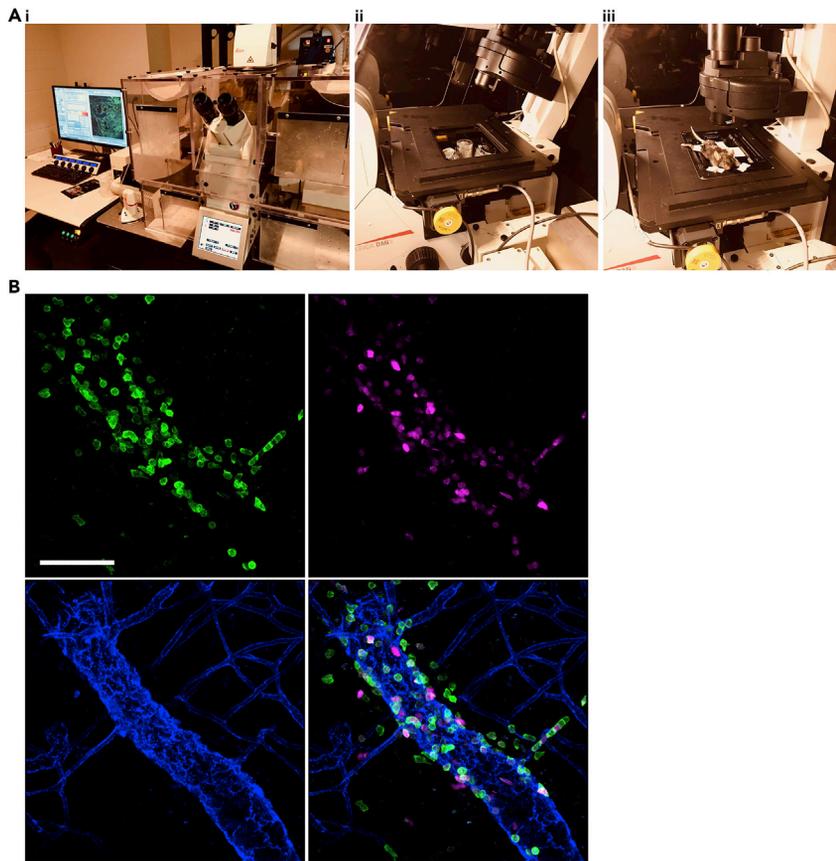
△ **CRITICAL:** Make sure to put a few droplets of 1 × PBS on the cover slip between the lymph node and glass to prevent the tissue from drying.

18. Start-up the imaging system by switching on the confocal microscope, lasers and computers. Allow the lasers and lamps to warm-up (Figure 2A).

19. Place the mouse preparation on the microscope stage, identify the lymph node and vasculature with wide-field settings on the microscope.

**Note:** The users should be looking for CD31 stained venules with abundant lymphocytes arriving and adhering at the site.

20. Switch to confocal mode and set up 4D imaging acquisition parameters (Figure 2B).



**Figure 2. Microscope and mouse set-up and example imaging result**

(Ai and Aii) SP8 DIVE with incubation chamber, fitted with 25x water immersion objective.

(Aiii) Placing mouse/insert set-up on imaging stage.

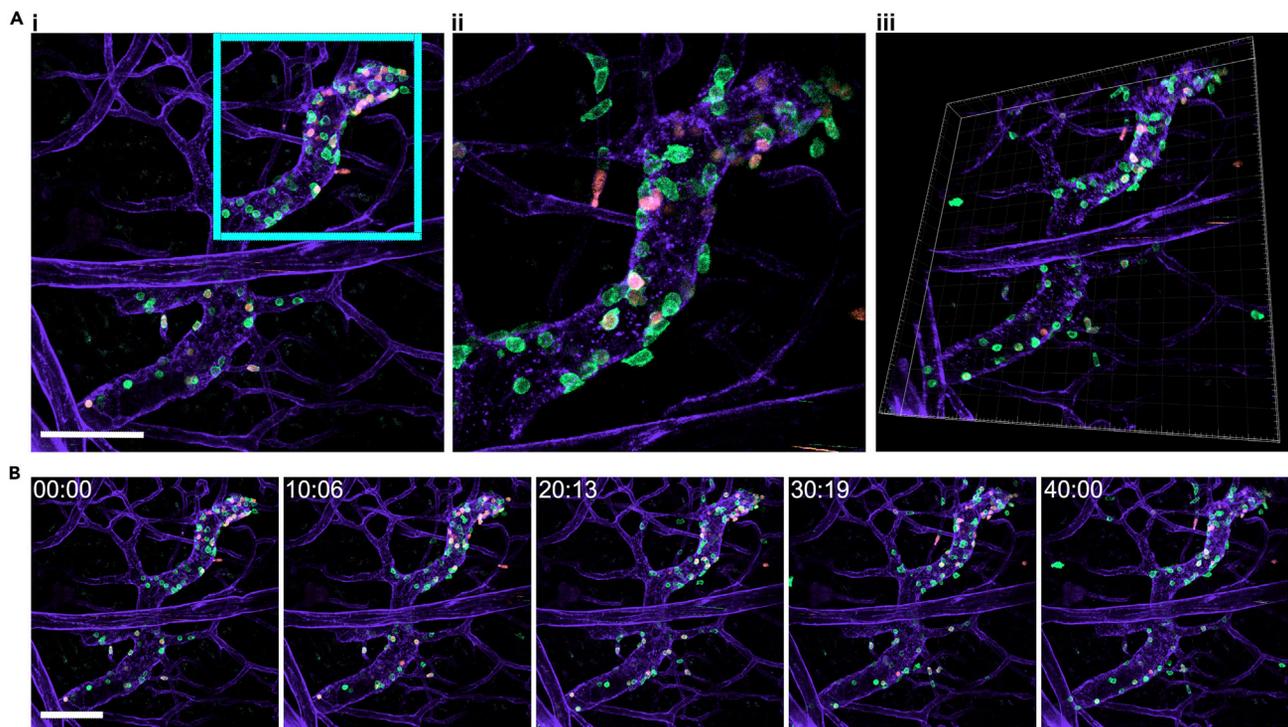
(B) Ideal confocal 4D imaging. Top left, single color channel for LifeAct-GFP cells. Top right, single color channel for CellTracker-orange labeled cells. Bottom left, single channel for CD31-AF647 labeled blood vessels (Bruggisser et al., 2020). Bottom right, all 3 channels combined. Scale bar = 60  $\mu$ m.

- a. Choose the desired laser settings based on excitation/emission wave lengths of the fluorochrome used.
  - b. Settings for intensity parameters such as gain and pinhole should be adjusted.
  - c. Time-lapse parameter settings should be adjusted to acquire desired frame rate ( $\sim$ 30 – 60 sec per interval) and overall acquisition duration ( $\sim$ 30 – 60 min) (Yan et al., 2019, Woodfin et al., 2011, Girbl et al., 2018).
21. After every 1–1.5 hr, inject half dose of initial Avertin to maintain mouse under anesthesia.

**Note:** Avoid shifts in the z-plane scan field during time-lapse acquisition by firmly stabilizing the skin flap with surgical tape.

**△ CRITICAL:** Minor shifts in the scan field, especially those in the x-y-plane often occur during the initial time-lapse image acquisition. This can be corrected by analysis software after the experiment. However, if shift becomes too severe, the experiments should be halted. Slight changes such as adjusting or repositioning the skin flap can help.

22. Use the appropriate software such as Imaris to create 4D reconstructions from the acquired raw data file (Yan et al., 2019, Park and Kehrl, 2019).



**Figure 3. 4D Imaris reconstructed images**

(Ai) Representative confocal image of an intravital microscopy experiment of wild-type host mouse immunostained with CD31-Alex Fluor 647 (violet), injected with LifeAct-GFP (green) and CellTracker-labeled (orange) lymphocytes. Scale bar = 80  $\mu\text{m}$ .

(Aii) Zoomed-in (2 $\times$ ) of (i).

(Aiii) Rotating of a representative 3D stack.

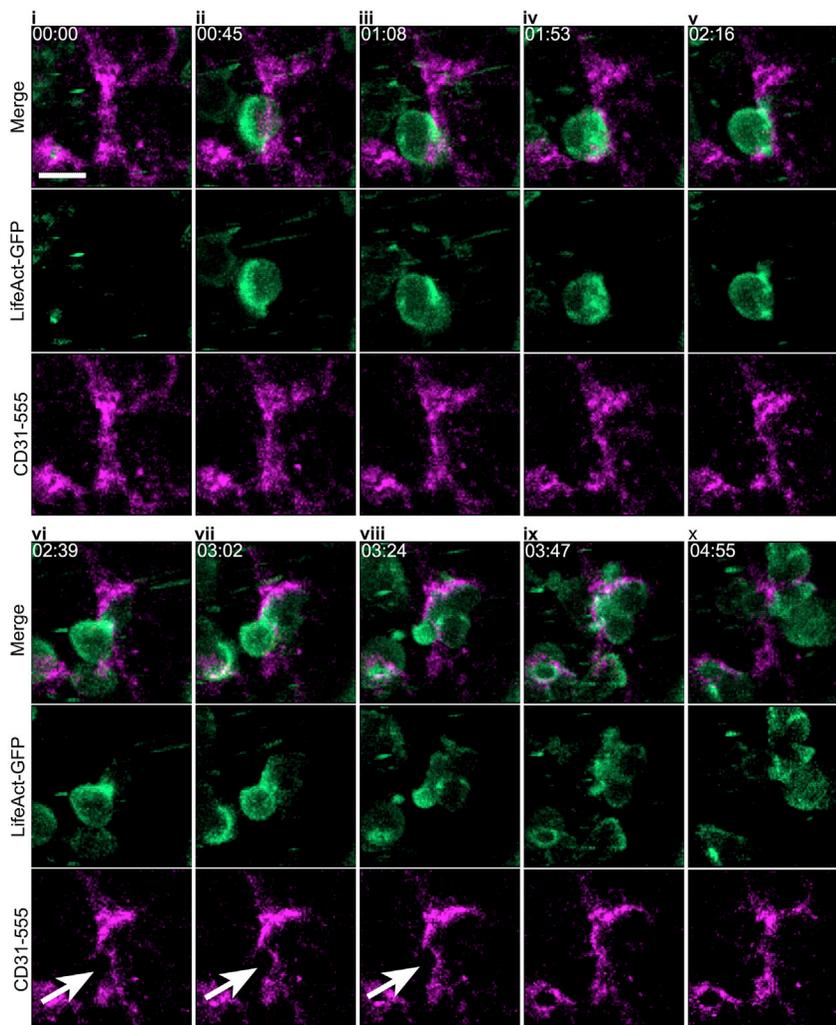
(B) Time-lapse confocal images (left to right) of 4D imaging experiment from start (00:00) to finish (40:00). Scale bar = 80  $\mu\text{m}$ . Time = mm:ss.

## EXPECTED OUTCOMES

By applying confocal intravital microscopy imaging to the mouse inguinal lymph node described here, it is possible to optically section through the superficial surface  $\sim 50 \mu\text{m}$  of the lymph node. These x-y data can be combined into a three-dimensional image stack by sequentially capturing successive x-y plane images (z-stack), and when repeated at fixed time intervals, depending on the experimental preference, this creates time-lapse images (Figure 3A).

The use of various suitable CellTracker Dyes and fluorescently labeled antibodies enables the visualization of immune cells *in vivo*, and their interactions with the lymph node vasculature. These images can then be further processed by image-analysis software into a movie, zoomed-in (Figure 3Aii) or rotated (Figure 3Aiii) to help better define cell positions/location around the vasculature. Using lymphocytes isolated from LifeAct-GFP mice, we observed the unique location and organization of F-actin filaments (Yan et al., 2019), and their location within the cell during naïve lymphocyte transmigration (Figure 4) and interstitial migration (Figure 5).

This protocol allows live imaging by laser scanning microscopy over extended periods of time (>4 h) and superior advanced spatial resolution compared to the conventional multi-photon intravital microscopy techniques as shown in Figure 6A (Mionnet et al., 2011, Kamenyeva et al., 2015). It is noteworthy that the murine inguinal lymph node has a better suitable vasculature for confocal intravital microscopy. This is evidenced by the fact that more vessels aligned to a z-plane can be found in the inguinal than the popliteal lymph node (Figure 6B). As a result, more distinct lymphocyte migration events can be observed during imaging of the inguinal lymph node during the steady states.



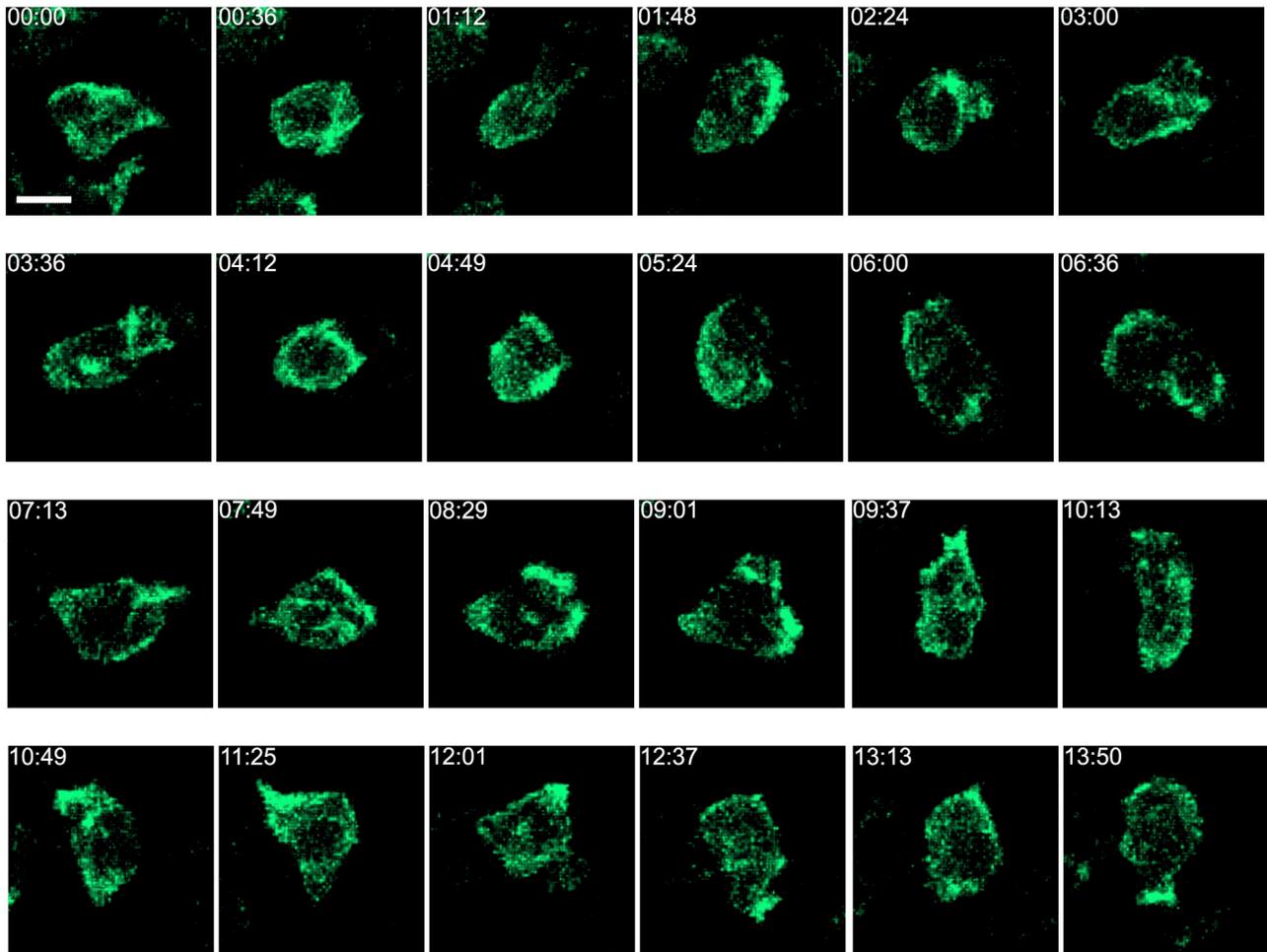
**Figure 4. F-Actin filament organization analysis during transmigration *in vivo***

(i–x) Representative time-lapse confocal images of a 4D intravital microscopy experiment showing changes in F-actin organization/location within a LifeAct-GFP (green) lymphocytes during transmigration. CD31-Alexa Fluor 555 labeled endothelial cell junctions of blood vessel (magenta). White arrows indicate endothelial cell breaching. Scale bar = 7  $\mu\text{m}$ . Time = mm:ss.

## LIMITATIONS

To obtain 4D information (x, y, z and time), confocal intravital imaging involves the process of capturing 3D image stacks at repeated time intervals. This acquisition technique requires considerations in terms of imaging depth and sampling frequency, in order to attain optimal spatial and temporal resolution. The furthest optical section through the superficial lymph node surface is  $\sim 50 \mu\text{m}$  of depth. If one needs to visualize lymph node deep tissue, multi-photon microscopy would be better suited. However, this technique will no longer yield the high resolution to visualize F-actin accurately (Figure 6A).

Typically, sampling at 30-s intervals between every image stack is sufficient to visualize the F-actin organization/localization and lymphocyte migration. It is more challenging to visualize fast-moving cells in the circulation due to the limitation in image acquisition rate. However, further refinement with the imaging parameters may enable the capture of rolling lymphocytes and even free-floating cells in the circulation. It is important to note that excessive liquid (such as interstitial fluid or blood)



**Figure 5. F-Actin filament organization during interstitial migration *in vivo***

Representative time-lapse confocal images of a 4D intravital microscopy experiment showing changes in F-actin organization/location within a LifeAct-GFP (green) lymphocyte during interstitial migration. Scale bar = 4  $\mu\text{m}$ . Time = mm:ss.

between the lymph node and the cover glass induced by surgery can reduce the imaging contrast and limit penetration depth of the laser light. In addition, careful precautions should be considered when removing excess fatty or connective tissues covering the inguinal lymph node, because perturbation can affect blood flow.

## TROUBLESHOOTING

### Problem 1

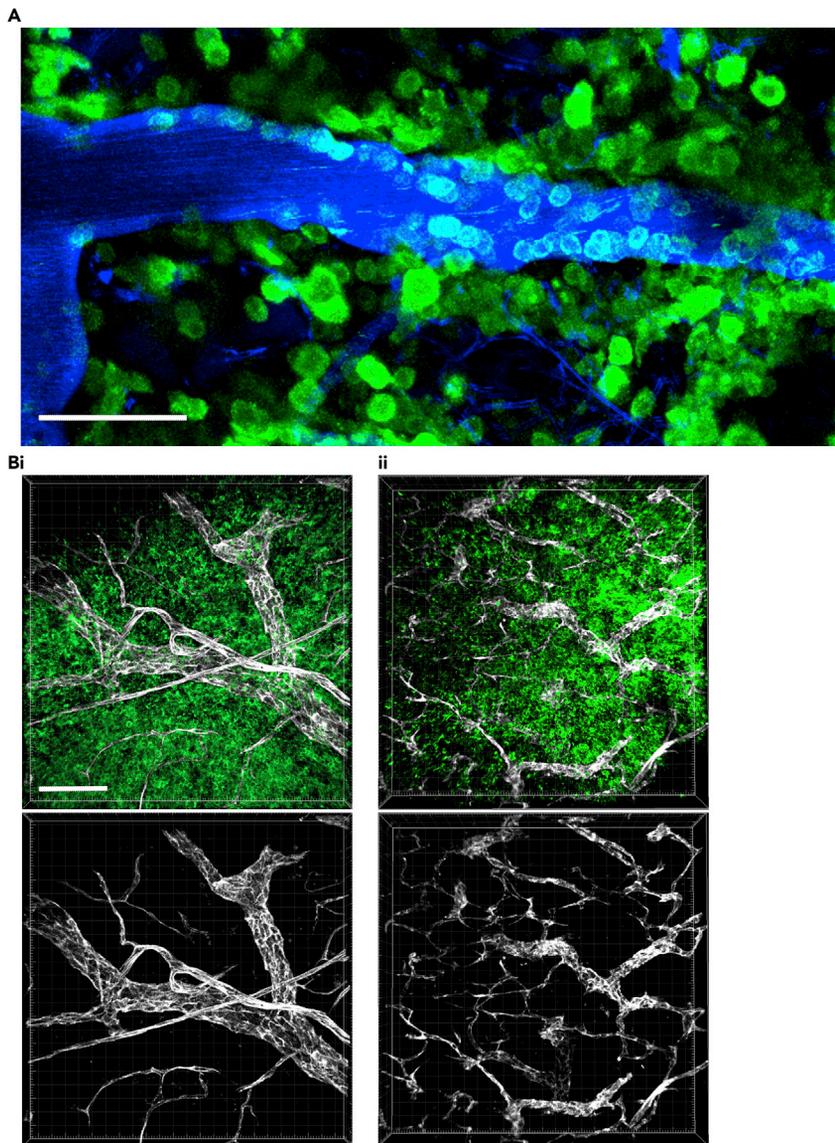
The initial x-y-plane shift at the start of image acquisition experiment (Confocal intravital imaging: step 17).

### Potential solution 1

Start the heated incubation chamber on the confocal microscope 20–30 min before placing the mouse under the scope. Having the incubation chamber pre-heated to 35°C–37°C can help reduce the initial x-y-plane shift.

### Problem 2

Decrease visibility and resolution (blurry image) during image acquisition experiment (Confocal intravital imaging: step 20).



**Figure 6. Multi-photon example imaging and lymph node comparisons**

(A) Representative multi-photon image of an intravital microscopy experiment of wild-type host mouse with Evans Blue Dye labeled vasculature (blue) and adoptive transferred LifeAct-GFP (green) cells. Scale bar = 50  $\mu\text{m}$ . Comparison of inguinal lymph node (Bi) and popliteal lymph node (Bii) vasculature layout. Lymph nodes are extracted from a LifeAct-GFP (green) mouse with CD31-Alexa Fluor 647 (white) immunostained vasculatures. The bottom panels (Bi-ii) shows the vasculature layout of each lymph node with single color channel for CD31-Alexa Fluor 647. Scale bar = 100  $\mu\text{m}$ .

### Potential solution 2

Place a few droplets of warm  $1 \times$  PBS on the inguinal lymph node after the surgery and mop up with Kimwipes, repeat this 3–5 times. This will likely clear away the interstitial fluid or blood arriving at the lymph node during surgery.

### Problem 3

Difficulties in removing excess fatty or connective tissues covering the inguinal lymph node (Mouse preparation and inguinal lymph node surgery: step 14).

### Potential solution 3

Use younger mice between ages 5–6 weeks of age.

### Problem 4

Difficulties in obtaining stable images if the mouse has irregular breathing pattern during image acquisition under the microscope (Confocal intravital imaging: step 20).

### Potential solution 4

If this problem occurs often, make a small incision in the lower trachea during surgical preparation phase and insert a small segment of surgical grade P50 tubing (~1–1.5 cm). This will likely create a better pathway for airflow, since upper trachea/airway can sometimes get mucus clogs during anesthesia.

### Problem 5

Failure of arriving lymphocytes transmigrating out of the high endothelial venules or migrating into the interstitial tissue (Confocal intravital imaging: step 20).

### Potential solution 5

Make sure the incubation chamber on the microscope is set to 37°C and the internal circulating air temperature within the chamber matches that setting. *In vivo* lymphocyte migration is extremely temperature sensitive, thus, it's critical to maintain physiological temperature.

## RESOURCE AVAILABILITY

### Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, John H. Kehrl, [jkehrl@niaid.nih.gov](mailto:jkehrl@niaid.nih.gov).

### Materials availability

This study did not generate any new reagents.

### Data and code availability

This paper does not generate any dataset or code

## ACKNOWLEDGMENTS

We would like to thank Olena Kamenyeva, Juraj Kabat, and Owen Schwartz of the NIAID Biological Imaging Facility for assistance in initial microscopy setup and data analysis setup; Larry Lantz of the NIAID Custom Antibody Facility for assistance in conjugating Alexa Fluor fluorochromes to antibodies. This work was supported by the Intramural Research Program of National Institute of Allergy and Infectious Diseases.

## AUTHOR CONTRIBUTIONS

Conceptualization, S.L.Y. and J.K.; investigation, S.L.Y. and J.K.; methodology, S.L.Y.; writing – original draft, S.L.Y.; writing – review & editing, S.L.Y. and J.K.; funding acquisition, J.K.; and supervision, J.K.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

## REFERENCES

- Bruggisser, J., Tarek, B., Wyder, M., Muller, P., Von Ballmoos, C., Witz, G., Enzmann, G., Deutsch, U., Engelhardt, B., and Posthaus, H. (2020). CD31 (PECAM-1) serves as the endothelial cell-specific receptor of clostridium perfringens beta-toxin. *Cell Host Microbe* 28, 69–78 e6.
- Bruggisser, J., Tarek, B., Wyder, M., Muller, P., Von Ballmoos, C., Witz, G., Enzmann, G., Deutsch, U., Engelhardt, B., and Posthaus, H. (2020). CD31 (PECAM-1) serves as the endothelial cell-specific receptor of clostridium perfringens beta-toxin. *Cell Host Microbe* 28, 69–78 e6.
- Catron, D.M., Pape, K.A., Fife, B.T., Van Rooijen, N., and Jenkins, M.K. (2010). A protease-dependent mechanism for initiating T-dependent B cell responses to large particulate antigens. *J. Immunol.* 184, 3609–3617.
- Girbl, T., Lenn, T., Perez, L., Rolas, L., Barkaway, A., Thiriot, A., Del Fresno, C., Lynam, E., Hub, E., Thelen, M., et al. (2018). Distinct compartmentalization of the chemokines CXCL1 and CXCL2 and the atypical receptor ACKR1 determine discrete stages of neutrophil diapedesis. *Immunity* 49, 1062–1076 e6.
- Kamenyeva, O., Boularan, C., Kabat, J., Cheung, G.Y., Cicala, C., Yeh, A.J., Chan, J.L., Periasamy, S., Otto, M., and Kehrl, J.H. (2015). Neutrophil recruitment to lymph nodes limits local humoral response to *Staphylococcus aureus*. *PLoS Pathog.* 11, e1004827.
- Mionnet, C., Sanos, S.L., Mondor, I., Jorquera, A., Laugier, J.P., Germain, R.N., and Bajenoff, M. (2011). High endothelial venules as traffic control points maintaining lymphocyte population homeostasis in lymph nodes. *Blood* 118, 6115–6122.
- Nakanishi, M., Grebe, R., Bhutto, I.A., Edwards, M., Mcleod, D.S., and Luty, G.A. (2016). Albumen

transport to bruch's membrane and RPE by choriocapillaris caveolae. *Invest. Ophthalmol. Vis. Sci.* 57, 2213–2224.

Park, C., and Kehrl, J.H. (2019). An integrin/MFG-E8 shuttle loads HIV-1 viral-like particles onto follicular dendritic cells in mouse lymph node. *Elife* 8, e47776.

Phan, T.G., Green, J.A., Gray, E.E., Xu, Y., and Cyster, J.G. (2009). Immune complex relay by subcapsular sinus macrophages and noncognate B cells drives antibody affinity maturation. *Nat. Immunol.* 10, 786–793.

Riedl, J., Flynn, K.C., Raducanu, A., Gartner, F., Beck, G., Bosl, M., Bradke, F., Massberg, S., Aszodi, A., Sixt, M., and Wedlich-Soldner, R. (2010). Lifeact mice for studying F-actin dynamics. *Nat. Methods* 7, 168–169.

von Andrian, U.H. (1996). Intravital microscopy of the peripheral lymph node microcirculation in mice. *Microcirculation* 3, 287–300.

Woodfin, A., Voisin, M.B., Beyrau, M., Colom, B., Caille, D., Diapouli, F.M., Nash, G.B., Chavakis, T., Albelda, S.M., Rainger, G.E.,

Meda, P., Imhof, B.A., and Nourshargh, S. (2011). The junctional adhesion molecule JAM-C regulates polarized transendothelial migration of neutrophils in vivo. *Nat. Immunol.* 12, 761–769.

Yan, S.L.S., Hwang, I.Y., Kamenyeva, O., and Kehrl, J.H. (2019). In vivo F-actin filament organization during lymphocyte transendothelial and interstitial migration revealed by intravital microscopy. *iScience* 16, 283–297.