Video Article

Non-invasive Imaging of Leukocyte Homing and Migration *in vivo*

Baomei Wang[1], Bernd H. Zinselmeyer[1,2], Jeremiah R. McDole[1], Peggy A. Gieselman[1], Mark J. Miller[1]

[1]Department of Pathology and Immunology, Washington University in St. Louis
[2]National Institute of Neurological Disorders and Stroke, NINDS, NIH - National Institute of Health

Correspondence to: Mark J. Miller at miller@pathology.wustl.edu

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Abstract

Two-photon (2P) microscopy is a high resolution imaging technique that has been broadly adapted by biologists. The value of 2P microscopy is that it provides rich spatiotemporal information regarding cell behaviors within intact tissues and in live mice. Leukocyte recruitment plays a significant role in host defense against infection and when unchecked, can contribute to inflammatory and autoimmune diseases. Studying leukocyte recruitment *in vivo* is technically challenging since cells are moving rapidly within vessels located deep within light scattering tissues. To date, most intravital imaging studies require surgical preparation to expose the blood vessels and tissues. To avoid the tissue damage and inflammation induced by surgery itself, here, we describe a non-invasive single-cell imaging approach that can be used to study leukocyte trafficking in the mouse footpad and phalanges. We discuss the technical aspects of our 2P imaging preparation and walk the reader through a typical experiment from initial set up to execution and data collection.

Protocol

1. Animal preparation:

This protocol uses adult female adult LysM-eGFP mice, in which neutrophils and macrophages are fluorescently labeled by the expression of eGFP [1].

a. Labeling blood vessels:
   To visualize blood vessels, 15µl of non-targeted quantum dots (655nm) are diluted into 100 µl of PBS and injected intravenously into the mouse 10-30 min before imaging.
b. Anesthesia:
   1. Place the mouse in the induction chamber of the veterinary inhalation anesthesia system (Noble Medical Service, Inc).
   2. Set the vaporizer to 3-4% Isoflurane and the 100% oxygen carrier gas flow rate to ~1 L/min. To prevent accidental anesthesia overdose, closely monitor the animal till the rear toe pinch reflex is lost.
   3. Anesthesia is maintained at 1.5~2% Isoflurane and fine tuned during imaging to minimize breathing artifacts as necessary. The breathing rate of the mouse is monitored carefully throughout the imaging session to insure an adequate plane of anesthesia.
   4. Puralube veterinary ointment is applied to the eyes to prevent drying.
   5. The mouse is placed on a regulated 36°C heating pad (Braintree Scientific) during the injections and imaging and to prevent hypothermia.
   6. To prevent dehydration, the mouse is given 100 µl of PBS s.c every 1-2 hours.

c. Delivering an inflammatory stimulus to induce leukocyte recruitment:
   1. Place the mouse on the surgery stage and swab the footpad with 70% ethanol.
   2. Bend the needle of an insulin syringe ~90 degrees near the bevel to facilitate injection and improve the precision of the delivery depth.
   3. Load the syringe with 0.5 µg of E.coli bioparticles diluted in 1µl PBS. This can be done in the lid of a microfuge tube to make it easier to load the droplet.
   4. Hold the toe with curved forceps and insert the needle gently through the skin with the bevel facing upward until it is just below the skin.
   5. Inject slowly and hold in place for 5 seconds to prevent any back flush.

2. Imaging stage setup:

The imaging platform consists of an aluminum plate with a circular hole cut through the center. A large cover glass is glued to the bottom of the plate and a rubber O-ring is glued on the top to create a fluid-tight recessed chamber to accommodate the rear paw. The aluminum plate is warmed to 36°C via two heating elements attached to the top of the plate.

a. Place the mouse on the pre-warmed imaging stage and maintain the mouse's core body temperature using the 36°C warming pad.
b. Secure the paw to the coverglass of the imaging chamber with a thin film of superglue.
c. Wash the paw twice to remove any floating bits of glue and then fill the chamber with fresh PBS (pre-warmed to 36°C).

Mice can be imaged for up to 4 hours without comprising the animal's viability. The foot can be gently released with small amount of acetone and mice revived for longitudinal imaging studies. However, in this case, we recommend the imaging period to < 2 hours and waiting 24-48 h between sessions to minimize the risk of anesthesia overdose or dehydration.

3. Imaging Acquisition

The two-photon microscope (2P microscope) used in this protocol is a custom-built dual-laser video-rate system consisting an upright microscope. The system is equipped with two Ti: Sapphire lasers, four head-on Bi-alkali PMTs for simultaneous 3 and 4 channel acquisitions and a 20x water immersion objective (Olympus, 20x/0.95 NA).

a. Tune the Chameleon XR Ti:sapphire laser (Coherent Inc.) to 890-900nm.
b. Use 480nm and 560nm dichroic mirrors (SemRoc) to separate three channels: blue (<480 nm, second harmonic generation signal), green (480-560nm, LysM-eGFP positive neutrophils) and red (>560 nm, quantum dot labeled blood vessels). Alternatively, the 560nm filter can be replaced with a 570nm filter to distinguish *E. coli* bioparticles (576nm bioparticles will appear orange) from 655nm quantum dots (will appear deep red).
c. Carefully lower the objective into the PBS and focus on a vessel of the toe.
d. With the image acquisition speed operating at video-rate (30f/sec) and using the second harmonic signal emanating from collagen as a tissue landmark, quickly survey the tissue (at video-rate acquisition speed) to locate a blood vessel of interest (one with visible lysM-eGFP neutrophils).
e. Adjust the gain on the PMTs to optimize color separation and minimize the amount of laser required to achieve sufficient signal over background (be careful not to saturate the image!).
f. Once area region of interests is located, begin time-lapse imaging. Time-lapse imaging can be performed with two different temporal resolutions. For imaging cell rolling and adhesion and in real-time within blood vessels, we acquire 30f/sec at a single z-plane. For documenting cell extravasation and migration in the parenchyma we perform 3D time-lapse imaging. A typical acquisition protocol will consist of averaging 15 frames for each Z-step and acquiring 21 sequential 2.5µm z-steps for a volume of 200-225 50µm at 30 sec intervals.
g. Once the data files are stored on the lab server, Imaris or Volocity software can be used to perform multi-dimensional rendering and cell tracking [2, 3].

4. Representative Results

1.Real-time (30f/sec) imagining of neutrophil rolling along the vessels at 200x. Time lapse imaging shows neutrophils, in green, rolling along the endothelium of blood vessels about 10mins after infection with Listeria monocytogenes, shown in red. Collagen fibers in the connective tissue appear in blue. Please click here to see the video.

2.Time-lapse 3D imaging of neutrophil recruitment dynamics at 200x. Typical neutrophil recruitment from circulation and migration through inflamed tissue is shown. Please click here to see the video.

Discussion

In this video protocol we demonstrate the procedures for non-invasive 2P imaging of leukocyte recruitment in response to inflammation.

The footpad is a classic physiological site for studying inflammation such as allergy, infection and autoimmune disease [4-7]. 2P imaging provides a detailed picture of distinct steps in leukocyte trafficking pathways, from rolling and adhesion in the blood vessels to chemotaxis to sites of effector function. We have found that the leukocyte recruitment varies in response to different types of inflammatory stimuli. The dose of the stimulus should be chosen carefully and every attempt made to recapitulate a physiological insult. If the dose is too high or delivered to a non-physiological site, the stimulus might overwhelm the host or lead to experimental artifacts and produce abnormal phenomena. We recommend titrated doses of stimuli for pilot experiments paired with a suitable control and using a bent needle to increase the precision of both the volume delivered and the depth of injection. It is also crucial that the site of injection can be easily located, especially for serial imaging experiments. This can be accomplished by injecting at a specific location (i.e., second digit on third toe of right hind paw) or by tattooing the skin and injecting nearby.

2P imaging provides a unique opportunity to visualize leukocyte trafficking and effector function *in vivo*. The majority of intravital imaging approaches used to study leukocyte recruitment require surgery to expose tissues and blood vessels in anesthetized mice [8-10]. The surgery itself induces inflammation that will impact leukocyte behavior. However, 2P imaging allows leukocytes to be studied non-invasively and at single-cell resolution deep within the native 3D tissue environment. Furthermore, since the imaging procedure itself does not harm the mouse, it is an excellent choice for performing longitudinal studies for models of cancer and arthritis. 2P imaging has the potential to provide and unprecedented view of a disease process from disease initiation and progression to resolution, all within an individual animal subject. This approach is a valuable tool for obtaining insight into the cellular and molecular mechanisms that underlie cellular immunity to infection and how these same responses, if improperly regulated, may lead to inflammatory and autoimmune diseases. Ultimately, a detailed picture of leukocyte behavior *in vivo* will be aid in the development of new therapeutics for treating inflammatory and infectious diseases.

Disclosures

All experiments were performed according to protocols approved by the Animal Studies Committee of Washington University in St Louis.

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References

1. Faust ,N., Varas, F., Kelly, L.M., Heck, S., Graf, T. Insertion of enhanced green fluorescent protein into the lysozyme gene creates mice with green fluorescent granulocytes and macrophages. Blood 96, 719-26 (2000).
2. Zinselmeyer, B.H., Lynch, J.N., Zhang, X., Aoshi, T., Miller, M.J. Video-rate two-photon imaging of mouse footpad - a promising model for studying leukocyte recruitment dynamics during inflammation. Inflamm Res. 57, 93-6 (2008).
3. Zinselmeyer, B.H., Dempster, J., Wokosin, D.L., Cannon, J.J., Pless, R., Parker, I., Miller, M.J. Chapter 16. Two-photon microscopy and multidimensional analysis of cell dynamics. Methods Enzymol. 461, 349-78 (2009).
4. Gray, D.F., Jennings, P.A. Allergy in experimental mouse tuberculosis. Am Rev Tuberc. 72, 171-95 (1955).

5. Lin, A., Loughman, J.A., Zinselmeyer, B.H., Miller, M.J., Caparon, M.G. Streptolysin S inhibits neutrophil recruitment during the early stages of Streptococcus pyogenes infection. Infect Immun. 77, 5190-201 (2009).
6. Smith, C.J., Zhang, Y., Koboldt, C.M., Muhammad, J., Zweifel, B.S., Shaffer, A., Talley, J.J., Masferrer, J.L., Seibert, K., Isakson, P.C. Pharmacological analysis of cyclooxygenase-1 in inflammation. Proc Natl Acad Sci U S A. 95, 13313-8 (1998).
7. Wipke, B.T., Allen, P.M. Essential role of neutrophils in the initiation and progression of a murine model of rheumatoid arthritis. J Immunol. 167, 1601-8 (2001).
8. Baez, S. An open cremaster muscle preparation for the study of blood vessels by *in vivo* microscopy. Microvasc Res. 5, 384-94 (1973).
9. Mempel, T.R., Scimone, M.L., Mora, J.R., von Andrian, U.H. *in vivo* imaging of leukocyte trafficking in blood vessels and tissues. Curr Opin Immunol. 16, 406-17 (2004).
10. Miller, M.J., Wei, S.H., Cahalan, M.D., Parker, I. Autonomous T cell trafficking examined *in vivo* with intravital two-photon microscopy. Proc Natl Acad Sci U S A. 100, 2604-9 (2003).

