

Video Article

Femur Window Chamber Model for *In Vivo* Cell Tracking in the Murine Bone Marrow

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URL: <https://www.jove.com/video/54205>

DOI: [doi:10.3791/54205](https://doi.org/10.3791/54205)

Keywords: Immunology, Issue 113, Intravital imaging, multiphoton fluorescence microscopy, window chamber, femoral bone marrow, neutrophils, cell tracking

Date Published: 7/28/2016

Citation: Chen, Y., Maeda, A., Bu, J., DaCosta, R. Femur Window Chamber Model for *In Vivo* Cell Tracking in the Murine Bone Marrow. *J. Vis. Exp.* (113), e54205, doi:10.3791/54205 (2016).

Abstract

Bone marrow is a complex organ that contains various hematopoietic and non-hematopoietic cells. These cells are involved in many biological processes, including hematopoiesis, immune regulation and tumor regulation. Commonly used methods for understanding cellular actions in the bone marrow, such as histology and blood counts, provide static information rather than capturing the dynamic action of multiple cellular components *in vivo*. To complement the standard methods, a window chamber (WC)-based model was developed to enable serial *in vivo* imaging of cells and structures in the murine bone marrow. This protocol describes a surgical procedure for installing the WC in the femur, in order to facilitate long-term optical access to the femoral bone marrow. In particular, to demonstrate its experimental utility, this WC approach was used to image and track neutrophils within the vascular network of the femur, thereby providing a novel method to visualize and quantify immune cell trafficking and regulation in the bone marrow. This method can be applied to study various biological processes in the murine bone marrow, such as hematopoiesis, stem cell transplantation, and immune responses in pathological conditions, including cancer.

Video Link

The video component of this article can be found at <https://www.jove.com/video/54205/>

Introduction

Bone marrow is an important organ involved in hematopoiesis and immune regulation. It consists of a hematopoietic component containing hematopoietic stem and progenitor cells (HSPCs), and a stromal component containing non-hematopoietic progenitor cells that give rise to mesenchymal cells¹. Two-thirds of hematopoietic activity is dedicated to the generation of myeloid cells². In particular, a large number of neutrophils are produced in the bone marrow, with $1-2 \times 10^{11}$ cells generated per day in a normal adult human². Neutrophils are the first line of defense against microbial infections and are mostly reserved in the bone marrow until stress triggers their mobilization to supplement peripheral neutrophils^{1,3}. In addition to their anti-microbial effects, recent studies suggest an important role of neutrophils in cancer biology, having both pro- and anti-tumorigenic phenotypes depending on transforming growth factor beta (TGF- β) signaling in the tumor microenvironment^{4,5}. Moreover, studies demonstrated that neutrophils that accumulate in primary tumors exert pro-tumorigenic and metastatic effects by suppressing the cytotoxic function of T cells^{6,7}, while neutrophils in circulation exert a cytotoxic, anti-metastatic effect⁸. As such, investigation of hematopoietic cells in the bone marrow, particularly neutrophils, is crucial to elucidating their role in immune and tumor regulation.

Histopathology and a complete peripheral blood count are routinely used to evaluate cellular and structural alterations in the bone marrow⁹. However, these methods only provide static information of different cell populations or tissue microstructures. Longitudinal *in vivo* imaging can be used in combination with the standard methods to assess the dynamics of multiple cellular, vascular and stromal components as well as cell-to-cell interactions in a longitudinal manner. Intravital microscopy (IVM), defined as imaging of living animals at microscopic resolution¹⁰, is particularly useful for assessing dynamic cellular processes over time in the same sample, reducing the number of experimental animal required. IVM is often combined with a chronically transplanted window chamber (WC) to access the organ of interest for imaging over a duration of weeks to months. Cranial and dorsal-skinfold WC models have the longest history of use dating back to the mid 1990s. More recently, other organ-specific WC models such as those of the mammary fat pad and various abdominal organs have been developed¹¹.

The typical approach for imaging bone marrow *in vivo* has mainly involved exposure of the calvaria of mice, where the thinned bone enables direct visualization of single cells with minimal surgical intervention¹²⁻¹⁴. However, the calvarial bone marrow may be distinct from that of other bones, such as the long bone, as demonstrated by a lower number of HSPCs and hypoxic cells in calvaria, which indicates reduced maintenance and development of HSPCs¹⁵. Therefore, alternative approaches for assessing cellular components in the long bone have been investigated. These include direct exposure of the femoral bone marrow¹⁶ and ectopic transplantation of split femur in the dorsal skinfold WC¹⁷.

However, the former is a terminal procedure that does not permit tracking of cellular, structural and functional alterations over longer time periods, and the latter likely disturbs normal bone marrow function due to the transplantation of femur to an ectopic site inside the dorsal skinfold WC. Another method that enables orthotopic serial imaging of femoral bone marrow over time is the use of a WC in the femoral bone. One previous report demonstrated long-term imaging of microcirculation in the femoral bone marrow using a femur WC in mice¹⁸. Additionally, the authors demonstrated visualization of tumor cells in the femur, indicating its utility in monitoring bone marrow metastasis. However, this WC design was limited by its large size (1.2 cm diameter) and relatively small imaging area (4 mm diameter), which was only suitable for large mice (26-34 g, 3-6 months of age) thereby making the approach impractical for routine use.

Therefore, a new WC with a smaller overall size and larger inner imaging area was designed for the purpose of this study. The goal of this study was to provide a method for imaging various cell types in the femoral bone marrow. The femur WC model was developed in-house and was used to visualize and track neutrophils within the 3D vascular network. Using this model, IVM of the bone marrow can be performed serially over 40 days. This approach can be applied to a variety of fields for elucidating the processes of hematopoiesis, immune regulation and tumor development.

Protocol

NOTE: All animal work was carried out under protocol #2615 approved by the University Health Network Institutional Animal Care and Use Committee.

1. Surgical Preparation of Mouse

1. Prior to surgery, sterilize all surgical instruments and the window chamber (WC) by autoclaving. Supplement the drinking water with 50 mg/kg body weight of amoxicillin 2 days prior to surgery. Inject the mouse with 0.1 mg/kg body weight of buprenorphine intraperitoneally 4 hr prior to surgery.
2. Anesthetize the athymic nude mouse by intraperitoneal injection of 350-400 μ l saline solution containing 80 mg/kg ketamine and 13 mg/kg xylazine (Note: This was approved by the Institutional Animal Care Committee, and we have not had any experience with adverse effects). Confirm proper anesthesia by testing for foot reflex. Continue observing for respiratory pattern, mucous membrane color and foot reflex during the procedure.
3. Perform the surgical procedures under a biosafety cabinet to maintain sterile conditions during the surgery. Place the mouse on an electric heating pad covered with a surgical drape to maintain body temperature during the surgery. Perform all surgical procedures under the stereomicroscope.
4. Apply eye ointment to keep the eyes moist during surgery. Repeat if necessary during the surgical procedure. Sterilize the operating area of the mouse by wiping with 7.5% povidone-iodine, 70% isopropyl alcohol and 10% povidone-iodine consecutively, and repeat this procedure twice.
5. Make a 10 mm longitudinal skin incision using scalpel with a blade (#15), approaching the femur from the lateral side. Expose the femur between the flexor and extensor muscles by blunt dissection using forceps and hemostats to ensure muscle functionality.
6. Insert the U-shaped bar under the bone, and install the WC (**Figure 1**). Secure the bar with the window using two nuts, and tighten the nuts with forceps. Fill the space between the bone and the WC with dental cement.
7. Grind down the cortical bone in a 6 x 2 mm² region with a micro-drill to gain optical access to the marrow cavity.
Note: Carefully inspect thinning of the cortical bone under the microscope while performing this procedure. Leave an intact, almost transparent periosteal layer of the bone.
8. Place the 8 mm coverglass onto the window, and secure the coverglass with a snap ring to keep it in place.
9. Readjust the musculature of the flexors and extensors back to their original locations by using forceps to maintain their function following the surgery. Suture the dermis using 5-0 suture and a needle holder.
10. Monitor the mouse for recovery, until it is conscious and is able to move freely. Put the animal into a new cage when recovered.
11. After recovery, perform image acquisition on the animal on the same day or the following days (see section 2). Monitor the physical condition of the mouse daily.
Note: Monitor physical conditions, such as weight bearing on limb, hunching, rapid or shallow breathing, hind limb swelling, self-mutilation, cellulitis and severe dermatitis.
12. Give intraperitoneal injection of 0.1 mg/kg body weight of buprenorphine for 3 days, and 1 mg/kg body weight of meloxicam for 5 days following the surgery for treatment of post-surgical pain and inflammation. Continue providing the amoxicillin-supplemented water for 5 days.
13. At the experimental endpoint, euthanize the animal by CO₂ narcosis followed by a secondary method (e.g., cervical dislocation).

2. Image Acquisition Using Combined Multi-photon and Confocal Microscopy

1. Prior to imaging, anesthetize the mouse by intraperitoneal injection of a 350-400 μ l saline solution containing 80 mg/kg ketamine and 13 mg/kg xylazine. Alternatively, use inhalable anesthetic, when available. Apply eye ointment.
2. Inject the mixture of intravascular dyes for labeling vasculature (0.65 mg of 2 MDa Fluorescein (FITC)-dextran) and neutrophils (4 μ g of Allophycocyanine (APC)-anti-Ly6G antibody).
3. Use an upright combined multi-photon and confocal microscope equipped with a multi-photon laser ranging from 705-980 nm for two-photon excitation, as well as single photon lasers that provides excitation wavelengths from blue to red.
4. Set a custom-made imaging stage onto the microscope (**Figure 1B**). Secure the mouse on the stage by clamping the two ends of the WC with alligator clips.
Note: Critical components of the stage are: two alligator clips to hold the WC, and a heating element to maintain physiological temperature (37 °C) of the mouse during imaging.
5. Turn on the two-photon laser and the confocal system, and launch the image acquisition software by clicking "Start System". A window with 4 tabs (Locate, Acquisition, Processing, and Maintain) will open.

6. Set up the imaging channels. Click "Smart Setup" under the Acquisition tab and choose the dyes that will be used for imaging: FITC (488 nm excitation, 493-588 nm emission) and APC (633 nm excitation and 638-743 nm emission). Choose "Best Signal" and click "Apply".
7. Add the second harmonic generation (SHG) image acquisition channel manually. Turn on the two-photon laser under the "Laser" window in the Acquisition tab. Under the Channels window, click on the "+" sign to add a track.
8. Set up the SHG track. Change the wavelength to 840 nm under Channels window using the up and down arrows, and choose the detection range to be 415-425 nm under "Light Path" window using the slider bar.
9. Click on "Oculars Online" under the Locate tab. Click FITC under the filter setting for FITC to view the region of interest and adjust the focus manually by turning the focus knob on the side of the microscope. When the region of interest is located, click "Oculars Offline" under the Locate tab.
10. Click on the Acquisition tab. Select FITC track under Channels window and click on "Live" to preview the image of FITC-dextran with 5X objective. Adjust the focus if necessary, and click "Stop" under Acquisition tab to stop the preview.
11. Manually turn to 20X water objective by moving a wheel above the lenses. View the image again for FITC-dextran using the 20X objective, and adjust Master Gain and Pinhole under the Channels window to achieve optimal signal and contrast. Repeat for other channels by selecting APC and SHG tracks under the Channels window.
12. Choose the imaging parameters. Click on "Acquisition Mode" to open its window. Set the frame size to be 1,024 x 1,024 pixels, and averaging of 4X to obtain high quality images.
13. Acquire a 2D snapshot. Select all channels under Channels window and click on "Snap" under the Acquisition tab. Check the quality of the image and change imaging parameters if necessary.
14. Acquire 2D time-lapse image.
 1. Check off "Time Series" in the Acquisition tab to open the Time Series window. Type in 0 under the intervals, and 40 for total number of scans to take 40 images continuously without time interval.
Note: 40 scans generates approximately 5-minute long time-series with a scan speed of 7.5 sec/frame and the image size of 600 μ m x 600 μ m. Adjust the interval and number of scans depending on the microscope, image acquisition settings and the frame rate of the camera. A fixed interval time (>0 sec) may be used to compare time-lapse images acquired at different time points. A longer time-lapse imaging duration may be required depending on the expected average velocity of neutrophils (or other cells of interest) in the experimental condition.
15. Acquire 3D image.
 1. Check off "Z-Stack" in the Acquisition tab to open the Z-Stack window. Start the "Live" preview mode for FITC-dextran. Focus on the top of the sample and click "Set First", and focus on the other end of the sample and click "Set Last" in the Z-stack window.
 2. Set the interval to be 10 μ m under the Z-stack window. Depending on the first and the last optical slice selected in the previous step, this will generate 10-20 slices, with overall optical thickness of 90-180 μ m.
Note: The z-stack interval is dependent on the size of the pinhole, which determines the thickness of the optical slice. Set the interval to be less than the thickness of the optical slice.
 3. Click "Start Experiment" under the Acquisition tab to start collecting images.
16. Acquire 3D time-lapse image.
 1. Check off "Time Series" and "Z-stack" in the Acquisition tab to open the Time Series and the Z-stack windows.
 2. Set up the Time Series and Z-stack acquisition parameters as described in steps 2.14 and 2.15. Click "Start Experiment" under the Acquisition tab to start collecting images.
17. Monitor the mouse during imaging to make sure it is stable and unconscious. If the mouse is unstable and/ or conscious during imaging, remove the mouse from the stage, inject additional 100-150 μ l of the anesthetic solution intraperitoneally, and continue imaging.
Note: Signs of instability include fast breathing and motion of the mouse, which can be seen as shifting artifacts in acquired images.
18. After image acquisition, take the mouse off the stage and return it to the cage. Use a heating lamp to keep it warm, and monitor the mouse until it is conscious.

3. Image Analysis and Quantification

1. Use image analysis software to analyze the 3D and time-series data. Open the software and add images by dragging them into the open screen, which is called "Arena" view. Double click on an image file to open it; the image will be opened under "Surpass" view.
2. Create a 2D/3D image.
 1. From the "More" bar found on the top right corner, click "Edit/ Show Display Adjustment" to open "Display Adjustment" window. Optimize the fluorescence display by adjusting the thresholds for each channel, and take a snapshot to save the image.
3. Generate object tracks using time-lapse images.
 1. Open the image to be analyzed under "Surpass" view. Click on the icon with an image of orange dots, found below the "Surpass" icon. This will open a new "Spots" window.
 2. Check off "Track Spots (over Time)" and click on the blue arrow to go to the next step. Select the channel containing the objects to track under "Source Channel", and set the estimated object diameter under "Estimated XY Diameter" by typing a number in. Click on the blue arrow to go to the next step.
Note: For neutrophil tracking, select the channel containing APC fluorescence for neutrophils and set the object diameter to 12 μ m based on the known size of neutrophils.
 3. In the window, find and select "Quality" filter by clicking on the down arrow. Observe the spots defined by the software and adjust the threshold manually to include or exclude spots. Once threshold is defined, click on the blue arrow to go to the "Tracking" step.
 4. Under Algorithm, find and select "Autoregressive Motion Algorithm" by clicking on the down arrow. Type in 10 μ m for "Max Distance" and 3 for "Max Gap Size" found under Parameters. Check off "Fill gaps with all detected objects" to start tracking, and click on the blue arrow to go to the next step.

Note: "Autoregressive motion algorithm" is used for objects that have directed movement, since it generates tracks based on predicted location from previous motion. "Max Distance" and "Max Gap Size" may be adjusted depending on the expected dimension and velocity of the object being tracked, as well as the overall duration of the time-lapse image.

5. Under Filter Type, select the "Track Duration" filter. Click on the green arrow to finish tracking, and check the tracks that were generated. Go back to the previous step if necessary to manually sort the tracks.
4. If the generated time-lapse image drifts, correct for translational drift.
 1. Click on the icon with an image of a pencil. This is an editing tab that opens "Edit Tracks" window. Examine the time-lapse image and find a reference spot that should not be moving for all the images.
 2. In the left upper corner of the screen, find "Pointer" window and check off "Select" to activate the cursor for selecting a spot. Click on the reference spot.
 3. Click "Correct Drift" in the "Edit Tracks" window. Check off "Translational Drift". The software will automatically generate the corrected series of images.
5. Measure cellular movement defined by the object tracks in time-lapse images.
 1. Under the "Edit Tracks" window, check off "Tracks" and select a track that represents the movement of a cell. The selected track will be highlighted in yellow in the time-series image.
 2. Click on the icon with an image of a graph to open the "Statistics" window (statistics tab).
 3. Under "Selection", select "Track Speed Mean" by clicking on the down arrow to generate the track speed mean for the selected track. Choose different tracks and repeat the steps to measure the track speed mean for all cells of interest.

Representative Results

Murine femoral bone marrow is successfully accessed using the WC to enable visualization of individual neutrophils and vascular networks.

Figure 1 shows the WC instrument and describes the surgical procedure, which involves exposure of the femoral bone and thinning of cortical bone to gain optical access inside the bone. The surgery is well-tolerated in mice; they were assessed for adverse physical reactions, such as hind limb swelling, non-weight bearing on limb, self-mutilation, cellulitis and dermatitis, as well as damage to the WC for 5 days post-surgery, with no report of distress. Notably, the window remains optically clear for IVM for up to 40 days, which may enable long-term assessment of cellular and structural alterations following an intervention.

Vasculature within the bone marrow can be visualized by intravascular dyes, such as FITC-Dextran (**Figure 2**). Since the contrast is dependent on the injection of the dye, the acquired image represents functional vasculature where there is sufficient blood flow. In addition to the vasculature, neutrophils can be stained *in vivo* using anti-Ly6G antibody. **Figure 3A** and **3B** represent the same area of the bone marrow observed at different time points, where neutrophils are seen both in the intravascular and extravascular space. These areas were characterized by the vascular landmarks. Rhodamine 6G may be used as an alternative to anti-Ly6G to stain neutrophils *in vivo*; however, it diffuses out of the vasculature and stains monocytes in the extravascular space in a non-specific manner. Therefore, anti-Ly6G is preferred because it enables specific imaging of neutrophils. **Figure 3C** represents imaging of the cortical bone, which is mainly composed of collagen fibers. The maximal depth for imaging achieved in this experiment is 180 μm (**Figure 3C**).

Time-lapse fluorescence imaging facilitates tracking and quantification of cellular movement *in vivo*. **Figure 4A** shows tracking of neutrophils in the bone marrow vascular niche. Imaging with no additional time interval between each scan allows for accurate tracking of cells; however, some user input is required to further improve the accuracy of tracks generated by the software. Tracking of spots (*i.e.*, cells of interest) enables quantification of various parameters, such as mean track speed (**Figure 4B**). Other parameters that can be measured include, but are not limited to, track length, change in spot size, shape and intensity. This assay provides quantification of various parameters that improve the characterization of cellular movements and interactions.

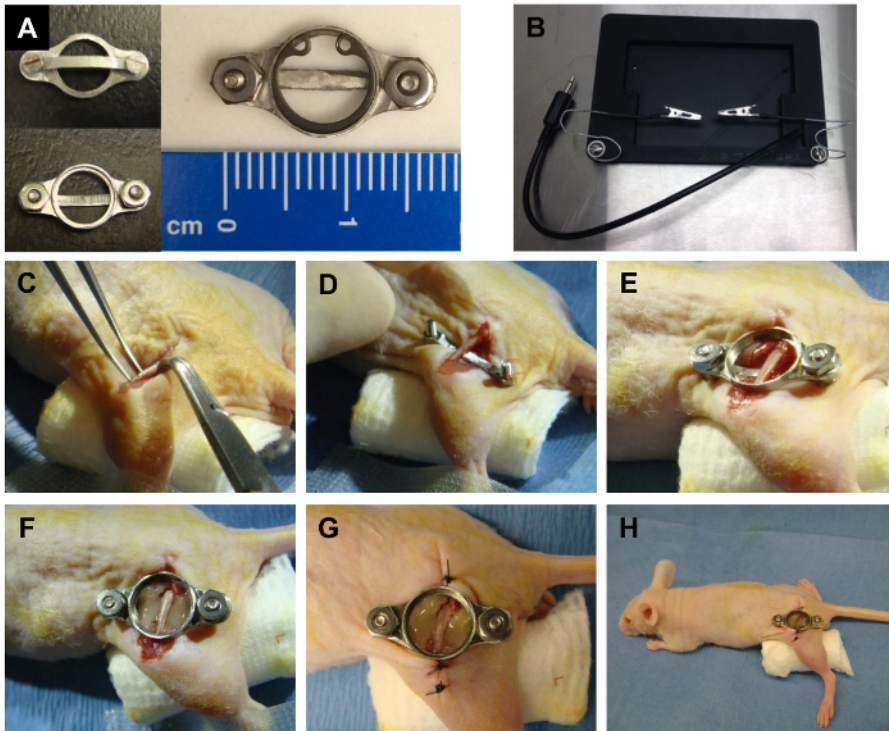


Figure 1: Femur window chamber (WC), imaging stage and the surgical procedure. (A) Custom-made titanium femur WC with a U-shaped bar that serves to fix the femur in place. The WC holds an 8 mm diameter cover glass, secured by a snap ring. (B) Custom-made imaging stage. A heating element is placed on the bottom of the stage to maintain the physiological temperature of the mouse during imaging. The two alligator clips are used to hold the WC. (C-H) Surgical procedure for installing the femur WC. (C) The femur is exposed between the flexor and extensor muscles. Muscles are not removed during the procedure. (D) The U-shaped bar is placed under the femur, and (E) the WC is installed and secured using two nuts. (F) Dental cement is applied between the bone and the WC to fill the space. (G) The dermis is sutured and (H) the cover glass is fixed in place with the retaining ring. [Please click here to view a larger version of this figure.](#)

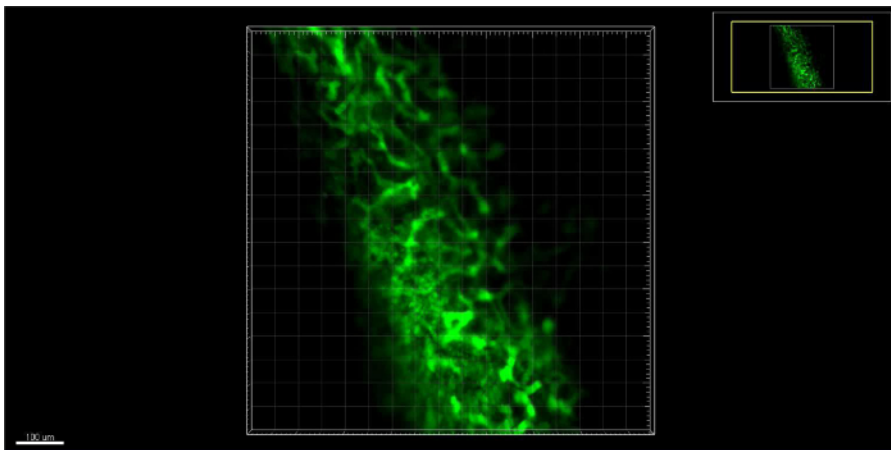


Figure 2: 3D fluorescence imaging of vascular network in the bone marrow *in vivo*. Representative image of vasculature in the bone marrow, imaged through the femur WC. Vasculature (green) is visualized by tail vein injection of 2 MDa FITC-Dextran. Scale bar = 100 μ m. [Please click here to view a larger version of this figure.](#)

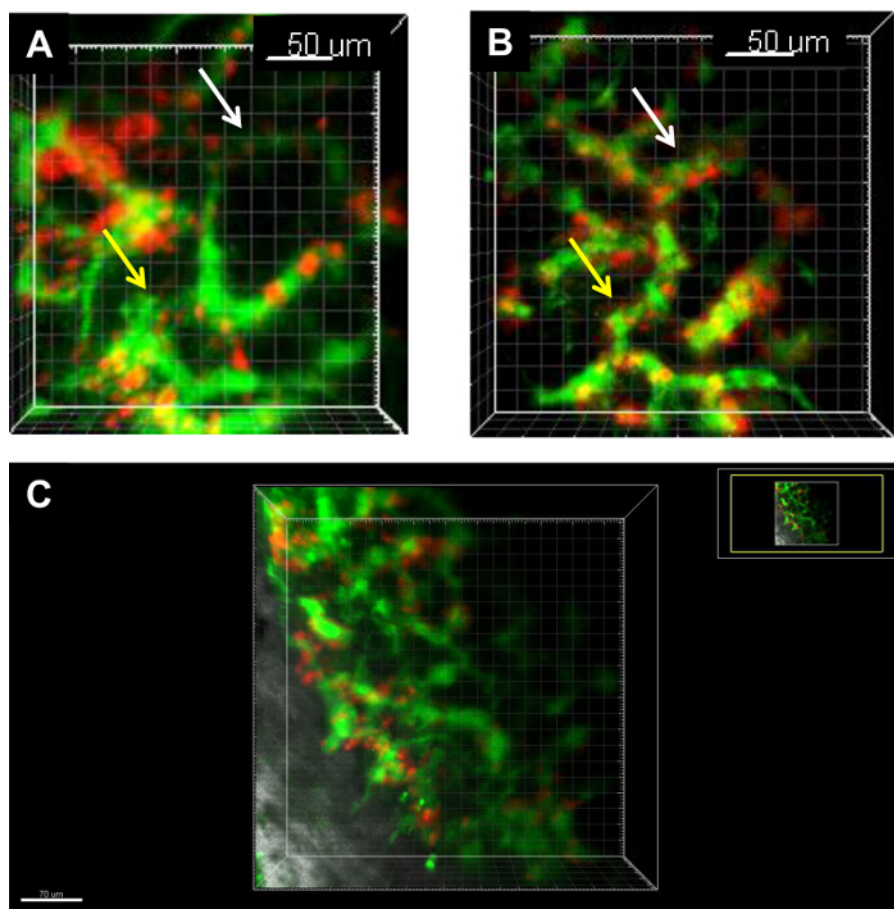


Figure 3: 3D imaging of vascular network, neutrophils and bone matrix *in vivo*. Images of vasculature and neutrophils in the bone marrow (A) 3 days and (B) 10 days after the femur WC surgery. Neutrophils, stained by tail vein injection of APC-labeled Ly6G antibody, are seen both in the intravascular and extravascular spaces. The arrows point to the vascular structure seen on both days, indicating the ability to track the same location over time. Scale bar = 50 μm. (C) Bone matrix can be visualized using SHG (white), in addition to vasculature (green) and neutrophils (red). The image was acquired 40 days after the WC surgery. Scale bar = 70 μm. [Please click here to view a larger version of this figure.](#)

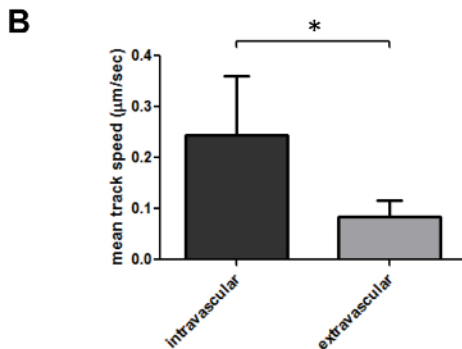
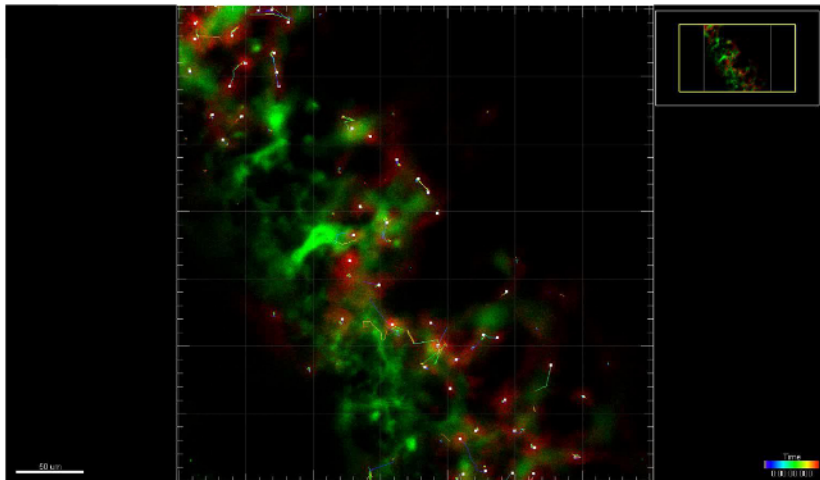


Figure 4: Tracking neutrophil movement within the vascular network of the bone marrow *in vivo*. (A) Track paths of neutrophils within the vascular network. Tracks are generated automatically using the software's built-in Autoregressive Motion algorithm. Each white dot represents a center point of a particle being tracked (neutrophils). Scale bar = 50 μm . (B) Mean track speed measured for intravascular and extravascular neutrophils, indicating difference in the movement of neutrophils (Error bar = mean + SD, * $p = 0.0176$, two-tailed t-test). [Please click here to view a larger version of this figure.](#)

Discussion

Real-time, serial imaging of the dynamic cellular processes in bone marrow provides information that is otherwise challenging to obtain using conventional techniques such as histology and total blood counts. The femur WC model described here provides unique opportunities to investigate cellular and structural alterations in the bone marrow over time. Although a femur WC model has been previously reported, our novel design provides a larger imaging field of view and smaller overall WC size, which are more compatible for use in adult mice. The femur WC model can be used in a variety of mouse strains including immunocompetent and/or transgenic reporter mice.

There are critical steps during the surgery that determine the overall duration and quality of imaging. When exposing the femur, only a blunt dissection of muscles between flexors and extensors is performed; removal of these muscles may result in reduced mobility of the mouse and must be avoided. Furthermore, grinding of the cortical bone must be performed carefully. Grinding approximately 50 μm is adequate for optical imaging while maintaining the structural integrity of the femoral vascular network. Application of dental cement around the bone is crucial for sealing the surgically exposed area, preventing fluid accumulation, and maintaining a clear optical imaging window for over a month while reducing the risk of infection. The dental cement also acts to fix the WC in place, preventing the WC from rotating around the femoral bone. Lastly, the snap ring used to hold the coverglass is suitable for use with water-immersion lens, providing water-seal. The snap ring is recommended over adhesives to attach the coverglass to the WC because addition of adhesives may increase the gap between the bone and the coverglass, or place the coverglass at a slight angle relative to the imaging plane, thereby reducing the achievable penetration depth for imaging. Furthermore, the snap ring is useful when the coverglass needs to be cleaned or changed.

In addition to the surgery, there are several critical steps to achieve successful optical imaging of the bone marrow. To image inside the femur WC, the WC is placed horizontally to the imaging stage for proper immersion of the objective lens. The custom-made imaging stage is suitable for this purpose as it allows for adjustments of the angle of the WC with respect to the lens. Moreover, the design of the WC, particularly the size of the U-shaped bar, maintains the bone in a horizontal position as close to the coverglass as possible to achieve optimal penetration depth. During imaging, breathing artifacts may be observed as horizontal lines in the fluorescence images. In addition to securing the WC to the imaging stage, the temperature of the heating element under the imaging stage may be adjusted to adequately warm the animal to minimize excessive breathing. A thin layer of gauze may be placed between the mouse and the heated stage to further help reduce breathing artifacts during imaging. Depending on the biological imaging target, acquisition parameters such as frame rate, imaging volume and imaging duration may also be adjusted to collect sufficient images to address experimental questions. For example, velocity of neutrophil migration and crawling

may differ under various experimental conditions, from 2.8 $\mu\text{m}/\text{min}$ for resident neutrophils under basal conditions to anywhere ranging from 5-10 $\mu\text{m}/\text{min}$ for neutrophils under inflammatory conditions¹⁹⁻²¹. Thus, the appropriate imaging speed and duration for neutrophil tracking *in vivo* would also differ based on experimental conditions. In addition, as seen from the results, tracking of cells within the vasculature would require a fast imaging camera with a high frame rate (30 frames/sec). However, if the target cells are residing in extravascular space, or if the cell-to-cell interaction of interest is expected to occur more slowly, image quality should be prioritized over imaging speed.

The influence of injected antibodies for *in vivo* cell staining on cellular function must also be considered. In our study, we used anti-Ly6G antibody (1A8 clone), which is typically used at high doses (150-250 μg) to deplete neutrophils from circulation and study their role in various disease models. Yipp and Kubes demonstrated that fluorochrome-labeled anti-Ly6G antibody does not disrupt leukocyte recruitment at low intravascular injection doses (1-40 μg)²². However, a more recent study suggests that fluorochromes, having great differences in molecular mass and chemical structure, can differentially influence the function of anti-Ly6G²³. The study demonstrated that FITC-labeled anti-Ly6G, but not PE- and APC-labeled antibodies, effectively depletes neutrophils *in vivo* at a low concentration (30 μg). In our study, we did not observe neutrophil depletion overtime using 4 μg of APC-labeled anti-Ly6G. Nevertheless, further characterization of the function of fluorochrome-labeled anti-Ly6G antibody *in vivo* is required to validate its use in intravital imaging. This also applies to other antibodies that have functional ability to alter cellular interactions. As an alternative to *in vivo* cell staining with antibodies, transgenic reporter mice²⁴ and/or labeled fluorescent cells²⁵ can be used to visualize neutrophils or other cell population of interest *in vivo*.

The femur WC model described in this protocol enables imaging for up to 40 days after the surgery. Some of the challenges for long-term imaging using this WC model include the delayed bone healing process and maintenance of a clean glass window. Notably, we did not observe signs of obvious bone healing following the WC implantation, which would include thickening of periosteum; this is not unexpected given that bone healing process typically occurs after 1 ½-3 months^{26,27}. Maintenance of a clean and optically-clear window can be achieved by the application of the dental cement, and the use of snap ring and coverglass which provide a firm water-seal with the tissue. The coverglass can be occasionally cleaned with cotton tips from the outside to remove dust and debris. The WC has a wide imaging field, almost covering the entire length of the diaphysis region (approximately 8.4-8.6 mm²⁸), which makes it superior to the previously published model¹⁸. However, access to the distal regions and the deeper marrow cavity is limited. In particular, the trabecular-rich metaphysis, located at the distal ends of the diaphysis, is known to be rich in HPSCs²⁹. These areas may be accessed histologically, or by IVM as a terminal procedure. Alternatively, different imaging modalities such as micro-computed tomography can be used to image the distal regions of the bone marrow *in vivo*³⁰. Such methods are complementary to IVM, providing an integrated approach for assessing anatomy and the various cellular and structural alterations and interactions within the bone marrow.

The protocol described here offers a novel approach for long-term spatio-temporal assessment of the femoral bone marrow in mice at cellular resolution. The methods presented enable *in vivo* serial imaging to track movements of a single cell or cellular populations over biologically relevant time scales, while simultaneously obtaining structural and functional information of the bone marrow. In addition, quantitative image analysis can be performed to further characterize cellular and functional alterations, such as tracking of cellular movements. Application of this femur WC model in various strains of mice, particularly in transgenic reporter mice³¹, may further enable investigation of multiple individually-labeled cell populations such as those involved in hematopoiesis. Therefore, the described protocol is widely applicable to a wide range of preclinical studies involving various biological processes within the living murine bone marrow, including investigations in hematopoiesis, leukemia, HPSC transplantation, immune regulation, the bone marrow microenvironment (e.g., hypoxic niche) and the contribution of immune cells to tumor progression and metastasis.

Disclosures

The authors have no competing financial interests.

Acknowledgements

The authors would like to thank the Advanced Optical Microscopy Facility (www.aomf.ca) at the University Health Network for assistance with microscopy, and Mr. Jason Ellis from the Princess Margaret Cancer Center Machine Shop for manufacturing the WC and the imaging stage. We would also like to thank Dr. Iris Kulbatski for manuscript editing.

References

1. Zhao, E. *et al.* Bone marrow and the control of immunity. *Cell Mol Immunol.* **9** (1), 11-19, (2012).
2. Borregaard, N. Neutrophils, from marrow to microbes. *Immunity.* **33** (5), 657-670, (2010).
3. Boxio, R., Bossenmeyer-Pourie, C., Steinckwich, N., Dournon, C., & Nusse, O. Mouse bone marrow contains large numbers of functionally competent neutrophils. *J Leukoc Biol.* **75** (4), 604-611, (2004).
4. Fridlender, Z. G., & Albelda, S. M. Tumor-associated neutrophils: friend or foe? *Carcinogenesis.* (2012).
5. Fridlender, Z. G. *et al.* Polarization of tumor-associated neutrophil phenotype by TGF-beta: 'N1' versus 'N2' TAN. *Cancer Cell.* **16** (3), 183-194, (2009).
6. Coffelt, S. B. *et al.* IL-17-producing [ggr][dgr] T cells and neutrophils conspire to promote breast cancer metastasis. *Nature.* **522** (7556), 345-348, (2015).
7. Kitamura, T., Qian, B. Z., & Pollard, J. W. Immune cell promotion of metastasis. *Nat Rev Immunol.* **15** (2), 73-86, (2015).
8. Granot, Z. *et al.* Tumor entrained neutrophils inhibit seeding in the premetastatic lung. *Cancer Cell.* **20** (3), 300-314, (2011).
9. Travlos, G. S. Normal structure, function, and histology of the bone marrow. *Toxicol Pathol.* **34** (5), 548-565, (2006).
10. Pittet, M. J., & Weissleder, R. Intravital Imaging. *Cell.* **147** (5), 983-991, (2011).
11. Alieva, M., Ritsma, L., Giedt, R. J., Weissleder, R., & van Rheenen, J. Imaging windows for long-term intravital imaging. *IntraVital.* **3** (2), e29917, (2014).

12. Hamon, P., Rodero, M. P., Combadiere, C., & Boissonnas, A. Tracking mouse bone marrow monocytes in vivo. *J Vis Exp.* (96), e52476, (2015).
13. Mazo, I. B. *et al.* Hematopoietic Progenitor Cell Rolling in Bone Marrow Microvessels: Parallel Contributions by Endothelial Selectins and Vascular Cell Adhesion Molecule 1. *J. Exp. Med.* **188** (3), 465-474, (1998).
14. Scott, M. K., Akinduro, O., & Lo Celso, C. In Vivo 4-Dimensional Tracking of Hematopoietic Stem and Progenitor Cells in Adult Mouse Calvarial Bone Marrow. *J. Vis. Exp.* (91), e51683, (2014).
15. Lassailly, F., Foster, K., Lopez-Onieva, L., Currie, E., & Bonnet, D. Multimodal imaging reveals structural and functional heterogeneity in different bone marrow compartments: functional implications on hematopoietic stem cells. *Blood.* **122** (10), 1730-1740, (2013).
16. Kohler, A., Geiger, H., & Gunzer, M. Imaging hematopoietic stem cells in the marrow of long bones in vivo. *Methods Mol Biol.* **750** 215-224, (2011).
17. Balan, M., & Kiefer, F. A novel model for ectopic, chronic, intravital multiphoton imaging of bone marrow vasculature and architecture in split femurs. *IntraVital.* **4** (2), e1066949, (2015).
18. Hansen-Algenstaedt, N. *et al.* Femur window—a new approach to microcirculation of living bone in situ. *J Orthop Res.* **23** (5), 1073-1082, (2005).
19. Xu, N., Lei, X., & Liu, L. Tracking Neutrophil Intraluminal Crawling, Transendothelial Migration and Chemotaxis in Tissue by Intravital Video Microscopy. *J. Vis. Exp.* (55), e3296, (2011).
20. Mizuno, R. *et al.* In vivo imaging reveals PKA regulation of ERK activity during neutrophil recruitment to inflamed intestines. *J. Exp. Med.* **211** (6), 1123-1136, (2014).
21. Kreisel, D. *et al.* In vivo two-photon imaging reveals monocyte-dependent neutrophil extravasation during pulmonary inflammation. *Proc. Natl. Acad. Sci.* **107** (42), 18073-18078, (2010).
22. Yipp, B. G., & Kubes, P. Antibodies against neutrophil LY6G do not inhibit leukocyte recruitment in mice *in vivo*. *Blood.* **121**(1) 241-2, (2013).
23. Bucher, K. *et al.* Fluorescent Ly6G antibodies determine macrophage phagocytosis of neutrophils and alter the retrieval of neutrophils in mice. *J Leukoc Biol.* **98** (3), 365-372, (2015).
24. Lammermann, T. *et al.* Neutrophil swarms require LTB4 and integrins at sites of cell death in vivo. *Nature.* **498** (7454), 371-375, (2013).
25. Prohazky, F., Dallman, M. J., & Lo Celso, C. From seeing to believing: labelling strategies for in vivo cell-tracking experiments. *Interface Focus.* **3** (3), (2013).
26. Koewler, N. J. *et al.* Effects of a Monoclonal Antibody Raised Against Nerve Growth Factor on Skeletal Pain and Bone Healing After Fracture of the C57BL/6J Mouse Femur. *J Bone Miner Res.* **22** (11), 1732-1742, (2007).
27. Garcia, P. *et al.* Rodent animal models of delayed bone healing and non-union formation: a comprehensive review. *Eur Cell Mater.* **26** 1-12; discussion 12-14 (2013).
28. Liu, K. *et al.* A murine femoral segmental defect model for bone tissue engineering using a novel rigid internal fixation system. *J Surg Res.* **183** (2), 493-502, (2013).
29. Morrison, S. J., & Scadden, D. T. The bone marrow niche for haematopoietic stem cells. *Nature.* **505** (7483), 327-334, (2014).
30. Ellis, S. L. *et al.* The relationship between bone, hemopoietic stem cells, and vasculature. *Blood.* **118** (6), 1516-1524, (2011).
31. Vacaru, A., Vitale, J., Nieves, J., & Baron, M. in *Mouse Genetics* Vol. 1194 *Methods in Molecular Biology*. eds Shree Ram Singh & Vincenzo Coppola) Ch. 16, 289-312, Springer New York, (2014).