

## ***In Vivo* Hematopoietic stem cell Imaging**

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**Abstract:**

Though immunohistochemistry of tissue sections has been the gold standard for analyzing tissue structure and cellular localization, this approach has significant shortcomings when it comes to analyzing complex and heterogeneous tissues such as the bone marrow or rare cells like hematopoietic stem cells. Hence studying rare cells and their relationship with the surrounding heterogeneous environment requires visualization of specifically labeled cells within large intact tissues in three dimensions. Here we describe a whole mount sternal bone imaging method which enables detailed quantitative and qualitative analysis of rare HSCs within the sternal tissue. The methodology is broadly applicable for examining the 3D architecture of niche cells in relation to HSCs.

**Key words:** Hematopoietic stem cells, Microenvironment/ niche, immunostaining, confocal imaging, whole mount, second harmonic generation, sternum

## 1. Introduction

Hematopoietic stem cells (HSCs) are rare, self-renewing immature blood cells present in bone marrow and capable of forming mature blood cells or hematopoiesis. HSCs occupy a specialized niche and receive signals for their maintenance(1,2). Though functional studies provide existence of the interactions between HSCs and niches, the localization of HSCs in the bone marrow is still unclear due to its rarity. Determining the location of HSCs would help us to further understand how niche components contribute to stem cell regulation in homeostatic as well as disease conditions. Earlier imaging studies are mainly dependent on 2-dimensional (2D) static imaging in tissue sections using antibodies. It has become increasingly evident that HSCs exist within the three-dimensional (3D) structure of niche, and sophisticated in-depth imaging is required to more completely reveal the stem cell-niche cell interaction. *In vivo* 3D imaging of whole mount tissue can resolve many of these issues and extend our understanding of stem cell behavior in homeostasis and stress(3). The 3D images can be assessed qualitatively and quantitatively to appreciate the distribution of cells with minimal perturbation of the tissues.

The sternum is an excellent location to study bone marrow regeneration due to its complete hematopoietic reconstitution, easy sectioning and large volume(4) (5-7). Here we demonstrate using confocal imaging and multiple immuno-labelling, high spatial resolution of HSCs at single cell level resolution in a whole mount sternal bone (Figure 1a & b). We include second harmonic generation (SHG) imaging to visualize bone in order to quantitate the distance of HSCs present in the bone marrow from the endosteal region. This technology enables qualitative and quantitative assessment of HSC and its location in a homeostatic condition and can be extended to other pathological conditions.

## 2 Materials

### 2.1 Mouse preparation

1. C57BL/6 strain can be used to detect the hematopoietic stem cells.
2. Phosphate buffered saline (PBS)- 1 x Phosphate buffered saline, pH 7.4+2% Fetal bovine serum (FBS)
3. 4',6-Diamidine-2'-phenylindole dihydrochloride (DAPI)- 1mg/ml of water
4. Surgical blade, Dissection board, forceps and scissors
5. Petri dishes

### 2.2 Whole mount immunostaining

1. Exclusion markers- Lineage cocktail (Biotinylated antibody against CD3, CD4, CD8, Mac1, Gr1, Ter119, IgM, B220), CD41 biotin and CD48 biotin. Mix equal concentration of antibodies present in lineage to make the cocktail.
2. Glass bottom dishes: Plastic petri dishes mounted with No. 1.5 coverglass bottoms, such as those sold by MatTek (Part No: P35G-1.5-14-C). The well must be large enough to easily accommodate the bone to be imaged.
3. Microscope: Fluorescence imaging may be performed on a typical confocal microscope setup with appropriate filters and an inverted stand. We have used a scanning confocal (Zeiss LSM780) and a spinning disk confocal (Perkin Elmer Ultraview) for fluorescence imaging.
4. If SHG imaging is required, a scanning confocal must be used and must be equipped with a mode-locked femtosecond multiphoton laser such as the Chameleon tunable (700–1000 nm) titanium sapphire oscillator laser from Coherent. It is possible to acquire SHG images (together with a fluorescent reference channel) separately and at

a lower resolution from the fluorescence images, and three dimensionally align the images in processing for distance quantification; thus the SHG-equipped microscope need not necessarily be the same instrument as the fluorescent microscope.

5. Stage: A motorized stage and Z motor must be used for tile scanning. Microscope software must be configured for tile scan (also called large image) acquisition.
6. Objective lens: The microscope objective should be selected carefully to balance a large field of view, long working distance for imaging the entire bone depth, and adequate resolution for identifying HSC morphology. A 20x magnification objective with numerical aperture of 0.8 is adequate. We have also used the Zeiss Plan-Apochromat multi-immersion 25x/NA 0.8 long working distance objective with good results.
7. Analysis workstation: A workstation installed with Fiji software (National Institutes of Health) and equipped with sufficient RAM to open the entire data set for stitching (we recommend 32 GB of RAM) is required. Fiji is free and may be installed at <https://imagej.net/Fiji/Downloads>. If three dimensional alignment of separately acquired fluorescence and SHG images will be performed, the required plugins may be obtained by following the Stowers Institute update site <http://research.stowers.org/imagejplugins/updates/>. Instructions on how to follow an update site are available in the FIJI help guide: [https://imagej.net/Following\\_an\\_update\\_site](https://imagej.net/Following_an_update_site)

### 3 Methods:

#### 3.1 Preparation of whole mount tissue:

1. Euthanize C57bl/6 (6-8 weeks) old mice as per IACUC institutional guidelines. Either male or female mice can be used. Harvest the sternal bone which lies in the midline of anterior chest wall.
2. Place the sternal bone in ice cold 1 xPBS +2% FBS. Clean the bone by removing the attached muscle, using a surgical blade to gently remove the attached muscle without destroying the bone marrow (note 1 &2).
3. Cut the sternal bone at the midline longitudinally using a sharp surgical blade to expose the bone marrow. Then cut transversely into 3 – 4 fragments approximately 2mm in length at the joint between two sternal fosse and image through the cut surface(4).  
Always keep the bone in 1xPBS with 2%FBS.
4. Gently place the tissue in 96-well plate containing at least 150-200ul of 4% PFA without disturbing the exposed marrow. Allow the tissue to be fixed room temperature for 60mins.
5. Wash with tissue with 1x PBS (3 X 15mins). Now the tissue is ready for immunostaining.

### 3.2 Immunostaining of HSCs in whole mount tissue

1. Carry out the following steps in a 96 well plate with gentle shaking.
2. Block and permeabilize the tissue using 1xPBS containing 20% normal goat serum and 0.5% Triton X-100 for 3hrs.
3. Dilute all your primary antibodies in 1 x PBS with 20% goat serum. To identify hematopoietic stem cell (HSC) by immunostaining, both exclusion and inclusion markers are needed.
4. The unconjugated CD150 antibody has shown limited success hence only a direct fluorescent conjugate CD150 PE at dilution of 1:100 is to be used for whole mount

immunostaining. Other exclusion markers include CD48-Biotin 1:100, CD41-Biotin 1:100, Lineage cocktail Biotin 1:50). Incubate the tissue at room temperature in darkness for 3 days (Note 3& 4).

5. Wash with PBS (3 X 15 mins)
6. Add secondary antibody streptavidin conjugated with FITC, Pacific Blue or APC for the Lineage. Again, add CD150 PE and if you want to see nuclei you can add Dapi at the same time. Incubate at room temperature for 2 hrs.
7. Wash with PBS (3 X 15 mins). Now the tissue is ready for imaging.

### 3.3 Sample orientation for Microscopy:

1. Mount the sample. Using forceps, place the bone cut side down onto the cover glass insert of the dish. Add a small amount of 1 x PBS to keep the bone from drying out (Note 5 & 6).
2. Apply immersion oil to objective (if needed), and carefully load sample dish into stage insert. Focus objective using transmitted light.
3. To minimize the total number of tiles, align the bone axis with the x or y axis of the stage.
4. The cut side of the bone with exposed marrow should be facing the cover glass. It can be difficult when placing the bone in the dish to be sure that the orientation of the bone is correct, with cut side down.
5. Confirm this by focusing up and down through the bone (use transmitted light to minimize unnecessary photobleaching). The characteristic appearance of marrow, with densely packed round cells, should appear all the way to the bottom of the bone, and the marrow surface should be level near the surface of the cover glass.

6. If you see endosteal region appear as you focus down toward the cover glass, the bone is likely in the wrong orientation. If the marrow surface is not level at the cover glass, there may be excess tissue projecting below the cut surface and disrupting the orientation of the bone. Readjust the bone positioning using forceps or remove the bone and trim the excess tissue.

### 3.4 Microscopy

1. Second harmonic generation (SHG) imaging allows identification of bone without antibody labeling. SHG imaging can take place concurrently with fluorescence imaging (this is preferred), however it is possible to perform SHG imaging separately on a different system (together with a reference channel) and three dimensionally align the SHG and multichannel fluorescence images in postprocessing. In that case, complete the fluorescent imaging of the sample first (by following the steps below), then carefully move the sample dish to the SHG microscope. Align the sample axis within the SHG imaging field of view as closely as possible to the orientation used for fluorescent imaging, and repeat the below steps in that system.
2. Set up imaging parameters. In order to minimize crosstalk, use single color labeled samples to test for crosstalk among channels: Image the single-labeled sample using multichannel settings, and check for signal in channels corresponding to longer wavelengths. It is especially important to avoid crosstalk of labels coding for HSC identity, such as CD150 and Lineage (note 7).
3. If acquiring SHG images separately from fluorescence images, one fluorescent channel must be acquired in both sets of data to act as a reference channel. CD150-PE works



well for this as the brightly stained megakaryocytes in that channel are easy to identify between images.

4. Set up SHG parameters if needed. The second harmonic light generated in SHG imaging is precisely half the wavelength of the incident pulsed laser light, so the wavelength of the tunable laser may be selected to minimize overlap of the second harmonic wavelength with emission spectra of fluorophores. We typically use 900nm for excitation of SHG signal and collect at 437-464 nm.
5. Next specify tile scan region and z stack dimensions in software. A pixel size of 0.33  $\mu\text{m}$  in xy, with a z-slice spacing of 4  $\mu\text{m}$  is adequate for resolving subcellular features. If acquiring SHG images separately at lower resolution, the SHG pixel size should be an integer multiple of the fluorescent pixel size, for example  $4 \times 0.33 = 1.32 \mu\text{m}$ .
6. Specifying larger, fewer tiles improves speed and reduces the frequency of stage movements as well as the number of stitched/overlapped pixels in the final image. If using a scanning confocal, the number of total tiles can be reduced by minimizing zoom during acquisition (on the Zeiss LSM-780, this is 0.6), while increasing image dimensions to maintain pixel size. The number of tiles can also be reduced by decreasing tile overlap to the minimum required for adequate stitching. We use 6-10% overlap.
7. Bone samples tend to be irregular in shape and topography. Switching between brightfield visualization through oculars and confocal imaging of fluorescence, move around the perimeter of the sample to mark its extremities in the x, y and z dimensions in the acquisition software. Check a few spots in the center of the sample to confirm that

the range of the z stack covers the sample over its entirety. Typically the final z stack is around 250-300  $\mu\text{m}$  in size.

8. For maximum speed and minimum stage movement, order of acquisition should be [z stack, channel/track, tile]. Tiles should be acquired in a snake-by-rows format if possible, with rows parallel to the short side of the bone sample. This pattern minimizes the time and stage movement between adjacent tiles and improves stitching.
9. If possible, set acquisition software to autosave each tile individually, and begin the scan.

### 3.5 Analysis

1. Analysis may be performed using Fiji software (National Institutes of Health).
2. Process images prior to stitching to avoid possible edge effects. To improve visibility of cellular features, perform a Gaussian blur with standard deviation 0.5 pixel and a rolling ball background subtract of radius 100 pixels.
3. Stitch tiles into a full 3D image using the Grid/Collection Stitching plugin(8).
4. If SHG/reference and fluorescent multichannel images were acquired separately, they must be three dimensionally aligned following stitching. First, resample the SHG/Reference image to match the pixel scaling of fluorescent images, and ensure that both images have the same bit depth. Crop images in x,y, and z so that SHG/Reference and fluorescent multichannel image stacks are the same dimensions, save these images separately (these will be needed for the final alignment), and then merge into a single multichannel image. Using the Channels toolbar, adjust the visualization such that only the reference channel and its corresponding fluorescent image channel are visible in a composite image of contrasting colors. You will now

need to generate a list of point ROIs marking the locations of ten specific landmarks (such as the top right corners of ten megakaryocytes) in both the fluorescence and reference images, marking one ROI for the landmark position in the fluorescence channel and a second for its position in the reference channel, until the ROI list contains twenty total ROIs. To place each point, choose the Point Selection tool and click on the image at the desired location, then hit “t” on the keyboard to add the point the ROI manager (the ROI manager window will appear when the first ROI is added). For best results choose landmarks that are distributed throughout the bone. Save the ROI set by choosing Deselect, then More → Save in the ROI manager.

Close the combined multichannel image, and re-open the previously saved, resolution-matched SHG/reference and fluorescence images. Run the plugin “manual transform 3D images jru v1” available through the Stowers Institute update site (see Materials section 2.2.7). Using the pull down menus, select the fluorescence image stack as the reference image, and the SHG stack as the image to be transformed.

When the plugin has finished, the SHG image will be three dimensionally aligned to the fluorescence image. Merge again into a single multichannel image and save.

5. Identify HSCs: Although CD150 is enriched in HSCs, it is also expressed in other blood cell types, making automatic quantitation impractical. To analyze by eye, samples should be blinded and (ideally) analyzed by a researcher unfamiliar with the hypotheses of the study. HSCs are identified by the presence of CD150 staining combined with the absence of exclusion markers. Cells may be considered positive for signal if the cell membrane can be discerned against background (**Figure 1B**).

6. After HSCs are identified, the distance of HSCs to features of interest can be measured by marking a point region of interest (ROI) at the edge of the cell nearest the feature of interest, and a second point ROI at the feature.
7. The locations of the points are then measured in Fiji and copied to an external program such as Excel. The distance between the points can then be calculated using the 3D Pythagorean Theorem.
8. Distance quantification of random ROIs: A macro for generating random ROIs can be found at [https://github.com/sarah-ellen-smith/image-processing-macros-fiji/blob/master/random\\_ROI\\_generator.ijm](https://github.com/sarah-ellen-smith/image-processing-macros-fiji/blob/master/random_ROI_generator.ijm) . Confirm that the random point ROI is in a region of bone marrow (presence of Lin<sup>+</sup> cells in the area may be used as a reference; generation of random ROIs can be restricted to a binary masked area using the macro available here: [https://github.com/sarah-ellen-smith/image-processing-macros-fiji/blob/master/random\\_point\\_ROI\\_generator\\_masked.ijm](https://github.com/sarah-ellen-smith/image-processing-macros-fiji/blob/master/random_point_ROI_generator_masked.ijm) ). Proceed with distance quantification as if the randomly generated point were an identified HSC.

#### 4. Notes

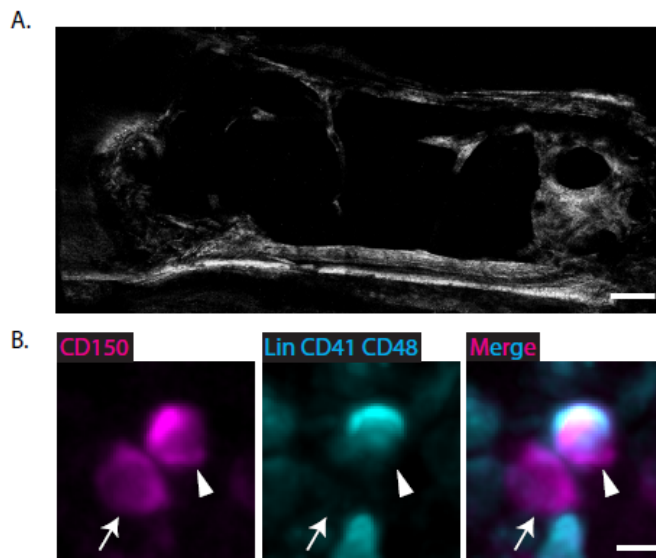
1. Throughout the procedure make sure the bone always is hydrated in buffer or staining solution. A dry sternal bone can give false positive results.
2. The bone should have no muscle attached during staining protocol. Muscle can interfere with the staining procedure.
3. For whole mount only CD150 PE conjugate works and not CD150 APC conjugate even though the antibody has identical clone number. Because of this care should be taken that no other secondary antibody for other proteins should be in PE range.

4. CD150(Slam) is enriched in HSC. However, CD150 is also expressed on other differentiated blood cells hence other exclusion markers needs to be combined with CD150.
5. The PBS should come to the top of the bone. If the PBS is too deep, however, there may be problems with the bone floating or tilting up away from the cover glass.
6. It is worthwhile to allow for extra scan time (either by increased exposure/pixel dwell time or averaging) to maximize signal-to-noise, especially if the staining combination in use must be analyzed by eye.
7. Testing for crosstalk is necessary when establishing settings for the first experiment but, can be omitted in future experiments if the same staining protocol and microscope settings are used.
8. It may be easiest to search for the plugin by name rather than attempting to hunt through menus. Type “1” on the keyboard to get the Fiji search bar.

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**Figure Legend**

Figure 1: A. Maximum projection image of SHG signal in a sternum bone. Scale bar 200µm. B. Two cells positive for CD150 staining. The cell indicated by the arrow is negative for Lin CD41 CD48 staining (and would be counted as an HSC) while the cell indicated by the arrowhead is positive for Lin CD41 CD48 staining and is not an HSC. Scale bar 5µm.