Chapter 6

Histological and In Vivo Microscopic Analysis of the Bone Marrow Microenvironment in a Murine Model of Chronic Myelogenous Leukemia

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Abstract

Imaging of the leukemic bone marrow microenvironment, also called the leukemic bone marrow niche, is an essential method to determine and to evaluate the progression of chronic myelogenous leukemia (CML) and other leukemias in murine models. In this chapter we introduce the murine model of CML primarily used in our laboratory by describing blood and bone marrow analysis as well as the method of histological sectioning and immunohistochemistry in combination with various stainings that can help to understand the complex interaction between leukemic cells, their normal hematopoietic counterparts, and the bone marrow microenvironment. We conclude with describing how to image the bone marrow niche using in vivo microscopy.

Key words Histological sectioning, Immunohistochemistry, In vivo microscopy, Bone marrow niche, Leukemic stem cell niche, Murine models of hematological malignancies, Chronic myelogenous leukemia

1 Introduction

Chronic myelogenous leukemia (CML) is a hematological malignancy caused by reciprocal translocation of chromosomes 9 and 22 that results in the generation of the *BCR/ABL1* fusion gene. This gene encodes for a dysregulated cytoplasmic tyrosine kinase with a size of 210 kDa and is therefore named p210-BCR/ABL1 [1].

In humans, CML is mainly characterized by elevated leukocytes (leukocytosis) in the peripheral blood, most of them being myeloid cells of different maturation stages, basophilia, eosinophilia, and splenomegaly. If untreated, CML progresses from a chronic phase (<5 % blasts) to an accelerated phase (5–19 % blasts) and blast crisis [1], which resembles an acute myeloid or lymphoblastic leukemia (>20 % blasts) [2].

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The characterization of a disease like CML requires specific laboratory techniques and a complex, faithful mouse model. There have been different approaches [3] on how to investigate the pathophysiology of CML so far, and most approaches have their own merit.

Some experiments can be modeled in vitro, for instance, with the BCR/ABL1+ cell line K562 (derived from a patient with CML in blast crisis), but an in-depth examination of the mechanisms of CML, especially when it pertains to the bone marrow microenvironment (BMM) [4], cannot be fully addressed with in vitro experiments only. While some promising in vitro methods to model the BMM are being developed, many lack factors such as shear forces, pH, oxygen status, the cytokine milieu, etc., all of which are thought to play a role in the BMM. For more detailed research and research on the BMM in particular, in vivo models probably provide a more comprehensive way to analyze disease development, progression, and response to therapy. One method to induce CML-like myeloproliferative neoplasia (MPN) in vivo is to transplant human CML cells into immunocompromised mice, for instance, NOD SCID interleukin-2 receptor gamma knockout (NSG) mice, in a so-called xenotransplantation [5]. However, this model is limited as the engraftment of BCR/ABL1+ cells is poor. Another attempt is the development of murine models that express the *BCR/ABL1* oncogene, for instance, in the form of a transgene [6] or in the way of transplantation of donor bone marrow retrovirally transduced with BCR/ABL1 [7, 8].

The murine CML model with the most history, which has also been used to model other hematological malignancies, is the murine retroviral transduction/transplantation model of BCR/ABL1+ CML [7, 8]. In this model, depending on the mouse strain, CML-like leukemia can be induced in 100 % of recipient mice within 30 days after transplantation. As the disease progresses, the malignant cells infiltrate the spleen and liver, causing hepatosplenomegaly (massive enlargement of both the spleen and liver). Due to infiltration of the lungs by myeloid cells, death occurs rapidly due to respiratory failure and pulmonary hemorrhages (acute bleeding affecting the lung tissue).

In order to test disease establishment, progression, and interaction with the BMM, histological analysis and immunohistochemistry can be performed on bone sections embedded in paraffin or on cryopreserved sections. In addition, in vivo microscopy is capable of imaging the microanatomy of the bone marrow niche in a very profound and detailed way.

2 Materials

2.1 Components for Blood Analysis

Microtainer tube with dipotassium EDTA (BD, Heidelberg, Baden-Württemberg, Germany).

Hematocrit capillaries (Hirschmann Laborgeräte GmbH & Co. KG, Eberstadt, Baden-Württemberg, Germany).

FACS tubes for flow cytometry (Sarstedt AG & Co., Nümbrecht, Nordrhein-Westfalen, Germany).

ACK Lysing Buffer (red cell lysis buffer) (Life Technologies, Darmstadt, Hessen, Germany).

PE anti-mouse/human Mac-1/CD11b antibody (BD Pharmingen, Heidelberg, Baden-Württemberg, Germany).

FACS buffer with 3 % fetal calf serum (FCS) and 2 mM EDTA: Add 15 ml FCS to 485 ml of DPBS so that the final concentration of FCS is 3 %. Add 2 ml of EDTA (stock solution 0.5 M) and mix the solutions by inverting the bottle a few times. Store at 4 °C.

2.2 Cytospins

Cytospin Cytocentrifuge (Thermo Scientific/Fisher Scientific, Schwerte, Nordrhein-Westfalen, Germany) or THARMACspin Cytocentrifuge (Tharmac GmbH, Marburg, Hessen, Germany).

DPBS containing 2 % FCS: Add 10 ml of FCS to a 490 ml of DPBS. Mix the solution by inverting the bottle a few times, store at 4 °C.

2.3 Histological Sections

10 % formalin, neutral buffered (Sigma-Aldrich Chemie GmbH, Taufkirchen, Bayern, Germany).

Xylene (AppliChem GmbH, Darmstadt, Hessen, Germany).

Paraffin wax/Paraplast® (Sigma-Aldrich Chemie GmbH, Taufkirchen, Bayern, Germany).

2.4 Histological Staining

Aluminum potassium sulfate, sodium iodate, and acetic acid (Sigma-Aldrich Chemie GmbH, Taufkirchen, Bayern, Germany).

Hematoxylin: either buy the Hematoxylin Solution (Mayer's) directly from Sigma-Aldrich (this is 100 mg of hematoxylin already dissolved in 100 ml of dH_2O) or buy the powdered form from Leica (Leica Mikrosysteme Vertrieb GmbH Mikroskopie und Histologie, Wetzlar, Hessen, Germany).

For mounting of your slides: Entellan (Merck Millipore, Darmstadt, Hessen, Germany).

2.5 Immunohistochemistry

0.01 M sodium citrate buffer pH 6.0: Combine 3 ml 100 mM citric acid and 3 ml 100 mM sodium citrate in a measuring cylinder. Fill up to 500 ml with ddH_2O. Check pH with a pH meter and adjust to a pH of 6.0, if necessary.

1× PBS/0.1 % Triton X-100 solution: For 500 ml, take one bottle of PBS and add 500 µl of Triton X-100, and double the amounts if necessary.

Whatman 3MM.

1× PBS/0.1 % Triton X-100 + 10 % goat serum: Prepare as above, adding 50 ml of normal goat serum.

2.6 Immunohistochemical Methods

Tartrate-resistant acid phosphatase/acid phosphatase leukocyte kit (Sigma-Aldrich Chemie GmbH, Taufkirchen, Bayern, Germany).

2.7 In Vivo Microscopy

C57/BL6N mice.

Lineage Cell Depletion Kit for mice (Miltenyi Biotec GmbH, Bergisch Gladbach, Nordrhein-Westfalen, Germany).

Lineage Cocktail: anti-CD5, B220, Gr-1, Ter119, F4/80 (BD Pharmingen, Heidelberg, Baden-Württemberg, Germany).

Methocel (Dow Wolff Cellulosics GmbH, Bomlitz, Niedersachsen, Germany).

3 Methods

Before explaining our most commonly used methods for analyzing disease progression in mice with CML, we would like to point out another alternative to the retroviral transduction/transplantation model: the transgenic mouse model. This system allows the user to model a BCR-ABL1-positive CML in mice (SCLtTA/BCR-ABL mice) without the need to irradiate and transplant [6]. It is a less time-consuming model which has the advantage that you need fewer mice. The disadvantage on the other hand is that you are limited to the use of the SCLtTA/BCR-ABL mice, while in the retroviral transduction/transplantation model, you can chose various genetically modified mice as donors or recipients depending on the scientific question being asked.

For the induction of CML, the "tet-off" system is used: tetracycline, an antibiotic, is put in the drinking water of the mice. Tetracycline will constantly suppress the translation of the *BCR-ABL1* oncogene in these mice when consumed. When removed from the water, tetracycline will no longer have a preventing effect, BCR-ABL1 can be translated, and leukemia will be induced about 4 weeks after tetracycline withdrawal. Death will occur within 50–120 days.

In order to closely follow disease progression in mice in the CML model (retroviral or transgenic), blood and bone marrow analysis are essential, as discussed below.

3.1 Blood Analysis via CBC

1. Collect blood (around 75 μl is sufficient) in an EDTA- or heparin-coated tube to stop the sample from clotting (*see* **Note 1**).
2. Analyze the sample by using an animal blood counter (the machine we use is the scil Vet abc, scil animal care company GmbH, Viernheim, Hessen, Germany, *see* **Note 2**).

3.2 Blood Analysis via FACS

1. Immediately after analyzing your sample by the animal blood counter, add 500–800 μl ACK Lysing Buffer to lyse the erythrocytes. Let the mixture sit on ice for at least 5 min.
2. Transfer your samples to polystyrene FACS tubes. Add 1 ml PBS and pellet at 320 × *g* for 5 min, preferably at 4 °C.
3. While the tubes are spinning, prepare the Mac-1 antibody. We generally dilute the appropriate antibody (if, for instance, phycoerythrin conjugated) 1:10 in FACS buffer and then take 3.3 μl from this dilution to reach a final dilution of 1:300. Incubate for 15 min on ice in the dark.
4. Add 1 ml DPBS to wash away unbound antibody before spinning with the same conditions again.
5. Remove supernatant and resuspend the pellet in 300 μl FACS buffer.
6. Keep dark and cold until the FACS analysis.

3.3 Cytospins

1. To assess the morphology of cells that may only be present in small numbers cytospins can be performed. Assemble your slide, filtercard, and the TPX cell funnel and put them into the metal clip provided with your machine (mostly, the TPX cell funnel system for the Shandon® Cytospin® is used). Perform the following steps on ice if not stated otherwise: Resuspend your cells in DPBS with 2 % FCS so that the final concentration is approximately 1–5 × 10^5 cells per ml.
2. Pipette 100 μl of your sample into the corresponding funnel. Spin at 70 × *g* for 3–5 min (*see* **Note 3**).
3. Remove the filter without disturbing the cells on the slide. Let the slide dry.
4. Check cell viability and morphology under a light microscope. One should aim for a rather confluent single layer distribution with cells lying flat on the slide.
5. Let the slide dry at RT or in a desiccation chamber, if at hand. Non-fixed slides can be stored at RT for up to 2 days.
6. The slides can now be stained with May-Grünwald or Giemsa stains.

3.4 Histological Sections

1. For a more detailed microscopic view of the bone marrow, its cellular constituents, and its surrounding tissues, histological sections can be very helpful. First, the femur of a deceased mouse must be isolated and then placed into 10 % buffered formalin, thereby preventing decay like autolysis. The formalin fixation works best if you keep the bone in the solution for at least 48 h. Then the bone needs to be decalcified by putting it in 0.5 M EDTA for 24 h. After 24 h, change the EDTA to fresh 0.5 M EDTA and let the bone sit until it is flexible without breaking. This might take up to 5 consecutive days.
2. Then the samples must be dehydrated, if the sample is to be embedded in paraffin. This is achieved by using a series of ascending ethanol concentrations:
   - 70 % ethanol for ½ h
   - 70 % ethanol for 1 h
   - 95 % ethanol for 1 h
   - 100 % ethanol for 1 h
   - 100 % ethanol for 1½ h
   - 100 % ethanol for 2 h
3. After incubating your samples in the different alcoholic baths, a clearing agent needs to be used to free the sample from the ethanol. Place the sample into Xylene for 1 h. Repeat this step (*see* **Note 4**).
4. The tissue can now be immersed in paraffin wax that has been heated to about 56–58 °C (this varies depending on the company the paraffin has been ordered from, we generally use Paraplast®). Let the sample sit in paraffin for at least 1 h and repeat in another paraffin bath.
5. When hard, the paraffin block can be further processed and sectioned into 2–4 μm thin slices that should be immediately placed into a 40–45 °C water bath for this will smoothen its surface.
6. After letting the thin paraffin section float in the water bath for 1–3 min, gently slide a glass object holder underneath it and let your specimen attach.
7. The sample is now ready for further procedures, e.g., different staining methods.

3.5 Histological Stainings

1. Hematoxylin and eosin (H&E) stain: This stain is the most widely used stain for histological sections [9, 10]. Properly done, it provides an excellent overview of your sample and its morphological structures. First, deparaffinize all specimen in two different xylene chambers for 5 min each. Prepare a

humidified chamber and put it in a 37 °C incubator while rehydrating your slides in EtOH baths:

- 100 % ethanol for 5 min
- 100 % ethanol for 5 min
- 95 % ethanol for 5 min
- 70 % ethanol for 5 min
- Distilled water for 5 min

2. Meanwhile, prepare your Hematoxylin Solution (Mayer):
   - Dissolve 50 g of aluminum potassium sulfate (= alum) in 1 l of distilled H_2O.
   - Add 1 g of Hematoxylin and wait for it to dissolve.
   - Add 0.2 mg of sodium iodate and 20 ml of acetic acid.
   - Heat the solution until it boils, letting it cool down afterward.
   - Filter into an appropriate container.
3. Add your Hematoxylin Solution dropwise on your slides. Incubate for 5 min (*see* **Note 5**).
4. Let any excessive Hematoxylin drip down the side of your slide by holding it in a slight angle, then wash the slide with dH_2O.
5. Rinse with tap water for 10 min (*see* **Note 6**).
6. Stain with 1 % eosin (make up this solution in distilled water; we recommend to make at least 100 ml) for 2–5 min; this depends on the thickness of your sample (*see* **Note 7**).
7. Rinse again with tap water for 5 min.
8. End your differentiation process and begin dehydrating your sample in ethanol baths of increasing concentrations, starting from 95 % for 2 min and then moving on to 100 % ethanol in two more containers. Finish with two different xylene baths, letting your slide sit for 5 min in each one.
9. Mount your slides.
10. Your samples are ready to be analyzed microscopically.

3.6 Immunohistochemistry

Immunohistochemistry (IHC) was first described by Coons et al. in 1941 when they found a method to detect antigens in mammalian tissue sections by using immunofluorescence. Now, a few decades later, IHC has become the go-to method in clinical diagnosis as well as in research in general. The technique behind IHC is fairly simple: antigens are detected with specific antibodies that are coupled with a detection system (reporter molecules, mostly used are enzymes like peroxidases, etc.) [11, 12]. These complexes that are either direct (antigen + coupled antibody) or indirect (antigen + primary antibody + coupled secondary antibody) can then be visualized under a

light microscope. In the following, we will describe an indirect method using avidin-biotin complexes (ABC) [13]:

1. Deparaffinize sections as done for the H&E stain, placing them into dH_2O afterward.
2. Retrieval of your antigen using the heat-inducing method (*see* **Note 8**): It is said that inducing heat to a sectioned tissue sample can reverse the interaction between proteins and formalin, at least slightly. High temperatures can also reverse the mask effect on some epitopes by hydrolysis of methylene cross-links; other scientists state that the retrieval happens by extraction of blocking proteins or by rehydration of the specimen which then eases antibody penetration. Most commonly used for heat-induced epitope retrieval (HIER) is a 0.01 M sodium citrate buffer with a pH of 6.0: Put your samples in a jar filled with the buffer and heat in a steamer at 90–95 °C for 20 min. Let the slides cool on bench for 20–30 min.
3. Transfer your samples back to dH_2O.
4. To block endogenous peroxidase activity in your specimen place your samples in a jar with 0.003–3 % H_2O_2 in 70 % methanol (prepare this solution fresh in ddH_2O every time) for 5 min (*see* **Note 9**).
5. Wash your sections again in dH_2O.
6. Blocking of unspecific antigen-antibody reactions: Incubate your sections for 15 min in a 1× PBS/0.1 % Triton X-100 solution.
7. Meanwhile, prepare wet chambers using, e.g. Whatman 3MM Paper that has been dipped into ddH_2O and put a stage for your slides above the wet paper.
8. Let the excess amount of the PBS/Triton solution drip off from your specimen and place them onto your stage. Add a few drops of your blocking solution (1× PBS/0.1 % Triton X-100 + 10 % goat serum) and incubate for 1 h at room temperature.
9. Take your sections and let the excess liquid drip off again. Add some of your primary antibody solution dropwise onto your samples and let them incubate in the fridge at 4 °C. Seal your container with your slides with Parafilm to prevent evaporation.
10. Get your samples out of the container, let the excess liquid drip off, and wash them in 1× PBS/0.1 % Triton X-100 for 10 min. Repeat.
11. Get rid of the excess liquid by dipping the edge of your slide gently on a paper towel. Place your slides onto the stage of your wet chamber again and add a sufficient amount of secondary

antibody to your specimen. Let them sit for 30–35 min at room temperature.

12. Prepare your ABC solution (avidin-biotin-complex solution) according to the manufacturer's instructions.
13. Wash your slides twice with 1× PBS/0.1 % Triton X-100, then wash them with 1× PBS for 5 min.
14. Place your slide back onto your stage after letting the excess liquid drip off again. Add the ABC solution dropwise to your slide. Incubate for 30–45 min at room temperature.
15. Put your slide into a jar filled with 1× PBS and wash them for 5 min. Repeat this step three times. Meanwhile, prepare your 3,3′-diaminobenzidine (DAB) solution according to the manufacturer's instructions.
16. Take the first slide, let the excess of the PBS drip off, and add the DAB dropwise onto your sample. Monitor this staining step closely under a microscope for 1–4 min. If you are satisfied with the result, place your slide into a container filled with ddH_2O. This will stop the staining process. Repeat this step for all your slides, keeping the finished ones in 1× PBS.
17. Wash your samples in 1× PBS for 5 min.
18. Wash again in ddH_2O for 5 min. Meanwhile, inactivate the excessive DAB-solution by adding 3 ml of 2 M H_2SO_4 and 3 ml of 0.2 M $KMnO_4$. Incubate overnight and then dispose of it.
19. Now that you stained for DAB, you have to make sure that you will not confuse DAB+ cells with normal/unstained cells. To achieve this, it is common to use a hematoxylin and eosin (H&E) counterstain (*see* Subheading 3.5).
20. Mount your slides. Your slides are ready for microscopic analysis.

3.7 TRAP Staining

1. TRAP staining using the SIGMA kit to visualize osteoclasts: First, deparaffinize specimen (blank sections) in two different xylene baths for 5 min each. Prepare a humidified chamber and put it in a 37 °C incubator while rehydrating your slides in EtOH baths:
   - 100 % ethanol for 5 min
   - 100 % ethanol for 5 min
   - 95 % ethanol for 5 min
   - 70 % ethanol for 5 min
   - Distilled water for 5 min
2. Preheat around 10 ml of distilled H_2O in a 37 °C water bath.
3. To a tube, add 0.5 ml Fast Garnet GBC Base solution and 0.5 ml sodium nitrite solution from the Sigma Kit, mix by

gently inverting the tube for 30 s, and let it incubate for 2 min at room temperature.

4. In a 100 ml beaker or Coplin jar, mix the following solutions:
   - 45 ml of distilled 37 °C H_2O
   - 1.0 ml of the Fast Garnet/sodium nitrite solution that was made up in **step 3**
   - 0.5 ml naphthol AS-BI phosphate solution
   - 2.0 ml acetate solution
   - 1.0 ml tartrate solution
5. Heat up your jar in a 37 °C water bath. Add your samples to the jar.
6. Incubate for up to 1 h but at least for 10–30 min and keep your slides protected from light.
7. Wash your slides in dH_2O for about 5 min.
8. Counterstain with Hematoxylin Solution, Gill No. 3 from the SIGMA kit for 2 min.
9. Rinse your slides for about 5 min with tap water and check for color changes (from red to blue/violet).
10. Let your slides air-dry. If desired, mount your slide with Aqua-Mount (be aware that the dye might fade after a certain period of time). Your samples are ready for microscopic analysis.

3.8 In Vivo Microscopy

In vivo microscopy entails the microscopy of the bone marrow cavity, the calvarium, in live mice [14]. While traditional 2D microscopy and histological sections provide information about cellular structures and tissues, they are not very suitable for more detailed observations on a single-cell level or for 3D imaging. 2D imaging has also not been performed on live mice; hence, traditional microscopy has been limited in imaging interactions between cells and their microenvironment in real time. Therefore the new method of in vivo microscopy, which is gaining increased visibility and importance, also due to its increasing sophistication, is described here: In vivo microscopy can visualize how leukemic cells are distributed in the osteoblastic or vascular niches of a diseased mouse compared to a healthy equivalent or how transplanted hematopoietic stem and progenitor cells (HSPCs) interact with their microenvironment upon homing into the niche. Osteoblastic cells, which are involved in bone formation, are part of the osteoblastic niche in the bone marrow microenvironment, and they are involved in the lodging of hematopoietic stem cells, as well as their regulation with regard to cell number, quiescence, and differentiation. The bone marrow microenvironment also consists of endothelial cells in the vascular niche, mesenchymal stem cells, neurons, and many other cell types. To further investigate the

relationship between bone marrow and injected leukemia cells, in vivo imaging of the calvarium of a live mouse can be performed using two-photon (or, ideally, confocal two-photon) microscopy as was shown by Lo Celso et al. in 2010 [14]. The obvious advantages of in vivo microscopy compared to other methods are single-cell observations that can be obtained in a live animal with minimally invasive techniques. However, there are some limitations to this method as the maximum depth of observation is only 100 μm with most microscopes. Although this, therefore, may pose a bias with regard to the structures being imaged, this method may help to understand complex interactions between different cell types that occur, e.g. while homing or during first stages of engraftment of hematopoietic stem and progenitor cells, as well as leukemia (-initiating) cells.

Depending on the scientific question, it is possible to image the homing of leukemia-initiating cells within 2–4 h of transplantation or to image leukemic growth at later time points. For imaging of short-term homing, leukemia-initiating cells can be labeled with the lipophilic dye DiD or DiR. For longer-term experiments (>72 h after injection), it is recommended to use permanently labeled cells, e.g., bone marrow cells from an actin-DsRed mouse. The following imaging protocol describes imaging in a short-term homing experiment in the retroviral transduction/transplantation model. Please, note that leukemic mice as donors of leukemia-initiating cells have to be generated first, as insufficient numbers of leukemia-initiating cells are retrieved from the 48-h in vitro culture system:

1. For your experimental setup with the retroviral transduction/transplantation model to generate donors of leukemia-initiating cells, follow common bone marrow transplant procedures involving irradiation of your recipient mice (generally, 450 cGy twice with a period of at least 2 h in between for C57/BL6N mice), harvest of donor bone marrow, and transduction with retrovirus. Between days 17–19 after transplantation, when the CML is fully established, sort for your desired cells by flow cytometry. In this model, the Lin^- c-Kit^+ $Sca\text{-}1^+$ ($CD48^-$ $CD150^+$) fraction will harbor the leukemia-initiating cells (*see* **Note 10**).
2. Label your cells (ideally, approximately 10,000 cells) with DiD (Thermo Fisher, Darmstadt, Hessen, Germany).
3. Anesthetize the recipient mouse, ideally a reporter mouse, with fluorescent bone marrow niche cells, e.g., Col2.3 kb GFP (osteoblastic cells), Tie2-GFP (endothelial cells), or nestin-GFP (mesenchymal stem cells) mice with ketamine/xylazine (ketamine 120 mg/kg body weight, xylazine 16 mg/kg body weight) by intraperitoneal injection. Place Methocel on the mouse's eyes to prevent them from drying out.

4. Remove the fur on the scalp. Cut the scalp open using a sterile pair of scissors. The incision should be made starting between the ears and descending in a straight line toward the nose on one side past the eye. Flap over the scalp and wash remaining hair off the calvarium with sterile PBS. Place Methocel onto the calvarium to allow an airtight seal between the calvarium and coverslip. Place the mouse onto your microscopic stage to hold the mouse's calvarium in a flat position. It is important to keep the mouse warm, either by fixing the mouse in a heatable device or by placing the mouse in an incubator-like structure while under the microscope.
5. Align the lasers on the intersection between the central and the frontal sinus and find the correct plane for imaging. Then find the intersection between the central and the coronal sinus (bottom reference), record those coordinates, and then find the intersection between the central and the frontal sinus (top reference) by following the course of the central sinus and record the coordinates. Start observing and imaging your labeled cells by meandering through the bone marrow cavity and taking stacks of up to 50 images (2 μm sections) of the cells. Vascular dyes can be injected to visualize the vasculature. Reporter mice make it easier to discern the location of the injected leukemia-initiating cells in relation to GFP^+ "landmarks" in the calvarium.
6. After finishing your imaging process either euthanize the animal or close the wound by using sutures or dermal glue. Up to one or possibly two more imaging sessions can be performed on the same mouse, but the scarring of the tissue can decrease the quality of the imaging process (*see* **Note 10**).
7. The analysis of the images in the way of measurement of distances between leukemia-initiating cells to bone, osteoblastic cells, or the vasculature, the counting of leukemic cells per leukemic "nest," or measuring the diameter of such a leukemic "nest" is performed by ImageJ software.

4 Notes

1. This can be done by different techniques: by puncturing the retro-orbital venous plexus or the facial vein or by making a small cut in the tail and then collecting the blood in a microtainer tube. Make sure to mix the blood with the EDTA or heparin in the tube to prevent coagulation.
2. In order to quantitate the tumor burden, it is recommended to focus on the white blood count (WBC, an elevated leukocyte number is a hallmark of CML (and possibly other leukemias))

and on the percentage of granulocytes (% GRA). The reference range for the WBC lies between 3000 and 15,000 cells per μl; the % GRA should be around 10–20 % for a healthy mouse. With disease progression, the WBC normally increases to more than 50,000 cells per μl (we even observe numbers above 400,000 cells per μl, but this is dependent on the type of leukemia and the mouse strain). The % GRA can be used to judge the frequency of granulocytes in comparison to lymphocytes. With disease progression, the % GRA increases due to the proliferation of myeloid cells of various maturation stages. However, the most quantitative assessment of tumor burden is performed by flow cytometry of GFP^+ (BCR-ABL1+) myeloid cells.

3. If you are concerned that your cell numbers are low, place 100 μl of the cold DPBS-FCS mixture into each funnel and spin for 1–2 min, as this will wet the filter and should allow more cells to be pelleted on the slide.
4. Performing this step is necessary because ethanol is polar compared to paraffin. Therefore, both solutions cannot be mixed and a clearing agent like xylene is necessary prior to further processing.
5. As hematoxylin is a basic dye and positively charged, it will react with negatively charged compounds. In this case, it will stain the nucleic acids found in the nucleus blue.
6. Tap water has a basic pH and will therefore change the color of the staining from red to blue. This step will also stabilize the hematoxylin.
7. Most proteins that are present in the cytoplasm are basic (positively charged); the dye will bind to those structures because of its own negative charge, eventually turning them red/pink. You can check the staining and your differentiation process by observing the sample under a microscope: If the cytoplasm has a pale/pink color, repeat your eosin-staining step for another 2–3 min. Check again under the microscope before moving on to the next step.
8. Formalin fixation changes the tertiary structure of antigens, therefore making your antibodies unable to detect them. Antigen retrieval will help in optimizing the antigen-antibody reaction. There are several methods and techniques that can be used for this purpose: using enzymes (protease-induced epitope retrieval (PIER)), which was introduced by Huang in 1975, or by using heat-induced epitope retrieval (HIER), first described in 1991 by Shi et al.
9. The LSAB method is an immunological stain that is based on enzyme-substrate reactions that transform chromogens into

colored end products. The LSAB method can be classified as a "three-step indirect method":

(a) Unconjugated primary antibody

(b) Biotinylated secondary antibody

(c) Peroxidase-labeled streptavidin

(d) Substrate/chromogen

(e) Visualization of color change.
If DAB (acts as an electron-donor) is present, the enzyme (peroxidase) and the substrate-chromogen mix will create a complex that will then, after oxidation of the DAB, change its color to an unsoluble brown end product. In most laboratories, horseradish peroxidase (HRP) is used as an enzyme to catalyze the chromogenic reaction. Streptavidin (derived from *Streptomyces avidinii*) is widely used because of its affinity to biotin.

10. Closing the wound by suturing should be performed if further imaging sessions with this animal are planned as this will lead to less scar tissue than after using dermal glue.

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