Video Article

Femoral Bone Marrow Aspiration in Live Mice

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Abstract

Serial sampling of the cellular composition of bone marrow (BM) is a routine procedure critical to clinical hematology. This protocol describes a detailed step-by-step technical procedure for an analogous procedure in live mice which allows for serial characterization of cells present in the BM. This procedure facilitates studies aimed to detect the presence of exogenously administered cells within the BM of mice as would be done in xenograft studies for instance. Moreover, this procedure allows for the retrieval and characterization of cells enriched in the BM such as hematopoietic stem and progenitor cells (HSPCs) without sacrifice of mice. Given that the cellular composition of peripheral blood is not necessarily reflective of proportions and types of stem and progenitor cells present in the marrow, procedures which provide access to this compartment without requiring termination of the mice are very helpful. The use of femoral bone marrow aspiration is illustrated here for cytological analysis of marrow cells, flow cytometric characterization of the hematopoietic stem/progenitor compartment, and culture of sorted HSPCs obtained by femoral BM aspiration compared with conventional marrow harvest.

Video Link

The video component of this article can be found at https://www.jove.com/video/51660/

Introduction

All blood cells are derived from hematopoietic stem cells (HSCs). Procedures which allow access to the bone marrow (BM), the site of the vast majority of HSC's in mice and men, without requiring sacrifice of animals, provide a resource to monitor the source of hematopoiesis serially. The overall goal of the procedure described here is to provide a detailed protocol for the sampling of BM cells from the femur of live mice which provides material representative of the cells which would be retrieved by conventional harvest of BM cells through sacrifice of the mice. The advantage of this procedure over conventional harvest of BM cells is that this procedure does not require the sacrifice of mice and therefore allows for longitudinal study of the bone marrow compartment of mice over time.

Although this murine bone marrow aspiration procedure has been used in a number of studies and described previously (see Sundberg *et al.* for a historical review[1]), formal step-by-step procedures illustrating this technique have not been previously published. This protocol enables routine serial sampling of the BM for purposes such as assessing engraftment in the BM of cells exogenously introduced into the mouse (as would be done in xenograft studies for instance[2,3]), analysis of chimerism in the BM for comparison with that of the peripheral blood (where results are not necessarily congruent), monitoring the abundance of specific cell types normally enriched in the marrow such as hematopoietic stem and progenitor cells, and retrieval of BM cells for *ex vivo* culture and/or transplantation. In addition, evaluation of marrow contents may be very useful in murine models of hematological disease as aspiration is helpful in assessing hematological disease burden at time points when disease may not be evident in the peripheral blood[3-5]. Moreover, this technique may be used to evaluate response of hematologic disease in the marrow to drugs administered in therapeutic studies. Thus, the procedure described here is ideal for investigators wishing to obtain access to bone marrow hematopoietic cells from mice for cytological analysis, flow cytometric analysis, *in vitro* culturing, and/or *in vivo* transplantation studies without requiring sacrifice or harm to the mice.

The protocol for sampling of BM described here is quite similar to the technique used for intra-femoral injection of material directly into the marrow cavity[3,6]. The key difference being that this protocol details the procedure for removal of cells from the marrow as opposed to instilling material directly into the marrow cavity space as is done with intrafemoral injection.

Protocol

All animal procedures described in this protocol were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee (IACUCs) at Memorial Sloan-Kettering Cancer Center.

1. Aspiration of Bone Marrow (BM) Cells from the Femur

1. Anesthetize the mouse which will undergo femoral bone marrow aspiration with isoflurane (1-4%) administered with a precision vaporizer. Depth of anesthesia should be monitored every 5-10 min throughout the procedure by observing that there is no change in respiratory rate associated with surgical manipulation and/or ear, toe, and tail pinch. Anesthesia is induced with isoflurane and also maintained throughout the procedure with isoflurane. A pre-emptive pre-procedural dose of buprenorphine at a dose of 0.05-0.1 mg/kg subcutaneously every 6-12 hr can be used to prevent pain associated with the procedure as an adjunct to the Carprofen.
2. Apply ophthalmic ointment to the eyes of the mouse following induction of anesthesia to prevent corneal drying.
3. The fur is carefully clipped from an area of skin approximately 150% larger than the area of the intended aspiration site. Loose fur is removed with a moist gauze pad. Please keep the mouse on a circulating warm water pad or other safe thermostatically controlled surface to prevent hypothermia during the procedure.
4. Disinfect the entire leg containing the femur which will undergo aspiration with three sets of alternating scrubs (alternating with either a povidone-iodine (Betadine) or a chlorhexidine (Nolvasan) scrub and 70% isopropyl alcohol or 70% ethanol soaked gauze sponges).
5. Wet a 0.5 ml Tuberculin Syringe (volume: 0.5 cc; gauge: 27.5 G) with sterile phosphate-buffered saline (PBS) before aspirating the BM. Fill the syringe with 200-500 µl of PBS and immediately expel the PBS. Repeat this procedure 2-3 times.
6. Keep the tibia bent from the femur by pushing the tibia with either the ring finger or the fifth finger. Confirm that a suitable anesthetic plane has been attained. The syringe is held using the thumb and the index finger. This allows the condyles to be exposed and facilitates insertion of the needle.
7. After wetting the syringe, insert the needle through the patellar tendon so that the needle is lodged securely between the two condyles of the femur. By holding the diaphysis close to the epiphysis of the femur with the thumb and the index finger, the needle is inserted into the shaft of the femur easily.
8. Swivel the needle outward and upward so that it is parallel with the shaft of the femur. This action facilitates retrieval of bone marrow contents from the femur shaft.
9. Turn the needle clockwise and counterclockwise while pushing it slowly into the femoral marrow cavity. Confirm the correct positioning of the needle by gently moving the syringe laterally.
10. Gently pull the needle plunger back, creating negative pressure, while moving the needle back and forth within the BM cavity. Note: The volume of BM aspirated will be approximately 5 µl which typically corresponds to 0.4-0.8 x 10^6 mononuclear cells. Successful aspiration will be confirmed visually by the appearance of blood in the top of the needle in the base of the syringe. If no blood is seen in the syringe it is likely that a small bone or tissue fragment is stuck in the needle. This can be removed from the needle by moving the plunger up and down in PBS (this is one reason why the syringe should be prefilled with PBS before aspirating the BM). If the tissue cannot be dislodged from the syringe, use a new needle and syringe (again wet the syringe with 200-500 µl of PBS).
11. Once BM is successfully aspirated from the femur, remove the needle and syringe from the femur and mouse.
12. Move aspirated BM to a microfuge tube prefilled with 500 µl of PBS. For most applications, the BM cells should then be kept on ice until further processing if possible.
13. Following completion of the procedure, administer analgesic with carprofen 5 mg/kg subcutaneously. Then remove mouse from the anesthesia and place on a heated pad until fully recovered. NOTE: There should be no complication or distress experienced following the aspiration procedure if done properly.
14. Before returning mice to the housing area, ensure they are able to ambulate and reach food and water. Observe the mice for signs of distress or infection post procedure in the next 24 hr. Signs include: constant bleeding, anemia, lethargy. If any of these signs are seen post procedure, the animal(s) should be euthanized. Note: BM aspiration/sampling can be repeated but the repeat procedure should be performed on the opposite femur to prevent repeated trauma to the same leg. There is little information available regarding the frequency that BM aspiration can be performed. Femoral bone marrow aspiration is generally repeated no more frequently than every 2 weeks.

2. Assessment of Cellular Content in Aspirated BM Cells

1. Pellet cells retrieved from BM aspiration and place in a microfuge tube by centrifugation at 300 x g for 5 min at 4 ^{o}C or RT.
2. Aspirate the supernatant and then resuspend the pellet in 500 µl of ACK red blood cell lysis buffer (“ammonium-chloride-potassium” lysis buffer).
3. Incubate the cells in red cell lysis buffer for 10 min and then add 1 ml of PBS and spin down the mixture again at 300 x g for 5 min at 4 ^{o}C or RT. Note: The red blood cell lysed pellet now consists of BM mononuclear cells. These can be resuspended for FACS staining, cell counting, transplantation, cytospin analysis and/or any other use (just as BM cells harvested from sacrificed mice would be used).

Representative Results

Femoral BM aspiration of a live C57/B6 mouse was utilized to obtain BM mononuclear cells followed by conventional BM harvest of the same mouse after sacrifice. BM mononuclear cells obtained by the two methods were then analyzed by (1) cytological analysis of BM cells, (2) determining the relative frequency of hematopoietic stem/progenitor cells (HSPCs), and (3) *ex vivo* culture of sorted HSPCs. In the latter experiment, lineage-negative Sca1+ c-KIT+ (LSK) cells were sorted from mononuclear cells obtained by BM aspiration as well as from conventional BM harvest. 150 LSK cells obtained by each method were then plated in methylcellulose semi-solid media containing myeloid-erythroid cytokines for 7 days in technical duplicate. The data shown in **Figure 1 A,B** illustrates that similar types and proportions of bulk cells and HSPCs were seen by morphologic and flow cytometric analysis using either femoral BM aspiration or conventional BM harvest.

In order to quantitatively determine the percentage of LSK and myeloid progenitor cells seen with conventional bone marrow harvest versus femoral bone marrow aspiration, we compared the percentage of LSK and MP subpopulations as a frequency of total live cells in 3 independent aspirations and conventional marrow harvests. This data, displayed in **Figure 1C**, reveals a decrease in LSK and MP subpopulations from bone marrow collected by femoral aspiration compared with conventional marrow harvest (although this difference was not statistically significant for any population).

Sorting of LSK cells followed by *ex vivo* culture in methylcellulose resulted in similar colony numbers and cell types obtained by femoral bone marrow aspiration and conventional BM harvest (as shown in **Figure 1D**). The colony types observed and enumerated include CFU-GEMM (the granulocyte, erythrocyte, monocyte, megakaryocyte colony-forming unit), CFU-GM (the granulocyte, monocyte colony forming unit), and BFU-E (erythroid burst-forming unit).

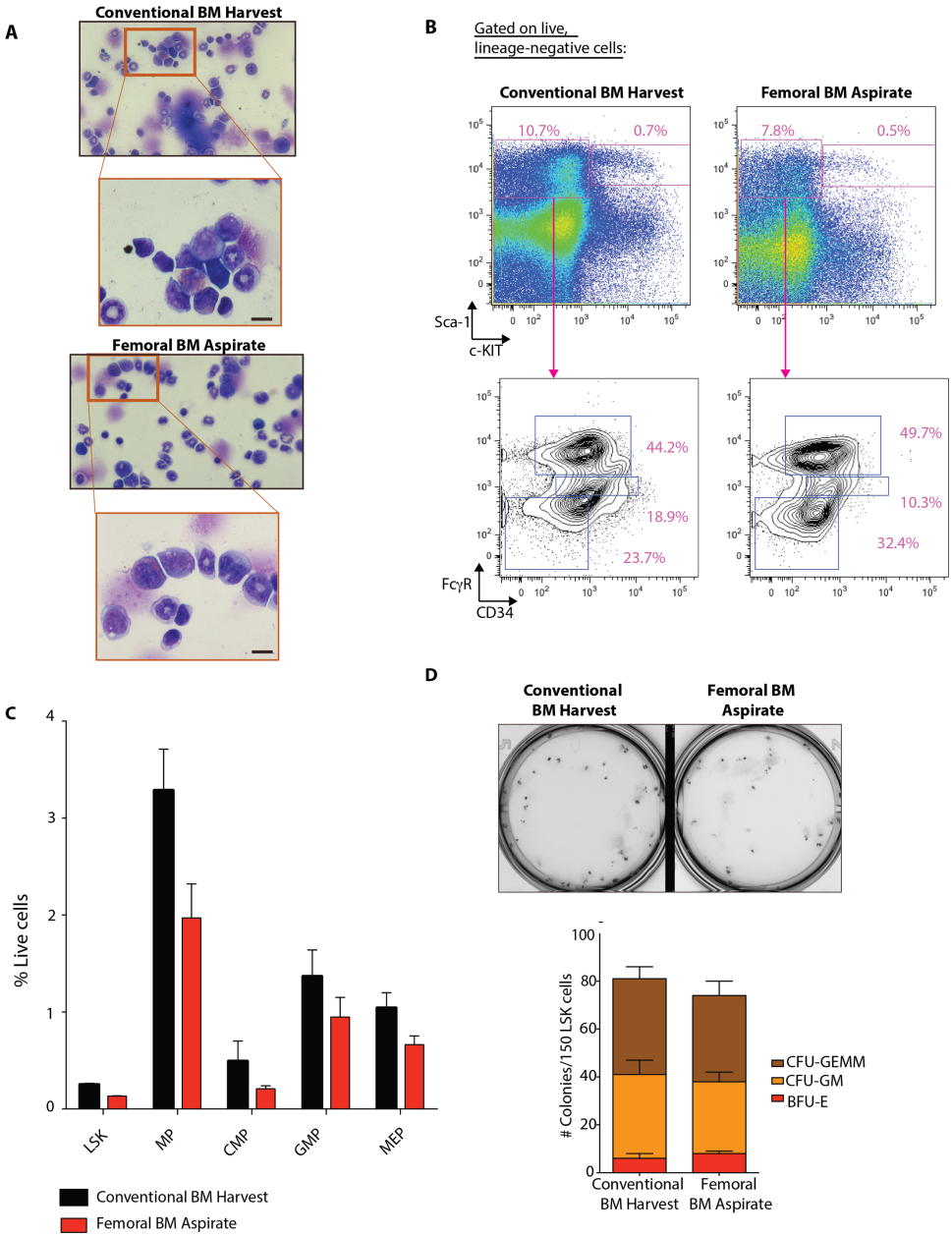

Figure 1. Femoral bone marrow (BM) aspiration in live mice to facilitate examination of marrow contents without sacrifice of mice. (A) Wright Giemsa stained cytospins of mononuclear cells obtained through BM aspiration of live mice. Approximately 0.4-0.8 x 10^6 mononuclear cells may be obtained in each individual femoral aspiration. Shown here are typically marrow contents of wildtype 6-week old C57/B6 mice obtained by femoral BM aspiration (right) versus conventional BM cell isolation through sacrifice of mice (left) (scale bar represents 10 μm). **(B)** Femoral BM aspiration provides an adequate number of cells for assessment of the hematopoietic stem/progenitor compartment as illustrated here (Right: cells from BM aspirate; Left: cells obtained by harvesting of marrow from sacrificed mice). Lineage-negative Sca-1+ c-KIT+ (LSK) cells containing hematopoietic stem cells as well as lineage-negative Sca-1- c-KIT+ myeloid progenitors and their subtypes as defined by CD34 and FcγR expression were analyzed (parent gate is living, lineage-negative cells). Percentages shown refer to percent of cells within gate. **(C)** Quantification of LSK (cells, myeloid progenitor (MP) cells, common myeloid progenitors (lineage-negative Sca-1- c-KIT+ CD34+ FcγR intermediate+), granulocyte-macrophage progenitor cells (lineage-negative Sca-1- c-KIT+ CD34+ FcγR+), and megakaryocyte-erythroid progenitor cells (lineage-negative Sca-1- c-KIT+ CD34- FcγR-), as a frequency of live cells from conventional marrow harvest (n=3) and femoral bone marrow aspiration (n=3). Error bars represent standard deviation. **(D)** Representative photographs of methylcellulose colony assay results one week after plating of 150 LSK cells in myeloid/erythroid containing methylcellulose (above) and numbers and types of colonies observed using LSK cells from each method. The colony types observed and enumerated include CFU-GEMM (the granulocyte, erythrocyte, monocyte, megakaryocyte colony-forming unit), CFU-GM (the granulocyte, monocyte colony forming unit), and BFU-E (erythroid burst-forming unit). Please click here to view a larger version of this figure.

Discussion

Serial BM aspiration is a routine procedure critical to clinical investigation of hematologic disorders in humans. The ability to perform an analogous serial sampling of BM in mice for characterization of the cellular composition and constituents of BM throughout lengthy experiments is likewise very valuable. This procedure is useful for characterization of HSPC's without sacrificing the mouse but also for detecting the presence of additional cell types in the BM in instances where the contents of the peripheral blood may not be reflective of the cells residing in the BM. Serial BM aspiration has been used very effectively for this later purpose in monitoring the presence of human cells xenografted into immunocompromised mice for instance[3]. Given that 0.4-0.8 x 10^6 cells are typically retrieved with each BM aspiration, these cells can be utilized for a number of purposes including cytological analysis (**Figure 1A**), diagnostic flow cytometry (**Figure 1B**), flow cytometric cell separation followed by further downstream use of sorted cells including culture of cells (**Figure 1C**), nucleic acid extraction, protein extraction for assays geared towards proteomic characterization of limited cell numbers, and even further transplantation of extracted cells. At the same time, it is important to note that the number of HSCs retrieved in each femoral aspiration is limited by the overall number of cells retrieved in this procedure. For instance, considering the definition of HSCs as lineage-negative Sca1+ cKIT+ CD150+ CD48- CD244- cells, the frequency of HSCs is approximately 10 HSCs/100,000 cells[7]. Based on this frequency, approximately 40-80 HSCs is expected to be retrieved with each aspiration procedure. Also, as noted in **Figure 1C**, the frequency of LSK and progenitor cells is lower, albeit not reaching statistical significance, in bone marrow cells obtained via femoral bone marrow aspiration compared with conventional bone marrow harvest. We believe this relative decrease in LSK and progenitor frequency seen with aspiration relative to surgical bone marrow harvest is related to contamination with peripheral blood that occurs with femoral bone marrow aspiration. These limitations of femoral bone marrow aspiration in evaluating rare cell populations in the marrow should be noted. One additional note is that we have typically performed this procedure in mice of 6 weeks of age or older. We believe that this procedure would be increasingly technically challenging with mice less than 6 weeks of age.

This technique is also well suited to assess the chimerism of the BM as opposed to the peripheral blood in the context of an ongoing competitive reconstitution experiment. In these assays, the functional potential of HSC's from an experimental set of mice is tested against a set known number of HSC's with the readout being peripheral blood chimerism as well as chimerism of HSC's (see review by Purton *et al.*[8]). Chimerism of the peripheral blood may contrast from the chimerism in the BM if perturbations affecting the hematopoietic stem cell compartment result in impaired differentiation of HSPC's into mature circulating blood cells. Given that definitive hematopoiesis is not established until at least 16 weeks following transplantation[9] and that chimerism may change after even longer periods of time[10], techniques which facilitate earlier access to the HSPC compartment such as illustrated here may be particularly useful. In one recent example, competitive transplantation of BM cells from mice with homozygous post-natal deletion of *Dnmt3a* by Challen *et al.* revealed reduced chimerism from *Dnmt3a*-null mice in the peripheral blood but a paradoxical increase in HSC chimerism of *Dnmt3a*-null HSC's in the BM[11]. This result was indicative of a differentiation defect of *Dnmt3a*$^{-/-}$ HSC's despite an increase in self-renewal. Moreover, this result was seen only at timed sacrifice of recipient transplanted mice in serial competitive transplantation experiments at intervals of every 16 weeks. Therefore assays which allow for sampling of BM cells in parallel with peripheral blood without requiring sacrifice of the mice and allowing ongoing observation of mice are quite useful.

As mentioned earlier, the technique demonstrated here is similar to the technique utilized for intrafemoral injection directly into the marrow cavity space of mice[2,3]. Direct intrafemoral injection has proven to be very useful in facilitating human xenograft studies in mice where it has been shown to provide improved engraftment in direct comparison with intravenous injection[6,12,13]. This technique has even allowed for identification of previously unknown class of rapidly populating human HSCs.

Currently it is not known whether repeated sampling of the BM in mice through the same femur influences the cellular composition of the BM or number of marrow cells retrieved with repeated aspirations. Moreover, the minimum amount of time advisable to perform repeat aspiration procedures from the same femur is not known. When performing repeated femoral bone marrow aspirations in the same mouse, alternate the femur used for BM aspiration and perform serial BM aspirates at intervals of >2 weeks. Further efforts focused on understanding the potential effects, if any, of serial femoral BM aspiration procedures in mice at specific intervals on the types and number of cells retrieved from BM or architecture of the BM are needed.

Disclosures

The authors have nothing to disclose.

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