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Preparation of Whole Bone Marrow for Mass Cytometry Analysis of Neutrophil-lineage Cells

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Abstract

In this article, we present a protocol that is optimized to preserve neutrophil-lineage cells in fresh BM for whole BM CyTOF analysis. We utilized a myeloid-biased 39-antibody CyTOF panel to evaluate the hematopoietic system with a focus on the neutrophil-lineage cells by using this protocol. The CyTOF result was analyzed with an open-resource dimensional reduction algorithm, viSNE, and the data was presented to demonstrate the outcome of this protocol. We have discovered new neutrophil-lineage cell populations based on this protocol. This protocol of fresh whole BM preparation may be used for 1), CyTOF analysis to discover unidentified cell populations from whole BM, 2), investigating whole BM defects for patients with blood disorders such as leukemia, 3), assisting optimization of fluorescence-activated flow cytometry protocols that utilize fresh whole BM.

Keywords

Immunology and Infection; Issue 148; Neutrophil-lineage Cells; Bone Marrow; BM; Mass Cytometry; CyTOF; Flow Cytometry; viSNE

Introduction

In the past few decades, cytometry methods have been a powerful tool to investigate the hematopoietic system in the BM. These methods include fluorescence-activated flow cytometry and the new method of CyTOF using heavy metal-labeled antibodies. They have led to discoveries of many cell types in a heterogeneous biological specimen by identification of their unique surface marker expression profiles. Increased spectrum overlaps that's associated with more channels leads to higher data inaccuracy in fluorescence-activated flow cytometry applications. Therefore, unwanted cells are routinely removed in order to enrich cell populations of interest for fluorescence-activated flow

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Video Link

The video component of this article can be found at https://www.jove.com/video/59617/

Disclosures

The authors have nothing to disclose.

cytometry analysis. For example, Ly6G (or Gr-1) and CD11b are considered mature myeloid cell markers and $Ly6G^+$ (or $Gr\text{-}1^+$) and $CD11b^+$ cells are routinely removed from BM samples by using magnetic enrichment kits prior to flow cytometry analysis of hematopoietic stem and progenitor cells (HSPCs) or by combining these markers in one dump cocktail channel[1,2,3]. Another example is that neutrophils are routinely removed from human blood specimen to enrich peripheral blood mononuclear cells (PBMC) for immunological studies. Whole bone marrow isolated from mouse or human, however, is rarely investigated intact for cytometry analysis.

Recently, CyTOF has become a revolutionary tool to investigate the hematopoietic system[4,5,6]. With CyTOF, the fluorophore-labeled antibodies are replaced by heavy element reporter-labeled antibodies. This method allows for the measurement of over 40 markers simultaneously without the concern of spectrum overlap. It has enabled the analysis of intact biological specimen without pre-depletion steps or a dump channel. Therefore, we can view the hematopoietic system comprehensively with high-content dimensionality from conventional 2-D flow cytometry plots. Cell populations omitted in the past during depletion or gating process can now be brought into light with the high-dimensional data generated by CyTOF[4,5]. We have designed an antibody panel that simultaneously measures 39 parameters in the hematopoietic system with a focus on the myeloid linage[7]. Compared to the conventional flow cytometry data, the interpretation and visualization of the unprecedented single-cell high-dimensional data generated by CyTOF is challenging. Computational scientists have developed dimensionality reduction techniques for the visualization of high-dimensional datasets. In this article, we used the algorithm, viSNE, which uses t-Distributed Stochastic Neighbor Embedding (t-SNE) technique to analyze the CyTOF data and to present the high-dimensional result on a 2-dimensional map while conserving the high-dimensional structure of the data[8,9,10]. On the tSNE plot, similar cells are clustered into subsets and the color is used to highlight the feature of the cells. For example, on Figure 1 the myeloid cells are distributed into several cell subsets based on the similarities of their expression patterns of 33 surface markers resulted from CyTOF (Figure 1)[4]. Here we investigated mouse bone marrow with our previously reported 39-marker CyTOF panel by viSNE analysis[7]. viSNE analysis of our CyTOF data revealed an unidentified cell population that showed both HSPC ($CD117^+$) and neutrophil ($Ly6G^+$) characteristics (Figure 2)[7].

In conclusion, we present a protocol to process fresh whole bone marrow for CyTOF analysis. In this article, we used mouse bone marrow as an example, while this protocol can also be used to process human bone marrow samples. The details specific to human bone marrow samples are also noted in the protocol as well. The advantage of this protocol is that it contains details such as incubation time and temperature that were optimized to preserve neutrophil-lineage cells in the whole bone marrow to enable investigation on the intact whole bone marrow. This protocol may also be easily modified for fluorescence-activated flow cytometry applications.

Protocol

All experiments followed approved guidelines of the La Jolla Institute for Allergy and Immunology Animal Care and Use Committee, and approval for the use of rodents was

obtained from the La Jolla Institute for Allergy and Immunology according to criteria outlined in the Guide for the Care and Use of Laboratory Animals from the National Institutes of Health.

1. Harvest Mouse Bone Marrow (BM)

1. Purchase C57BL/6J mice from a commercial vendor. Feed the standard rodent chow diet and house in microisolator cages in a pathogen-free facility.
2. Use male mice, 6–10 weeks of age, for experimental purpose. Euthanize by CO_2 inhalation followed by the cervical dislocation.
3. Place the mouse onto a sterile surgical pad with the abdomen side up. Sterilize the skin of the abdomen and hindlimbs area by spraying 70% ethanol. Use a pair of dissecting surgical scissors to cut the abdominal cavity open.
4. Remove the skin to expose hindlimbs. Use a pair of blunt-tip dressing forceps to hold the mouse tibia right below the ankle. Use another pair of curved dressing forceps to stabilize the tibia below the blunt-tip dressing forceps. Break the tibia and expose the bone by ripping off the muscle with the blunt-tip dressing forceps.

   NOTE: The tibia is loosely attached to the knee joint and can be easily picked out by using the blunt-tip dressing forceps.
5. Place the tibia in cold 1x PBS.
6. Next, move the stabilizing curved dressing forceps downward to the femur. Slide the blunt-tip dressing forceps below the knee joint and hold the kneecap. Dislocate the kneecap by gently pulling it up. Expose the femur by ripping off the muscle attached to the kneecap. Hold the exposed femur by the curved dressing forceps and cut the femur off from the bottom of the bone using dissecting surgical scissors.
7. Place the femur in cold 1x PBS.
8. Punch a hole in 0.5 mL microcentrifuge tube with an 18 G needle.
9. Place both tibia and femur into the same 0.5 mL microcentrifuge tube with the open end of the bones facing downwards to the hole.
10. Place the 0.5 mL tube containing both tibia and femur into a 1.7 mL microcentrifuge tube.
11. Spin the double-layered tubes containing both tibia and femur at 5,510 *x g* for 30 s in the micro-centrifuge.
12. Ensure that the BM is extracted from the bones and pelleted at the bottom of the tube. Toss the 0.5 mL tube containing the hallowed bones. The mouse BM is ready for next steps.

    NOTE: Human BM is harvested at clinical resources as previously described[11].

2. Stain BM Cells for CyTOF

1. Resuspend the BM in 1 mL 1x Red Blood Cell (RBC) lysis buffer. For human BM, resuspend the whole BM in a 10x volume of 1x Red Blood Cell (RBC) lysis buffer. Incubate for 10 min at room temperature (RT).
2. Spin the tube at 350 *x g* for 5 min at 4 °C. For human BM, repeat step 2.1 and 2.2 before proceeding to step 2.3.
3. Carefully aspirate the supernatant and leave the pellet undisrupted. Resuspend the pellet with 1 mL of cold 1x PBS. Filter the pellet into a 15 mL conical tube through a 70 μm cell strainer. The BM cells are now completely isolated into the tube from the muscle and bone debris. Wash the cells by adding 9 mL of cold 1x PBS into the tube.
4. Spin the 15 mL tube at 350 *x g* for 5min at 4 °C.
5. Carefully aspirate the supernatant and resuspend the BM cells with 10 mL cold PBS. Take an aliquot of cells for counting. Count cells using a hemocytometer.
6. Aliquot 5×10^6 BM cells into a new 15 mL tube for CyTOF staining.
7. Spin the 15 mL tube aliquot at 350 *x g* for 5 min at 4 °C.
8. Carefully aspirate the supernatant and resuspend the BM cells with 125 nM Cisplatin in 1 mL of CyTOF Staining Buffer as a viability indicator for the sample. Incubate for 5 min at RT.
9. After incubation, add 4 mL of CyTOF staining buffer to the tube. Spin the tube at 350 *x g* for 5 min at 4 °C. For human BM, add 10% human AB serum into the CyTOF staining buffer.
10. Carefully aspirate the supernatant and resuspend the BM cells with 50 μL of Fc Receptor blocking solution. Incubate for 10 min at 4 °C. Skip this step for human BM.
11. Add 50 μL of the homemade CyTOF antibody cocktail[5] to the sample so the total staining volume is 100 μL. Gently pipette to mix. Incubate for 30 min at 4 °C. The final volume of the antibody cocktail is 100 μL for both mouse BM and human BM samples.
12. Add 2 mL of CyTOF staining buffer to each tube following the incubation to wash the cells, spin the tube at 350 *x g* for 5 min at 4 °C.
13. Repeat step 2.12 for a total of two washes.
14. Prepare a fresh 1.6% formaldehyde solution from the 16% stock ampule. Dilute 1 part of the stock formaldehyde with 9 parts of 1x PBS.
15. Carefully aspirate the supernatant and resuspend the pellet with 1 mL fresh 1.6% FA solution. Incubate for 15 min at RT.
16. Spin the tube at 800 *x g* for 5 min at 4 °C.

17. Carefully aspirate the supernatant and resuspend the cell pellet with 125 nM intercalation solution in 1 mL fix/perm buffer.

18. Incubate the sample in intercalation solution overnight at 4 °C.

3. Prepare Cells for CyTOF Acquisition

1. Gently vortex and spin cells at 800 *x g* for 5 min at 4 °C.

2. Wash cells by adding 2 mL of CyTOF staining buffer, spin cells at 800 *x g* for 5 min at 4 °C and remove the supernatant by aspiration.

3. Resuspend cells in 1 mL of diH_2O. Reserve a small volume (approximately 10 μL) from each tube to count cells.

4. Spin cells at 800 *x g* for 5 min at 4 °C.

5. Repeat step 3.3 and 3.4.

6. Carefully aspirate the supernatant and leave the cells in the pellet. The BM cells are now ready for resuspension to the concentration of 1×10^6 cells/mL for CyTOF acquisition.

Representative Results

Figure 1 is presented as an example result from CyTOF experiments. On this tSNE plot the cells across multiple mouse tissues were clustered into subsets based on the similarity of their surface marker expression profiles measured by a 33-parameter CyTOF panel. Cells with more similar properties were automatically clustered together such as the neutrophils, macrophages, or the DCs based on the expression of the 33 markers on each cell.

Figure 2 is presented as an example result for the mouse BM CyTOF experiment using the protocol presented in this study. The protocol preserved the integrity of the whole BM, leading to the discovery of a previously unknown cell population that co-expresses neutrophil ($Ly6G^+$, Figure 2A) and HSPC ($CD117^+$, Figure 2B) signature surface markers simultaneously. This cell population shows a distinct pattern of the surface marker expression measured by our CyTOF panel (Figure 2C) and was omitted by previous myeloid progenitor research due to $Ly6G^+$ cell depletion[1,2,3]. More importantly, this result led to the discovery of the small subset of the cluster to the left side of the viSNE map that doesn't express Ly6G however was clustered closely to the $CD117^+Ly6G^+$ cells, which suggests the similarity of these cells to the neutrophil-lineage based on the expression of the 39 markers used for this CyTOF experiment.

We used the marker expression profile from the CyTOF data shown in Figure 2C to build a 13-color FACS (fluorescence-activated cell sorting) panel that allows us to isolate the neutrophil progenitors by flow cytometry for downstream functional assays (Figure 3).

Discussion

In past decades, fluorescence-based flow cytometry was used as the main method to study cellular lineages and heterogeneity[1,2,3]. Although flow cytometry has provided multi-

dimensional data, this method is limited by choices of parameters and spectral overlap. To overcome the weakness of flow cytometry we took advantage of CyTOF, which uses heavy metal isotopes instead of fluorophores to label antibodies that eliminates crosstalk between detector channels or autofluorescence of the cells, therefore, can measure many more parameters simultaneously at the single-cell level and generate a much deeper assessment of cellular diversity[12]. Cell populations omitted in the past during depletion or gating process, especially important immune cells like neutrophil-lineage cells which were for long considered homogeneous[13,14], can be better characterized by CyTOF[2,3,7].

To accommodate this powerful cytometry tool of CyTOF to study cell heterogeneity and characterize rare cell populations, protocols that preserve the integrity of biological specimens are extremely practical for heterogeneity studies. Here we described a protocol to process whole BM for CyTOF that enables comprehensive characterization of new cell populations in intact whole BM, which may be used for mouse or human studies. Whole bone marrow isolated from mouse and human contains cell populations, especially neutrophil-lineage cells that are highly fragile and sensitive to environmental changes such as temperature and culture conditions. In this protocol, we have optimized the temperature and incubation conditions for each step in order to preserve these sensitive populations to the maximum level. By doing so the integrity of the whole bone marrow cells is well protected. With personalized marker panel incorporated to this protocol, researchers may identify more cells of interest in different research fields.

Although CyTOF is a powerful end-point analytical tool for discovery of new cell populations and is able to predict the new populations' function by their marker characteristics, this technology is limited for further downstream functional studies. To enable downstream functional studies, we used viSNE to comprehensively characterize each cluster by examine their expression level of each 39 markers and found the distinct marker combinations as shown in Figure 2C. Although CyTOF data could not be applied to current sorting technologies, its advantage on the measurement of many parameters simultaneously could assist us to identify the best combination of markers within limited parameters. This critical step of data analysis assisted us to take full advantage of CyTOF results to design a FACS-compatible fluorescence-based sorting panel. For example, in this experiment, we used this information in Figure 2C to build a 13-color FACS panel that allows us to isolate the neutrophil progenitor (NeP) by flow cytometry for downstream functional assays (Figure 3). By using CyTOF and FACS in a pipeline, these methods together could enable the isolation of newly discovered cell types for functional studies such as morphology, versatility, in vitro assays, and in vivo studies.

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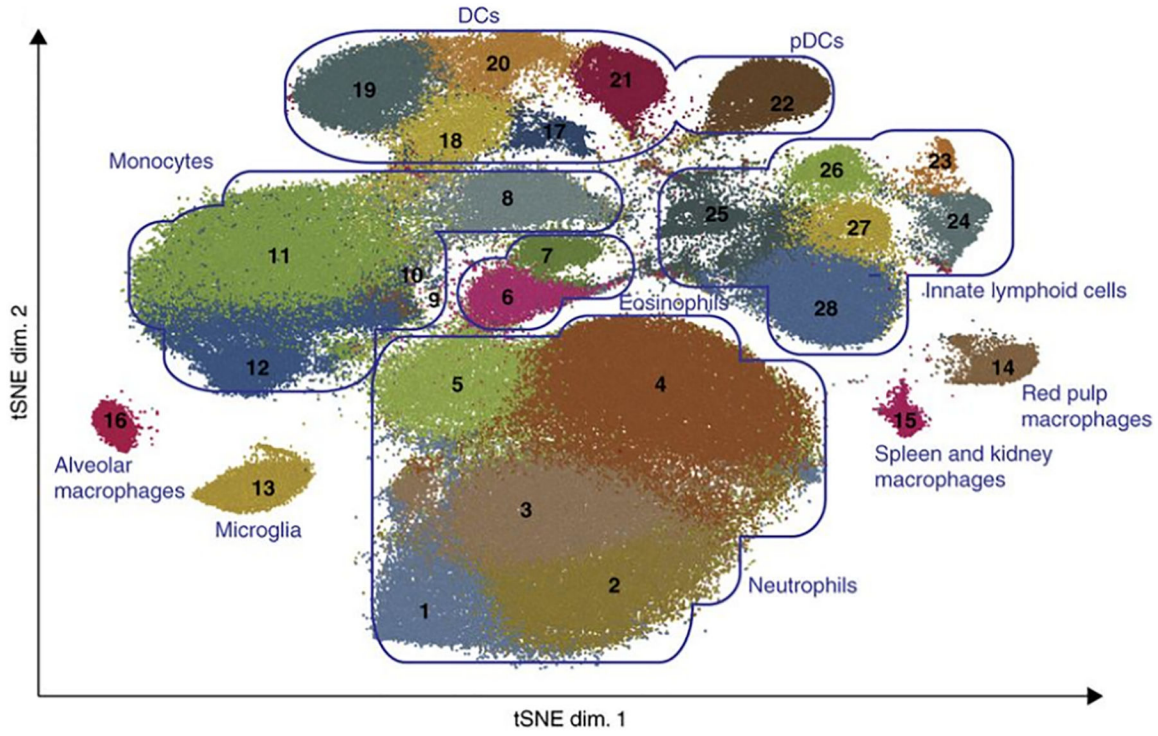

Figure 1: Example of the tSNE plot resulted from CyTOF.

Aggregate tSNE dimensionality reduced single-cell data from all mice tissues analyzed were plotted and color coded by the 28 'unsupervised' clusters. The coarse identities of each cluster were annotated based on various published analyses. This figure has been modified from reference[4].

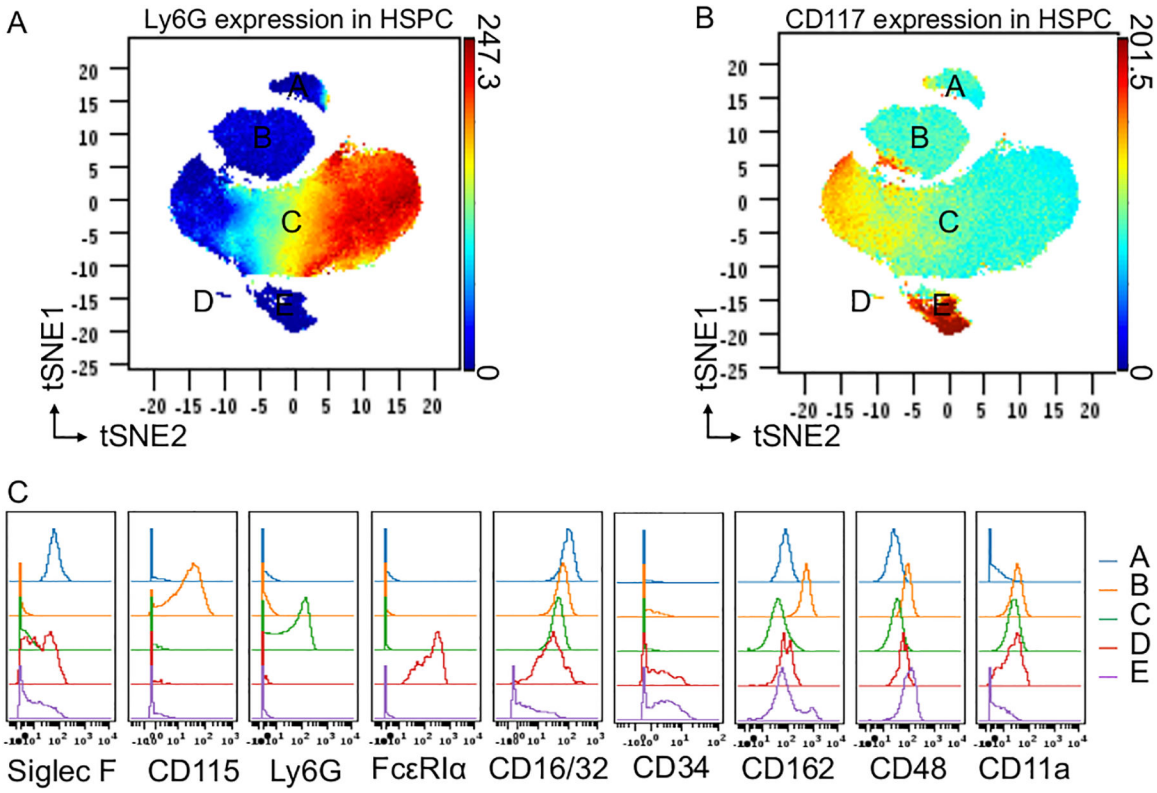

Figure 2: Automated single-cell analysis of Lin$^-$CD117$^+$Ly6A/E$^-$ HSPC cells in bone marrow identifies a distinct neutrophil progenitor population.

viSNE maps of Lin$^-$CD117$^+$Ly6A/E$^-$ HSPC cells are shown as dot overlays to display the 5 automated clusters. (**A**) Ly6G and (**B**) CD117 expression pattern is shown on viSNE map of Lin$^-$CD117$^+$Ly6A/E$^-$ HSPC cells as spectrum colored dots. (**C**) The expression patterns of the indicated markers are shown as histogram overlays of each cluster. This figure has been modified from reference[7].

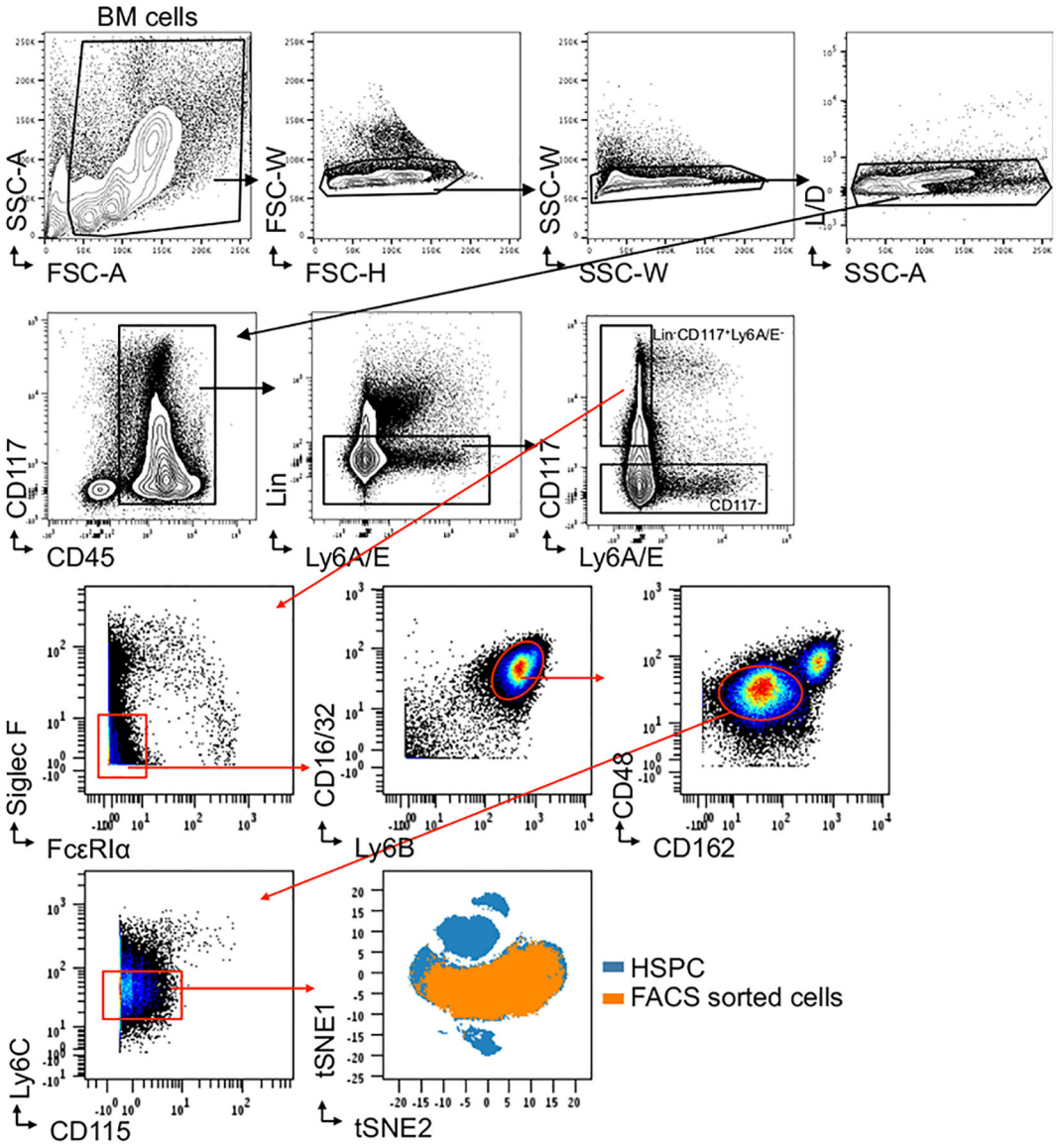

Figure 3: FACS gating strategy demonstrated with mass cytometry (CyTOF) dataset.

Manually gated target population was back gated to automated viSNE map for validation. This figure has been modified from reference[7].