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

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Cover Letter

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Vineeta Bajaj, Ph.D.
Review Editor
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Dear Vineeta:

Thank you for your thorough editorial comments and helpful suggestion for the 2nd revision of this manuscript. According to your suggestions, we have revised this manuscript for easier film production. We have changed the focus of this manuscript to sample preparation for mass cytometry. The modifications appear on both of the title and the context of the manuscript. The title is now changed into 'Preparation of Whole Bone Marrow for Mass Cytometry Analysis of Neutrophil-lineage Cells'. To accommodate this change, the previous section of the data analysis including Figure 4 and Figure 5 were removed from the current revision. We are submitting this revised manuscript for your consideration. We kept the editorial changes and our changes traceable in this revised manuscript.

We thank you for your time and efforts and thank you for your consideration of publishing our manuscript on JoVE.

Warm Regards,

Yanfang Peipei Zhu, Ph.D.
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TITLE:

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

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KEYWORDS:

Neutrophil-lineage Cells, Bone Marrow, BM, Mass Cytometry, CyTOF, Flow Cytometry, viSNE

SUMMARY:

Here, we present a protocol to process fresh bone marrow (BM) isolated from mouse or human for high-dimensional mass cytometry (Cytometry by Time-Of-Flight, CyTOF) analysis of neutrophil-lineage cells.

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.

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 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[1-3]. 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[4-6]. 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[8-10]. 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).

1.1. Purchase C57BL/6J mice from a commercial vendor. Feed the standard rodent chow diet and house in microisolator cages in a pathogen-free facility.

1.2. Use male mice, 6-10 weeks of age, for experimental purpose. Euthanize by CO_2 inhalation followed by the cervical dislocation.

1.3. 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.

1.4. 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.

1.5. Place the tibia in cold 1x PBS.

1.6. 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.

1.7. Place the femur in cold 1x PBS.

1.8. Punch a hole in 0.5 mL microcentrifuge tube with an 18 G needle.

1.9. 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.

1.10. Place the 0.5 mL tube containing both tibia and femur into a 1.7 mL microcentrifuge tube.

1.11. Spin the double-layered tubes containing both tibia and femur at 5510 *x g* for 15 s in the micro-centrifuge.

1.12. 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**

2.1. 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).

2.2. 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.

2.3. 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.

2.4. Spin the 15 mL tube at 350 *x g* for 5min at 4 °C.

2.5. 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.

2.6. Aliquot 5 x 10^6 BM cells into a new 15 mL tube for CyTOF staining.

2.7. Spin the 15 mL tube aliquot at 350 *x g* for 5 min at 4 °C.

2.8. 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.

2.9. 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.

2.10. 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.

2.11. 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.

2.12. 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.

2.13. Repeat step 2.12 for a total of two washes.

2.14. 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.

2.15. Carefully aspirate the supernatant and resuspend the pellet with 1 mL fresh 1.6% FA solution. Incubate for 15 min at RT.

2.16. Spin the tube at 800 *x g* for 5 min at 4 °C.

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

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

3. **Prepare cells for CyTOF acquisition**

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

3.2. 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.

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

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

3.5. Repeat step 3.3 and 3.4.

3.6. Carefully aspirate the supernatant and leave the cells in the pellet. The BM cells are now ready for resuspension to the concentration of 1 x 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[1-3]. 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**).

FIGURE AND TABLE LEGENDS:

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 [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].

DISCUSSION:

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

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

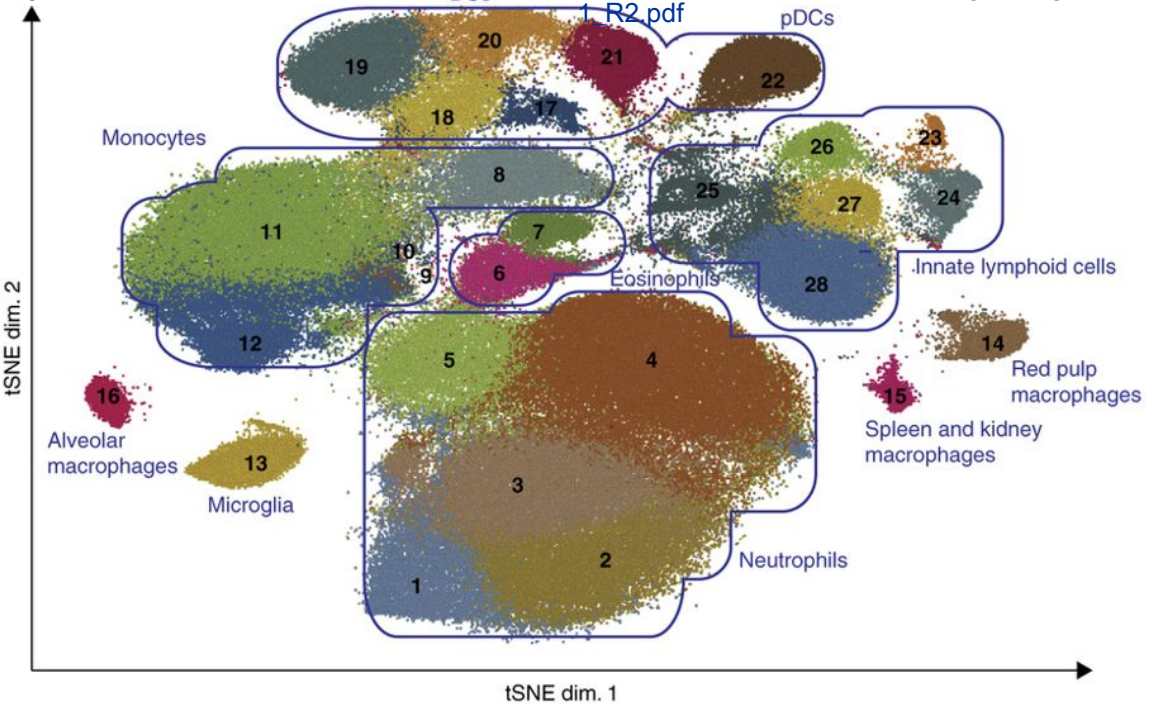

Figure 2

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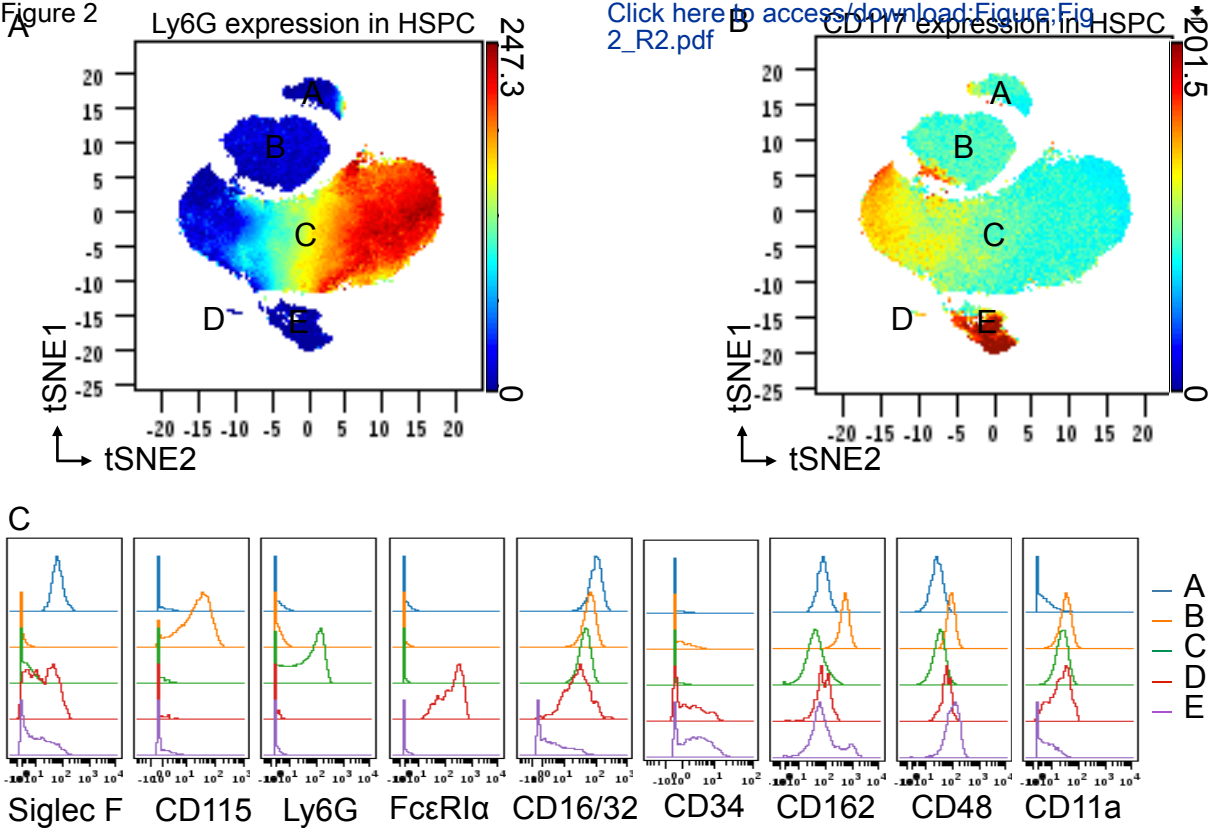
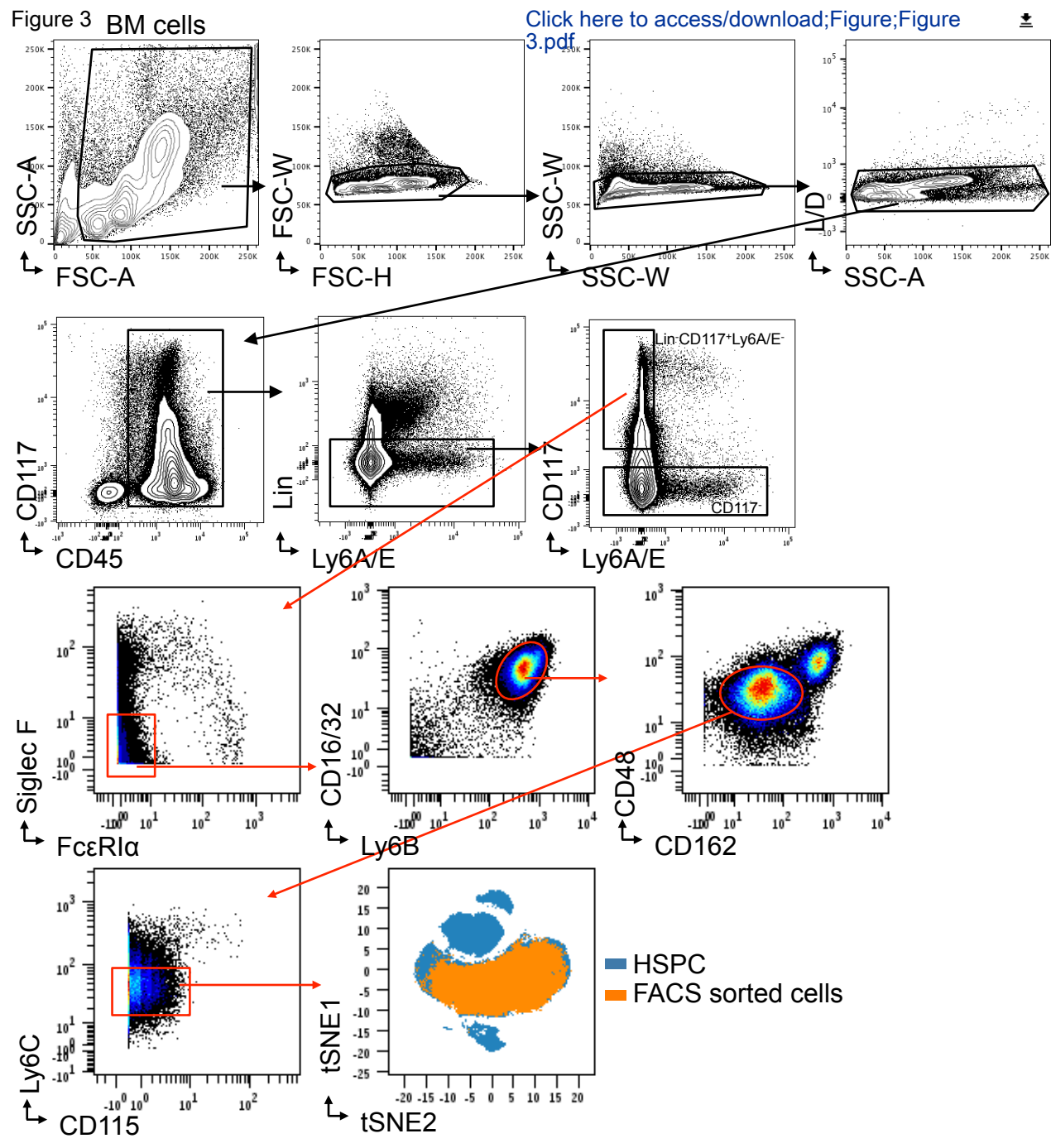


Figure 3



Click here to access/download;Figure;Figure 3.pdf

Name of Material/ Equipment	Company
CyTOF Antibodies (mouse)	
Anti-Mouse CD45 (Clone 30-F11) -89Y	Fluidigm
Anti-Human/Mouse CD45R/B220 (Clone RA36B2)-176Yb	Fluidigm
Anti-mouse CD105 (Clone MJ7/18)-Purified	Biolegend
Anti-mouse CD115 (CSF-1R) (Clone AFS98)-Purified	Biolegend
Anti-Mouse CD117/c-kit (Clone 2B8)-166Er	Fluidigm
Anti-mouse CD11a (Clone M17/4)-Purified	Biolegend
Anti-Mouse CD11b (Clone M1/70)-148Nd	Fluidigm
Anti-Mouse CD11c (Clone N418)-142Nd	Fluidigm
Anti-mouse CD127 (IL-7Rα) (Clone A7R34)-M	Biolegend
Anti-Mouse CD150 (Clone TC1512F12.2)-167Er	Fluidigm
Anti-mouse CD16.2 (FcγRIV) (Clone 9E9)-Purif	Biolegend
Anti-Mouse CD162 (Clone 4RA10 (RUO))-Purified	BD Biosciences
Anti-mouse CD169 (Siglec-1) (Clone 3D6.112)-Purified	Biolegend
Anti-mouse CD182 (CXCR2) (Clone SA044G4)-Purified	Biolegend
Anti-mouse CD183 (Clone CXCR3-173)-Purified	Biolegend
Anti-mouse CD335 (NKp46) (Clone 29A1.4)-M	Biolegend
Anti-mouse CD34 (Clone MEC14.7)-Purified	Biolegend
Anti-mouse CD41 (Clone MWReg30)-MaxPar	Biolegend
Anti-Mouse CD43 (Clone S11)-146Nd	Fluidigm
Anti-Mouse CD48 (Clone HM48.1)-156Gd	Fluidigm
Anti-mouse CD62L (Clone MEL-14)-MaxPar Re	ThermoFisher
Anti-mouse CD71 (Clone RI7217)-Purified	Biolegend
Anti-mouse CD90 (Clone G7)-Purified	Biolegend
Anti-Mouse F4/80 (Clone BM8)-159Tb	Fluidigm
Anti-mouse FcεRIα (Clone MAR-1)-MaxPar Ready	Biolegend
Anti-mouse GM-CSF (MP1-22E9 (RUO))-Purifi	BD Biosciences
Anti-Mouse I-A/I-E (Clone M5/114.15.2)-174Yb	Fluidigm
Anti-Mouse Ki67 (Clone B56 (RUO))-Purified	BD Biosciences
Anti-Mouse Ly-6A/E (Sca-1) (Clone D7)-169Tm	Fluidigm
Anti-Mouse Ly6B (Clone 7/4)-Purified	abcam
Anti-mouse Ly-6G (Clone 1A8)-MaxPar Ready	Biolegend
Anti-Mouse NK1.1 (Clone PK136)-165Ho	Fluidigm
Anti-Mouse Siglec-F (Clone E50-2440 (RUO))-Purified	BD Biosciences
Anti-Mouse TCRβ (Clone H57-597)-143Nd	Fluidigm

Anti-mouse TER-119/Erythroid Cells (Clone TE	Biolegend
Chemicals, Peptides and Recombinant Proteins	
Antibody Stabilizer	CANDOR Bioscience
Bovine Serum Albumin	Sigma-Aldrich
Cisplatin-194Pt	Fluidigm
eBioscience 1X RBC Lysis Buffer	ThermoFisher
eBioscience Foxp3 / Transcription Factor Staining Buffer Set	ThermoFisher
EQ Four Element Calibration Beads	Fluidigm
Ethylenediaminetetraacetic acid (EDTA)	ThermoFisher
Fetal Bovine Serum	Omega Scientific
HyClone Phosphate Buffered Saline solution	GE Lifesciences
Intercalator-Ir	Fluidigm
MAXPAR Antibody Labeling Kits	Fluidigm
Paraformaldehyde	Sigma-Aldrich
Sodium azide	Sigma-Aldrich
Triton X-100	Sigma-Aldrich
Trypsin EDTA 1X	Corning
Experimental Model: Organism/Strains	
Mouse: C57BL/6J	The Jackson Laboratory
Software Alogrithm	
Bead-based Normalizer	Finck et al., 2013
Cytobank	Cytobank
Cytofkit v1.r.0	Chen et al., 2016
t-SNE	van der Maaten and Hinton, 200

Catalog Number	ents/Description
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https://bioconductor.org/packages/release/bioc/html/cytofkit.html	
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Title of Article: Single-cell Analysis of Neutrophil Progenitor Cells in Whole Bone Marrow via Mass Cytometry and 10x Single-cell RNA-3' End

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Reviewers' comments:

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Reviewer #1:
Manuscript Summary:
This methods paper describes how CyTOF, cell sorting, and single-cell transcriptional profiling can enable high-dimensional characterization of discrete populations in complex tissue. This pipeline overcomes the technological hurdles of flow cytometry that depends on marker selection and spectral overlaps.

Major Concerns:
A resource should be provided for the viSNE algorithm (Line 178).

We appreciate your careful review. The manuscript is revised accordingly and highighted in Green.

Minor Concerns:
Line - no comma

We appreciate your careful review. The manuscript is revised accordingly and highighted in Green.

Line 47 - delete "had"
Adoptive transfer experiments confirmed that the newly discovered cell

We appreciate your careful review. The manuscript is revised accordingly and highighted in Green.

Line 60 - delete "a" and replace "had" with "has"
In the past few decades, the development of multi-channel flow cytometry has led to

We appreciate your careful review. The manuscript is revised accordingly and highighted in Green.

Line 65 - comma after "(GMP)" and delete "could be identified"
Granulocyte Monocyte Progenitor (GMP), by differential expression of

Line 69 - delete "accordingly" and add comma
complicated, leading to higher data inaccuracy.

Lines 69-70 - replace "this reason" with "these reasons," "a lot" with "many," and "were" with "are"
Due to these reasons, many of the cellular markers are omitted by depleting certain fractions of the specimen prior to flow

We appreciate your careful review. The manuscript is revised accordingly and highighted in Green.

Line 71 - replace "maximum" with "maximize"
cytometry analysis in order to maximize available fluorescent channels for analysis of the cells

We appreciate your careful review. The manuscript is revised accordingly and highighted in Green.

Lines 72-73 - replace "were" with "are" (twice) and delete "as"
For example, Ly6G (or Gr-1) and CD11b are considered mature myeloid cell markers therefore the Ly6G+ (or Gr-1+) and CD11b+ cells are routinely removed from bone

We appreciate your careful review. The manuscript is revised accordingly and highighted in Green.

Line 75 - delete comma
cytometry analysis of hematopoietic stem and progenitor cells (HSPCs) or by putting these

We appreciate your careful review. The manuscript is revised accordingly and highighted in Green.

Line 77 - replace "helps" with "is," comma with semicolon, and "led" with "lead"; insert "can" before "also"
is to enrich the cells of interest; however, it can also lead to depletion of certain subtypes of the

We appreciate your careful review. The manuscript is revised accordingly and highighted in Green.

Line 78 - replace comma with period; start new sentence; replace "could be" with "would have been"
target cell. For example the HSPCs that co-express Ly6G (or Gr-1) or CD11b would have been eliminated

We appreciate your careful review. The manuscript is revised accordingly and highighted in Green.

Line 79 - delete "in the past" and "the" before depletion
from the analysis due to depletion

We appreciate your careful review. The manuscript is revised accordingly and highighted in Green.

Line 81 - replace "were" with "are"
antibodies were replaced by heavy element reporter-labeled antibodies

We appreciate your careful review. The manuscript is revised accordingly and highighted in Green.

Line 82 - replace "makers" with "markers"
measurement of over 40 markers simultaneously without the concern of spectrum overlap

We appreciate your careful review. The manuscript is revised accordingly and highighted in Green.

Line 83 - add "a" before 'dump'
has enabled analysis of intact biological specimen without pre-depletion steps or a 'dump'

We appreciate your careful review. The manuscript is revised accordingly and highighted in Green.

Lines 84-85 - revise
Therefore, we are able to view the hematopoietic system comprehensively with high-content dimensionality from conventional 2-D flow cytometry plots

We appreciate your careful review. The manuscript is revised accordingly and highighted in Green.

Lines 85-87 - revise
Cell populations omitted in the past during depletion or the gating process can now be brought to light with the high-parameter data generated by CyTOF.

We appreciate your careful review. The manuscript is revised accordingly and highighted in Green.

Lines 87-88 - revise
Here we have designed an antibody panel that simultaneously measures 39 parameters in the hematopoietic system with a focus on the myeloid

We appreciate your careful review. The manuscript is revised accordingly and highighted in Green.

Line 93 - replace "had" with "have"
Computational scientists have developed dimensionality reduction techniques for the

We appreciate your careful review. The manuscript is revised accordingly and highighted in Green.

Line 94 - replace "an" with "the"
In this article we used the algorithm, viSNE, which

We appreciate your careful review. The manuscript is revised accordingly and highighted in Green.

Line 96 - replace "yet conserve" with "while conserving"
and to present the high-dimensional result on a 2-dimensional map while conserving the high-

We appreciate your careful review. The manuscript is revised accordingly and highighted in Green.

Line 97 - add "the" before viSNE
On the viSNE map

We appreciate your careful review. The manuscript is revised accordingly and highighted in Green.

Line 99 - lowercase viSNE
viSNE analysis of the CyTOF data we generated

We appreciate your careful review. The manuscript is revised accordingly and highighted in Green.

Line 103-104 - replace "identify" with "identifying" and "to" with "with"
Although CyTOF is powerful in identifying cell populations, current FACS sorting software is not compatible with viSNE plots.

We appreciate your careful review. The manuscript is revised accordingly and highighted in Green.

Line 104-105 - comma after "cluster", replace "makers" with "marker", replace "that suits" with "suitable"
Thus, in order to study the functions of the newly identified cell cluster, we examined the CyTOF dataset to find the best marker combination suitable for 13-

We appreciate your careful review. The manuscript is revised accordingly and highighted in Green.

Line 107 - replace "the following" with "proceeding with"
to the CyTOF viSNE map to ensure the purity of the sorted cells before proceeding with functional

We appreciate your careful review. The manuscript is revised accordingly and highighted in Green.

Line 112 - add "the" before "molecular"
the cellular complexity of NeP at the molecular level under homeostatic and cancer conditions

We appreciate your careful review. The manuscript is revised accordingly and highighted in Green.

Line 113 - replace "index" with "perform"
Genomics uses a 10x GemCode technology to deliver a scalable microfluidic platform to perform

We appreciate your careful review. The manuscript is revised accordingly and highighted in Green.

Line 117 - delete comma, revise
mice and found that the NeP population consists of several distinct subpopulations that changed in quantities with cancer

We appreciate your careful review. The manuscript is revised accordingly and highighted in Green.

Line 118 - replace "this result" with "these subpopulations" and "predict potential transcriptome" with "potentially lead to"; delete "and will not be discussed here."

Further investigation of these subpopulations could potentially lead to targets in NeP cancer treatment.

We appreciate your careful review. The manuscript is revised accordingly and highighted in Green.

Line 121-122 - replace "Overall" with "In conclusion"; add "the" before "NeP"
In conclusion, we have presented a pipeline using CyTOF, FACS sort, and 10x scRNA-seq methods to discover, isolate, and investigate a new cell type, the NeP.

We appreciate your careful review. The manuscript is revised accordingly and highighted in Green.

Line 210 - Lowercase viSNE

We appreciate your careful review. The manuscript is revised accordingly and highighted in Green.

Line 213 - replace "of" with "for"
markers are shown as histogram overlays for each cluster

We appreciate your careful review. The manuscript is revised accordingly and highighted in Green.

Line 222-223 - replace "NeP" with "NePs" and "its" with "their"
ScRNA-Seq reveals the heterogeneity of NePs and their neutrophil precursor progeny in healthy vs. tumor-bearing mice

We appreciate your careful review. The manuscript is revised accordingly and highighted in Green.

Line 235-236 - replace "for" with "characterize" and "characterization" with "populations"
Single-cell technologies have become powerful tools to study cell heterogeneity and characterize rare cell populations.

We appreciate your careful review. The manuscript is revised accordingly and highighted in Green.

Line 237 - replace "greatly advanced so far" with "flow cytometry has provided multi-dimensional data"
Although flow cytometry has provided multi-dimensional data,

We appreciate your careful review. The manuscript is revised accordingly and highighted in Green.

Line 238 - replace "numbers of independent" with "choice of"
method is limited by the choice of parameters

We appreciate your careful review. The manuscript is revised accordingly and highighted in Green.

Line 239 - pluralize "fluorophores"

We appreciate your careful review. The manuscript is revised accordingly and highighted in Green.

Line 241 - replace "much more numbers of" with "many more"
This feature allows CyTOF to measure many more parameters simultaneously

We appreciate your careful review. The manuscript is revised accordingly and highighted in Green.

Line 245-246 - delete "Overall"; add "in" before "discoveries"; replace "new" with "previously unidentified"; replace "population" with "populations"
CyTOF is a revolutionary cytometry method that could assist in discoveries of previously unidentified rare cell populations by cellular expression of unique marker proteins.

We appreciate your careful review. The manuscript is revised accordingly and highighted in Green.

Line 249 - add "a" before "FACS"
to design a FACS-compatible

We appreciate your careful review. The manuscript is revised accordingly and highighted in Green.

Line 251 - replace "the discovered new" with "newly discovered"; delete "actual"
together could enable isolation of newly discovered cell types for functional studies

We appreciate your careful review. The manuscript is revised accordingly and highighted in Green.

Line 252 - list should maintain parallel structure by retaining the same grammatical form

We appreciate your careful review. The manuscript is revised accordingly and highighted in Green.

Line 253 - pluralize NePs, replace "transcriptome" with "the transcriptional", replace "further" with "conducting"
investigate the heterogeneity of FACS-sorted NePs at the transcriptional level before conducting

We appreciate your careful review. The manuscript is revised accordingly and highighted in Green.

Line 259 - replace "advances" with "makes advancements"
This method also makes advancements in its

We appreciate your careful review. The manuscript is revised accordingly and highighted in Green.

Line 260 - add "the" before "collection"
droplet-based collection method that lowers the stress to single cells during the collection

We appreciate your careful review. The manuscript is revised accordingly and highighted in Green.

Line 261 - replace "preserving" with "preserve"
procedure, which could help preserve RNA quality of the sample

We appreciate your careful review. The manuscript is revised accordingly and highighted in Green.

Line 264-265 - replace "Overall" with "In summary"; replace "enables" with "enable"; replace "transcriptome heterogeneity studies" with "single-cell transcriptional profiling"
In summary, we have described a pipeline for discovery of new cell types and methods to isolate the cells that enable downstream analysis including single-cell transcriptional profiling.

We appreciate your careful review. The manuscript is revised accordingly and highighted in Green.

Reviewer #2:
Manuscript Summary:
This description for a JoVE manuscript/presentation describes methods used by a lab group to use single cell technologies to identify immune cell subsets for downstream flow sorts, molecular characterization by 10X single cell RNAseq and suggests that this platform can be used to study the function of cell subsets that are identified by this workflow. The groups uses mouse bone marrow analysis as an example for their workflow. The authors provide a brief overview of how they used CyTOF, FACS, and scRNAseq to characterize a bone marrow PMN progenitor population call NeP. Overall, the workflow is logical and not really innovative for research groups that have expertise with these methods. There are some points that should be addressed to strengthen the impact of the article.

Major Concerns:

1. This workflow does not seem to be novel or difficult to work out for groups that know how to use these technologies. If the goal is to demonstrate how to do CyTOF, FACS sorts, and scRNAseq, then this is not really going to be all that helpful to others.

Novelty is not a requirement for publication on JoVE but we appreciate your review and revised the discussion section and added a few highlights in the manuscript to make the goal of this manuscript more clear and helpful to others.

2. The abstract indicated that they were going to present an analytical approach to develop FACS sorting panels. That was not described in the manuscript.

We thank you for this reminder and added one more panel in Figure 2 along with a brief discussion in the text to describe how the FACS sorting panel is developed.

3. The results with scRNAseq were not related back to the CyTOF findings. Was this their intention or did the author want to use the tumor model as an example for how to use this method.

We thank you for this reminder. We revised the manuscript to make it clear that the tumor model is an example of using this method.

4. Why didn't they begin comparing normal and tumor-bearing mice by CyTOF and then use CyTOF data to drive the development of FAC panels for sorts, followed by detailed molecular analysis by scRNAseq?

We appreciate your review. We revised the manuscript to make it clear that the tumor model is an example of using this method.

5. It is not clear how this work will be useful when presented in video format. Are they going to show stains, sorts, and RNAseq OR are they going to describe their analytical methods OR both. This should be clarified.

We appreciate your review. We added one more panel in Figure 2 and more discussion in the manuscript to make the goal of this manuscript more clear.

Minor Concerns:

6. Minor, Ly6G is not GR-1. GR-1 is an antibody reagent that recognized Ly6C/G. We do not use GR-1 in CyTOF. Instead we use anti-Ly6C and anti-Ly6G.

We appreciate your review. We did not use Gr-1 for CyTOF. Gr-1 was only mentioned in the context of citing previous literatures using Gr-1 before it was replaced by Ly-6G.

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