Neutrophil Analysis in 10x Genomics Single Cell Gene Expression Assays

Introduction

Neutrophils are the most abundant white blood cells in circulation. They are the first responders of the innate immune system that release cytotoxic compounds to kill bacteria and phagocytose foreign particles. Analysis of neutrophils and granulocytes is challenging in single cell transcriptomic data due to the low RNA content in these cells along with relatively high levels of RNases and other inhibitory compounds. Furthermore, neutrophils are sensitive to degradation after collection, requiring careful sample handling for preservation. This Technical Note describes the successful isolation and analysis of neutrophil populations from whole blood.

Methods

Whole blood was collected from healthy donors in K_2EDTA Vacutainer Tubes (BD 366643) and transported at room temperature to the processing site. Whole blood leukocytes (i.e. PBMCs and granulocytes) were isolated from blood samples 3-6 hours post collection as described in the Demonstrated Protocol for Isolation of Leukocytes, Bone Marrow and Peripheral Blood Mononuclear Cells for Single Cell RNA Sequencing (Document CG000392). All samples were handled at room temperature as neutrophils are sensitive to cold temperatures. Red blood cell (RBC) lysis was performed twice for efficient RBC removal.

Isolated cells were counted using AO/PI on the Celleca MX automated counter and were processed using the Chromium Next GEM Single Cell 3' Reagent Kits v3.1 (Dual Index) User Guide (Document CG000315), targeting 8,000 cells. An additional amplification cycle was added to the recommended cDNA Amplification PCR cycles in Step 2.2d, to account for reduced RNA content in the granulocytes. All libraries were sequenced using paired-end sequencing of 28 x 91 bps on an Illumina Novaseq with a target of ~35,000 read pairs per cell. Raw sequencing data were processed with Cell Ranger 6.1 and downstream analysis was performed using Loupe Browser 5.1 and 3rd party analysis tools. The Capturing Neutrophils in 10x Single Cell Gene Expression Data Tutorial provides step by step instructions for performing some of the analysis steps described in this Technical Note using Cell Ranger and Loupe Browser for Chromium Single Cell 3' v3.1 and Chromium Single Cell 5' v2 libraries.

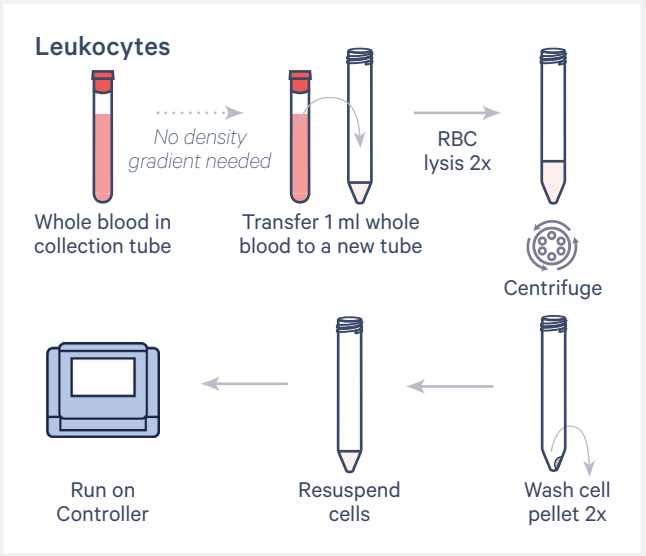


Figure 1. Isolation of leukocytes from whole blood samples for use in 10x Single Gene Expression assays. Analysis can be performed using Cell Ranger and Loupe Browser Software.

Results

Modified Cell Calling is Necessary to Capture Neutrophils

Neutrophil populations are low in RNA content, have a lower number of UMIs per barcode than RNA rich cells, and are often excluded along with background by default Cell Ranger cell-calling algorithms. Below is the barcode rank plot from a neutrophil dataset run with the default Cell Ranger pipeline. A second "knee" is present on the barcodes called as "background", which contains the low UMI neutrophil population. In order to include these in the downstream analysis and given that 8,000 cells are expected to be recovered for this sample, we used the --force-cells=8000 parameter to analyze this dataset.

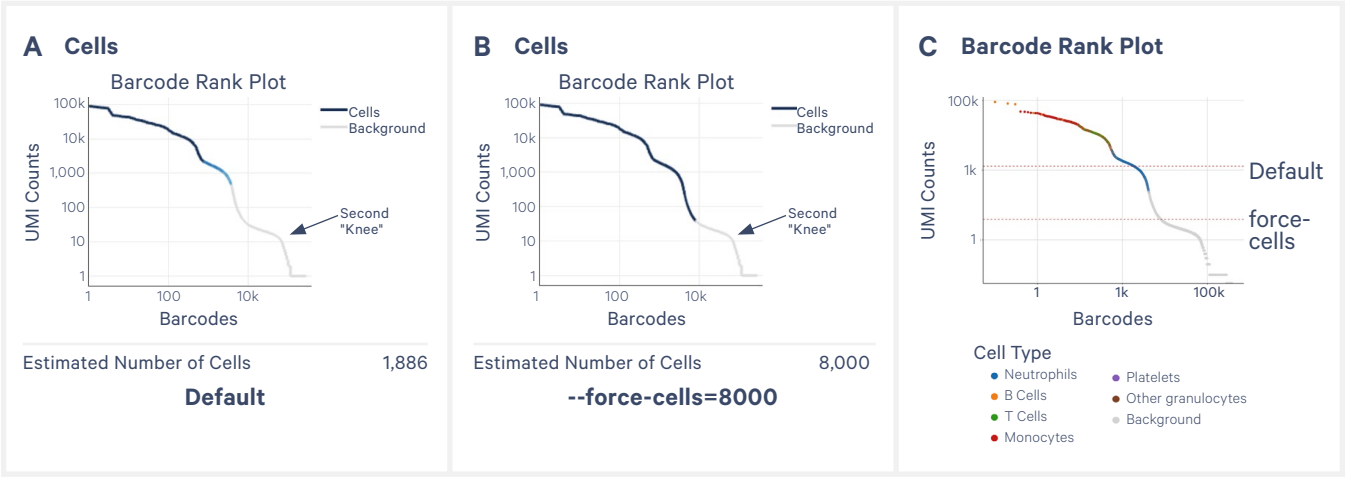


Figure 2. Barcode rank plots derived from whole blood sample data processed using the Single Cell 3' v3.1 workflow. Plots (A) and (B) compare data analyzed with default vs. --force-cells=8000 parameters, respectively, using Cell Ranger Software. The second "knee" region of the barcode rank plots represent the background barcodes with the low UMI neutrophil population. Barcode rank plot (C) with barcodes is colored by cell population. Dashed red lines represent threshold for cell calling with and without introns.

Intron Mapping Increases Neutrophil Sensitivity

Neutrophils retain a high percentage of introns as part of their differentiation mechanisms (Ullrich and Guigó, 2020; Wong et al., 2013). Intronic reads were mapped using the Cell Ranger "include-introns" option, an approach commonly applied where higher intronic reads are expected (i.e. nuclei). The inclusion of intronic reads improved the median number of genes detected per cell in neutrophils from 230 genes detected without intron mapping to 593 genes detected.

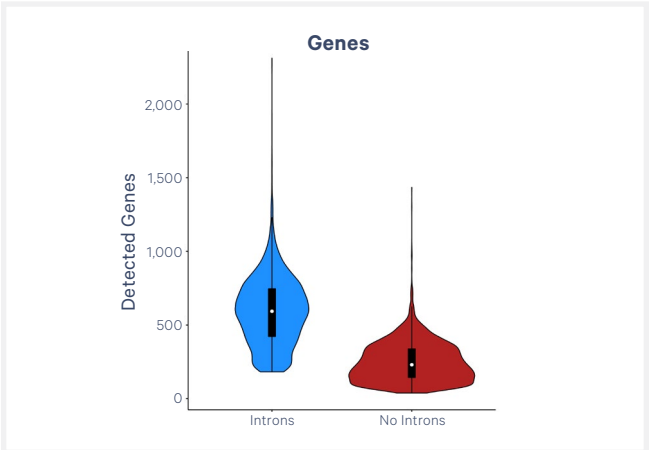


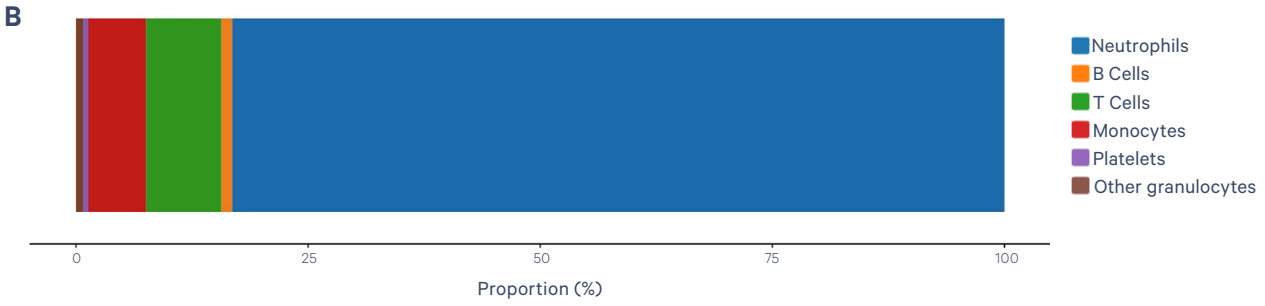
Figure 3. Violin plots displaying genes mapped with introns (blue) and without introns (red) within the neutrophil population.

Distinct Leukocyte Populations can be Detected

Following the analysis tutorial, major blood cell types (e.g. Neutrophils, T cells, B cells, Monocytes, Platelets, and other Granulocytes like Eosinophils and Basophils) were identified using known cell markers. A large cloud of neutrophils was observed, which made up 83.18% of the total cell population. Interestingly, distinct clusters were observed within the large neutrophil cloud.

A

B



C

Cell Type	%
Neutrophils	*83.18*
B Cells	*1.17*
T Cells	*8.11*
Monocytes	*6.22*
Platelets	*0.55*
Other Granulocytes	*0.77*

Figure 4. UMAP plot displaying leukocyte cell type proportions. The large cloud of neutrophils (blue) makes up 83.18% of the total cell population (A). Boxplot showing leukocyte cell proportions (B). Table with leukocyte cell type proportions listed as a percentage of the total cell population (C).

Neutrophil Subpopulations

To gain further insight on neutrophil subpopulations, the expression of common neutrophil markers was assessed within the large neutrophil cloud after running Recluster. Two major types of neutrophils can be identified, immature and mature.

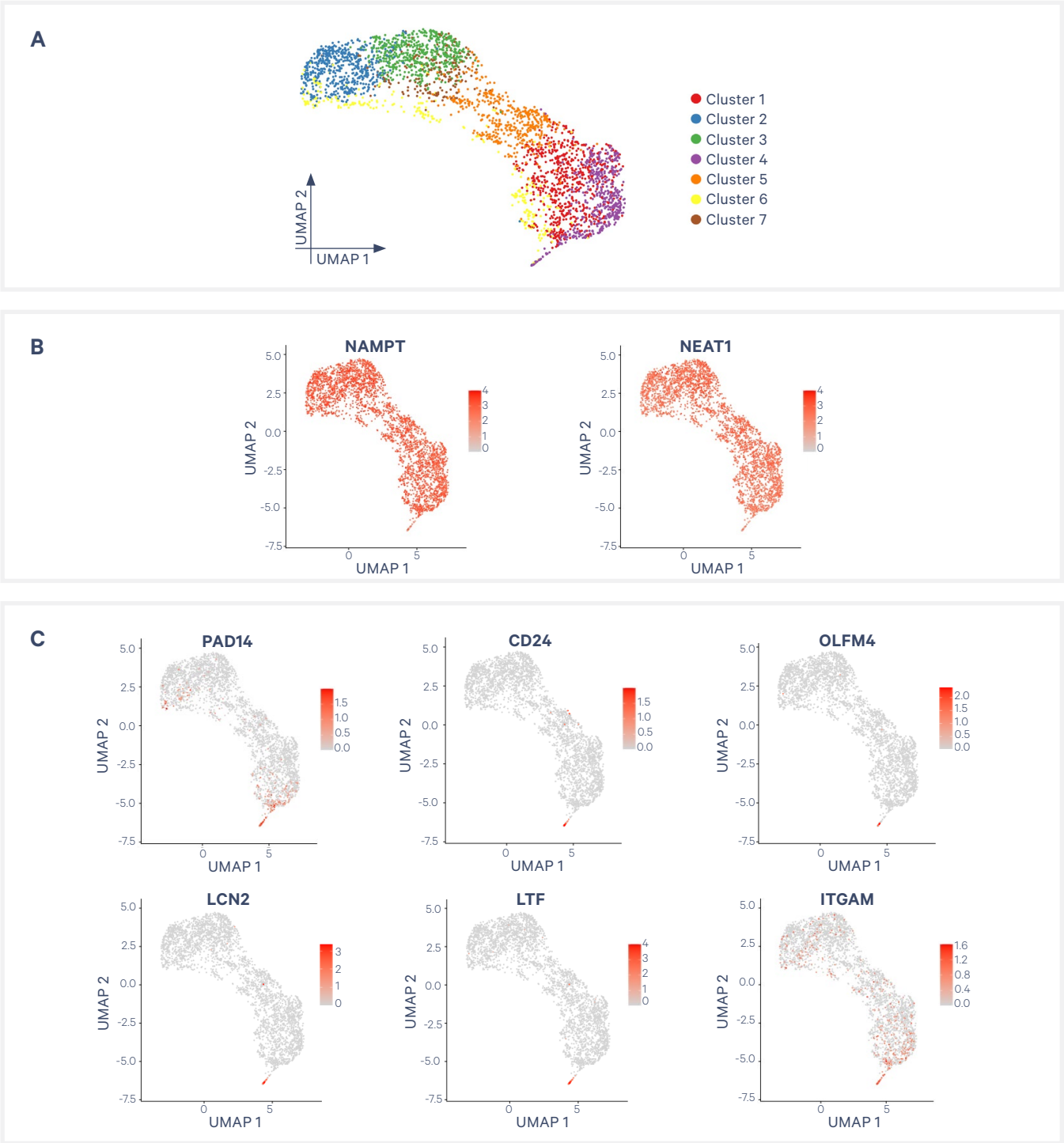


Figure 5. UMAP displaying clusters of neutrophil subpopulations characterized by color (A). NAMPT and NEAT are markers for mature neutrophils (B) and PADI4, LCN2, CD24, LTF, OLFM4, and ITGAM are markers for immature neutrophils (C).

Differential Gene Expression of Mature Neutrophils

The expression of common immune markers differs throughout the neutrophil cloud. The differential expression of common apoptosis genes was observed, hinting that these mature neutrophil subclusters may be neutrophils at different stages of maturation (Rosales et al., 2018).

Figure 6. The expression of apoptosis markers differentiates immature neutrophils from mature neutrophils. The UMAP plots display immature neutrophils (A) and neutrophils expressing various apoptotic markers (B).

Neutrophil Maturation is Captured by Single Cell Analysis

To gain further insight into the different neutrophil subclusters, trajectory analysis was performed using scVelo (Bergen et al., 2020). This tool allows us to estimate and represent trajectories involved in different biological processes such as maturation or differentiation. In order to represent the trajectory, arrows are plotted in the UMAP to show the directionality of the involved transitions. Also, the algorithm estimates latent time values per cell in order to better distinguish immature and mature neutrophils. The results demonstrate the maturation path from immature neutrophils (Cluster 4) to the most mature neutrophil population, Neutrophil Cluster 2.

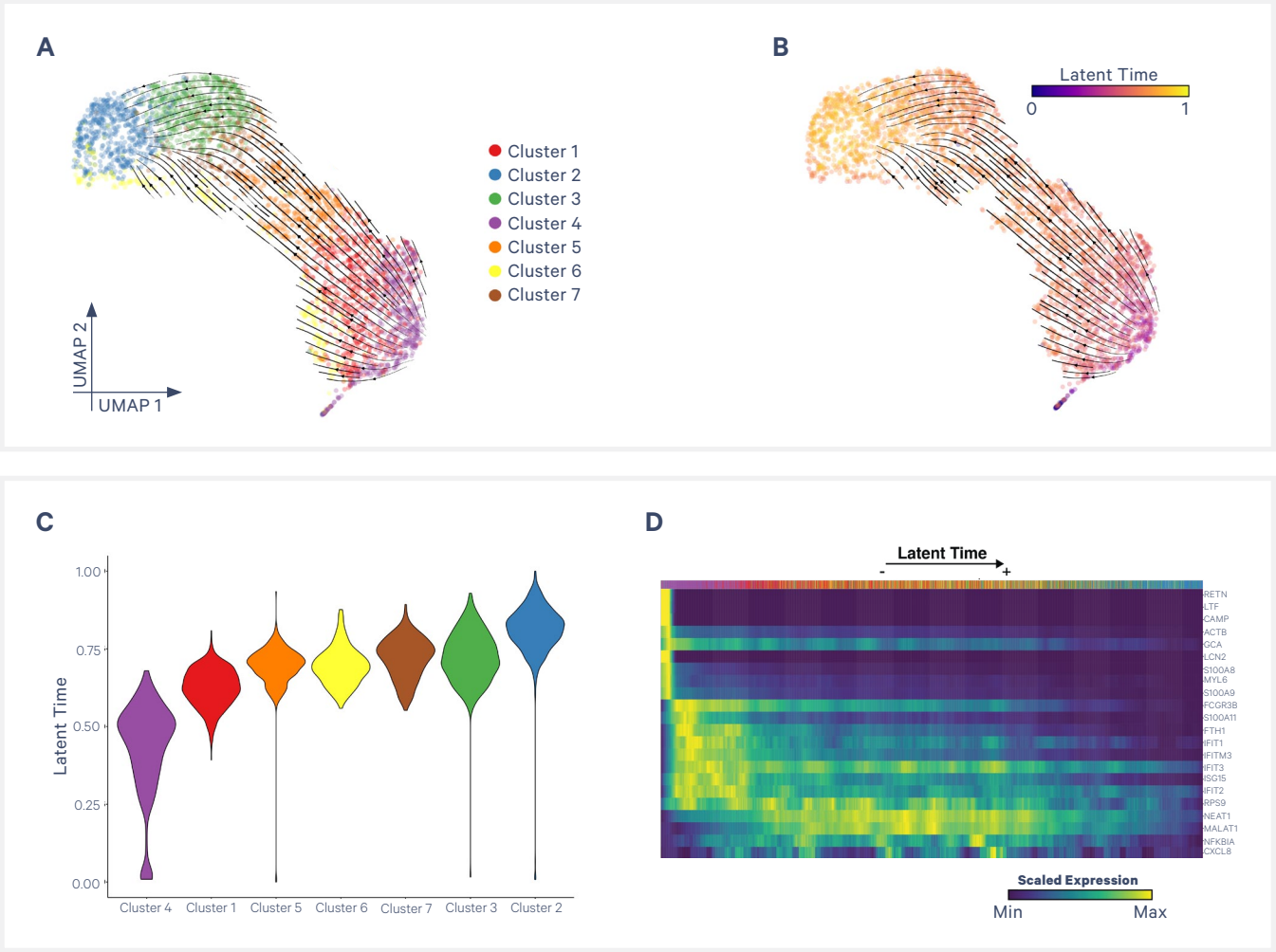


Figure 7. UMAP plots with the identified trajectories of neutrophil subclusters show the maturation path from immature to mature neutrophils with cells colored by cluster (A) and retrieved latent times (B). Violin plots (C) with the latent time values per cluster show an increase fom cluster 4 (immature) to cluster 2 (mature). Heatmap (D) with cells ordered by latent time values display gene expression dynamics of markers previously identified in neutrophil maturation.

Conclusion

Neutrophils are important cell types involved in immune responses to disease. Single cell analysis can be a powerful tool for characterizing neutrophil subpopulations and the biology of their mechanisms of action (Combes et al., 2021). Proper sample preparation and handling is key to successfully isolate neutrophils. To accurately detect neutrophils within a given single cell dataset, cell calling and mapping analysis steps can be modified to fit the unique characteristics of neutrophils (e.g. low RNA, intron retaining, etc.). Neutrophils captured in this single cell experiment from a healthy donor show gene signatures consistent with normal cell maturation pathways. However, further insights may be gained by assessing whole blood samples from diseased patients using a similar approach.

References

1. A Combes et. al., Global Absence and Targeting of Protective Immune States in Severe COVID-19. *Nature*. 591, 124–130 (2021).
2. C Rosales et. al., Neutrophil: A Cell with Many Roles in Inflammation or Several Cell Types?. *Front Physiol*. 9, 113 (2018).
3. J Wong, et. al., Orchestrated Intron Retention Regulates Normal Granulocyte Differentiation. *Cell*. 154(3), 583-95 (2013).
4. S Ullrich and R Guigó. Dynamic Changes in Intron Retention are Tightly Associated with Regulation of Splicing Factors and Proliferative Activity during B-Cell Development. *Nucleic Acids Research*. 48, 1327-1340 (2020).
5. V Bergen, et. al., Generalizing RNA velocity to transient cell states through dynamical modeling. *Nat Biotechnol*. 38, 1408–1414 (2020).
6. Chromium Single Cell 3' v3.1 (Dual Index) Reagent Kits User Guide (CG000315).
7. Chromium Single Cell 5'v2 (Dual Index) Reagent Kits User Guide (CG000331).
8. Demonstrated Protocol for Isolation of Leukocytes, Bone Marrow and Peripheral Blood Mononuclear Cells for Single Cell RNA Sequencing (CG000392).

Contact:
support@10xgenomics.com
10x Genomics
6230 Stoneridge Mall Road
Pleasanton, CA 94588 USA