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Single-Cell Transcriptome Analysis Workflow for Splenic Myeloid-Derived Suppressor Cells from Murine Breast Cancer Models

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Abstract

Single-cell transcriptomics is a powerful tool to study previously unrealized cellular heterogeneity at the resolution of individual cells. Most of the previous knowledge in cell biology is based on data generated by bulk analysis methods, which provide averaged readouts that usually mask cellular heterogeneity. This approach is challenging when the biological effect of interest is limited to a subpopulation within a cell type. This may particularly apply immune cell populations as these cells are highly mobile and swiftly respond to changes in cytokines or chemokines. For example, in cancer certain subset of myeloid immune cells may acquire immunosuppressive features to suppress antitumor immune responses, and thus described as myeloid-derived suppressor cells (MDSCs). Advances in single-cell RNA sequencing (scRNAseq) allowed scientists to overcome this limitation and enable in-depth interrogation of these subsets of immune cells including MDSCs. Here, we provide a detailed protocol for using scRNAseq to explore MDSCs in the context of splenic myeloid cells from breast tumor-bearing mice in comparison to wildtype controls to define the unique molecular features of immunosuppressive myeloid cells.

Keywords

Single-cell RNA sequencing; Cellular heterogeneity; Breast cancer; Neutrophils; Monocytes; Myeloid-derived suppressor cells

1 Introduction

In the past decade, scientific studies have widely used bulk profiling methods to explore gene expression at population level. This method provides average gene expression across thousands of cells that generally masks cellular heterogeneity. The recent development of robust single-cell RNA sequencing (scRNAseq) technologies has now enabled us to explore large scale gene expression at the single-cell resolution, and thus advanced our understanding of how biological systems function particularly in the areas of immunology, neurobiology, stem cell biology, and cancer research [1, 2]. This approach is particularly useful to define changes in poorly defined cell populations when there is a lack of specific cell surface receptors for prospective enrichment. Our recent work utilized scRNAseq to reveal the cellular and molecular properties of myeloid-derived suppresser cells (MDSCs)

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in breast cancer and demonstrating distinct MDSCs clusters that stand out from normal spectrum of myeloid cells [3]. Here, we discuss tissue dissociation and single-cell isolation, cell enrichment, quality control approaches optimized for scRNAseq analysis of myeloid cell populations to diminish batch effects and technical variation that may overshadow true biological insights. We also provide details on computational analysis pipelines and settings used to analyze immune cell single-cell transcriptomics datasets.

2 Materials

2.1 Tissues and Reagents

1. Spleens from FVB/n and Transgenic PyMT (MMTV-PyMT) mice were purchased from The Jackson (JAX) Laboratory.
2. RPMI (Corning, 10-040-CV).
3. Fetal bovine serum (FBS) (Omega Scientific, FB-12).
4. 1×PBS (Corning® DPBS (Dulbecco's Phosphate-Buffered Saline) MT21031CV).
5. FACS buffer (1×PBS, 3% FBS).
6. EasySep™ Mouse MDSC ($CD11b^{+}Gr1^{+}$) Isolation Kit, Stem cell Technologies, Cat.No.19867.
7. Fc-receptor blocking with anti-mouse FcγR (CD16/CD32) (BioLegend, 101301).
8. SYTOX Blue viability dye (Life Technologies, S34857).
9. Anti-mouse-CD45 (30-F11) (BioLegend).
10. Anti-mouse-CD11b (M1/70) (BioLegend).
11. Anti-mouse-Gr1 (Rb6-8C5) (BioLegend).
12. 70-μm cell strainer (Fisher Scientific, 22363548).
13. Automated cell counter Countess™ II (ThermoScientific, AMQAX1000).
14. 5 mL culture tubes with closures (VWR, 211-0061).
15. 5 mL polystyrene round bottom with cell-strainer cap (Thermo Scientific, 352235).
16. FlowJo software v 10.0.7 (Tree Star, Inc).
17. BD FACSAria™ Fusion.

2.2 Single-Cell RNA Sequencing Reagents

1. Chromium™ Single Cell 3′ Library & Gel Bead Kit v2, 16 rxns (10× Genomics 120237).
2. Chromium Single Cell A Chip Kit, 48 rxns (10× Genomics 120236).

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3. Chromium i7 Multiplex Kit, 96 rxns (10× Genomics 120262).
4. TempAssure PCR 8-tube strip (USA Scientific 1402-4700).
5. DNA LoBind Tubes, 1.5 mL (Eppendorf 022431021).
6. DynaBeads MyOne Saline Beads (Thermo Fisher 37002D).
7. Nuclease-Free Water (Thermo Fisher AM9937).
8. Low TE Buffer 10 mM Tris–HCL pH 8.0, 0.1 mM EDTA (Thermo Fisher 12090-015).
9. Ethanol (Sigma 459836-500ML).
10. SPRIselect Reagent Kit (Beckman Coulter B23318).
11. 10% Tween 20 (Bio-Rad 1610781).
12. Glycerin 50% (v/v) Aqueous Solution (Ricca Chemical Company 3290-32).
13. Qubit Fluorometer/dsDNA HS Assay Kit (Thermo Fisher Q33216/Q32854).
14. Illumina Library Quantification Kit (KAPA Biosystems KK4824).
15. 2100 Bioanalyzer Laptop Bundle/High Sensitivity DNA Kit (Agilent G2943CA/ 5067-46726).
16. Chromium Controller (10× Genomics 1000202).
17. C1000 Touch Thermal Cycler with 96-Deep Well Reaction Module (Bio-Rad 1851197).
18. *See* Note 1.

2.3 Computational Analysis

1. Recommended computing requirements (Mac, Linux, or Windows OS installed), 8 GB RAM or higher.
2. R software suite installed version 3.5.0 or higher.
3. R software packages installed (Seurat version 2.3.1 or higher, Monocle version 2.8.0 or higher).

3 Methods

3.1 Tissue Dissociation and Single-Cell Preparation for scRNAseq

1. Spleens were collected from tumor-bearing mice and FVB/n in small volume of RPMI with 3%FBS under sterile conditions.

[1]. Reagents and methods are specific to the methods used in the recently published article by Alshetaiwi et al. [3]. We recommend following 10× Genomics Chromium Single Cell User Guide for latest material list. Chromium Single Cell 3′ Reagent Kits User Guide (v2 Chemistry) https://support.10xgenomics.com/single-cell-gene-expression/library-prep/doc/user-guide-chromium-single-cell-3-reagent-kits-user-guide-v2-chemistry.

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2. Place spleen in a 70-μm cell strainer in the top of a 50 mL conical tube and gently disaggregate using the plunger of a 5-mL syringe and push through the filter (Fig. 1 (1)).
3. Wash the 70-μm cell strainer with 5 mL of RPMI with 3%FBS to create a cell suspension of splenocytes into the 50 mL conical tube.
4. Repeat **step 3** one more time to collect all splenocytes into the 50 mL conical tube.
5. Centrifuge cell suspension at 500 × *g* at 4 °C for 5 min and discard the supernatant.
6. Lyse red blood cells by adding a volume of 5 mL of RBCs lysis buffer (for each spleen) and incubate at room temperature for min.
7. Quench cells with at least three times of RBCs lysis buffer volume with RPMI with 3%FBS and gently pipet up and down.
8. Centrifuge cell suspension at 500 × *g* at 4 °C for 5 min and discard the supernatant.
9. Resuspend cell pellet in 10–15 mL of RPMI with 3%FBS.
10. Count the cells with trypan blue solution (10 μL of cells + 10 μL of trypan blue) loading samples into Countess™ II and record live/dead and viability.
11. Transfer 1×10^8 live cells from spleen tumor-bearing mice and FVB/n to new 15 mL conical tube and add 10 mL of FACS buffer to wash the cells.
12. Centrifuge cell suspension at 500 × *g* at 4 °C for 5 min and discard the supernatant.
13. Preform EasySep™ Mouse MDSC ($CD11b^+Gr1^+$) Isolation Kit to enrich for MDSCs (*if you are using this kit ignore* ***steps 6*** *and 7 of the isolation kit protocol*). Follow the manufacturer protocol.
14. Repeat **step 10**, to count the cells post MDSC isolation kit.
15. Desired number of cells should be transferred into FACS tubes (culture tubes with closures 12 × 75 MM, 5 mL) for staining.
16. Centrifuge cells at 500 × *g* at 4 °C for 5 min and discard the supernatant.
17. Incubate cells with blocking reagent (1:100) FcγR (CD16/CD32) in FACS buffer at room temperature for 10 min.
18. Add mixture of antibodies containing anti-CD45 (1:100), anti-CD11b (1:100), and anti-Gr-1 (1:100) and incubate at 4C for 20–30 min.
19. Wash cells 1–2 times with FACS buffer and centrifuge cells at 500 × *g* at 4 °C for 5 min and discard the supernatant.
20. After labeling the cells, resuspend cells with 500 μL of FACS buffer.

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21. Transfer cells into FACS tubes 5 mL tube polystyrene round bottom with cell-strainer cap.
22. Add 0.5 μL of SYTOX Blue viability dye to the cells.
23. Process your samples to BD FACSAria™ Fusion to sort your MDSCs.
24. FACS gating strategies are illustrated in Fig. 1 (2).
25. Sorting MDSCs in FACS buffer.

3.2 Cell Enrichment

Tissue dissociation and single-cell preparation for scRNAseq is described in Subheading 3.1. There are numerous methods for enriching a specific cell population and depleting unwanted cells of specific tissue types. Magnetic beads have been utilized to enrich CD11b/Gr1-positive MDSC populations (Stem cell technologies, Cat.No.19867). Fluorescence-activated cell sorting (FACS) is a high throughput method widely used to enrich specific cell types, such as neutrophils or monocytes. The following steps demonstrate how MDSC-containing myeloid populations should be gated in FACS for isolation and subsequent scRNAseq (Fig. 1 (2))

1. All cells should be gated based on SSC-A vs. FSC-A.
2. To avoid doublets, two gating strategies should be performed:
   a. SSC-W vs. SSC-H.
   b. FSC-W vs. FSC-H.
3. Gate for live/dead cells.
4. Gate for $CD45^+$ positive cells.
5. Gate for $CD11b^+Gr\text{-}1^+$ double positive cells.
6. Sorting MDSCs in FACS buffer.
7. Quality control (*see* Subheading 3.3).

3.3 Quality Control

After isolating MDSC-containing myeloid cells by FACS, quality control measurements should be executed prior to droplet-enabled scRNAseq performance. A useful metric can be acquired using microscopic imaging of cells and assessing viability using the countess platform (Fisher Scientific). Also, FACS is an additional valuable metric to measure cell viability and purity. The following steps are useful measurements prior to subjecting isolated myeloid cells to droplet-enabled scRNAseq

1. Take small aliquot of sorted MDSCs and mix them with trypan blue solution (10 μL of MDSCs + 10 μL of trypan blue).
2. Load MDSCs into Countess™ II and record live/dead and viability of cells after FACS sorting.

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3. Take small aliquot of sorted MDSCs ~10 μL and do post sort analysis in flow cytometry to evaluate MDSCs population and their viability.
4. Perform functional characterization of MDSCs such as T cell suppression and reactive oxygen species (ROS) assays and other biochemical and molecular parameters associated with MDSC characterization that has been described in recent review [4] to validate MDSCs prior scRNAseq.
5. After MDSCs viability, purity, and characterization were confirmed, pure sorted MDSCs will be introduced into droplet-enabled scRNAseq.

3.4 Single-Cell RNA Sequencing

Single-Cell RNA libraries were prepared according to: Chromium Single Cell 3′ Reagent Kits v2 User Guide (10× Genomics CG00052 Rev. B) (*see* Note 1). In short, libraries were prepared by the following procedure:

1. Wash FACS isolated cells in 0.04% BSA in PBS solution.
2. Resuspend cell pellet to achieve approximately 1000 cell/μL.
3. Count the cells with trypan blue solution (10 μL of cells + 10 μL of trypan blue) by loading samples into Countess II. Note actual cell concentration for chip loading.
4. Prepare Single-Cell A Chip according to 10× Chromium protocol and load cells for Targeted Cell Recovery of 10,000 cells.
5. Generate Gel Beads in Emulsion (GEMs) using the Chromium Controller.
6. Collected GEMs were processed according to 10× Chromium protocol into cDNA libraries.
7. Check cDNA libraries concentration using Qubit dsDNA HS Assay Kit and Qubit Fluorometer.
8. Check fragment distribution using High Sensitivity DNA kit and 2100 Bioanalyzer.
9. Quantify library for Illumina indexed fragments using Illumina Library Quantification Kit.

3.5 Library Sequencing

1. Single Cell 3′ libraries were sequenced on the Illumina HiSeq 2500, using Rapid Run. (*see* Note 2).
2. Sequencing run was performed using the following cycles for each read:
    a. Read1 26 cycles
    b. Read2 98 cycles

[2]. Sequencing can be performed on any next-generation sequencing instrument capable of performing the necessary cycles described.

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c. i7 Index 8 cycles

d. i5 Index 0 cycles

3. Approximately 50,000 reads per cell were targeted for our sequencing depth. (*see* Note 3).

4. FastQ files were aligned using Cell Ranger Count 2.1.0.

5. Aligned libraries were aggregated to normalization based on mapped reads per cell using Cell Ranger Aggr 2.1.0.

3.6 Quality Control

Sequencing data should be checked to ensure appropriate read depth and cell calling before downstream analysis is performed. Useful metrics to consider can be obtained from the Cell Ranger alignment software (10× Genomics), such as sequencing saturation, which may vary for each specific cell type that is sequenced. For proper read depth, the balance between mean reads per cell and library complexity is important. The sequencing saturation curve in Fig. 1 (3) shows observed library complexity for the projected mean reads per cell. As the sequencing saturation approaches 1, a larger portion of the converted mRNA has been sequenced. Cells that have lower complexity (such as neutrophils) will approach this saturated library complexity faster, and therefore will require lower mean reads per cell. Cell types with higher gene expression complexity will require more increased reads per cell to capture more of the converted mRNA.

Cell calling by Cell Ranger is done by comparing UMI counts and barcodes detected. It is important to note that for a library with many different cell types including cells with high and low library complexity, cell calling may be skewed. Neutrophil granulocytes in particular commonly have lower numbers of genes detected. In the presence of cells with higher gene expression, complexity of these neutrophils may be improperly called as an empty droplet and filtered out from further analysis (*see* Note 4).

3.7 Bioinformatic Analysis

Here, we present a general overview of the independent steps encompassing useful computational analysis approach to study scRNA-seq libraries from MDSC-containing immune cell populations. For detailed instructions please refer to the original Seurat (https://satijalab.org/seurat/vignettes.html) and Monocle (http://cole-trapnell-lab.github.io/monocle-release/) vignettes.

1. Load R, R packages and their dependencies (e.g., Seurat, Monocle).

[3]. Initial sequencing can be done at lower depth (e.g., approximately 5000 reads per cell) to first estimate the number of cells captured. This will allow for calculation of optimal sequencing depth to obtain appropriate number of reads per cell in a second sequencing run.

[4]. Manually increasing cell calling by Cell Ranger will include droplets with lower RNA contents. This might alleviate the problem of improper calling of lower complexity cells. More stringent analysis and validation will be needed to ensure cell calling is accurate. Validation such as checking marker genes from any new clusters that may form will be necessary to ensure detection of an actual cell type. If new cluster does not have any distinct marker genes that correspond to known cell types, it might be made up of droplets that contain ambient RNA.

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2. Read in Cell Ranger filtered gene matrix for each library (Wild Type and PYMT mouse cells) into R environment.
3. For Each: Create Seurat object with gene matrix, trimming out genes not expressed in at least three cells, and trimming out cells expressing less than 500 unique genes (*see* Note 5).
4. Calculate percent of counts corresponding to genes on mitochondrial genome per cell (percent.mito).
5. Remove cells from analysis that have a percent.mito above 8% or those that have more than 5000 unique genes expressed (*see* Note 6).
6. Scale Seurat Object.
7. Find highly variable genes to use for canonical correlation analysis (CCA) (*see* Note 7).
8. Take intersect of highly variable genes and perform CCA out to 30 components for the two objects.
9. Select number of components to align subspace (*see* Note 8) and align the two objects.
10. Perform dimensionality reduction (tSNE) and clustering, keeping the number of components used as input consistent with those used to align the subspace (Fig. 1 (4)).
11. Find marker genes for resultant clustering, iterating through clustering resolutions until good separation of cell types and states is achieved (*see* Note 9).
12. Explore differential expression between cell types and states of interest in the analysis (*see* Note 10).
13. Identify candidates for pseudotemporal analysis of cell types and states and subset the cell groups into their own objects.

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5. These cutoffs represent approximate guidelines for trimming out lowly captured genes and cell barcodes that did not have many unique genes expressed. These can be adjusted depending on cell type, as some cell types may naturally express less genes than others and thus can be empirically revisited.

6. The numbers used here are a recommendation to eliminate cells that elicit stress response due to cell isolation and capture, as well as potential doublets cells with the highly unique gene cutoff. As before, these are subject to empirical adjustments depending on the source of these cells.

7. Seurat's default settings to select variable genes for downstream clustering analysis is a valuable starting point; however, the selection settings may be adjusted to move forward with genes representing pathways in which much of the expected heterogeneity of the dataset is captured.

8. This is a selection that often needs to be reiterated during an analysis. A useful functionality within Seurat (the MetageneBicorPlot) can be helpful in this context. In more recent versions of the Seurat Package, the data integration workflow seeks to combine multiple Seurat objects into a single analysis. The goal is to have generalizable commentary on the presence of cell types and states among different batches/conditions.

9. The clustering resolution is another parameter that requires multiple iterations. A useful metric to select the appropriate resolution can be gauged based on the distinctness of marker genes identified for the resultant clusters. These are then visualized via heatmap to assess how exclusive their expression is found across the clusters; if the resolution is too high there will be too many clusters and the marker gene expression is not exclusive enough, while if the resolution is too low not enough clusters are detected and additional potentially important biological diversity may remain undefined.

10. The metadata annotating cells in the analysis are increasing in dimensionality based on batch/condition/tissue/clustering, etc., and this information can be leveraged when performing tests for differential expression between groups. Depending on the question at hand, a test can be designed to compare between cell types within a condition, between conditions, or across batches. Organization is critical here.

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14. Using Monocle, create a CellDataSet (Monocle object) of cells of interest. Maintain metadata from Seurat analysis and convert format to a phenodata object for Monocle (Fig. 1 (4)).
15. Preprocess Monocle object by calculating size factors and dispersion for genes.
16. Select genes to use for pseudotemporal ordering of cells and reduce dimensions (*see* Note 11).
17. Select the origin of pseudotime (*see* Note 12).
18. Analyze gene expression changes through pseudotime using Monocle's differential expression test or use Seurat's marker gene functionality to interrogate state expression.

3.8 Validation Analysis

To validate the scRNAseq data do the following (Fig. 1 (5)):

1. Quantitative PCR (qPCR): CD11b^{+}Gr1^{+} cells from spleens of WT and tumor-bearing mice should be sorted by FACS and subject to (qPCR) [3].
2. FACS: profile your findings by FACS [3].
3. Preform ROS assay [3].
4. Preform T cell suppression assay [5].

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References

1. Wagner A, Regev A, Yosef N et al. (2016) Revealing the vectors of cellular identity with single-cell genomics. Nat Biotechnol 34:1145–1160 [PubMed: 27824854]
2. Nguyen QH, Pervolarakis N et al. (2018) Experimental considerations for single-cell RNA sequencing approaches. Front Cell Dev Biol 6:1–7 [PubMed: 29417046]
3. Alshetaiwi H, Pervolarakis N et al. (2020) Defining the emergence of myeloid-derived suppressor cells in breast cancer using single-cell transcriptomics. Sci Immunol 5:eaay6017 [PubMed: 32086381]
4. Bronte V, Brandau S et al. (2016) Recommendations for myeloid-derived suppressor cell nomenclature and characterization standards. Nat Commun 7:1–10
5. Solito S, Pinton L et al. (2019) Methods to measure MDSC immune suppressive activity in vitro and in vivo. Curr Protoc Immunol 124:e61 [PubMed: 30303619]

[11]. The genes used for pseudotemporal ordering can be selected in a variety of ways. Analysis using pseudotime is a way to linearly cluster cells based on a spectrum of similarity of expression for a given set of genes. These genes can be differentially expressed genes as calculated using Monocle, marker genes for cell types from Seurat, or genes curated that have an association with a phenotype with which the cells are to be stratified. Each will produce different results, and the interpretation of the trajectory is dependent on what was used as input to order the cells.

[12]. Pseudotime as a calculation in Monocle is contingent on the choice of an origin. Typically, this will be informed with prior knowledge of what a more immature cell type/state expresses, and so where those genes are highly expressed in the trajectory calculated will be chosen as the origin. In other cases of a stratification based on acquisition of a phenotype or expression program, researchers can choose to pick those cells that have not acquired expression of those genes as the start.

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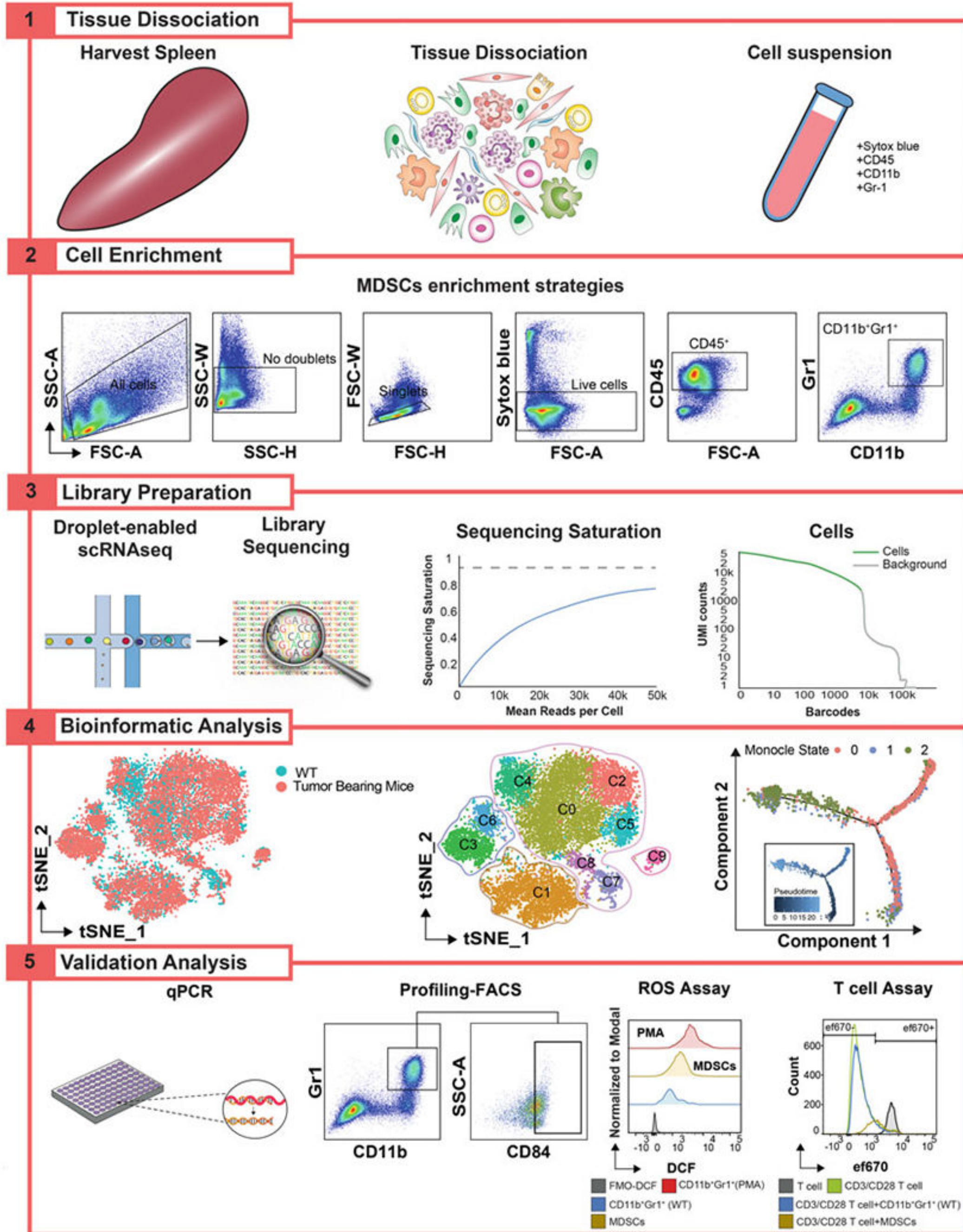

Fig. 1.

Illustration of scRNAseq protocol (*1*) Tissue dissociation and single-cell suspension (*2*) MDSCs gating and enrichment strategies (*3*) Library preparation and sequencing for scRNAseq include sequencing saturation and cell calling plot (*4*) tSNE plots for; combined WT and tumor-bearing mice, and various distinct clusters of CD11b^{+}Gr1^{+} cells. Monocle analysis on subset of MDSCs clusters resulted in branched trajectory with three distinct

Monocle states (color code for each state is indicated) (*5*) qPCR, FACS profiling, ROS, and T cell suppression assays for validation

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