Application Note

Dissociation of tumor tissue samples for single-cell genomics

An examination of the impact of sample preparation methods on genomic data quality

In cooperation with:

10x GENOMICS

Introduction

Unraveling the diversity of cell types and understanding the complexity of the tumor microenvironment are critical milestones to advance cancer research and the development of both diagnostics and therapeutics for cancer. Within the tumor microenvironment there are numerous cells types, such as infiltrating immune cells, inflammatory cells, cancer-associated fibroblasts, vascular cells, stromal cells, extracellular matrix, and tumor cells. Tumor cells are highly evasive due to their elasticity and ability to adapt to environmental modifications. As a result of these properties, improving our understanding of a tumor's dynamic cellular composition and gene expression profile will enhance the research community's ability to develop more effective diagnostics and treatments for cancer.

In recent years, single-cell RNA sequencing (scRNA-seq) has become a popular analytical tool to significantly augment characterization of the tumor microenvironment. In contrast to RNA sequencing of bulk cell populations, scRNA-seq allows for the transcriptomic profiling of hundreds, thousands, or potentially millions of individual cells.[1,2] The ability to detect transcriptomic signatures unique to an individual cell greatly increases the diversity of transcripts that can be detected in a sample. This is in comparison to bulk sequencing, whereby the average signal across the entire sample obscures transcripts from rare cell populations. As a result, the use of scRNA-seq has the potential to facilitate advances, such as the discovery of new cell subpopulations and the identification of clonal tumor cell subsets responsible for resistance to treatment.

The dissociation of a tumor in order to release intact individual cells is a critical step in every scRNA-seq experiment. Ideally, the sample for a scRNA-seq experiment is a suspension consisting of fully dissociated, intact, viable cells. Attributes of a poor quality sample include clumps, dead or dying cells, extracellular debris, and ambient free-floating RNA. These cellular and molecular contaminants can negatively impact data quality and increase overall sequencing costs. Therefore, it is important to consider the sample preparation process and utilize an approach that does not produce a low-quality sample that adversely impacts scRNA-seq data.

To address this challenge, Miltenyi Biotec and 10x Genomics designed a collaborative study to demonstrate the impact of sample preparation on scRNA-seq. For this work, our research teams combined methodologies for sample storage, tumor tissue dissociation, and sample cleanup from Miltenyi Biotec, with the 10x Genomics technology for single-cell sequencing. As a result, we validated an end-to-end workflow for preparing solid tumor tissue for single-cell RNA sequencing. For this study, solid tumors from syngeneic mouse models were collected and preserved in MACS® Tissue Storage Solution, then dissociated using the gentleMACS™ Octo Dissociator with Heaters and the Tumor Dissociation Kit, mouse. Following dissociation, a series of steps was performed to remove debris, red blood cells (RBCs), and dead cells prior to analysis of the samples using the 10x Genomics Chromium System and an Illumina sequencing platform.[3]

Materials and methods

Tumor storage and dissociation materials

- MACS Tissue Storage Solution
- gentleMACS Octo Dissociator with Heaters
- gentleMACS C Tubes
- Tumor Dissociation Kit, mouse

Sample clearance materials

- MACS SmartStrainers (70 µm)
- Red Blood Cell Lysis Solution
- Dead Cell Removal Kit

Flow cytometry materials

- MACSQuant® Analyzer 10
- Anti-Ter-119-PE, mouse (clone: #Ter-119)
- FcR Blocking Reagent, mouse
- Propidium Iodide Solution

Single-cell RNA sequencing materials

- Chromium Controller or Chromium Single Cell Controller
- Chromium Single Cell 3′ v2 Library Kit
- Chromium Single Cell 3′ v2 Gel Bead Kit
- Chromium Single Cell A Chip Kit
- Chromium i7 Multiplex Kit

Tumor storage and dissociation methods

Breast (4T1), colon (WT26), and melanoma (B16 F10) tumors from syngeneic mouse tumor models were obtained from Charles River Laboratories (Durham, NC) and shipped to the 10x Genomics research and development facility (Pleasanton, CA). For transport, tumors were shipped in MACS® Tissue Storage Solution and maintained at 4°C until processing for single-cell sequencing. Three tumors (n=3) from each tumor type (breast, colon, and melanoma) were used in this study. Tumors were dissociated using the gentleMACS™ Octo Dissociator with Heaters and the Tumor Dissociation Kit, mouse, as per manufacturer's instructions.

Removal of RBCs and dead cells methods

Immediately following dissociation, all single-cell suspensions were filtered using a MACS SmartStrainer (70µm). They were then treated with Red Blood Cell Lysis Solution and one or two rounds of dead cell removal using the Dead Cell Removal Kit depending on the amount of dead cells in each sample. All samples were processed as per manufacturer's instructions.

Flow cytometry

Flow cytometry was used for quality control of samples and cell counting. Cells were stained as per manufacturer's instructions using the suggested concentration of antibody, but incubated in a slightly lower total final volume. Up to 10^6 cells were resuspended in a total of 50µl (consisting of phosphate-buffered saline (PBS) containing ethylenediaminetetraacetic acid (EDTA) and a panel of antibodies as per manufacturer's recommendations) and incubated for 10 minutes at 4°C. Cells were then washed, resuspended in PBS containing bovine serum albumin (BSA), and flow cytometric analysis performed with the MACSQuant® Analyzer 10. Data was analyzed regarding cell viability, proportion of singlets, and number of RBCs and dead cells within the sample (data not shown).

Single-cell RNA sequencing methods

Cells suspended in PBS were processed using the Chromium Single Cell v2 Gene Expression Solution, as per manufacturer's instructions. Triplicate samples from each tumor were sequenced to a depth of >20,000 reads per cell using an Illumina HiSeq® 4000. Sequencing data was processed using Cell Ranger software, and results were visualized using Loupe Cell Browser software, both from 10x Genomics.

Results

Sample preparation and single-cell sequencing workflow

The workflow for gene expression analysis of single cells from tumor samples included the following steps: tumor dissociation using the gentleMACS Octo Dissociator with Heaters and the Tumor Dissociation Kit, mouse and subsequent passage through a 70µm filter; RBC removal; one or two rounds of dead cell removal (fig. 1, steps A-D). Subsequently, gene expression of single cells was analyzed using the Chromium Single Cell v2 Gene Expression Solution and an Illumina sequencer.

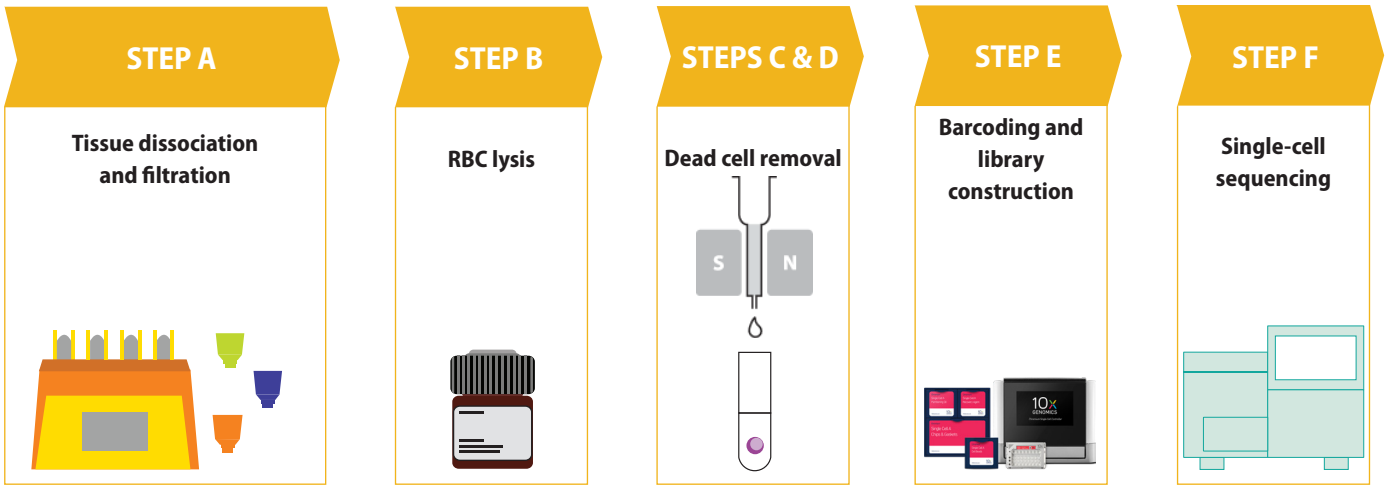

Figure 1. Tumor tissue single-cell sequencing workflow: generation of optimal single cell suspensions from tumors for single cell gene expression analysis, including tumor dissociation and filtration (A), red blood cell lysis (B), one or two rounds of dead cell removal (C & D), and subsequent single-cell gene expression analysis (E and F).

Impact of sample preparation method on single-cell sequencing data quality

In order to evaluate the quality of the samples, an aliquot of cells was collected after each cleanup step (fig. 1, steps A-D) and single-cell gene expression analysis was performed. Sequencing data was analyzed using 10x Genomics Cell Ranger software.

This software combines transcriptome alignment with cellular barcoding to rapidly generate expression profiles of the individual cells. Using this analysis, we evaluated the quality of the sequencing results using standard single-cell sequencing parameters such as library cleanliness, library complexity, and cell recovery.

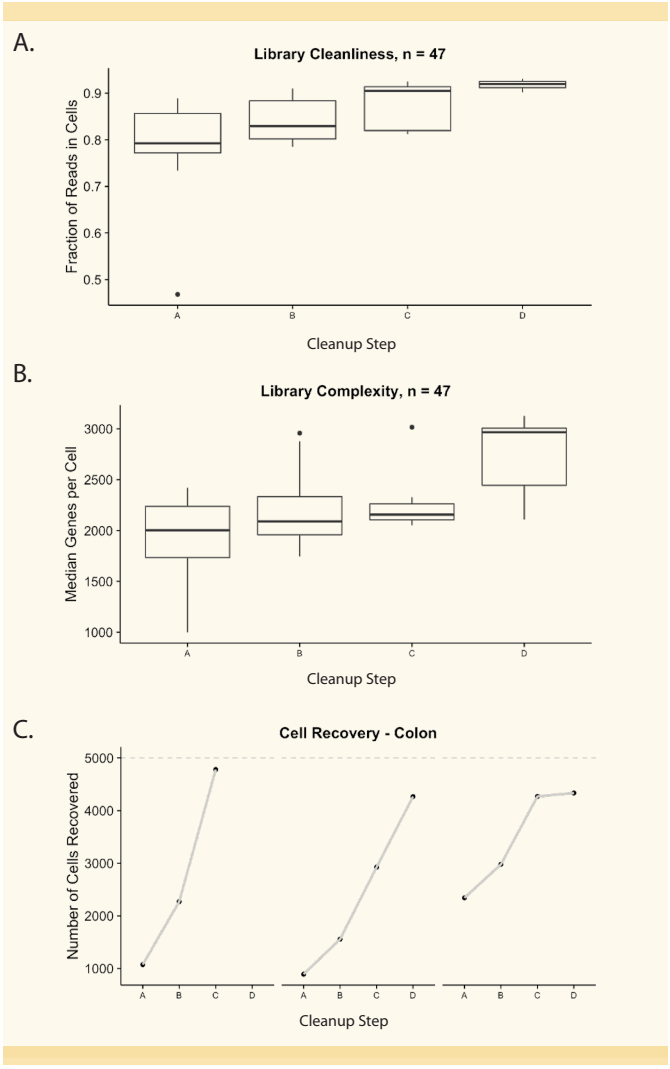

Figure 2. To evaluate the impact of sample cleanup, scRNA-seq was performed on aliquots of cells after sample preparation steps A-D as shown in figure 1 and indicated on the x-axis in figure 2: step A = dissociated cells filtered through SmartStrainer only; step B = step A plus RBC removal; step C = steps A and B, plus one round dead cell removal; step D = steps A, B, and C, plus additional round of dead cell removal. For library cleanliness figure 2A and library complexity figure 2B, the data was combined across all tumor samples and replicates (47 libraries). For cell recovery figure 2C, the data of colon samples was combined across all replicates. Data was analyzed using the Cell Ranger software. The sequencing depth was 20,000 reads per cell. For cell recovery, we aimed for 5,000 cells.

Library cleanliness indicates the percentage of reads that can be confidently mapped back to a single cell. Additional cleanup steps post dissociation improved library cleanliness from 80% before cleanup to 90% after cleanup (fig. 2A). In addition, the number of genes detected per cell (library complexity) was significantly increased in samples in which cleanup steps A-D were performed. To determine cell recovery, we looked at the dataset from each tumor type and aimed for 5,000 recovered cells for colon and melanoma tumors and 2,000 recovered cells for breast tumors. The number of recovered cells indicates the number of cells that were expected to generate functional libraries. The cell recovery increased with each cleanup step for all tested tumor entities (fig. 2C; breast and melanoma data not shown).

Reproducibility

Samples were visually evaluated for reproducibility using the Loupe Cell Browser. This suite of gene expression analysis tools allows easy identification of distinct cellular sub-populations, isolation of significant genes, and the measurement of gene expression levels. Similar tumor type samples from different mice and technical replicates showed a high degree of reproducibility across samples after all cleanup steps (fig. 3A-C).

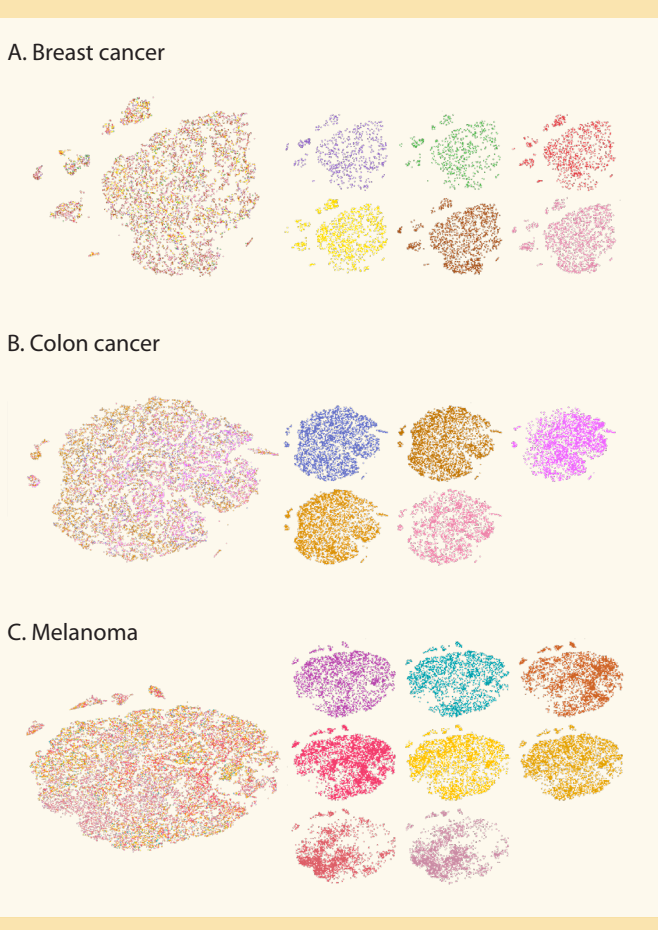

Figure 3. Samples were visually evaluated for reproducibility using the software program Loupe Cell Browser. This figure depicts samples following RBC lysis and dead cell removal, grouped by cancer model A. Breast cancer, B. Colon cancer, C. Melanoma. Individual samples are separated by color, corresponding to different mice and technical replicates from the same mouse. Visual inspection confirms a high degree of reproducibility across samples.

Data from intermediate cleanup steps in the workflow was also analyzed for reproducibility. Single-cell sequencing data from samples subjected to only dissociation and filtering were analyzed as described above. For these samples, reproducibility was observed across all samples for the breast and colon cancer models, but not for the melanoma model, owing to a high degree of necrosis in the tumors, and low viability of the cell suspensions (data not shown).

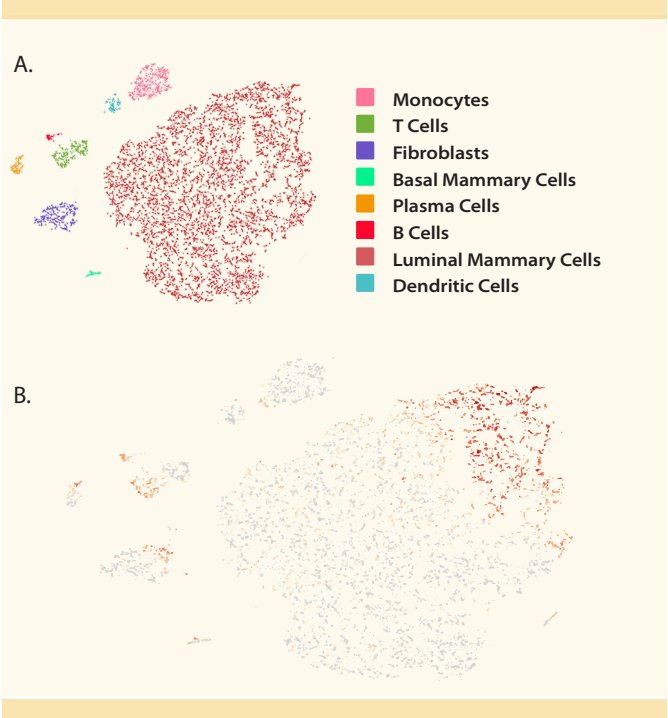

Figure 4. Example of cell identification using mammary tumor sample. Clustering was performed using Cell Ranger and viewed in Loupe Cell Browser; cell type classification was added to 'Graph-Based' clusters based on manual curation using known marker genes. (A) 83% of identified cells are luminal tumor cells. Marker genes used for annotation: Monocyte cluster: $CD68^{+}$; T Cell cluster: $CD3e^{+}$, $CD4^{+}$, $CD8^{+}$; Fibroblast cluster: $CD34^{+}$, $Pdgfra^{+}$, $Pdpn^{+}$; Basal Mammary cluster: $Krt5^{+}$, $Krt14^{+}$; Luminal mammary (tumor) cluster: $Krt18^{+}$, $epcam^{+}$, cldn4+; B Cell Cluster: $CD79a^{+}$, $CD19^{+}$; Plasma Cell Cluster: $CD79a^{+}$, $CD19^{-}$, $CD38^{+}$, $Sdc1(CD138)^{+}$; Dendritic cluster: $Flt3^{+}$, $CD68^{+}$, $CD24a^{+}$, $CD14^{-}$, $ly6c2^{-}$. (B) Proliferating cells identified by expression of known proliferation marker genes Cdc20, Cenpe, Top2a, Mki67. Proliferating cells are observed in the tumor cells, fibroblasts, and immune cells.

Tumor heterogeneity

To analyze tumor heterogeneity and identify the different cell types present in the sample, data sets corresponding to each tumor type were combined and clustered using Cell Ranger software and visualized using the Loupe Cell Brower software program. Using manual curation of known marker genes, we identified the different cell types in each tumor. An example of this analysis for the breast tumor samples is shown in figure 4A. In this analysis, data from 6,335 cells was used.

In figure 4A, each color indicates a different cluster. Eight cell types were identified by looking at distinct marker genes. These cell populations include immune cells, fibroblasts, plasma cells, basal mammary cells, and luminal mammary (tumor) cells. To extend this analysis, we also looked at markers for cell proliferation (fig. 4B). Proliferating cells were observed in immune cell, fibroblasts, and tumor cell populations (fig. 4B).

Conclusions

Starting with the MACS® Tissue Storage Solution for tumor sample collection and transport, followed by sample processing with gentleMACS™ Octo Dissociator with Heaters and the Tumor Dissociation kit, mouse, we were able to produce high quality single-cell suspensions with adequate performance in scRNA-seq. However, additional cleanup steps including RBCs and dead cell removal significantly improved different quality metrics for single-cell gene expression data generated using the 10x Genomics Chromium Platform. Our data indicates that the reduction of RBCs and dead cells from the dissociated tumor tissue samples leads to:

- An increased number of reads confidently mapped back to one cell
- A higher number of genes detected per cell
- Maximal cell recovery with the 10x Chromium System

References

1. Zheng, G. *et al.* (2017) Massively parallel digital transcriptional profiling of single cells. Nat. Commun. 8: 14049.
2. 10x Genomics. Transcriptional profiling of 1.3 million brain cells with the chromium single cell 3' solution.
3. 10x Genomics. Tumor Dissociation for Single Cell RNA Sequencing.

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