

TECHNOTE 2 - PREPARATION OF MOUSE AORTIC TISSUE FOR SINGLE-CELL RNA SEQUENCING: METHODOLOGICAL INSIGHTS FOR BENCHMARKING

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1. Background

In cardiovascular research, the ability to dissect the cellular composition of disease target tissues, i.e. aorta, has recently increasingly relied on the use of multi-omics approaches, as a crucial mean to understand the underlying pathophysiological processes in conditions like atherosclerosis and ageing. To this end, single-cell RNA sequencing (scRNA-seq) has been an important technological milestone that has revolutionised the study of complex tissues by enabling high-resolution characterisation of cellular heterogeneity (1). The current technical note outlines a workflow designed for the preparation of mouse aortic tissue for scRNA-seq, with a focus on maximising cell quality, RNA integrity, and sequencing depth.

While traditional methods for single-cell analysis often rely on bulk tissue samples or less efficient technologies, we combine cutting-edge approaches that streamline cell sorting, barcoding, and sequencing. This presented note integrates several advancements in the field of single-cell transcriptomics, such as droplet-based barcoding (using 10X Genomics Chromium), cell-based isolation, and sample multiplexing strategies. The 10X Genomics Chromium platform enables high cell capture efficiency and low multiplet rates, making it ideal for profiling heterogeneous cardiovascular tissues, such as aorta. Compared with alternative techniques, such as SPLiT-seq (Parse Biosciences) or BD Rhapsody, the 10X system offers more robust data for rare cell populations due to its high sensitivity and deep sequencing capacity (2, 3).

By optimising protocols for cell isolation and subsequent high-throughput sequencing, this workflow addressed specific challenges of vascular tissue, such as fibrous tissue components, large cell sizes, and the need for high sample throughput. Through these efforts, we have ensured that large cell sizes, fragile and heterogeneous populations, such as primary fibroblasts (FBLs), endothelial cells (ECs) and smooth muscle cells (SMCs), can be efficiently captured, profiled, and analysed.

The above technical note outlines a detailed step-by-step protocol for cells isolation from mouse aortic tissue for single-cell RNA sequencing (scRNA-seq). The protocol includes procedures for tissue extraction, enzymatic digestion, cell sorting, barcoding, library preparation, sequencing, and data analysis. Special emphasis is placed on preserving high cell viability and recovering adequate cell numbers for meaningful analysis. By utilising advanced technologies and bioinformatics tools such as TrailMaker, researchers can generate high-quality single-cell RNA-seq data to explore the cellular dynamics associated with vascular complications (like atherosclerosis) and in various disease models.

2. Experimental Design and Sample Preparation

2.1 Steps for Cohort Planning and Pooling Strategy

STEP1: To maximise cell yield, and ensure reliable biological representation, aortae from multiple mice are pooled for each experimental condition.

STEP2: A typical experiment involves three mice per condition.

STEP3: The tissue is harvested fresh during dissection, which is crucial for maintaining high cellular integrity.

STEP4: To avoid batch effects and tissue degradation, careful attention is paid to tissue handling and timing during the dissection process.

STEP5: To prevent contamination from blood cells, tissue is perfused with saline solution before dissection, ensuring a cleaner sample suitable for subsequent RNA analysis. Aortas are cleaned of periaortic fat and stored in fresh cell medium without supplements, for short-term.

2.2 Tissue Dissociation and Nuclei Preparation

Digestion of aortic tissue using enzymatic digestion protocol is designed to release intact cells while minimising damage (see STEP 3.1 and reference (4)). Cell size in mouse aorta can be quite large (up to 30 μm), making proper dissociation critical to ensure that viable, intact cells are recovered. The dissociation protocol was optimised to balance cell yield and viability, preserving the common cell types within the tissue.

3. Quality Control and Sorting

3.1 Cell Viability

Cell viability is a critical parameter throughout the process, and it is assessed by fluorescence-activated cell sorting (FACS). Only samples with >70% viability are used for library preparation.

4. Barcoding, Multiplexing, and Droplet Generation

4.1 Barcoding Approach for Multiplexing

Each sample is assigned a unique barcode using modified cholesterol-conjugated oligonucleotides, providing distinct molecular identifiers for each sample and each cell within a droplet (5). This approach facilitates the individual identification of samples during analysis. The 10X Genomics Chromium system encapsulates cells into droplets along with beads that carry oligos with unique barcodes. This enables efficient single-cell RNA capture, ensuring high-resolution gene expression profiles from a diverse range of cells. This adaptability is beneficial in experiments with multiple biological replicates or time points, as it reduces per-sample costs without compromising the overall data quality.

4.2 Reverse Transcription and Storage Conditions

After the generation of droplets, reverse transcription (RT) is performed in the droplets, whereby mRNA from each cell is converted into complementary DNA (cDNA). This is a critical step where most sequencing artefacts can occur. Post-RT, droplets are stored either at 4°C for up to 48 hours, or at -20°C for longer-term storage (up to a week) before library preparation. This ensures flexibility in scheduling and allows for high-throughput sequencing without compromising sample integrity.

5. Library Preparation and Sequencing Parameters

5.1 Library Preparation

Following RT, libraries are prepared according to the standard 10X Genomics protocol (6), which includes amplification of cDNA, fragmentation, and indexing (7). Each pooled sample generates two libraries: **1.** for individual cell barcodes and **2.** for pooled barcodes. This dual-library approach allows for robust identification of cell types and the deconvolution of pooled samples during the analysis.

5.2 Sequencing Strategy

Sequencing is conducted using Illumina platform, aiming to obtain approximately 40,000 reads per cell. This sequencing depth is deemed adequate for downstream analysis, particularly when working with rare or underrepresented populations, of cells (like ECs) in the tissue. For a typical sample containing 30,000 cells, there is a requirement for approximately 1.2 billion reads per single sequencing lane. Multiple samples can be pooled per lane, to achieve cost-efficiency. Two pools in each lane generate ~600 million reads per sample. This pooling strategy helps maximise throughput while maintaining high-quality data.

6. Data Analysis and Downstream Considerations

After sequencing, data is processed using an *in-house* bioinformatics pipeline (see step 8.1.) and commercially available software for multiplexing, deconvolution, and downstream analysis. The processing steps comprise of clustering and differentially expressed gene (DEG) analysis to study the temporal and spatial changes in cell populations, particularly the endothelial and smooth muscle cell compartments. Cell quality and library complexity are assessed using standard metrics to confirm that the sequencing results reflect the true biological diversity of the aortic tissue.

DETAILED PROTOCOL

STEP 1. Sample Size

Each experimental condition involves the use of 3 mice per pool, depending on cell yield and specific needs.

STEP 2. Isolation of aortas

- 1. Tissue Collection:** Mice are euthanised by cervical dislocation, and aorta is carefully exposed under the surgical microscope. Tissue is perfused with phosphate-buffered saline (PBS) to remove excess blood. Collection starts from the root, including aortic arch, ascending, descending, thoracic and abdominal regions. Aorta is very carefully cut out, at the edge, on each side, by the same person for all replicates, to maintain consistency. Aorta is best extracted, as a whole piece (~3cm length) from each animal together with the heart, and stored in 3ml DMEM medium without serum, on ice, until dissection.
- 2. Tissue Dissection:** Cleaning the tissue is performed in a Silastic-coated dish filled with DMEM medium under the sterile conditions to prevent contamination. This process is done under a bench top microscope using a pair of narrow pattern curved forceps and micro-dissecting spring scissors (Fine Science Tools). The perivascular fat is cleaned off each aorta while pinned down. Cleaned aorta is stored in fresh cell medium without supplements, on ice, for short-term.
- 3. Tissue processing:** Aorta is carefully placed in 12-well plate, ensuring minimal mechanical stress, which can affect the viability of cells. Full length aorta is cut into small segments (<0.5cm length), for optimal digestion and kept in DMEM medium as in STEP2.

STEP 3. Tissue Dissociation and Enzymatic Digestion

- 1. Mechanical Dissociation:** The tissue is finely chopped at room temperature using spring scissors to create a single-cell suspension based on an adapted previously published protocol (4). This process increases the surface area of the tissue, which allows for more efficient enzymatic digestion later.
- 2. Enzymatic Digestion:** Tissue is exposed to enzyme digestion buffer (400 U/mL collagenase type I, 120 U/mL collagenase type XI, 60 U/mL hyaluronidase and 20mM HEPES in Dulbecco PBS containing calcium), as reported before (4), for 50 minutes at 37°C. The dissociation

process is carefully monitored under a microscope every 20min to ensure that the cells are adequately dissociated without causing excessive damage. The subsequent cell suspension is then strained through a 100 μ m filter, attached to a 2ml syringe, and treated with FACs buffer (PBS with 2% FBS) for 10 minutes at 4°C. After centrifugation at 300xg, the cells are resuspended in 1mL FACs buffer for FACS analysis.

STEP 4. Cell Counting and Viability

- 1. Cell counting and viability** are initially assessed using a haemocytometer and the trypan blue exclusion method. Cell suspensions are washed and resuspended in phosphate-buffered saline (PBS) at 4°C before being analysed with a BD LSR Fortessa flow cytometer and data acquired using BD FACSDiva software (BD Biosciences). To exclude dead cells, DAPI is added prior to the flow cytometry analysis. A viability rate of above 60-70% is required to ensure the quality of the downstream scRNA-seq analysis.

STEP 5. Preparation of Samples for scRNA-seq

- 1. Cell preparation:** cells (in suspension as in 4.1) are processed using 10X Genomics Chromium Next GEM Single Cell 3' Reagent Kits v3.1 with Feature Barcode technology for cell multiplexing (CG000389), following manufacturer's instructions (6).
- 2. Multiplex labelling:** Isolated single cells are immediately labelled with oligonucleotides that are attached to a modified cholesterol molecule (10X Genomics CellPlex CMO oligos). Each cell is assigned a unique barcode, ensuring that the samples can be tracked individually throughout the analysis process.

STEP 6. Library Preparation

A maximum of 60,000 cells are loaded onto a 10X Genomics Chip G using V3.1 gel beads and partitioning oil. The cells are then encapsulated with gel beads using a 10X Genomics Chromium X instrument. Each droplet contains one gel bead, one cell, and a unique barcode, ensuring that individual cells are being captured and their mRNA could be sequenced separately.

After encapsulation and cDNA synthesis, each reaction is amplified by PCR. The resulting products are purified into low- and high-molecular weight (MW) fractions. The high-MW fraction, containing cDNA, is fragmented, ligated with sequencing adapters, and amplified to generate gene expression (GEX) libraries. The low-MW fraction is further amplified with

sequencing adapters to produce multiplexing (MP) libraries. All libraries are then pooled in a molar ratio of 10:1, of GEX to MP respectively, and sequenced on Illumina NextSeq 2000.

STEP 7. Sequencing

- 1. Sequencing Setup:** The libraries are loaded onto the high-throughput sequencing platform, Illumina NextSeq 2000. Typically, sequencing involves 40,000 reads per cell for a comprehensive overview of gene expression.
- 2. Multiplexing:** Multiple samples can be pooled together using unique barcodes, enabling more efficient sequencing and reduced costs. For example, up to four samples can be multiplexed per lane in a sequencing run.

STEP 8. Raw Data Processing

- 1. Data Preprocessing:** After sequencing, the raw data undergoes processing to filter out low-quality cells and reads. The data is aligned with the mouse genome, and gene expression levels are quantified based on the unique molecular identifiers (UMIs) associated with each cell. The single-cell RNA-Seq data are analysed using the “cellranger multi” command from 10X Genomics Cell Ranger v7.1.0, with a MAC value of 0.5, to generate demultiplexed, cell-specific gene expression data (<https://support.10xgenomics.com/docs/intron-mode-rec>) (8). As an additional step, which does not take into account the CellPlex CMO barcodes, the nf-core scRNA-Seq pipeline ([10.5281/zenodo.3568187](https://doi.org/10.5281/zenodo.3568187)) v2.3.0 is also executed on the data, utilising both Salmon v1.10.0 in combination with simpleaf v0.10.0, and “cellranger count” from 10X Genomics Cell Ranger v7.1.0 (7). The output is subsequently converted into an expression matrix, which serves as input for further analysis.
- 2. Trailmaker™** is used to process and visualise the single cell RNA-sequencing data analysis. Trailmaker™ Parse Biosciences, Seattle, U.S.A. is available at <https://app.trailmaker.parsebiosciences.com/>.

Trailmaker is an open-source online platform that enables biologists to analyse scRNA-seq datasets without requiring extensive computational experience. It includes features for project management as well as data processing, quality control, and visualisation of scRNA-seq data. It facilitates an in-depth data exploration through differential expression analysis, pathway analyses, and the creation of fully customisable plots suitable for publication. Key functionalities of Trailmaker include cell quality control, clustering, differentially expressed

gene (DEG) and trajectory analyses. As a result, users can visualise gene expression patterns across different cell types within tissues and identify changes in differentially expressed genes in cell populations over time.

STEP 9. Data Deposition

- 1. Data Sharing and Repository Submission:** Following analysis, raw data can be deposited into public repositories, such as GEO (Gene Expression Omnibus) (<https://www.ncbi.nlm.nih.gov/geo/>) or ArrayExpress (<https://www.ebi.ac.uk/biostudies/arrayexpress>), to ensure data integrity, facilitate broader access, and promote reproducibility. Relevant documentation and metadata are provided with each submission, ensuring that the data can be correctly interpreted by wider research community.

Conclusions

Above protocol represents a robust and scalable approach for processing mouse aortic tissue samples for scRNA-seq. By leveraging state-of-the-art technologies, such as 10X Genomics Chromium, and adopting flexible strategies for nuclei isolation, cell sorting, and multiplexing, this workflow provides efficient means of generating high-quality, reproducible transcriptomic data from challenging tissues. The integration of key advances in the field, such as droplet-based barcoding and sample multiplexing, ensures that even rare cell populations can be captured and analysed with high resolution. These methodologies, when applied to cardiovascular models, can enhance our understanding of cellular dynamics in vascular diseases, like atherosclerosis, offering valuable insights into disease progression and informing therapeutic strategies.

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Conflicts of interest

The authors have no conflicts of interest to declare

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