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🌐 Single Cell – RNA Sequencing FFPE Heart Tissue Using Fixed RNA Profiling

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We use this protocol and it's working

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Abstract

By preserving cellular integrity at the point of collection, formalin-fixed paraffin-embedded (FFPE) captures fragile transcriptomic signatures across diverse cardiac cell types. This protocol supports multiplexed processing of fixed cells, enabling the concurrent single-cell RNA sequencing (scRNA-seq) of multiple heart tissue samples within a single experimental run. This advancement facilitates a deeper understanding of cardiac biology underlying heart disease at single-cell resolution, even in archived or clinical specimens.



Materials

- *Xylene, Reagent Grade
- *Ethyl Alcohol, 200 Proof, anhydrous
- *Ethanol absolute $\geq 99.5\%$
- *Phosphate-buffered saline, 1X without Calcium and Magnesium
- *15 ml polypropylene Centrifuge Tubes
- *Nuclease-free Water (not DEPC-Treated)
- *gentleMACSTM C Tubes
- *gentleMACSTMOcto Dissociator with Heaters
- *Liberase TH
- *Pre-Separation Filters (30 μ m)
- *Countess II FL Automated Cell Counter
- *Ethidium Homodimer-1

scRNA-seq was conducted following the Demonstrated Protocols and User Guide provided by 10x Genomics:

1 Assess the RNA quality of FFPE heart tissue samples and select based on a DV200 value of $\geq 30\%$ and visual inspection of H&E-stained sections.

2 **Sample Preparation from FFPE Tissue Sections for Chromium Fixed RNA Profiling** according to Demonstrated Protocol CG000632, Rev D by 10x genomics.

Deparaffinization:

2.1 Prepare two intact sections, each 50 μm from the rehydrated tissue block and transfer them into a gentleMACS C Tube.

2.2 Add 3 mL of xylene to the gentleMACS C Tube and incubate at room temperature (RT) for 10 minutes.

2.3 Carefully remove the xylene without disrupting the tissue scrolls.

2.4 Repeat the xylene wash two more times (steps 1–2).

2.5 Add 3 mL of 100% ethanol, incubate for 30 seconds at RT, then remove.

2.6 Add 1 mL of 100% ethanol, incubate for 30 seconds at RT, then remove.

2.7 Repeat the ethanol washes sequentially with 1 mL each of 70% and 50% ethanol, incubating for 30 seconds at RT and removing the liquid after each step.

2.8 Add 1 mL of nuclease-free water, incubate for 30 seconds at RT, then remove.

2.9 Add 1 mL of PBS and keep the sample on ice.

2.10 Remove PBS from the gentleMACS C Tube.



- 2.11 Prepare the Dissociation Enzyme Mix by combining 420 μ L of 5 mg/mL Liberase TH with 1,680 μ L of RPMI medium.
- 2.12 Add 2 mL of the enzyme mix to the tube.
- 2.13 Place the tube on the gentleMACS Octo Dissociator with heating units attached and run the program 37C_FFPE_1 (~48 minutes).
- 2.14 After the run, remove the tube from the instrument.
- 2.15 Centrifuge at ~300 rcf for 1 minute and gently resuspend the pellet in the supernatant.
- 2.16 Filter the suspension through a 30 μ m Pre-Separation Filter into a 15 mL tube on ice.
- 2.17 Rinse the original tube with 2 mL of cold PBS and use it to wash the filter, collecting all filtrate in the same tube.
- 2.18 Centrifuge the cell suspension at 850 rcf at 4 °C for 5 minutes.
- 2.19 Carefully remove the supernatant without disturbing the pellet.
- 2.20 Resuspend the pellet in 0.5 mL of chilled Quenching Buffer, pipetting gently 5 times. Keep on ice. Note: Prepare the Quenching Buffer in advance and store at 4 °C. For one reaction, mix 437.5 μ L of nuclease-free water with 62.5 μ L of 8X Concentrated Quench Buffer (10x Genomics PN 2000516).
- 2.21 Determine cell concentration using an automated cell counter.
- 3 Proceed immediately to appropriate **Chromium Fixed RNA for Multiplexed Samples using 16 Probe Barcodes** – Probe Hybridization step 1.1 through to step 4 Fixed RNA-Gene Expression library construction (see [User Guide CG000527 Rev D by 10x Genomics](#)).

- a. Perform the post library construction quality control on an Agilent Bioanalyzer Instrument High Sensitivity chip.
- b. Select the region between 150-300 bp and determine the average size and concentration of the library.

4 **Sequencing libraries.**

Perform the library sequencing on an Illumina NovaSeq X Series 25B Reagent Kit (300 Cycle); PN 20104706 according to Fixed RNA-Gene expression library **sequencing parameters** based on 10x Genomics User Guide:

Sequencing depth of 15,000 read pairs per cell.

Sequencing type: paired-end, dual indexing.

Sequencing Read, Number of cycles: Read 1, 28; i7 index, 10; i5 index, 10; Read 2, 90.

5 **Data analysis.**

- 5.1 The FASTQ files from sequencing will be processed using Cell Ranger 7.1.0 from 10x Genomics. The Cell Ranger count feature will be employed for reading alignment and count calculation, utilizing the human reference genome Chromium Human Transcriptome Probe Set v1.0.1 GRCh38-2020-A.
- 5.2 Treat each sample as an individual batch. Conduct all post-alignment analyses within R 4.2.3 environment and Bioconductor 3.17. Specifically, generate R objects for each different slide using Seurat 5.03.
- 5.3 Calculate the percentage of genes related to mitochondria, ribosome, and hemoglobin. and remove cells with outliers.
- 5.4 Normalize individual samples using the negative binomial model SCTransform and then merge the Seurat objects.
- 5.5 Employ the anchor identification method to integration, considering 3000 genes as highly variable. Determine anchors for each sample using the FindIntegrationAnchors() function and then proceed with integration using Harmony().
- 5.6 Identify the principal components (PCs) of our integrated object using the RunPCA() function. Define the number of dimensions using an Elbow plot with the most significant PCs for further analysis.
- 5.7 Reduce the dimensions of the data using the most significant PCs with the UMAP method. Afterward, find the nearest neighbors and perform clustering.
- 5.8 The clusters will be annotated according to the markers suggested by Tucker and Farah.



Protocol references

1. Farah, E.N., Hu, R.K., Kern, C. et al. Spatially organized cellular communities form the developing human heart. *Nature* 627, 854–864 (2024).
2. Tucker NR, Chaffin M, Fleming SJ, Hall AW, Parsons VA, Bedi KC Jr, Akkad AD, Herndon CN, Arduini A, Papangelis I, Roselli C, Aguet F, Choi SH, Ardlie KG, Babadi M, Margulies KB, Stegmann CM, Ellinor PT. Transcriptional and Cellular Diversity of the Human Heart. *Circulation*. 2020 Aug 4;142(5):466-482.