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Single-cell Fixed RNA sequencing from Formalin-Fixed, Paraffin-Embedded (FFPE)-Lung Tissue by Chromium Fixed RNA Profiling for Multiplexed Samples

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TriState SenNet | Cellular Senescence Network (SenNet) Method Development Communit

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ABSTRACT

Fixed RNA Profiling provides an approach to FFPE-lung tissue samples that were previously inaccessible. Fixation at the point of sample collection preserves fragile biology to provide high-quality data on gene expression of multiple cell types in the lung, generating critical and novel insights. This protocol describes the new platform for single cell RNA-seq from Fixed RNA, which uses fixed cells isolated from paraffin-embedded tissue and multiplexed samples to analyze multiple samples in a single run to generate DNA libraries for sequencing.

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Protocol status: Working
We use this protocol and it's working

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MATERIALS

*Xylene, Reagent Grade

*Ethyl Alcohol, 200 Proof, anhydrous

*Ethanol absolute ≥99.5%

*Phosphate-buffered saline, 1X without Calcium and Magnesium

*15 ml polypropylene Centrifuge Tubes

*Nuclease-free Water (not DEPC-Treated)

*gentleMACS^{TM} C Tubes

*gentleMACS^{TM}Octo Dissociator with Heaters

*Liberase TH

*Pre-SeparationFilters (30µm)

*Countess II FL Automated Cell Counter

*Ethidium Homodimer-1

1 **Isolation of Cells from FFPE Tissue Sections for Chromium Fixed RNA Profiling** According to Demonstrated Protocol CG000632 by 10x genomics.

Protocol

2 Prepare two 50 µm sections from the rehydrated FFPE tissue block and transfer them into a gentleMACS^{TM} C Tube while keeping the scrolls intact.

3 Add 3 ml xylene to the gentleMACS^{TM} C Tube and incubate for 10 min at RT.

4 Remove the liquid from the tube without breaking the scrolls.

5 Repeat steps b-c twice.

6 Add 3 ml 100% ethanol to the gentleMACS^{TM} C Tube and incubate for 30 sec at RT.

7 Remove the liquid from the tube without breaking the scrolls.

8 Add 1 ml 100% ethanol and incubate for 30 sec at RT.

9 Remove the liquid from the tube without breaking the scrolls.

10 Add 1 ml 70% ethanol and incubate for 30 sec at RT.

11 Remove the liquid from the tube without breaking the scrolls.

12 Add 1 ml 50% ethanol and incubate for 30 sec at RT.

13 Remove the liquid from the tube without breaking the scrolls.

14 Add 1 ml nuclease-free water to the tube and incubate for 30 sec at RT.

15 Remove the liquid from the tube without breaking the scrolls.

16 Add 1 ml PBS and maintain on ice.

17 Remove the PBS from the gentleMACS™ C Tube without breaking the scrolls.

18 Add 2 ml Dissociation Enzyme Mix to the gentleMACS™ C Tube and close securely.

Dissociation Enzyme Mix	Stock	Final	1 rxn (µl)	4 rxn + 10% (µl)
Liberase TH (mg/ml)	5	1	420	1848
RPMI	-	-	1680	7392
Total Volume (µl)			**2100**	**9240**

19 Place the tube on the gentleMACS™ Octo Dissociator, apply Heating units, and run the gentleMACS™ Program 37C_FFPE_1. Run time ~48 min.

gentleMACS Program 37C_FFPE_1	
1	temp ON
2	spin - 20 rpm, 5' 0"
3	loop 3X
4	spin 20 rpm, 14' 0"
5	spin 1700 rpm, 7"
6	spin 1700 rpm, 1"
7	spin -1700 rpm, 2",
8	spin 1700 rpm, 1"
9	spin 1700 rpm, 4"
10	end loop
11	end

20 At the end of the run, detach the tube from the gentleMACS™ Octo Dissociator.

21 Centrifuge at ~300 rcf for 1 min and resuspend the cell pellet in the supernatant.

22 Pass the suspension through a Pre-Separation Filter (30 µm) placed on a 15-ml tube on ice.

23 Rinse the original gentleMACS™ tube with 2 ml chilled PBS and use that rinse for an additional wash of the 30 µm filter to minimize cell loss. Collect the filtrate in the same tube.

24 Centrifuge the cell suspension at 850 rcf at 4°C for 5 min.

25 Remove the supernatant without disturbing the pellet.

26 Resuspend the pellet in 0.5 ml chilled Quenching Buffer, pipette mix 5x, and maintain on ice.

Quenching Buffer *(Maintain at 4°C)*	Stock	Final	1 rxn (µl)	4 rxn + 10% (µl)
Nuclease-free Water	-	-	437.5	1925
Conc. Quench Buffer* (10x Genomics PN 2000516)	8X	1X	62.5	275
Total Volume (µl)			**500**	**2200**

27 Determine the cell concentration of the fixed sample using an Automated Cell Counter.

28 Proceed immediately to appropriate Chromium Fixed RNA for Multiplexed Samples using 16 Probe Barcodes – Probe Hybridization step 1.1 (**see User Guide CG000527 Rev D by 10x Genomics**)

Data analyis

29 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 read alignment and count calculation, utilizing the human reference genome (GRCh38, GENCODE v32/Ensembl 98).

30 Treat each sample as an individual batch. Conduct all post-alignment analyses within an R 4.2.3 environment and Bioconductor 3.17. Specifically, generate R objects for each different slide using Seurat 4.3.0.

31 Calculate the percentage of genes related to mitochondria, ribosome, and hemoglobin. and remove cells with outliers.

32 Normalize individual samples using the negative binomial model SCTransform, then merge the Seurat objects.

33 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 IntegrateData().

34 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.

35 Reduce the dimensions of the data using the most significant PCs with the UMAP method. Afterward, find the nearest neighbors and perform clustering.

36 The clusters will be annotated according to the markers suggested by the Human Lung Cell Atlas and LungMAP[1,2].