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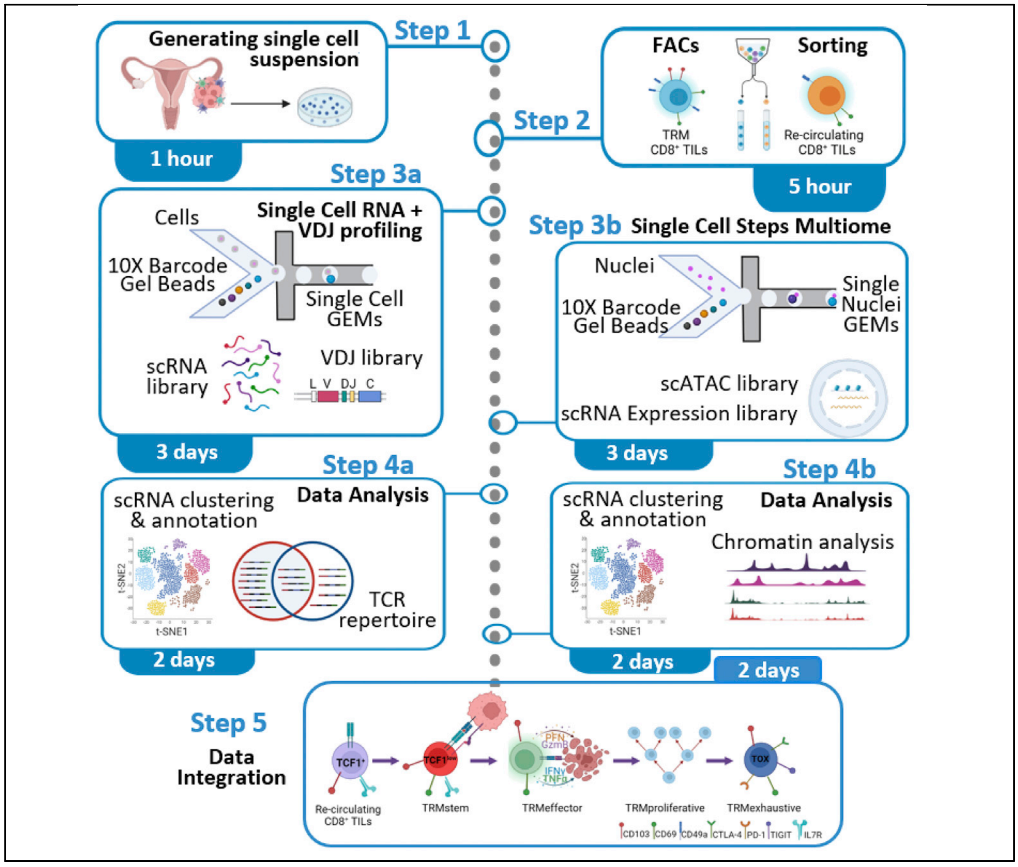
Protocol

Protocol for the isolation of CD8+ tumor-infiltrating lymphocytes from human tumors and their characterization by single-cell immune profiling and multiome



Understanding the heterogenicity of tumor-infiltraing lymphocyte (TIL) populations and the immunobiology in human cancer is a key to establish efficient immunotherapies. Here, we have established a protocol for the characterization of CD8^{+} TILs in tumors by single-cell RNA-seq paired to VDJ profiling and chromatin structure including dissociation of tumor biopsies. We have also provided guidance for subsequent fluorescence-activated cell sorting (FACS), single-cell encapsulation, bioinformatics analysis, and troubleshooting.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Single-cell suspension preparation and TILs sorting from frozen human ovarian tumors

Single-cell isolation, library preparation, and quality control for scRNA-seq + VDJ

Single nucleus isolation, library preparation, and quality control for 10× Multiome

scRNA-seq + VD and Multiome data integration and TIL trajectory identification

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Protocol for the isolation of CD8+ tumor-infiltrating lymphocytes from human tumors and their characterization by single-cell immune profiling and multiome

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SUMMARY

Understanding the heterogenicity of tumor-infiltraing lymphocyte (TIL) populations and the immunobiology in human cancer is a key to establish efficient immunotherapies. Here, we have established a protocol for the characterization of CD8$^+$ TILs in tumors by single-cell RNA-seq paired to VDJ profiling and chromatin structure including dissociation of tumor biopsies. We have also provided guidance for subsequent fluorescence-activated cell sorting (FACS), single-cell encapsulation, bioinformatics analysis, and troubleshooting.

For complete details on the use and execution of this protocol, please refer to Anadon et al. (2022).

BEFORE YOU BEGIN

The protocol below describes the specific steps for characterization of CD8$^+$ TILs using human ovarian tumor samples. However, we have also used this protocol with other human tumors such as lung cancer or. cutaneous T-cell lymphoma. For lung cancer, tumor processing was done in the same way and for cutaneous T-cell lymphina we sorted malignat cells directly from peripheral blood.

Institutional permission

This study has received institutional regulatory approval. Human high grade serous ovarian carcinoma (HGSOC) tissues were procured under protocols approved by the Committee for the Protection of Human Subjects at Dartmouth–Hitchcock Medical Center (no. 17702) or under a protocol approved by the H. Lee Moffitt Cancer Center (MCC no. 18974). Informed consent was obtained from all them.

Researchers should be aware of the legal requirements for the access to human specimens applicable at their institution.

Preparation for tumor processing and generating single-cell suspension from tumors

Timing: ~30 min

1. Prepare fresh R10 medium as described in the materials and equipment section.
2. Prepare RBC lysis buffer.

3. Pre-scour dish with two scalpels to help the dissociation.
4. Prepare freezing medium.
5. Be sure you have cryofreezing chambers (Mr. Frosty) at room temperature ready to use.

⚠ CRITICAL: Cell viability decrease with time, therefore try to process the sample quickly, centrifuge at 4°C and keep the sample on ice. Always keep tumor sample wet with a few mL of R10 media.

Preparation before processing the sample for FACs sorting and encapsulation

Timing: 30–60 min

6. Be sure that you have EasySep™ Dead Cell Removal (Annexin V) Kit - STEMCELL.
7. Prepare fresh dead cell removal buffer.
8. Prepare fresh sorting buffer and filtered FBS (0.2 μm) for recollection.
9. Prepare fresh single cell resuspension solution.

⚠ CRITICAL: Filter the single cell resuspension solution (0.2 μm) to avoid particles that can affect to the encapsulation process.

⚠ CRITICAL: Cell viability is the measurement of propotion of live and healthy cells within the single cell resuspection solucion. Cell viability is critical for the encapsulation to work and get good quality of RNA and DNA for post sequencing. The viability suggested from 10× Genomix is >85% but viability >70% is acceptable. We highly recommended to measure the viability again after sorting.

Preparation before encapsulation

Timing: ~1 h

10. Prepare diluted nuclear buffer.
11. Prepare wash buffer.
12. Prepare lysis buffer.
13. Prepare transposition buffer.
14. Prepare GEM master mix.

⚠ CRITICAL: All buffers and mix must be made fresh.

⚠ CRITICAL: Cells may have varied cells and be sure that enough wash buffer is made ahead. Cell lysis time is fixed once the optimized lysis is determined. However, if the cell number is between 100,000–1,000,000, the step 2 in the protocol of nuclei isolation for single cell multiome ATAC + gene expression sequencing is followed. Otherwise, the steps in Appendix should be followed instead in order to minimize the cell loss.

⚠ CRITICAL: The cell lysis time may need be optimized and the optimized lysis should be followed for all the samples.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse anti-human CD45 (Working dilution: 5:200)	BD Biosciences	BD Biosciences Cat# 563716, Clone H130, RRID: AB_2716864

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Mouse anti-human CD3 (Working dilution: 5:200)	Tonbo Biosciences	Tonbo Biosciences Cat# 65-0037, Clone OKT3, RRID: AB_2621873
Mouse anti-human CD8 (Working dilution: 5:200)	BD Biosciences	BD Biosciences Cat# 564628, Clone SK1, RRID: AB_2744464
Mouse anti-human CD4 (Working dilution: 5:200)	BD Biosciences	BD Biosciences Cat# 563552, Clone SK3, RRID: AB_2738275
Mouse anti-human CD69 (Working dilution: 5:200)	BioLegend	BioLegend Cat# 310942, Clone FN50, RRID: AB_2564277
Mouse anti-human CD103 (Integrin alpha E) (Working dilution: 5:200)	BioLegend	BioLegend Cat# 350205, Clone Ber-ACT8, RRID: AB_10642026
Biological samples		
Human ovarian carcinoma tissues	H. Lee Moffitt Cancer Center	N/A
Human ovarian carcinoma tissues	Darmouth Hitchcock Medical Center	N/A
Chemicals, peptides, and recombinant proteins		
RPMI 1640 Medium	Gibco	Cat# 11875093
Penicillin/Streptomycin	Lonza	Cat# 17602E
L-glutamine	Genesee Scientific	Cat# 25509
Sodium pyruvate	Thermo Fisher Scientific	Cat# 11360070
Dulbecco's Phosphate Buffered Saline 1×	VWR Life Science	Cat# 0201190500
Dulbecco's Phosphate Buffered Saline (DPBS), 1×, with calcium, magnesium	Thermo Fisher Scientific	Cat# 16777-257
Gamma-Globulins from Human Blood	Sigma	Cat# G4386
Human TruStain FcX™ (Fc Receptor Blocking Solution)	BioLegend	Cat# 422301
DAPI	Sigma	Cat# 8417
EDTA 0.5 M pH8.0	Invitrogen	Cat# 15575038
NH_4Cl	Sigma	Cat# A9434
$KHCO_3$	Fisher Chemical	Cat# 298146
DMSO	Fisher Chemical	Cat# D1284
HEPES (1 M)	Gibco	Cat# 15630080
Fetal Bovine Serum	Biowest	Cat# S1620
Ultrapure BSA	Invitrogen	Cat# AM2616
Nuclei Buffer (20×)	10× Genomics	Cat# 2000207
DTT	Sigma-Aldrich	Cat# 646563
RNase inhibitor	Sigma-Aldrich	Cat# 333502001
Nuclease-free Water	Invitrogen	Cat# AM9937
Tris-HCl (pH 7.4)	Sigma-Aldrich	Cat# T2194
NaCl	Sigma-Aldrich	Cat# 59222C
MgCl2	Sigma-Aldrich	Cat# M1028
BSA	Miltenyi Biotec	Cat# 130-091-376
Tween-20	Bio-Rad	Cat# 1662404
Nonidet P40 Substitute	Sigma-Aldrich	Cat# 74385
Digitonin	Thermo Fisher Scientific	Cat# BN2006
TAC Buffer B	10× Genomics	Cat# 2000193
TAC Enzyme B	10× Genomics	Cat# 2000265
Barcoding Reagent Mix	10× Genomics	Cat# 2000267
Template Switch Oligo	10× Genomics	Cat# 3000228
Reducing Agent B	10× Genomics	Cat# 2000087
Barcoding Enzyme Mix	10× Genomics	Cat# 2000266
Trypan Blue Solution, 0.4%	Thermo Fisher Scientific	Cat# 15250061
ViaStain AO/PI Staining Solution	Nexcelom Bioscience	Cat# CS2-0106-5
Critical commercial assays		
EasySep™ Dead Cell Removal (Annexin V) Kit	STEMCELL Technologies	Cat# 17899
Chromium™ Next GEM Single Cell 5′ Library and Gel Bead Kit v1.1	10× Genomics	Cat#1000165

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chromium™ Single Cell V(D)J Enrichment Kit, Human T Cell	10× Genomics	Cat#1000005
Chromium™ Single Cell 5′ Library Construction Kit	10× Genomics	Cat# 1000020
Chromium™ Next GEM Chip G Single Cell Kit	10× Genomics	Cat# 1000127
Chromium Next GEM Single Cell Multiome ATAC + Gene Expression Reagent Bundle	10× Genomics	Cat# 1000285
Chromium Next GEM Chip J Single Cell Kit	10× Genomics	Cat# 1000230
Deposited data		
Custom Source Code	This paper: GitHub folder: xxy39/TRM_OV_sc	GitHub: https://github.com/xxy39/TRM_OV_sc
Software and algorithms		
FlowJo v10.7.2	FlowJo LLC	N/A
CellRanger v5.0	10× Genomics	http://10xgenomics.com/
Seurat v4.0 (R package)	Stuart et al. (2019)	https://satijalab.org/seurat/
AUCell (R package)	Aibar et al. (2017)	https://github.com/aertslab/AUCell
Cell Ranger VDJ v3.0	10× Genomics	http://10xgenomics.com/
Fossil (R package)	Vavrek (2011)	https://cran.r-project.org/web/packages/fossil/index.html
Signac v1.3.0 (R package)	Stuart et al. (2021)	https://docs.signac.io/projects/core/en/v1.3.0/
harmony (R package)	Korsunsky et al. (2019)	https://github.com/immunogenomics/harmony
JASPAR2020 v0.99 (R package)	JASPAR	http://bioconductor.org/packages/release/data/annotation/html/JASPAR2020.html
TFBStools v1.31.2 (R package)	Tan and Lenhard (2016)	https://github.com/ge11232002/TFBSTools
FastIntegration v1.0.0	Li et al. (2022)	https://github.com/JinmiaoChenLab/FastIntegration
TRM_OV_sc	Current Manuscripts	https://github.com/xxy39/TRM_OV_sc https://doi.org/10.5281/zendo.6828033
Other		
BD FACSAria™ III Cell Sorter	BD Biosciences	N/A
Cellometer K2 instrument	VWR	Cat# NEXCMK2
Advanced Cell Strainers, 100 µm	Genesee Scientific	Cat # 25-375
Advanced Cell Strainers, 70 µm	Genesee Scientific	Cat # 25-376
Sartorius Minisart NMLSyringe Filters,0.45 µm, Yellow	Fisher Scientific	Cat# 14555308
Sartorius Minisart NMLSyringe Filters,0.2 µm, Blue	Fisher Scientific	Cat# 14555306
Fisherbrand™ Petri Dishes with Clear Lid	Fisher Scientific	Cat# FB0875712
CellPro™ 15 mL Conical Tubes, Centrifuge Tubes	Alkali Scienti	Cat# CN5601
CellPro™ 50 mL Conical Tubes	Alkali Scientific	Cat# CN5603
Disposable Serological Pipets 5 mL	VWR	Cat# 89130-896
Disposable Serological Pipets 10 mL	VWR	Cat# 89130-898
Disposable Serological Pipets 25 mL	VWR	Cat# 89130-900
Corning™ Falcon™ Round-Bottom Polystyrene Test Tubes	Fisher Scientific	Cat# 14-959-2A
Thermo Scientific™ Mr. Frosty™ Freezing Container	Fisher Scientific	Cat# 15-350-50
Neubauer Chamber (Hausser Scientific™ Bright-Line™ Counting Chamber)	Fisher Scientific	Cat# 02-671-51B

MATERIALS AND EQUIPMENT

R10

Reagent	Final concentration	Amount
RPMI-1640	N/A	440 mL
FBS	10%	50 mL
L-Glutamine	2 mM	5 mL
Penicillin/Streptomycin	100 μg/mL	5 mL
Sodium Pyruvate	0.5 mM	2.5 mL
Total	**N/A**	**500 mL**

Storage at 4°C. The medium can be stored for a month.

RBC Lysis

Reagent	Final concentration	Amount
NH_4Cl	N/A	4.01 g
$KHCO_3$	N/A	0.5 g
EDTA (0.5 M)	70 μM	70 μL
dH2O	N/A	500 mL
Total	**N/A**	**500 mL**

Storage at 4°C. It can be at this temperature for a week.

Freezing medium

Reagent	Final concentration	Amount
FBS	N/A	450 mL
DMSO	10%	50 mL
Total	**N/A**	**500 mL**

Storage at 4°C. The medium can be stored for a month.

Dead cell removal buffer

Reagent	Final concentration	Amount
PBS with Calcium	1 mM	490 mL
FBS	2%	10 mL
Total	**N/A**	**500 mL**

Storage at 4°C. The medium can be stored for a month.

⚠ CRITICAL: Ca^{++} is required for product performance.

Sorting buffer

Reagent	Final concentration	Amount
PBS (100 μM)	N/A	477.5 mL
FBS (10%)	20%	10 mL
HEPES (1 M)	25 mM	12.5 mL
Total	**N/A**	**500 mL**

Storage at 4°C. The medium can be stored for a month.

Single-cell resuspension solution

Reagent	Final concentration	Amount
PBS	N/A	49.6 mL

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Continued		
Reagent	Final concentration	Amount
BSA (50 mg/mL)	0.04%	400 µL
Total	**N/A**	**50 mL**
Storage at 4°C. The medium can be stored for 1 week.		

Diluted nuclei buffer		
Reagent	Final concentration	Amount
Nuclei Buffer (20×)	1×	50 µL
DTT (1,000 mM)	1 mM	1 µL
RNase inhibitor (40 U/ µL)	1 U/µL	25 µL
Nuclease-free Water	N/A	924 µL
Total	**N/A**	**1 mL**
Make solution immediately before use.		

Wash buffer		
Reagent	Final concentration	Amount
Tris-HCl (pH 7.4, 1 M)	10 mM	40 µL
NaCl (5 M)	10 mM	8 µL
MgCl2 (1 M)	3 mM	12 µL
BSA (10%)	1%	400 µL
Tween-20 (10%)	0.10%	40 µL
DTT (1 M)	1 mM	4 µL
RNAse inhibitor (40 U/ µL)	1 U/µL	100 µL
Nuclease-free Water	N/A	3.40 mL
Total	**N/A**	**4 mL**
Make solution immediately before use.		

Lysis buffer		
Reagent	Final concentration	Amount
Tris-HCl (pH 7.4, 1 M)	10 mM	20 µL
NaCl (5 M)	10 mM	4 µL
MgCl2 (1 M)	3 mM	6 µL
Tween-20 (10%)	0.10%	20 µL
Nonidet P40 Substitute (10%)	0.10%	20 µL
Digitonin (5%)	0.01%	4 µL
BSA (10%)	1%	200 µL
DTT (1 M)	1 mM	2 µL
RNase inhibitor (40 U/ µL)	1 U/µL	50 µL
Nuclease-free Water	N/A	1.67 mL
Total	**N/A**	**2 mL**
Make solution immediately before use.		

Transposition mix	
Reagent	Amount
TAC Buffer B	7 µL
TAC Enzyme B	3 µL
Total	**10 µL**
Make solution immediately before use.	

GEM master mix	
Reagent	Amount
Barcoding Reagent Mix	49.5 μL
Template Switch Oligo	1.1 μL
Reducing Agent B	1.9 μL
Barcoding Enzyme Mix	7.5 μL
Total	60 μL
Make solution immediately before use.	

STEP-BY-STEP METHOD DETAILS

Processing of ovarian tumor to single-cell suspensions

Timing: ~1 h

1. Pour fresh tumor sample into plastic petri dish previously pre-scoured with scalpels (Figure 1).
2. Use the plastic part of a plunger from a 10 mL syringe to gently press the minced tumor on the scoured petri dish, trying to be as careful as possible using the plastic part and not the rubber part of the plunger.
3. Filter tumor mash through a 100 μm nylon cell strainer. Use plunger from the 1 mL syringe to press through.

⚠ CRITICAL: Pass media through strainer also to help mash through filter.

4. Centrifuge sample at 500 × *g* at 4°C for 5 min and aspirate/pour off media to remove debris and dead cells.

⚠ CRITICAL: All media/material waste from sample is treated in at least 10% bleach.

5. Add 5–10 mL of RBC lysis buffer and resuspend the pellet. Incubate for 5 min with rotation at RT.

⚠ CRITICAL: Longer incubation times could significantly affect to the viability of the immune cells.

6. Add 10 mL R10 media to stop the RBC lysis reaction.
7. Centrifuge sample at 500 × *g* at 4°C for 5 min and aspirate/pour off media to remove debris and dead cells.
8. Next, fully re-suspend pellet in fresh R10 media and pass through a 70 μm strainer in a new 50 mL conical tube.
9. Calculate the total viable cell number using trypan blue solution and the Neubauer chamber. Spin down sample at 500 × *g* at 4°C for 5 min while counting cells.
10. Remove supernatant completely and re-suspend in freezing media.

⚠ CRITICAL: We recommend to resuspend the pellet at a density of maximum 5×10^6 cells/mL in freezing medium (from 5×10^5 to 10×10^6 per cryovial).

11. Store samples in Mr. Frosty, remove from −80°C the next morning and transfer into LN2 tank.

Prepare sample for FACs sorting prior to encapsulation

Timing: ~5 h

12. Warm ~5 mL of complete culture medium in a 37°C water bath.

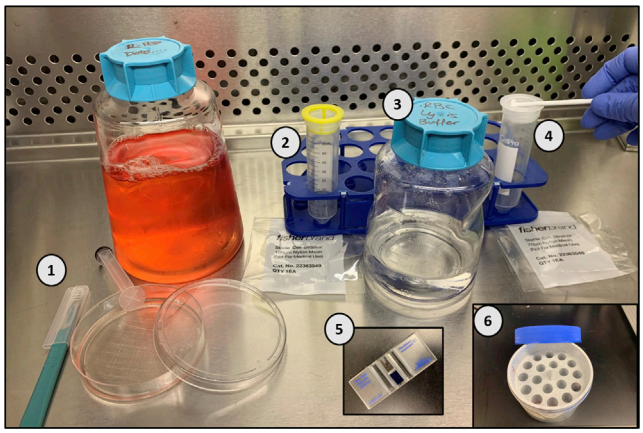

Figure 1. Steps for the generation of single-cell suspension from tumor biopsies

Representative photographs of the used materials. 1) Scoured petri dish to press the tumor. 2) Filter first through a 100 μm nylon cell strainer. 3) Add RBC lysis buffer for 5 min. 4) Filter after centrifugation through a 70 μm nylon cell strainer.5) Count the single cell suspension. 6) Freeze in Mr. Frostis.

13. Thaw one vial of ovarian tumor cells rapidly in a 37°C water bath with gentle agitation.
    a. Immediately upon thawing, remove from the water bath and wipe the outside of the vial with 70% ethanol.
    b. Unscrew the top of the vial slowly and, using 1 mL pipet, transfer the contents of the vial to a 15-mL conical centrifuge tube containing ~5 mL of prewarmed medium.
    c. Mix gently.
14. Remove the freezing media.
    a. Centrifuge at 500 × *g* for 5 min, 4°C.
    b. Carefully aspirate the supernatant, and gently resuspend the cells in ~5 mL of complete medium.
    c. Filter the single cell suspension through a 70 μm nylon cell strainer. Pass another 5 mL of media through the filter.
15. Wash the cells a second time.
    a. Centrifuge at 500 × *g* for 5 min, 4°C.
    b. Carefully aspirate the supernatant, and gently resuspend the cells in ~5 mL of complete medium.
    c. Filter the single cell suspension through a 45 μm nylon cell strainer. Pass another 5 mL of media through the filter.
16. Calculate the total cell number.
    a. Centrifuge at 500 × *g* for 5 min, 4°C.
    b. Carefully aspirate the supernatant, and gently resuspend the cells in ~5 mL of complete medium.
    c. Count the total number of cells.

⚠ CRITICAL: Keep the cells on ice while counting.

17. Fill the tube with dead cell removal Kit buffer and centrifuge at 500 × *g* for 5 min, 4°C and resuspend the pellet at a density of at 1 × 108 cells/mL.
18. Follow the instructions of the manufacturer: https://www.stemcell.com/products/easysep-dead-cell-removal-annexin-v-kit.
19. Pipette the 2.5 mL of enriched cell suspension into a new 15 mL tube and fill up the tube with 10 mL of sorting buffer.
20. Centrifuge and resuspend the pellet.
    a. Take an aliquot for counting and wash again with sorting buffer.

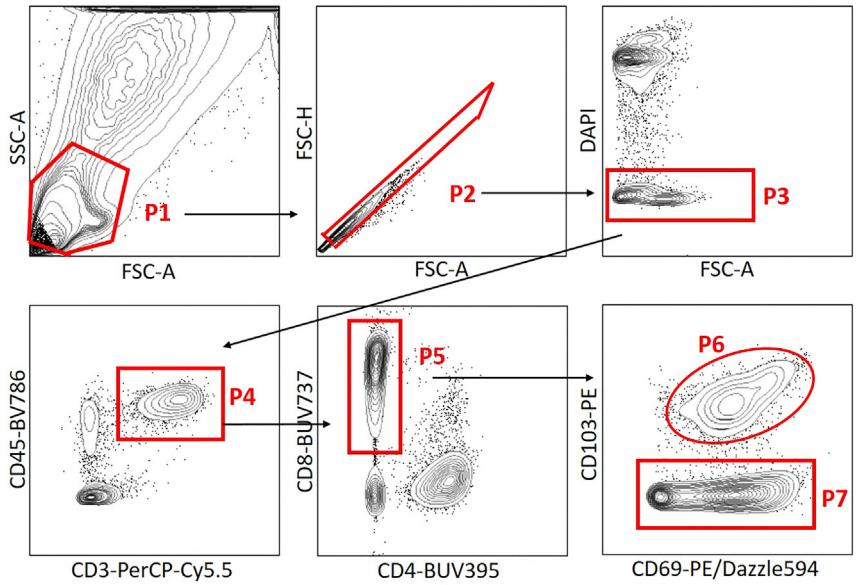

Figure 2. FACS sorting gating strategy for TRM CD8$^+$ TILs and their re-circulating counterpart

Dot plots show gates in a hirarchical order: P1 (SSC-A/FSC-A; lymphocytes according to size and granularity); P2 (FSC-H/FSC-A; singlets); P3 (DAPI/FSC-A; live cells); P4 (CD45/CD3; T cells); P5 (CD8/CD4; CD8$^+$ T cells); P6 and P7 (CD103/CD69; TRM CD8$^+$ TILs and re-circulating CD8$^+$ T cells, respectively).

b. Resuspend the pellet at 1 × 106 cells in 100 µL of sorting buffer with Human TruStain FcX (5 µL/100 µL).

⚠ CRITICAL: Since the viability is very important, centrifuge always at 4°C and keep the sample on ice during waiting times, unless otherwise specified.

21. Incubate 10 min at RT and add the antibodies for sorting: CD45, CD3, CD4, CD8, CD69 and CD103. Incubate for 1 h at 4°C in the dark.

Note: Prepare a mix with antibodies in sorting buffer at 5:100, then add to the samples (1 × 10^6 cells in 100 µL) for final antibody dilution of 5:200.

22. After 1 h of incubation, wash the cells with sorting buffer.
    a. Resuspend the cells at approx. 5 × 106 cells/mL.
    b. Filter again through a 40 µm filter to avoid clumps and add DAPI at a final concentration 100 ng/mL.

Note: We use BD FACS ARIA for sorting but another sorter like MACSQuant® Tyto® could be also.

23. Setup the sorter. We use 20 psi with a 100 µm nozzle.
24. Set the gates for singlets, viable, CD45+, CD3+ CD8+; CD69+CD103+ for TRM cells and CD69+/-CD103- for recirculating T cells (Figure 2).

⚠ CRITICAL: Use 1.5 mL Eppendorf for recollection since the number of sorted cells is low. Use filtered FBS as a recollection buffer to increase cell viability of sorted cells. However, it is important to have everything ready that you need after sorting to proceed and not leave the cells on ice for long.

Note: If number of cells is high, it is good to verify the cell purity using flow cytometry.

Table 1. Cell suspension volume calculator table

Cell stock concentration (cells/μL)	Targeted cell recovery										
	500	1,000	2,000	3,000	4,000	5,000	6,000	7,000	8,000	9,000	10,000
700	1.2	2.4	4.7	7.1	9.4	11.8	14.1	16.5	18.9	21.2	23.6
800	1	2.1	4.1	6.2	8.3	10.3	12.4	14.4	16.5	18.6	20.6
900	0.9	1.8	3.7	5.5	7.3	9.2	11	12.8	14.7	16.5	18.3
1,000	0.8	1.7	3.3	5	6.6	8.3	9.9	11.6	13.2	14.9	16.5
1,100	0.8	1.5	3	4.5	6	7.5	9	10.5	12	13.5	15
1,200	0.7	1.4	2.8	4.1	5.5	6.9	8.3	9.6	11	12.4	13.8

Prepare cells for encapsulation and following steps of single-cell RNA-seq + VDJ profiling

Timing: ~10 min

25. After sorting, fill up the Eppendorf tube with cells with 1 mL resuspension solution.
26. Centrifuge 300 g for 5 min and resuspend the pellet to the concentration of 700–1,200 cells/uL.
27. Add 10 ul cell suspension to 10 ul AO/PI staining solution and visualize on Nexcelon cellometer Auto 2K. Repeat the cell count two more times for a total of 3 times if cell number permits. The viability should be at least >70%.
28. Adjust the cell concentration with resuspension buffer if needed.

⚠ CRITICAL: If total cell number is less than required input cell number, centrifuge the cell suspension and remove the supernatant to make the final volume around 25 ul and use all for encapsulation.

⚠ CRITICAL: Do not exceed the number of cells allowed by 10× per encapsulation (10,000 cells). The doublets rate increase by 0.8% every 1,000 cells.

29. Follow the cell suspension volume calculator table (Table 1) to load the cells onto the 10× Genomics Chromium Single Cell Controller.
30. Follow the manufacturing recommendations: https://www.10xgenomics.com/support/single-cell-gene-expression/documentation/steps/library-prep/chromium-single-cell-3-reagent-kits-user-guide-v-3-1-chemistry. Encapsulate the single cells, reagents, and 10× Genomics gel beads into individual nanoliter-sized Gelbeads in Emulsion (GEMs) and then perform reverse transcription of poly-adenylated mRNA inside each droplet.
31. Complete the cDNA and VDJ-enriched libraries in a single bulk reaction using the 10× Genomics Chromium NextGEM Single Cell 5′ v 1.1 and V(D)J Reagent Kits.

⚠ CRITICAL: Run 1 μL library on the Agilent Bioanalyzer High Sensitive DNA chip to determine fragment size. Low molecular weight product (=<150 bp) and /or a high molecular weight product (> = 200 bp) may be present. This does not affect sequencing.

⚠ CRITICAL: Generate 50,000 or 5,000 sequencing reads per cell for scRNA-seq or VDJ libraries, respectively, on the Illumina NextSeq 500 or NextSeq 2000 instruments.

32. Perform demultiplexing, barcode processing, alignment, and gene counting using the 10× Genomics CellRanger v6.1.2 software.

Prepare cells for encapsulation and following steps of Multiome

Timing: ~3 days

Table 2. Nuclei concentration table

Targeted nuclei recovery	Nuclei concentration (nuclei/ul)
500	160–400
1,000	320–810
2,000	650–1,610
3,000	972–2,420
4,000	1,290–3,230
5,000	1,610–4,230
6,000	1,940–4,840
7,000	2,260–5,650
8,000	2,580–6,450
9,000	2,900–7,260
10,000	3,230–8,060

33. After sorting, prepare the cell suspension the same way as single cell RNAseq+VDJ protocol: use 100,000–1,000,000 cells for lysis.
34. Lyse the cells, and isolate and dilute nuclei in diluted nuclei buffer according to the manufacturer's protocol: https://www.10xgenomics.com/support/single-cell-multiome-atac-plus-gene-expression/documentation/steps/sample-prep/chromium-nuclei-isolation-kit-sample-prep-user-guide.
35. Add 10 ul nuclei suspension to 10 ul AO/PI staining solution and visualize on Nexcelon cellometer Auto 2K. Repeat the nuclei count two more times for a total of 3 times if nuclei number permits.

⚠ CRITICAL: After lysis, the optimal viability should be 2%–10%. The cells are underlysed if viability >10%, overlysed if viability <2%.

36. Adjust the nuclei concentration with Diluted Nuclei buffer following the table (Table 2) if needed:
37. Encapsulate the nuclei, reagents, and 10× Genomics gel beads into individual nanoliter-sized Gelbeads in Emulsion (GEMs) and then perform reverse transcription of poly-adenylated mRNA and 10× barcode attachment of transposed DNA inside each droplet.
38. Following pre-amplification, complete the cDNA and ATAC-seq libraries in a single bulk reaction using the 10× Genomics Chromium NextGEM Single Cell Multiome ATAC + Gene Expression Reagent Kit.
39. Generate 50,000 or 25,000 sequencing reads per cell for scRNA-seq or ATAC-seq libraries, respectively, on dedicated Illumina NextSeq 500 or NextSeq 2000 sequencing runs according to the vendor protocol: https://support.illumina.com/downloads/nextseq-500-user-guide-15046563.html and https://support.illumina.com/downloads/nextseq-2000-sequencing-system-guide-1000000109376.html.
40. Perform demultiplexing, barcode processing, alignment, and gene counting using the 10× Genomics CellRanger v6.1.2 software.

Integration of single-cell RNA-seq data from different samples

Timing: 2 days

41. The sorted TRM and recirculating CD8+ T cells from different patients were encapsulated individually, which posed an unique challenge to the downstream analysis.
    a. It is important to correct for the batch effects while maintaining the biological differences between TRM and recirculating CD8+ T cells within the same patient.

b. Below we provide a two-stage analytical plan for integrating sorted TRM and recirculating CD8+ T cells across patients using R package Seurat (Stuart et al., 2019).

⚠ CRITICAL: For this analysis the filtered gene-barcodes matrices generated by CellRanger are required. These matrices are formatted either as filtered_feature_bc_matrix.h5 or a folder named as filtered_feature_bc_matrix.

⚠ CRITICAL: This script first merges data from TRM and recirculating CD8+ T cells within the same patient, and then integrates merged data across patients. This is based on the assumptions that all patients have similar level of heterogeneity in the merged TRM recirculating CD8+ T cells. It is advised to examine whether the assumption holds in data, before using this script.

42. Processing the individual single-cell RNA-seq data. For each sample:
    a. Load the filtered gene-barcodes matrices generated by CellRanger to Seurat.
    b. Remove cells with less than 200 genes expressed or more than 10% UMIs originated from mitochondrial genes.
    c. Remove genes expressed in less than three cells.

```
>install.packages("Seurat")

>library(Seurat)

>P1_ReCir_h5 <- Read10X_h5("filtered_feature_bc_matrix.h5")

>P1_ReCir <- CreateSeuratObject(P1_ReCir_h5, min.cells = 3)

>P1_ReCir$percent.mt <- PercentageFeatureSet(P1_ReCir, pattern = "^MT-")

>P1_ReCir <- subset(P1_ReCir, subset = nFeature_RNA > 200 & percent.mt < 10
```

43. Repeat above steps for all TRM and recirculating single-cell RNA-seq data.
44. Merge TRM and recirculating data from the same patient.

```
>P1 <- merge(P1_TRM, P1_ReCir, add.cell.ids = c("P1_TRM", "P1_ReCir"), project = "P1'')

>P1 <- NormalizeData(P1)

>P1 <- FindVariableFeatures(P1, selection.method = "vst", nfeatures = 5000)

Vgene <- VariableFeatures(P1)

P1[["RNA"]]@var.features <- Vgene[!grepl("^IG",Vgene) & !grepl("^TR",Vgene))]
```

⚠ CRITICAL: To avoid cell clustering by V(D)J transcripts, it is important to remove T cell receptor and immunoglobulin genes from variable gene list of each merged data.

45. Repeat above step for all patients.
46. Integrate merged data across different patients.

```
>anchors <- FindIntergrationAnchors(object.list = list(P1, P2, P3, P4), dims = 1:40,
anchor.features = 8000)

>integrated <- IntegrateData(anchorset = anchors, dims = 1:40)
```

47. Scale the integrated data and perform clustering analysis.

```
>cc.genes <- readLines(con = ''regev_lab_cell_cycle_genes.txt'')

>integrated <- CellCycleScoring(object = integrated, s.features = cc.genes[1:43], g2m.features = cc.genes[44:97])

>integrated <- ScaleData(object = integrated, vars.to.regress = c("percent.mt","S.Score","G2M.Score"))

>integrated <- RunPCA(integrated, verbose = FALSE, npcs = 50)

>integrated <- RunUMAP(integrated, dims = 1:40, reduction = "pca",verbose = FALSE)

>integrated <- FindNeighbors(object = integrated, dims = 1:40)

>integrated <- FindClusters(object = integrated, reduction = "pca", resolution =1)

>saveRDS(integrated, "integrated.rds")
```

⚠ CRITICAL: The FindClusters parameter *resolution* should be decided based on heterogeneity levels within user's own datasets. For datasets of sorted CD8+ tumor resistant memory T cells, we recommend resolution ranges from 0.8 to 1.2. For more heterrogenious samples (for example, sorted tumor infiltrating T cells), larger resolution (1.5–2) might be used.

Identification of T cell trajectory

Timing: 2 days

The following steps include the analysis of T cell differentiation trajectory: Recirculating → TRMstem → TRMeffector → TRMproliferative → TRMexhaustive. Please see https://github.com/xxy39/TRM_OV_sc (https://doi.org/10.5281/zendo.6828033) for the detailed scripts.

48. Assign T cell differentiation states to clusters using markers derived from Anadon et al. (Anadon et al., 2022), following script deposited at GitHub https://github.com/xxy39/TRM_OV_sc/blob/main/DiffPlot.R.
    a. Calculate gene set activity scores for stemness, effector, proliferative, and exhaustive T cell differentiation states using R package *AUCell* (Aibar et al., 2017). Marker genes for each differentiation states are shown in table (Table 3) below.
    b. Generate a cluster-based dotplot for activity scores (Figure 3A). Determine cell type of each cluster based on the average activity score and percentage of cells expressing the genes.
    c. Plot the relationship between stemness and exhaustive scores for clusters (Figure 3B). This step helps to exam major cell type (TRM or Recirculating) in each cluster. The proliferative

Table 3. Marker genes for each differentiation state

States	Genes
Stemness	SELL, LEF1, CD28, CD27, CCR7, IL7R, CXCR5, TCF7, BACH2, JUNB, EGR1, KLF2
Activation	IL2, CD38, ENTPD1, ICOS, TNFRSF9, XCL1
Effector	GZMK, GZMH, GZMB, PRF1, GNLY, IFNG, FASLG, FGFBP2
TRM differentiation	ITGA1, ITGAE, ITGA2, ITGAL, ITGB2
Proliferative	TOP2A, MKI67, CDK1, STMN1, DNMT1, MCM7
Exhaustive	PDCD1, TIGIT, HAVCR2, LAG3, CTLA4, CD74, MIR155HG, CXCL13, LAYN, MYO7A, HLA-DRB1, HLA-DQA1, TOX, TOX2, BATF, ETV1, ID2, ZNF683, RBPJ, TBX21, RUNX1, RUNX3, RUNX2, EZH2

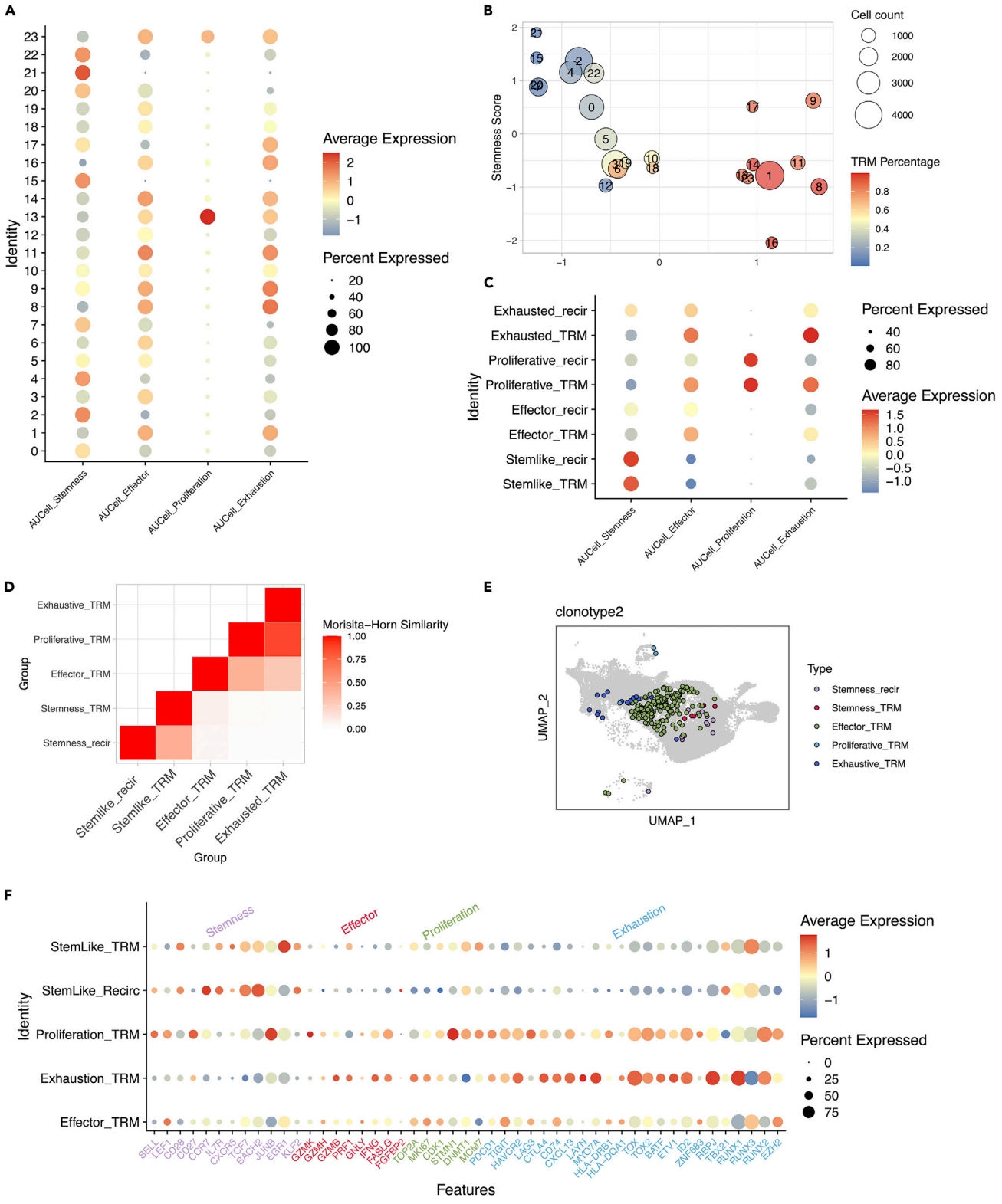

Figure 3. Identify T cell differentiation trajectory from TRM and recirculating CD8+ T cells using single-cell RNA/TCR/ATAC-seq data

(A) AUCell scores for T cell states stemness/effector/proliferative/exhaustive in clusters. Color of dot presents the average normalized expression from high (red) to low (blue). Size of dot represents the percentage of positive cells for each gene.

Figure 3. *Continued*

(B) Stemness score versus Exhaustion score from all the clusters. Color represents the percentage of TRM cells within each cluster. Size represents number of cells of each cluster.

(C) AUCell scores by 8 groups. Within each of groups "Stemlike", "Effector", "Proliferative", and "Exhausted", cells were separated based on cell types (TRM or recirculating).

(D) Heatmap of Morisita-Horn similarity of the 5 major groups identified based on gene expression.

(E) Example of clonotype with full trajectory. Cells are projected onto the UMAP generated from gene expression and cells of a particular clonotype are colored by their groups.

(F) Gene activity of T cell differentiation states genes measured in ATAC assay of Multiome data. Color of dot presents the average normalized gene activity from high (red) to low (blue). Size of dot represents the percentage of positive cells for each gene.

and exhaustive clusters tend to have higher TRM %, while stemness clusters tend to have lower TRM %. In the example data shown in https://github.com/xxy39/TRM_OV_sc, the proligerative and exhaustive clusters have TRM% > 60% while the stemness clusters have TRM% < 40%.

d. Assign groups to cells based on cell type (TRM or Recirculating) and differentiation states (Figure 3C).

49. The following steps include the analysis of T cell clonotypes, following script https://github.com/xxy39/TRM_OV_sc/blob/main/Clonotype.R.
    a. Add V(D)J clonotypes identified by VDJ assay from the same cells to meta data of Seurat object *integrated*.
    b. Measure between-group clonotype similarity by Morisita-Horn similarity index (Rempala and Seweryn, 2013) using R package *fossil* (Vavrek, 2011). This index compares overlap between clonotypes of any two groups and ranges from 0 (no overlap) and 1 (complete overlap). The similarity index can be visualized by heatmap (Figure 3D).
    c. Plot trajectory of individual clonotypes onto UMAP projection generated by step 48 (Figure 3E).
50. Validate the T cell trajectory using ATAC assay in Multiome data. Refer the scripts at GitHub https://github.com/xxy39/TRM_OV_sc/blob/main/ATAC.plot.R.
    a. Process the ATAC data generated from CellRanger-arc workflow using R package *Signac* (Stuart et al., 2021). Merge TRM and recirculating cells from the same patient.

⚠ CRITICAL: For this analysis following files generated by CellRanger-arc are required: atac_fragments.tsv.gz, atac_fragments.tsv.gz.tbi, atac_peaks.bed, per_barcode_metrics.csv.

    b. Remove low-quality cells with total number of fragments in peaks < 3,000 or > 20,000, fraction of fragments in peaks < 15%, ratio of mononucleosomal to nucleosome-free fragments > 4, or transcriptional start site (TSS) enrichment score < 2.

⚠ CRITICAL: These filtering criteria are dataset specific. We recommend choosing the cut-off after careful examination of the data.

    c. Perform a latent semantic indexing (LSI) dimension reduction on the filtered cells.
    d. Integrate merged data across patients and perform batch correction using RunHarmony() function implemented in R package *harmony* (Korsunsky et al., 2019).
    e. Create a gene activity matrix by counting reads mapped to gene body and promoter regions (extended 2 kb upstream from gene coordinates) using GeneActivity() function in *Signac*, and further log-normalize the matrix using NormalizeData() function in *Seurat* with default settings.
    f. Assign cells into major groups based on their grouping information obtained from Multiome GEX data following steps 42–48.
    g. Visualize the activity of T cell differentiation states markers in each group (Figure 3F). This step is to further confirm the T cell trajectory using chromatin accessibility data.

EXPECTED OUTCOMES

This protocol is intended for isolation of CD8 TILs from ovarian tumors frozen as single cell suspension. Using dead cell removal kit and quick processing of the sample, sorting and encapsulations, viabilities around 70%–90% can be obtained.

ScRNAseq + scVDJseq can encapsulate 10,000 cells/ sample. TRM cells in ovarian tumors normally represent around 60% of CD8 TILs. Therefore, we reach this number easily. However, depending on the tumor size, or the frequency of your target population, this number may not be reached. Lower numbers can also be used to encapsulate and study the heterogeneity of TILS.

This protocol also provides scripts for process single-cell RNA/TCR/ATAC-seq data from sorted TRM and recirculating CD8+ T cells. Following these scripts, it is possible to integrate the multiomic single-cell data, remove batch effects, and identify T cell developmental trajectory: Recirculating→TRMstem → TRMeffector → TRMproliferative → TRMexhaustive, and generate plots as shown in Figure 3.

LIMITATIONS

Cell viability is extremely important. Therefore, the faster you process the sample before encapsulation, the higher the possibility to get good results. For this reason, try to process few samples at the same time, and try to encapsulate the sample as soon as the FACS sort is done. To minimize sorting time, we use Dead Cell removal Kit. However, other kits to enrich your target population could be also a good option to minimize this time.

The number of cells that you need for encapsulation is low, but If your population has low frequency try to start with more material and use enrichment kits to reduce the sorting time.

TROUBLESHOOTING

Problem 1

The T cell numbers of some samples turn out to be much less than expected (related to step 28).

Potential solution

FACS cannot guarantee that all the recovered cells are T cells, and may not recover all the T cells effectively. The varying percentages of T cells among TILs samples depend on samples sources and enrichment. Therefore, it is worthy to analyze the sample by flow cytometry to choose the best possible sample before the final sorting.

Problem 2

The final recovered cell number is way off the targeted cell number (related to step 27).

Potential solution

- The input cell concentration is not accurately counted. We strongly suggest measuring the cell concentrations on cellometer using AO/PI staining solution three times and using the average.
- The cell concentration is either too low or too high. Our experience indicates that the cell concentration of <600 cells or >2,000 cells/ul will impact the accuracy of both concentration and the viability.
- Cell heterogeneity impacts the accuracy of cell counting.
- Red cell lysis is a must as the red cell will be encapsulated. Cell counting using single staining solution such as trypan and counting on hemocytometer likely overestimates the cells since the red blood cells will be included.

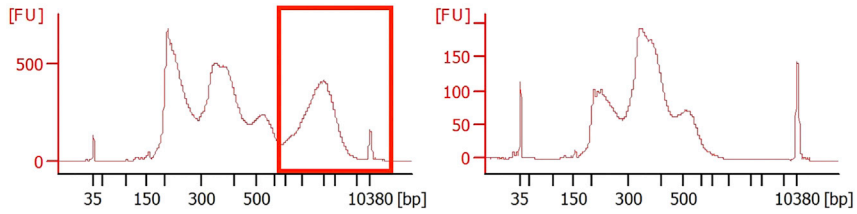

Figure 4. Bioanalyzer run profile for DNA

- Remove the debris since debris sometime are considered as cells on some counting device. To remove small suspension particles we filter by 0.2 μm FBS used for sample collection after sorting and single cells resuspension solution.
- Prevent cell aggregate and clump as much as possible.

Problem 3

Sorted cell viability decreases quickly which leads to a poor data.

Potential solution

- Minimize the sorting time as much as possible (related to step 24).
- Process no more than 4 samples at one encapsulation (related to step 30).
- Do multiple encapsulations if more than 4 cell samples are processed in one experiment.

Problem 4

A large size peak exit in the Multiome library as shown in Figure 4 (related to step 31):

Potential solution

The causes of the large size peak are under extensive discussion and may be due to, but not limited to multiple factors, such as low cell viability, DNA contamination, failure of RNase inhibitor, cell overlysis and inappropriate bead size selection etc., so the potential solutions can be:

- Perform the dead cell removal to remove the ambient DNA as much as possible.
- DNase treatment of the cell suspension to remove the ambient DNA if needed, and then wash thoroughly to remove DNase.
- Prepare fresh diluted nuclei buffer, wash buffer and lysis buffer.
- Optimize the cell lysis time.
- Follow exactly the protocol to perform the DNA size selection.
- Use the verified brands of RNase inhibitors.

Problem 5

Integration step of bioinformatics analysis is slow and memory consuming when dealing with large number of single-cell RNAseq samples (related to step 46).

Potential solution

Use R package FastIntegartion (Li et al., 2022), a faster and hig-capcacity version of Seurat Integration function.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Xiaoqing Yu (xiaoqing.yu@moffitt.org).

Materials availability

This study did not generate new unique reagents.

Data and code availability

The scripts used in this protocol can be retrieved: https://github.com/xxy39/TRM_OV_sc/. The data used as example are available at Gene Expression Omnibus (GEO) under accession ID GSE195486. Description for analysis of single-cell omics data can also be found in Methods section of Anadon et al. (Anadon et al., 2022).

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AUTHOR CONTRIBUTIONS

C.M.A., C.Z., J.R.C.-G., and X.Y. designed and optimized protocols. C.M.A. and C.Z. performed experiments. X.Y., X.W., and L.C. performed bioinformatic analyses. J.R.C.-G. obtained fundings. All authors contributed to writing and editing the manuscript.

DECLARATION OF INTERESTS

J.R.C.-G. has stock options in Compass Therapeutics, Anixa Biosciences, and Alloy Therapeutics; has sponsored research with Anixa Biosciences; receives honorarium from Alloy Therapeutics and Leidos; and has intellectual property with Compass Therapeutics and Anixa Biosciences.

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