Protocol

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

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 heterogeneity of tumor-infiltrating 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 lymphoma we sorted malignant 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

Ø 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 the encapsulation process.

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

Preparation before encapsulation

Ø 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: 1:200) | Tonbo Biosciences | Cat# 65-0037, Clone OKT3, RRID: AB_2621873 |
| Mouse anti-human CD8 (Working dilution: 1:200) | BD Biosciences | Cat# 564628, Clone SK1, RRID: AB_2744464 |
| Mouse anti-human CD4 (Working dilution: 1:200) | BD Biosciences | Cat# 563552, Clone SK3, RRID: AB_2738275 |
| Mouse anti-human CD69 (Working dilution: 1:200) | BioLegend | Cat# 310942, Clone FN50, RRID: AB_2564277 |
| Mouse anti-human CD103 (Integrin alpha E) (Working dilution: 1:200) | 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 1x | VWR Life Science | Cat# 0201190500 |
| Dulbecco’s Phosphate Buffered Saline (DPBS), 1x, 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 pH 8.0 | Invitrogen | Cat# 15575038 |
| NH4Cl | Sigma | Cat# A9434 |
| KHCO3 | 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 (20x) | 10x 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 | 10x Genomics | Cat# 2000193 |
| TAC Enzyme B | 10x Genomics | Cat# 2000265 |
| Barcoding Reagent Mix | 10x Genomics | Cat# 2000267 |
| Template Switch Oligo | 10x Genomics | Cat# 3000228 |
| Reducing Agent B | 10x Genomics | Cat# 2000087 |
| Barcoding Enzyme Mix | 10x Genomics | Cat# 2000266 |
| Trypan Blue Solution, 0.4% | Thermo Fisher Scientific | Cat# 15250061 |
| ViaStain AQ/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 | 10x Genomics | Cat# 1000165 |

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### Continued

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Chromium™ Single Cell V(D)J Enrichment Kit, Human T Cell | 10x Genomics | Cat#1000005 |
| Chromium™ Single Cell 5’ Library Construction Kit | 10x Genomics | Cat# 1000020 |
| Chromium™ Next GEM Chip G Single Cell Kit | 10x Genomics | Cat# 1000127 |
| Chromium Next GEM Single Cell Multiome ATAC + Gene Expression Reagent Bundle | 10x Genomics | Cat# 1000285 |
| Chromium Next GEM Chip J Single Cell Kit | 10x 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
- **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** (10x Genomics) https://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

### 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 NML Syringe Filters, 0.45 μm, Yellow** (Fisher Scientific) Cat# 14555308
- **Sartorius Minisart NML Syringe 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 Scient) Cat# CN5601
- **CellPro™ 50 mL Conical Tubes** (Alkali Scient) Cat# CN5603
- **Disposable Sterological Pipets 5 mL** (VWR) Cat# 89130-896
- **Disposable Sterological Pipets 10 mL** (VWR) Cat# 89130-898
- **Disposable Sterological 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 (Hauser 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   |
|-----------------|---------------------|----------|
| NH₄Cl           | N/A                 | 4.01 g   |
| KHCO₃           | 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: Ca²⁺ 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**          |                     | 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**                        |                     | 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**                            |                     | 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**                            |                     | 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.
STEP-BY-STEP METHOD DETAILS
Processing of ovarian tumor to single-cell suspensions

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 × 10⁶ cells/mL in freezing medium (from 5 × 10⁵ to 10 × 10⁶ 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

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

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### 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.
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
      medium 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
      medium 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.

    \[ \text{△ CRITICAL: Keep the cells on ice while counting.} \]

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.
b. Resuspend the pellet at 1 × 10^6 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 × 10^6 cells/mL.
   b. Filter again through a 40 µm filter to avoid clumps and add DAPI at a final concentration 100 ng/mL.

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

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: We use BD FACS ARIA for sorting but another sorter like MACSQuant® Tyto® could be also.
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/μL.
27. Add 10 μL cell suspension to 10 μL 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 μL and use
   all for encapsulation.
   △ CRITICAL: Do not exceed the number of cells allowed by 10x 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 10x Geno-
   mics 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 10x 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 10x Geno-
   mics 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 deter-
   mine 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 li-
   braries, respectively, on the Illumina NextSeq 500 or NextSeq 2000 instruments.
32. Perform demultiplexing, barcode processing, alignment, and gene counting using the 10x Ge-
   nomics CellRanger v6.1.2 software.

Prepare cells for encapsulation and following steps of Multiome

© Timing: ~3 days

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   |
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](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%, overlyised 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 10x Genomics gel beads into individual nanoliter-sized Gelbeads in Emulsion (GEMs) and then perform reverse transcription of poly-adenylated mRNA and 10x 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 10x 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](https://support.illumina.com/downloads/nextseq-500-user-guide-15046563.html) and [https://support.illumina.com/downloads/nextseq-2000-sequencing-system-guide-1000000109376.html](https://support.illumina.com/downloads/nextseq-2000-sequencing-system-guide-1000000109376.html).

40. Perform demultiplexing, barcode processing, alignment, and gene counting using the 10x 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.

43. Repeat above steps for all TRM and recirculating single-cell RNA-seq data.

44. Merge TRM and recirculating data from the same patient.

   >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

45. Repeat above step for all patients.

46. Integrate merged data across different patients.

   △ 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.

   >anchors <- FindIntergrationAnchors(object.list = list(P1, P2, P3, P4), dims = 1:40, anchor.features = 8000)
   >integrated <- IntegrateData(anchortset = anchors, dims = 1:40)
47. Scale the integrated data and perform clustering analysis.

```r
> 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 heterogeneous 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/zenodo.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

| States               | Genes                                      |
|----------------------|--------------------------------------------|
| Stemness             | SELL, LEF1, CD28, CD27, CCR7, IL7R, CXC5, 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, CYCL13, \( \text{LAYN, MYO7A, HLA-DRB1, HLA-DQA1, TOX, TOX2, BATF, ETv1, ID2, ZNF683, RBPj, TBX21, RUNX1, RUNX3, RUNX2, EZH2} \) |

Table 3. Marker genes for each differentiation state
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.
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 proliferative 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.

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.
EXPECTED OUTCOMES
This protocol is intended for isolation of CD8 TILs from ovarian tumors frozen as single cell suspen-
sion. 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 multi-
omic single-cell data, remove batch effects, and identify T cell developmental trajectory: Recirculat-
ing→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 encapsu-
lation, 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 con-
 centration 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 so-
  lution such as trypan and counting on hemocytometer likely overestimates the cells since the red
  blood cells will be included.
• 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 high-capacity 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/xxxy39/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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