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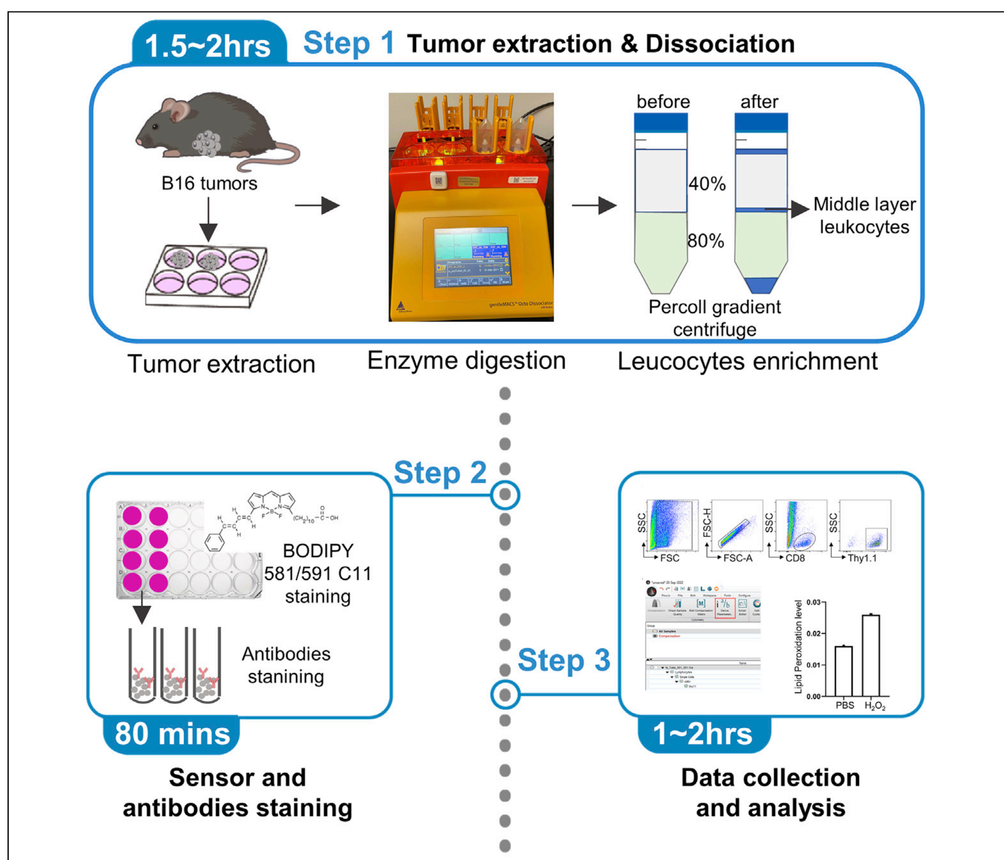
Isolation of adoptively transferred CD8$^+$ T cells in mouse tumor tissues for lipid peroxidation detection



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Highlights

Dissociation of mouse tumors with efficiency and preserved epitope

Enrichment for low-abundant tumor-infiltrating CD8$^+$ T cells

Measurement of the cellular lipid peroxidation level by flow cytometry

Ratiometric analysis of the cellular lipid peroxidation level in FlowJo

The lipid peroxidation level of tumor-infiltrating CD8$^+$ T cells is crucial for its activity and longevity. Here, we describe a protocol for effective and epitope-preserving dissociation of mouse tumors and subsequent leukocyte purification and lipid peroxidation staining of adoptively transferred CD8$^+$ T cells. We use BODIPY 581/591 C11 to monitor the cellular lipid peroxidation level and detect its fluorescent change by flow cytometry, followed by analysis in FlowJo. This protocol is adaptable to intrinsic CD8$^+$ T cells in tumors as well.

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

Xiao & Yi, STAR Protocols 4, 101945
March 17, 2023
https://doi.org/10.1016/j.xpro.2022.101945

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Isolation of adoptively transferred CD8$^+$ T cells in mouse tumor tissues for lipid peroxidation detection

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https://doi.org/10.1016/j.xpro.2022.101945

SUMMARY

The lipid peroxidation level of tumor-infiltrating CD8$^+$ T cells is crucial for its activity and longevity. Here, we describe a protocol for effective and epitope-preserving dissociation of mouse tumors and subsequent leukocyte purification and lipid peroxidation staining of adoptively transferred CD8$^+$ T cells. We use BODIPY 581/591 C11 to monitor the cellular lipid peroxidation level and detect its fluorescent change by flow cytometry, followed by analysis in FlowJo. This protocol is adaptable to intrinsic CD8$^+$ T cells in tumors as well.
For complete details on the use and execution of this protocol, please refer to Xiao et al. (2022)[1] and Ma et al. (2021).[2]

BEFORE YOU BEGIN

The following protocol describes the specific steps to detect lipid peroxidation on adoptively transferred CD8$^+$ T cells in mouse B16 subcutaneous tumors, which is also applicable to B16 lung metastasis tumors and MC38 colon tumors. Lipid peroxidation, a process by which lipids are attacked by oxidants,[3] can be detected by lipid peroxidation sensor BODIPY 581/591 C11.[4] Lipid peroxidation sensor changes its fluorescence from PE to FITC upon peroxidation by lipid ROS in cells, thus enabling ratiometric measurement of lipid peroxidation by fluorescence microscopy as well as flow cytometry. As the ratio of FITC/PE increases, the level of lipid peroxidation increases.[4] In this protocol, we focus on the analysis of lipid peroxidation by flow cytometry.

Institutional permissions

All mice experiments complied with protocols approved by the Institutional Animal Care and Use Committee of the Houston Methodist Research Institute. Anyone who follow this protocol should acquire permissions from the relevant institution.

Prepare items for tumor extraction and dissociation

Timing: 10 min

1. Prepare 500 mL 75% ethanol, clean scissors and forceps.
2. Prepare 6-well plates for extracted tissues by filling with 3 mL cold serum-free RPMI and place on ice.
3. Prepare stock components of the Tumor Dissociation Kit as outlined in manufacturer's datasheet (https://www.miltenyibiotec.com/upload/assets/IM0001973.PDF) and they are reiterated below.
   a. Prepare Enzyme D by reconstitution of the lyophilized powder in each vial with 3 mL of RPMI 1640 and aliquot to avoid repeated freeze-thaw.

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b. Prepare Enzyme R by reconstitution of the lyophilized powder in the vial with 2.7 mL RPMI 1640 and aliquot to avoid repeated freeze-thaw.
c. Prepare Enzyme A by reconstitution of the lyophilized powder in the vial with 1 mL of Buffer A supplied with the kit and aliquot to avoid repeated freeze-thaw. Do not vortex.

4. Prepare 10 mL working enzyme mix for 1 g tumor by adding 9.51 mL of RPMI, 400 μL of Enzyme D, 40 μL of Enzyme R, and 50 μL of Enzyme A into 15 mL centrifuge tube. Prepare immediately prior to beginning tumor extraction and keep on ice until use.

Prepare percoll

Timing: 5 min

5. Prepare 100% isotonic Percoll by mixing 1 mL 10× PBS (without $CaCl_2$ and $MgCl_2$) with 9 mL Percoll, mix well by inverting or vertexing.
6. Prepare 80% Percoll by mixing 8 mL of 100% isotonic Percoll with 2 mL of 1× PBS (without $CaCl_2$ and $MgCl_2$), mix well by inverting or vertexing.
7. Prepare 40% Percoll by mixing 6 mL of 100% isotonic Percoll with 4 mL of 1× PBS (without $CaCl_2$ and $MgCl_2$), mix well by inverting or vertexing.

CRITICAL: Always prepare Percoll solutions freshly.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
BV421 Anti-Mouse CD8a	BioLegend	Cat# 100737; RRID: AB_10897101
APC Anti-Rat CD90/Mouse CD90.1	BioLegend	Cat# 202526; RRID: AB_1595470;
Chemicals, peptides, and recombinant proteins		
BODIPY™ 581/591 C11	Thermo Fisher Scientific	Cat# D3861
Tumor Dissociation Kit	Miltenyi Biotec	Cat# 130-096-730
Phosphate buffered saline (PBS), 1×, -Ca, -Mg, -Phenol Red	Cytiva Life Sciences	Cat# SH3025602
PBS, phosphate buffered saline, 10× solution	GE Healthcare	Cat# BP3994
Percoll	GE Healthcare	Cat# 17-0891-02
RPMI 1640	Thermo Fisher Scientific	Cat#11875093
UltraPure™ 0.5 M EDTA, pH 8.0	Thermo Fisher Scientific	Cat# 15575020
Penicillin-streptomycin	Thermo Fisher Scientific	Cat# 15140122
L-Glutamine (200 mM)	Thermo Fisher Scientific	Cat# A2916801
Fetal bovine serum (FBS)	R&D Systems	Cat# S11150
DMSO	MilliporeSigma	Cat# D2650
Hydrogen peroxide	Thermo Fisher Scientific	Cat# 411885000
Experimental models: Cell lines		
Mouse Melanoma B16-F10	ATCC	Cat # CRL-6475
Experimental models: Organisms/strains		
8-week-old female C57BL/6 mice	Jackson Laboratories	Cat# 000664
Software and algorithms		
FlowJo_V10 software	TreeStar	N/A
Other		
Centrifuge	Eppendorf	Cat# 5810 R
Countess II	Thermo Fisher Scientific	Cat# AMQAX1000

(Continued on next page)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Cell culture CO_2 incubator	Thermo Fisher Scientific	Cat# 3310 Steri-Cult
Symphony flow cytometer	BD	Cat# BD FACSymphony A3
GentleMACS Octo Dissociator with Heaters	Miltenyi Biotec	Cat# 130-096-427
GentleMACS C Tube	Miltenyi Biotec	Cat# 130-093-237
MACS® SmartStrainers (70 μm)	Miltenyi Biotec	Cat# 130-110-916
Kimberly-Clark Kimtech Kimwipes EX-L Wipes	Kimberly-Clark	Cat# KCP34155

MATERIALS AND EQUIPMENT

Stock Enzyme D of Tumor Dissociation Kit

Reagent	Final concentration	Amount
Lyophilized Enzyme D	N/A	1 vial
serum-free RPMI 1640	N/A	3 mL
Total	N/A	3 mL

Store at −20°C for up to 6 months.

Stock Enzyme R of Tumor Dissociation Kit

Reagent	Final concentration	Amount
Lyophilized Enzyme R	N/A	1 vial
serum-free RPMI 1640	N/A	2.7 mL
Total	N/A	2.7 mL

Store at −20°C for up to 6 months.

Stock Enzyme A of Tumor Dissociation Kit

Reagent	Final concentration	Amount
Lyophilized Enzyme A	N/A	1 vial
serum-free RPMI 1640	N/A	1 mL
Total	N/A	1 mL

Store at −20°C for up to 6 months.

Enzyme mix of Tumor Dissociation Kit

Reagent	Final concentration	Amount
serum-free RPMI 1640	N/A	9.51 mL
Enzyme D	N/A	400 μL
Enzyme R	N/A	40 μL
Enzyme A	N/A	50 μL
Total	N/A	10 mL

Freshly prepare.

100% isotonic Percoll

Reagent	Final concentration	Amount
Percoll (original stock)	90% (relative to original stock)	9 mL
10× PBS	1×	1 mL
Total	N/A	10 mL

Store at room temperature for 6 h.

⚠ CRITICAL: Prewarm original stock to room temperature before use.

80% Percoll		
Reagent	Final concentration	Amount
100% isotonic Percoll	80% (relative to 100% isotonic Percoll)	8 mL
1× PBS	20%	2 mL
Total	N/A	10 mL
Store at room temperature for 6 h.		

40% Percoll		
Reagent	Final concentration	Amount
100% isotonic Percoll	40% (relative to 100% isotonic Percoll)	4 mL
1× PBS	60%	6 mL
Total	N/A	10 mL
Store at room temperature for 6 h.		

BODIPY 581/591 C11 stock solution		
Reagent	Final concentration	Amount
BODIPY™ 581/591 C11	10 mM	1 mg
DMSO	N/A	198 μL
Total	N/A	198 μL
Store at −20°C for up to 1 year.		

FACS buffer		
Reagent	Final concentration	Amount
FBS	2%	2 mL
0.5 M EDTA	2 mM	0.4 mL
1× PBS	N/A	97.6 mL
Total	N/A	100 mL
Store at 4°C for up to 1 month.		

Flow cytometry antibody mix (per 100 μL/sample, each sample includes 1–10 million cells)		
Reagent	Working dilution	Amount
FACS buffer	N/A	1.1 mL
APC Anti-Mouse CD8a	1:200	5.5 μL
BV421 Anti-Rat CD90/Mouse CD90.1	1:200	5.5 μL
Total	N/A	1.1 mL
Freshly prepare before staining.		

Cell culture medium for isolated T cells		
Reagent	Final concentration	Amount
RPMI 1640 medium	90%	450 mL
Heat inactivated FBS	10%	50 mL
Penicillin-streptomycin (10,000 U/mL)	100 U/mL	5 mL
L-glutamine (200 mM)	2 mM	5 mL
Total	N/A	510 mL
Store at 4°C for up to 3 months.		

BODIPY 581/591 C11 working solution		
Reagent	Final concentration	Amount
BODIPY 581/591 C11 stock (10 mM)	500 μM	5 μL
Cell culture medium	N/A	95 μL
Total	N/A	100 μL
Freshly prepare.		

STEP-BY-STEP METHOD DETAILS

Tumor extraction and dissociation

Timing: 1.5~2 h

This step will detailly illustrate how to extract tumor tissues and get single-cell suspensions.

1. Euthanize the mouse using carbon dioxide (CO_2) inhalation.
2. Take the tumor tissues and place them on 6-well plate that contain 3 mL cold RPMI 1640 on ice until processing (Figure 1A).
3. Remove fat, fibrous and necrotic areas from the tumor samples.
4. Blot the tumors on Kimtech wipes and weigh them.
5. Cut the tumors into small pieces of 2–4 mm with scissor in a 2 mL tube filled with 1 mL pre-prepared enzyme mix on ice.
6. Transfer the tumor tissues into the gentleMACS C Tube and fill the tubes with pre-prepared enzyme mix (1 mL enzyme mix for 100 mg tumor, Figure 1B).
7. Tightly close C Tube and attach it upside down onto the sleeve of the gentleMACS Octo Dissociator and inserted the heaters around the tube (Figure 1C).
8. Choose "Soft/medium 37C_m_TDK_1 Program" that preexist in the machine and run the selected program.
9. After termination of the program, detach C Tube from the gentleMACS Octo Dissociator.
10. Perform a short centrifugation (500 g for 10 s) step to collect the sample materials remain on the lids of C tube.
11. Resuspend sample and check digestion condition of tumor tissues by using a 1 mL micropipette to pipette up and down.
    a. Check if tumor tissues are well digested by checking that there are no obvious debris present and micropipette can pipette smoothly.
    b. Stop the digestion by adding 1 mL cold FBS.
12. Filter digested sample suspension on a 70 μm MACS SmartStrainer placed on a 15 mL tube.
13. Wash cell MACS SmartStrainer with RPMI 1640 until the liquid volume up to 14 mL.
14. Centrifuge cell suspension at 50 × *g* for 5 min and collect the supernatant. This step is used to remove part of tumor cells and debris.
15. Centrifuge supernatant at 500 g for 5 min. Aspirate supernatant completely and collect cell pellet.
16. Resuspend cells with 6 mL 40% percoll by pipetting up and down.
17. Transfer 6 mL of 80% Percoll into a 15 mL centrifuge tube and carefully layer a total of 6 mL of tumor cell suspension onto the 80% Percoll with a pasteur pipette without intermixing of the two Percoll densities (Figure 1D).
18. Centrifugate samples at 1,260 g for 20 min at room temperature with the acceleration at the lowest setting and no break.
19. Using a vacuum aspirator connected to a p200 micropipette tip to carefully remove top layer of the sample, and then collect the middle interface layer (Figure 1D) by a p1000 micropipette and transfer them into a new 15-mL tube containing 10 mL of FACS buffer.
20. Centrifuge at 500 g for 5 min, discard the supernatant and resuspend with 2 mL T cell culture medium.

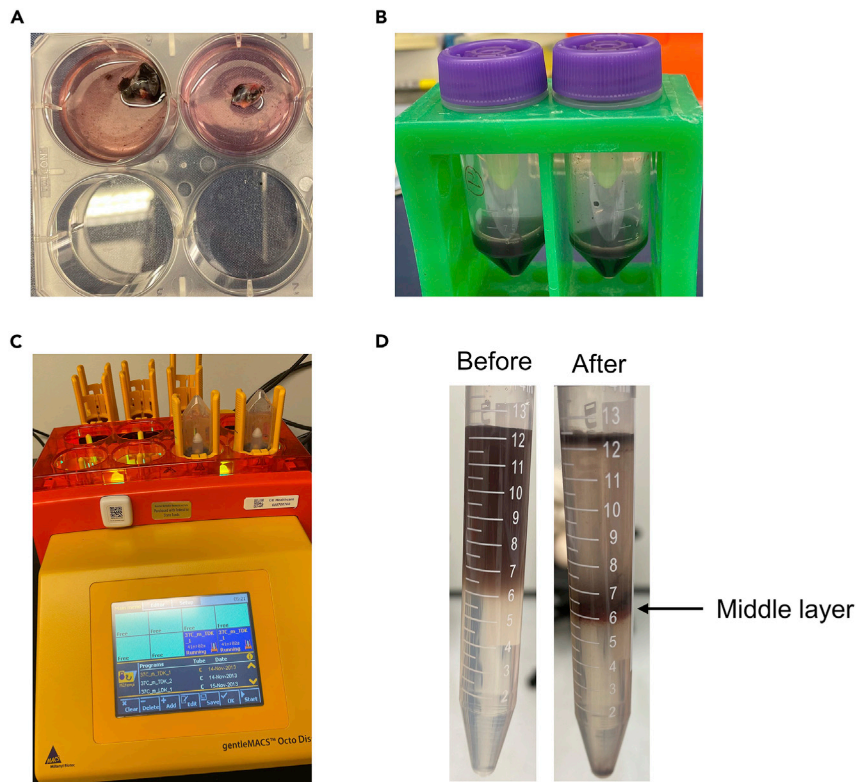

Figure 1. Processing the tissues
(A) Extraction of B16 tumor tissues.
(B) GentleMACS C Tube that transferred with tumor tissue suspension.
(C) GentleMACS Octo Dissociator that used to digest tumor tissues.
(D) Image of cell suspension of tumor tissues before and after gradient centrifugation.

⚠ CRITICAL: For the analysis of tumor infiltrating leukocytes (TILs), it is recommended to make the content of enzyme R to 0.4% in the enzyme mix, which will help to preserve cell surface epitopes but may lead to lower cell yields and viability of endothelial cells, epithelial cells, and tumor-associated fibroblasts.

⚠ CRITICAL: When placed the tube C into the gentleMACS Octo Dissociator, it has to be ensured that the sample material is located in the area of the rotor/stator.

Lipid peroxidation sensor and antibody staining

Timing: 80 min

This step details how to stain single cell suspension in tumor tissues with lipid peroxidation sensor and indicated surface antibodies.

21. Count acquired single cell suspension from tumor tissues.
22. Seed 5 million single cell suspension into one well of 24-well plate with 1.9 mL T cell culture medium for each sample. In addition, seed two extra wells for unstained or H_2O_2 treatment.
23. Treat cells with PBS or 5 μL 30% H2O2.
24. Add 100 μL lipid peroxidation sensor BODIPY 581/591 C11 working solution into cell culture medium in step 22, except for the unstained well.
25. Incubate the cells at 37°C with 5% CO_2 cell incubator for 30 min.

26. Label 5 mL FACS tubes for each sample and transfer cells in each well to the tubes.
27. Add 1 mL FACS buffer into each tube and centrifuge at 500 g for 5 min at 4°C.
28. Discard the supernatant and resuspend the cells with 100 μL flow cytometry antibody mix (see materials and equipment part), or 100 μL FACS buffer for unstained sample, incubate at 4°C for 15 min in the dark.
29. Wash stained cells with 1 mL of FACS buffer, vortex briefly and centrifuge at 500 g for 5 min at 4°C.
30. Discard the supernatant and resuspend cell pellet with 500 μL FACs buffer, placed cells on the ice.

Alternatives: if BODIPY™ 581/591 C11 is out of order, Lipid Peroxidation Assay Kit (Cell-based) (ab243377) from Abcam is a good choice.

Data collection and analysis

Timing: 1∼2 h

This step illustrates how to collect data on a BD symphony flow cytometer and analyze data on FlowJo software.

31. Collect data on a BD Symphony flow cytometer.
    a. Open the machine and software, choose FITC, PE, APC and BV421 laser.
    b. Use unstained sample and H_2O_2 treated positive control sample to set appropriate PMT voltages.
    c. Gate Thy1.1$^+$ CD8$^+$ T cells as showed in Figure 2A and collect data from each sample.
32. Data analysis.
    a. Export collected fcs files and opened it in the FlowJo software.
    b. Gating Thy1.1$^+$ CD8$^+$ T cells in tumor tissues (Figure 2A).
    c. To get the ratiometric measurement of lipid peroxidation level, choose "a/b derive parameters" in "Workspace" (if FlowJo is Experienced mode) or "Tools" (if FlowJo is Advanced mode) menu. For more information, please see the FlowJo website (https://docs.flowjo.com/flowjo/experiment-based-platforms/plat-derived-overview/) and it is reiterated below (Figure 2B).
       i. Click on a sample in the workspace, go to the Instrumentation band under the "Tools" tab and select "a/b Derived Parameters" (left panel in Figure 2B-i). This will bring up the "Derived Parameter window" as right panel in Figure 2B.
       ii. At the top of "Derived Parameter window", give your new parameter a name (default is Derived). Spaces are not allowed in the new parameter name (Figure 2B-ii).
       iii. At the bottom of "Derived Parameter window", there are several dropdown menus. First, choose "BB515-A" (equal to FITC) in the dropdown menu named Insert Reference; Second, choose "/division" function in the dropdown menu named Insert Function; Third, choose "PE-A" in the dropdown menu named Insert Reference (Figure 2B-iii).
       iv. In the dropdown menu named Scale, choose "Logarithm" for the new parameter and set the X-axis to an appropriate range by changing the values in Min and Max (Figure 2B-iv).
       v. Apply the new parameter to a single sample by clicking OK, or to a group by dragging the Derived Parameter node to the group in the workspace.
    d. Choosing statistical analysis of gated cells for "Median-Derived" to get lipid peroxidation value (Figure 2C).

Optional: Fluorescence microscope can be used to detect lipid peroxidation level by using traditional Texas Red® (590 nm) and FITC (510 nm) emission filters.

Note: In this experiment, we analyzed adoptively transferred Thy1.1 (also named as CD90.1)$^+$ CD8$^+$ T cells in tumors tissues. For lipid peroxidation detection, it's better to collect cells by flow cytometer within 2 h after incubation in case of fluorescence quenching.

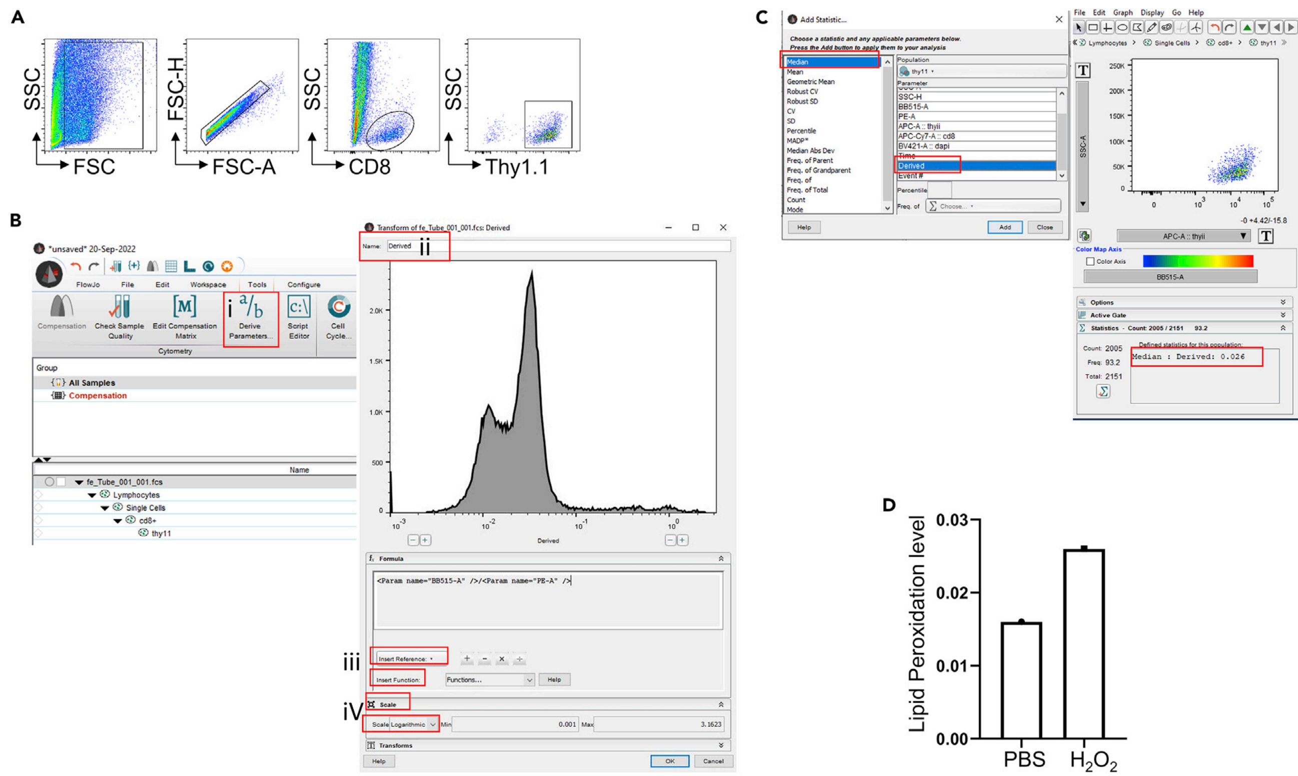

Figure 2. Analysis of lipid peroxidation level of adoptively transferred CD8$^+$ T cells in tumor tissues

(A) The gating strategy of Thy1.1$^+$ CD8$^+$ T cells in tumor tissues.

(B) Choose "a/b derive parameters" in "workspace" menu.

(C) Get lipid peroxidation value of Thy1.1$^+$ CD8$^+$ T cells.

(D) Lipid peroxidation levels on PBS or H_2O_2 treated adoptively transferred CD8$^+$ T cells.

Note: Researchers who are not familiar with flow cytometry can refer to Cossarizza et al. for the guidelines.[5]

Note: In order to detect lipid peroxidation of cells within 2 h after incubation, researchers should reserve flow cytometry machine earlier.

Pause point: Researchers can pause the experiment after step 31.

EXPECTED OUTCOMES

In this protocol, we developed strategies for detection of lipid peroxidation in adoptively transferred CD8$^+$ T from the mouse tumor tissues. Overwhelming lipid peroxidation level is the major feature of ferroptosis and play important role in maintaining CD8$^+$ T cell function.[1,2,6,7] The TME, which is commonly acidic, hypoxic, depleted of critical nutrients, and rich in immunosuppressive compounds, is deleterious for the accumulation of lipid peroxidation in CD8$^+$ T cells and impairs the longevity of adoptively transferred T cells.[1,8]

By applying this protocol, we will obtain clear CD8$^+$ T cells and Thy1.1$^+$ CD8$^+$ T cells (adoptively transferred CD8$^+$ T cells) clusters with CD8a and Thy1.1 antibodies (Figure 2A). Critically, we will get ratiometric measurement of lipid peroxidation level by using the derived parameter analysis in the FlowJo software (Figures 2B and 2C) and H_2O_2 treated positive control sample have higher level of lipid peroxidation level than PBS treated samples (Figure 2D).

LIMITATIONS

This protocol meets the basic needs for analyzing lipid peroxidation of $CD8^+$ and $CD4^+$ T cells in tumor tissues from several soft tumor models (including MC38 colon cancer, B16 lung metastatic melanoma and B16 subcutaneous melanoma), however, there is still some limitation. First, this protocol is not suitable for tough tumors (e.g., 4T1 cell line induced breast tumors). For tough tumors, other tumor digestion program should be chosen and more detailed can be found in manufacturer's datasheet (https://www.miltenyibiotec.com/upload/assets/IM0001973.PDF). Second, to keep best activity of T cells, we used special Tumor Dissociation kit and related gentleMACS™ Octo Dissociator, which may not apply to all labs and researchers can digest tumor tissues by using Collagenase I and DNase I instead.[7] Third, lipid peroxidation level in T cells can be detected by Liperfluo staining besides BODIPY 581/591 C11.[9]

TROUBLESHOOTING

Problem 1

Larger pieces of tissue remain after tumor dissociation (step 11).

Potential solution

Several reasons may result in this problem. 1) the tumor tissues were not well minced in step 4. To overcome this problem, further cut the tumor tissues into indicated size. 2) tumor heterogeneity. When working with tough tumors, some larger pieces of tissue may remain. To further increase the cell yield, allow the remaining tissue to settle and transfer the supernatant to a new tube. Add 4 mL RPMI 1640 to the C Tube with the remaining tissue pieces. Insert tube onto the sleeve of the gentleMACS Dissociator. Run the program preexist in the machine named "m_impTumor_01". Combine the resulting cell suspension with the previously removed supernatant.

Problem 2

There is no apparent middle interface layer after gradient centrifuge (step 19).

Potential solution

Several reasons may result in this problem: percoll layers blend together or centrifuge temperature is not correct. When this situation happens, collect up-layer that contain 40% percoll and cells and transfer them to a new 50 mL centrifuge tubes, then add 30 mL PBS to the 50 mL tube and centrifuge at 500 g for 5 min to pellet the cells. After that, resuspended the cell pellet in 40% percoll and repeat steps 15 and 16 and make sure the machine temperature is correct.

Problem 3

Inadequate cell number in step 22.

Potential solution

To avoid this problem, digest at least 50 mg tumor tissues. If there are less than 1 million cells, use all the cells for staining and incubate them on 96 wells.

Problem 4

No obvious FITC fluorescence in step 31.

Potential solution

Several reasons may result in this problem. 1) Inappropriate PMT voltages. To overcome this problem, use H_2O_2 treated cells as a positive control and unstained cells as a negative control to set an appropriate PMT voltage; 2) The staining of BODIPY 581/591 C11 is not bright. To overcome this problem, use higher concentration (10 μM) of the sensor or extended the incubation time to 45 min- 1 h; 3) Low cellular viability. To avoid this situation, we recommend processing the tissues gently and quickly and preparing necessary items before the experiment; 4) Fluorescence

quenching. To avoid this situation, we recommend collecting stained cells by flow cytometer within 2 h after incubation.

Problem 5

No positive staining of CD8a and Thy1.1 antibodies in step 31.

Potential solution

As these two antibodies are easy to stain on the cells, the potential reason for this problem might be that antibodies don't be added into the antibody mix. Freshly prepare the antibody mix and confirm that all the antibodies are included.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Qing Yi (qyi@houstonmethodist.org).

Materials availability

This study did not generate new materials.

Data and code availability

This protocol did not generate new data or employ custom code.

ACKNOWLEDGMENTS

This work was funded by National Cancer Institute (NCI) grants R01 CA200539 and R01 CA239255 and the Cancer Prevention & Research Institute of Texas Recruitment of Established Investigator Award (RR180044).

AUTHOR CONTRIBUTIONS

L.X. and Q.Y. designed experimental protocols. L.X. performed experiments and analyzed data. L.X. and Q.Y. wrote and revised the protocol. Q.Y. supervised the study.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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