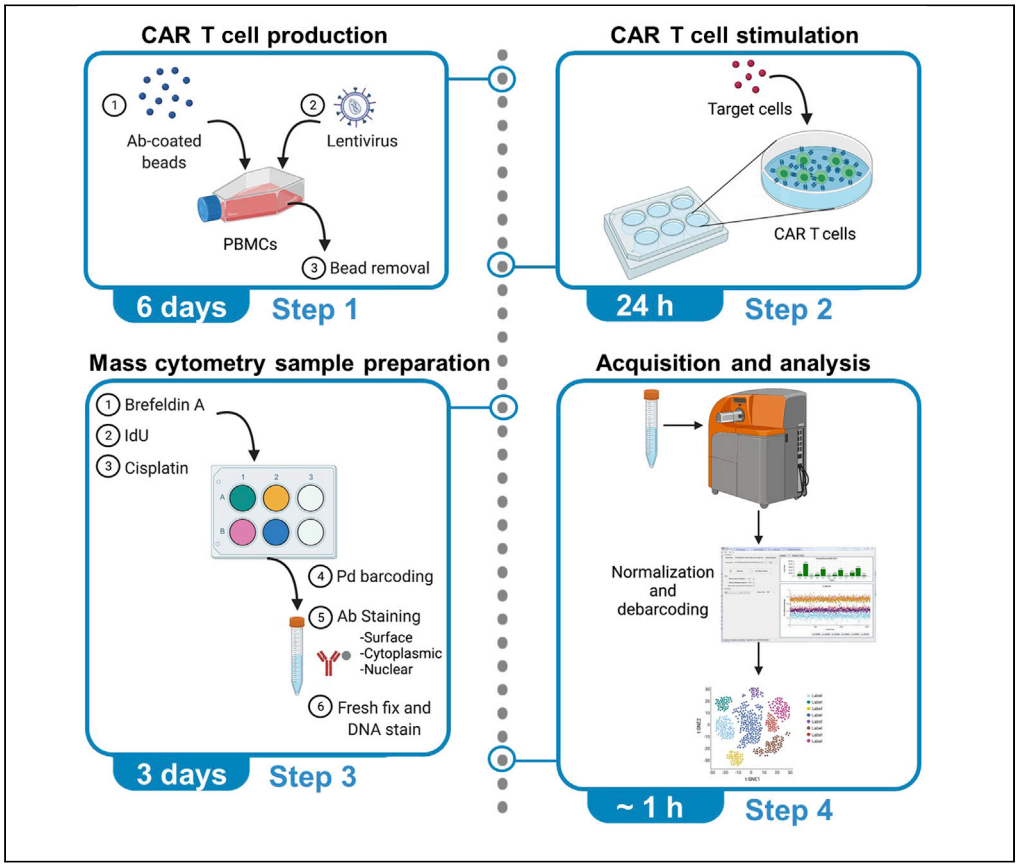
STAR Protocols

CellPress
OPEN ACCESS

Protocol

High-dimensional functional phenotyping of preclinical human CAR T cells using mass cytometry



Here, we present a comprehensive protocol for the generation and functional characterization of chimeric antigen receptor (CAR) T cells and their products by mass cytometry in a reproducible and scalable manner. We describe the production of CAR T cells from human peripheral blood mononuclear cells. We then detail a three-step staining protocol with metal-labeled antibodies and the subsequent mass cytometry analysis. This protocol allows simultaneous characterization of CAR T cell intracellular signaling, activation, proliferation, cytokine production, and phenotype in a single assay.

Ilaria M. Michelozzi, Jahangir Sufi, Thomas A. Adejumo, Persis J. Amrolia, Christopher J. Tape, Alice Giustacchini

i.michelozzi@ucl.ac.uk (I.M.M.)
a.giustacchini@ucl.ac.uk (A.G.)

Highlights

Efficient generation of CAR T cells from healthy donors' peripheral blood

Co-culture system to test CAR T cell activation *in vitro*

Assessment of CAR T cell product composition using mass cytometry

Functional and signaling phenotyping of CAR T cell using mass cytometry

Michelozzi et al., STAR Protocols 3, 101174
March 18, 2022
https://doi.org/10.1016/j.xpro.2022.101174

STAR Protocols

CellPress
OPEN ACCESS

Protocol

High-dimensional functional phenotyping of preclinical human CAR T cells using mass cytometry

Ilaria M. Michelozzi,[1,5,*] Jahangir Sufi,[2] Thomas A. Adejumo,[3] Persis J. Amrolia,[1,4] Christopher J. Tape,[2] and Alice Giustacchini[1,6,*]

[1]Molecular and Cellular Immunology Section, UCL Great Ormond Street Institute of Child Health, Zayed Centre For Research into Rare Disease in Children, WC1N 1DZ London, UK

[2]Cell Communication Lab, Department of Oncology, University College London Cancer Institute, WC1E 6DD London, UK

[3]Fluidigm Corporation, South San Francisco, CA, USA

[4]Department of Bone Marrow Transplant, Great Ormond Street Hospital for Children, WC1N 3JH London, UK

[5]Technical contact

[6]Lead contact

*Correspondence: i.michelozzi@ucl.ac.uk (I.M.M.), a.giustacchini@ucl.ac.uk (A.G.)
https://doi.org/10.1016/j.xpro.2022.101174

SUMMARY

Here, we present a comprehensive protocol for the generation and functional characterization of chimeric antigen receptor (CAR) T cells and their products by mass cytometry in a reproducible and scalable manner. We describe the production of CAR T cells from human peripheral blood mononuclear cells. We then detail a three-step staining protocol with metal-labeled antibodies and the subsequent mass cytometry analysis. This protocol allows simultaneous characterization of CAR T cell intracellular signaling, activation, proliferation, cytokine production, and phenotype in a single assay.

BEFORE YOU BEGIN

This protocol describes the generation of anti-CD19 CAR T cells from healthy donor-derived peripheral blood mononuclear cells (PBMCs) and their functional phenotyping by mass cytometry. Using a three-step staining procedure with metal-labeled antibodies, CAR T cells are characterized for the expression of surface and intracellular (cytoplasmic and nuclear) proteins relevant to the definition of their molecular and biochemical features.

Anti-CD19 CAR T cells are generated by lentiviral vector transduction, while untransduced T cells are used as non-CAR T cell controls. The resulting two experimental conditions (CAR T and non-CAR T) are next cultured without or with antigen-expressing NALM6 cell line (unstimulated and stimulated conditions, respectively) and analyzed 24 h post antigen-stimulation.

We provide a step-by-step guide for generating and analyzing four experimental conditions (CAR T cells and non-CAR T cells in presence or absence of stimulation) from one donor in a single experiment. The protocol can be easily adapted to include additional conditions and it has been successfully applied to phenotyping up to 90 experimental conditions from 5 donors in one single assay.

The recommended number of cells in the protocol refers to the four experimental conditions described above per single donor. Additional experimental conditions can be included by adjusting the number of cells accordingly. For instance, this protocol can be adapted to compare different CAR constructs and to test the effects of drugs/inhibitors on CAR T cell functionality (an example of this latter application is reported in the section "expected outcomes" of this manuscript).

Herein, we carry out CAR T cell immunophenotyping using an optimized panel of 39 metal-labeled antibodies designed to readout CAR T cell signaling and functionality. The panel can be adapted to meet the user's requirements, for example with particular focus on T cell memory subsets or exhaustion markers.

The applicability of this protocol can be extended to any CAR construct by selecting the appropriate antigenic stimulation.

The mass cytometry-related protocol can be applied to clinical CAR T cell products for extensive characterization. For this purpose, we recommend the inclusion in the antibody panel of one metal-labeled antibody that specifically recognizes the CAR T cells (e.g., anti-biotin CD19Fc), as proposed by (Corneau et al., 2021).

NALM6 thawing and cell culture

Timing: 1 h for thawing; cells are kept in culture for 8 days

In this part of the protocol, antigen-positive target cells are thawed and kept in culture until their use in the CAR T cell stimulation step (day 8). Here, we utilize NALM6, a B-Acute Lymphoblastic Leukemia cell line expressing CD19. To measure the effect of antigen levels on CAR T cell activation, cell lines genetically engineered to express varying levels of the target antigen can be employed (Ghorashian et al., 2019).

Note: The centrifuge in this part of the protocol is set at 4°C.

1. The day before the beginning of the experiment (day 0), thaw cryopreserved NALM6 cells.
   a. Prepare an ice bucket filled with ice.
   b. Fill a 15 mL Falcon tube with 9 mL of complete RPMI medium supplemented with 20% of FBS (recipe details in "materials and equipment") and place it on ice.
   c. Place one vial of cryopreserved cells at 37°C in the water bath for approximately 90 s, until its content is partially thawed (with just a small piece of ice left).

   Note: This timing refers to vials with 1 mL of cryopreserved cellular content. If the content volume of the vial is larger than 1 mL, the thawing process may take a little bit longer.

   d. Quickly transfer the vial content dropwise into the 15 mL Falcon tube filled with ice-cold medium, by pipetting it down the side of the 15 mL Falcon tube.
   e. Wash the vial twice with 1 mL of the 15 mL Falcon tube content to harvest any residual cell left.
   f. Gently invert the 15 mL Falcon tube with the thawed cells and centrifuge it at 200×*g* for 5 min at 4°C.
   g. Carefully aspirate and discard the supernatant without dislodging the cell pellet.
   h. Resuspend the cell pellet in a volume of pre-warmed complete RPMI medium suitable for the count.
   i. Count the viable cells using Trypan Blue (1:2 dilution, 10 µL cells in 10 µL Trypan Blue).
   j. Adjust the volume to seed the cells at a concentration of 1×10^6/mL. Place the cells in an incubator at 37°C and 5% CO_2.

2. The day after the thawing (day 1), count the cells and dilute them to 0.5×10^6/mL.
3. Keep the cells in culture at a concentration of 0.5×10^6/mL until day 8 of the experiment. Check the cell growth and add fresh medium twice a week.

   Note: When the cell culture growth is stabilized, it is possible to switch to complete RPMI supplemented with 10% FBS.

Note: This part of the protocol is optimized for NALM6 cell line , for which 3 to 5 passages from thawing are recommended before setting up the co-culture. The culture conditions, centrifuge speed and cellular density need to be adapted by the user according to the cell line used for CAR T cell antigenic stimulation

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-Cleaved Caspase 3 (D3E9)-142Nd (wd 1.00:50.00)	Cell Signaling Technology	Cat#9579
Anti-Human CD127/IL-7Ra (A019D5)-168Er (wd 1.50:50.00)	Fluidigm	Cat#3168017B
Anti-Human CD19 (HIB19)-165Ho (wd 1.00:50.00)	Fluidigm	Cat#3165025B
Anti-Human CD22 (HIB22)-151Eu (wd 3.00:50.00)	BioLegend	Cat#302511
Anti-Human CD25 (2A3)-169Tm (wd 1.00:50.00)	Fluidigm	Cat#3169003B
Anti-Human CD3 (UCHT1)-154Sm (wd 1.00:50.00)	Fluidigm	Cat#3154003B
Anti-Human CD4 (RPA-T4)-145Nd (wd 1.00:50.00)	Fluidigm	Cat#3145001B
Anti-Human CD44 (IM7)-111Cd (wd 4.00:50.00)	BioLegend	Cat#103051
Anti-Human CD69 (FN50)-115In (wd 3.00:50.00)	BioLegend	Cat#310939
Anti-Human CD8 (RPA-T8)-146Nd (wd 1.50:50.00)	Fluidigm	Cat#3146001B
Anti-Human CyclinB1 (GNS-1)-153Eu (wd 1.25:50.00)	Fluidigm	Cat#3153009A
Anti-Human Foxp3 (PCH101)-162Dy (wd 1.50:50.00)	Fluidigm	Cat#3162011A
Anti-Human GM-CSF (BVD2-21C11)-159Tb (wd 1.50:50.00)	Fluidigm	Cat#3159008B
Anti-Human Granzyme B (GB11)-173Yb (wd 1.50:50.00)	Fluidigm	Cat#3173006B
Anti-Human HLA-DR (L243)-170Er (wd 1.50:50.00)	Fluidigm	Cat#3170013B
Anti-Human IFNγ (B27)-158Gd (wd 1.50:50.00)	Fluidigm	Cat#3158017B
Anti-Human IL-17A (N49-653)-164Dy (wd 1.50:50.00)	Fluidigm	Cat#3164002B
Anti-Human IL-2 (MQ1-17H12)-166Er (wd 1.50:50.00)	Fluidigm	Cat#3166002B
Anti-Human NFAT1 (D43B1)-143Nd (wd 1.50:50.00)	Fluidigm	Cat#3143023A
Anti-Human pBad [S112] (40A9)-161Dy (wd 1.25:50.00)	Fluidigm	Cat#3161006A
Anti-Human Perforin (B-D48)-175Lu (wd 1.50:50.00)	Fluidigm	Cat#3175004B
Anti-Human TGFbeta (TW4-6H10)-163Dy (wd 1.50:50.00)	Fluidigm	Cat#3163010B
Anti-Human Tim-3 (F38-2E2)-144Nd (wd 4.00:50.00)	BioLegend	Cat#345019
Anti-Human TNFa (Mab11)-152Sm (wd 1.50:50.00)	Fluidigm	Cat#3152002B
Anti-mCherry (16D7)-155Gd (wd 1.00:50.00)	Invitrogen, Thermo Fisher Scientific	Cat#M11217
Anti-p38 [T180/Y182] (D3F9)-156Gd (wd 1.50:50.00)	Fluidigm	Cat#3156002A
Anti-p4E-BP1 [T37/T46] (236B4)-149Sm (wd 1.25:50.00)	Fluidigm	Cat#3149005A
Anti-pAMPKa [T172] (40H9)-160Gd (wd 2.50:50.00)	Cell Signaling Technology	Cat#2535
Anti-pBTK [Y551] (24a/BTK)-147Sm (wd 3.00:50.00)	BD Biosciences	Cat#558034
Anti-pCREB [S133] (87G3)-176Yb (wd 1.50:50.00)	Fluidigm	Cat#3176005A
Anti-pERK1/2 [T202/Y204] (20A)-167Er (wd 0.75:50.00)	BD Biosciences	Cat#612359
Anti-pHistone H2A.X [S139] (JBW301)-209Bi (wd 2.00:50.00)	Merck	Cat#80312BF
Anti-pHistone H3 [S28] (HTA28)-89Y (wd 1.50:50.00)	BioLegend	Cat#641007
Anti-pMKK3/MKK6 [S189/207] (D8E9)-157Gd (wd 1.00:50.00)	Cell Signaling Technology	Cat#12280
Anti-pPDPK1 [S241] (J66-653.44.22)-141Pr (wd 2.50:50.00)	BD Biosciences	Cat#558395
Anti-pRb [S807/S811] (J112-906)-150Nd (wd 1.50:50.00)	Fluidigm	Cat#3150013A
Anti-pS6 [S235/S236] (N7-548)-172Yb (wd 1.50:50.00)	Fluidigm	Cat#3172008A
Anti-pSRC [Y418] (SC1T2M3)-148Nd (wd 2.50:50.00)	Thermo Fisher Scientific	Cat#14-9034-82
Anti-pZAP70 [Y319]/pSyk [Y352] (17a)-171Yb (wd 1.50:50.00)	Fluidigm	Cat#3171005A

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Human Peripheral Blood Mononuclear Cells (age range: 19–41yr, sex: M/F)	N/A	N/A
Chemicals, peptides, and recombinant proteins		
Brefeldin A Solution (1,000×)	BioLegend	Cat#420601
Cell-ID™ 127 IdU	Fluidigm	Cat#201127
Cell-ID™ Cisplatin-100 µL	Fluidigm	Cat#201064
Cell-ID™ Intercalator-Ir-125 µM	Fluidigm	Cat#201192A
CliniMACS® PBS/EDTA Buffer	Miltenyi Biotec	Cat#700-25
CTS™ (Cell Therapy Systems) Dynabeads™ CD3/CD28	Gibco™, Thermo Fisher Scientific	Cat#40203D
DPBS, no calcium, no magnesium	Gibco™, Thermo Fisher Scientific	Cat#14190250
eBioscience™ Foxp3/Transcription Factor Staining Buffer Set	Invitrogen™, Thermo Fisher Scientific	Cat#00-5523-00
EQ™ Four Element Calibration Beads-100 mL	Fluidigm	Cat#201078
Fetal Bovine Serum (FBS), qualified, heat inactivated, Brazil	Gibco™, Thermo Fisher Scientific	Cat#10500064
Ficoll-Paque™ PLUS	GE Healthcare	Cat#17-1440-03
Human TruStain FcX™ (Fc Receptor Blocking Solution)	BioLegend	Cat#422301
Maxpar® Cell Acquisition Solution-200 mL	Fluidigm	Cat#201240
Maxpar® Cell Staining Buffer-500 mL	Fluidigm	Cat#201068
Maxpar® Fix and Perm Buffer-100 mL	Fluidigm	Cat#201067
Maxpar® PBS-500 mL	Fluidigm	Cat#201058
Maxpar® Water-500 mL	Fluidigm	Cat#201069
Methanol	VWR Chemicals	Cat#20847.307
Penicillin-Streptomycin	Sigma-Aldrich, Merck	Cat#P4333
Pierce™ 16% Formaldehyde (w/v), Methanol-free	Thermo Scientific™, Thermo Fisher Scientific	Cat#28906
RPMI 1640 Medium, GlutaMAX™ Supplement	Gibco™, Thermo Fisher Scientific	Cat#61870044
TexMACS™ Medium	Miltenyi Biotec	Cat#130-097-196
Critical commercial assays		
Cell-ID™ 20-Plex Pd Barcoding Kit	Fluidigm	Cat#201060
Experimental models: Cell lines		
NALM6, human cell line	DSMZ	ACC 128
Software and algorithms		
Cytobank	Cytobank, Inc., Beckman Coulter	https://cytobank.org/index.html
CyTOF Software Version 7	Fluidigm	https://www.fluidigm.com/products-services/software#helios-anchor
Other		
Automated hematology analyzer	Sysmex	Model XE-5000
Cell culture Flask 175 cm^2, treated	Greiner Bio-One	Cat#660160
Cell culture Flask 25 cm^2, treated	Greiner Bio-One	Cat#690160
Cell culture Flask 75 cm^2, treated	Greiner Bio-One	Cat#658175
Cell culture incubator CellXpert®	Eppendorf	Model C170i
Centrifuge	Eppendorf	Model 5920 R
Countess™ Cell Counting Chamber Slides	Invitrogen™, Thermo Fisher Scientific	Cat#C10228
Countess™ II Automated Cell Counter*	Invitrogen™, Thermo Fisher Scientific	Cat#AMQAX1000
DynaMag™-15 Magnet	Invitrogen™, Thermo Fisher Scientific	Cat#12301D

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Falcon® 15 mL Polypropylene Conical Tube	Corning Inc.	Cat#352096
Falcon® 5 mL High Clarity Polypropylene Round-bottom Tubes	Corning Inc.	Cat#352063
Falcon® 5 mL Polystyrene Round-bottom Tubes, with Cell Strainer Cap	Corning Inc.	Cat#352235
Falcon® 50 mL Polypropylene Conical Tube	Corning Inc.	Cat#352070
Helios™ CyTOF 3 Mass Cytometer	Fluidigm	N/A
Irradiator	CIS	Model IBL 437 C
Labnet Prism™ R Refrigerated Microcentrifuge	Labnet International, Inc.	Cat#C2500-R-230V
Liquipette® Fine point micro extended	ELKay	Cat#127-P423-STR
Microscope	Leica Microsystems CMS	Model DMIL LED Fluo
Nunc™ MicroWell™ 96-Well, Nunclon Delta-Treated, Flat-Bottom Microplate	Thermo Scientific™, Thermo Fisher Scientific	Cat#167008
SciQuip Vortex VariMix	SciQuip	Cat#821200060035
SciSpin MINI Microfuge	SciQuip	Cat#9011001012
Super Sampler	Victorian Airships	N/A
Trypan Blue stain 0.4%	Invitrogen™, Thermo Fisher Scientific	Cat#T10282

wd: working dilution

*This item has been discontinued and replaced by Countess™ 3 Automated Cell Counter Cat#AMQAX2000

MATERIALS AND EQUIPMENT

Human peripheral blood samples

Peripheral blood from healthy donors was acquired from Cambridge Bioscience, through the London based Research Donors tissue bank, under an ethically-approved protocol.

The day before the start of the experiment (day 0), collect fresh human whole blood samples (30 mL) from adult healthy donors after informed consent. Keep samples overnight (16–18 h) at room temperature (20°C–22°C) on a rocker in tubes containing the anti-coagulant dipotassium (K2) Ethylene Diamine Tetra Acetic Acid (EDTA).

Complete RPMI

Reagent	Final concentration	Amount (mL)
RPMI 1640 Medium GlutaMAX™ Supplement	n/a	197.5
FBS	20%	50
100× Penicillin-Streptomycin	1×	2.5
Total	**n/a**	**250**

Store at 4°C for up to 1 month.

Brefeldin A stock solution aliquoting

Dispense 1,000× (5 mg/mL) brefeldin A solution into single-use aliquots and store at 4°C for no more than 3 months.

Cell-ID™ cisplatin aliquoting

Dispense Cell-ID™ Cisplatin (5 mM) into single-use aliquots and store at −20°C for no more than one year from delivery.

Cell-ID™ Intercalator-Ir aliquoting

Dispense Cell-ID™ Intercalator-Ir (125 μM) into single-use aliquots and store at −20°C for no more than one year from delivery.

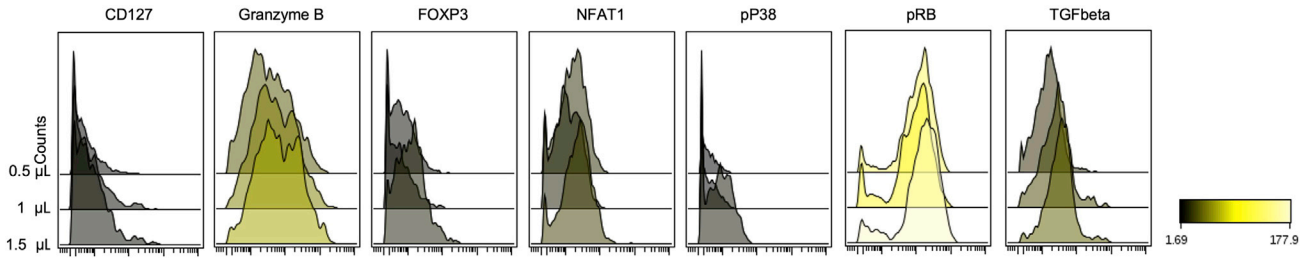


Figure 1. Antibody titration

Representative histograms showing CD127, Granzyme B, FOXP3, NFAT1, pP38, pRB and TGFbeta expression in stimulated live cells using 3 different antibody dilutions.

Alternatives: Here, we prepare fresh antibody mixes in steps 91, 100 and 112. However, these can be prepared, stored at 4°C, and used within 8 h. Alternatively, it is possible to store the mixes at −80°C or in liquid nitrogen for several months (Schulz et al., 2019).

Note: A good antibody panel design is important to reduce deconvolution and clean-up. Here, we provide an optimized panel of 39 metal-labeled antibodies. We advise users to optimize their own metal-labeled antibody panels by using Maxpar® Panel Designer Software (https://www.fluidigm.com/singlearticles/maxpar-panel-designer-v2-0) and to titrate their panels.

An example of antibody titration is provided below, testing 3 different dilutions (0.5–1–1.5 µL, as per Fluidigm recommendation) for 7 representative antibodies in our panel (Figure 1).

⚠ CRITICAL: Formaldehyde (FA) is carcinogenic and toxic if swallowed, inhaled or if in contact with skin. Handle FA in a fume hood using personal protective equipment.

Note: The timing in the protocol is relative to the handling of samples derived from one single donor. If using multiple donors, scale up the timing accordingly.

Note: Keep the samples and the reagents at room temperature (about 20°C) unless otherwise specified (steps 110–117; 130–137).

Note: All the centrifuges are at room temperature. However, it is also possible to perform centrifuges at 4°C after permeabilization to stabilize the surface antibody-antigen complexes.

STEP-BY-STEP METHOD DETAILS

PBMC isolation from human healthy donor whole peripheral blood

Timing: ~1 h 30 min

The starting material for CAR T cell manufacture can vary according to the clinical protocol followed. Unsorted white blood cells (WBCs), WBCs depleted from monocytes or natural killer cells, or enriched for specific T cell subsets (e.g., CD4+, CD8+ or central memory T cells), or purified CD3+ T cells can be used (Vormittag et al., 2018).

Here, we generate CAR T cells from freshly isolated healthy donor-derived PBMCs.

Alternatives: Alternatively, it is possible to apply this protocol (from step 12) to thawed PBMCs.

1. On day 0, collect fresh human whole blood sample from an adult healthy donor in tubes containing an anti-coagulant and keep the sample overnight (16–18 h) at room temperature on a rocker.
2. On day 1, dilute the peripheral blood sample 1:2 in PBS and invert gently. For instance, if the volume of the blood is 30 mL, add 30 mL of PBS to it.
3. Aliquot 15 mL of Ficoll-Paque™ PLUS density gradient medium into new 50 mL Falcon tubes.
4. Slowly layer the diluted peripheral blood sample (30 mL) onto the Ficoll-Paque™ PLUS medium by pipetting it down the side of the Falcon tube.

⚠ CRITICAL: It is important to layer the diluted blood sample on top of the Ficoll-Paque™ PLUS medium for a successful gradient separation. While adding the diluted blood sample, proceed carefully, hold the Falcon tube at an angle (45°) and avoid mixing the two components (Figure 2A). Gently handle the tubes.

Note: If the volume of the diluted blood sample is larger than 30–35 mL, prepare multiple 50 mL Falcon tubes filled with Ficoll-Paque™ PLUS medium and the diluted blood sample. The number of 50 mL Falcon tubes varies according to the diluted blood sample volume.

5. Quickly centrifuge the Falcon tubes with the diluted blood sample and the density gradient medium at 700×*g* for 20 min at room temperature, turning off the centrifuge brake.

Note: With the brake off, the centrifuge takes approximately 30–40 min to complete.

Note: Once centrifuged, gently handle the tubes to avoid the disruption of the density gradient formed.

Note: The density gradient centrifugation separates the diluted blood sample into four phases: a bottom layer with granulocytes and erythrocytes, an intermediate layer with Ficoll-Paque™ PLUS medium, a top layer containing plasma and platelets, and a mononuclear layer at the plasma/Ficoll-Paque™ PLUS interface (Figure 2B).

6. Carefully aspirate and discard most of the top layer (about 2/3 of it).
7. Prepare two 50 mL Falcon tubes for the PBMC isolation.
8. Slowly aspirate the mononuclear layer with a Pasteur pipette and transfer it to the new 50 mL Falcon tubes.

⚠ CRITICAL: While harvesting the mononuclear layer, avoid aspirating the underlying Ficoll-Paque™ PLUS medium.

9. Top up the 50 mL Falcon tubes containing the harvested PBMCs with PBS, invert the tubes gently, and centrifuge them at 220×*g* for 5 min.
10. Repeat the washing step for a total of 2 washes.
11. Discard the supernatant without disturbing the PBMC pellet and resuspend it in 10 mL of TexMACS™ medium, an optimized T cell medium.

Alternatives: Instead of using TexMACS™ medium for CAR T cell culture, it is possible to use X-VIVO 15 medium (Lonza) (Medvec et al., 2018).

12. Take an aliquot of the resuspended cells and count them using a hematology automated analyser. Record the WBC and the lymphocyte counts.

Alternatives: If the hematology automated analyser is not available, use an automated cell counter or a hemocytometer. However, extra-time will be required for the count by hemocytometer.

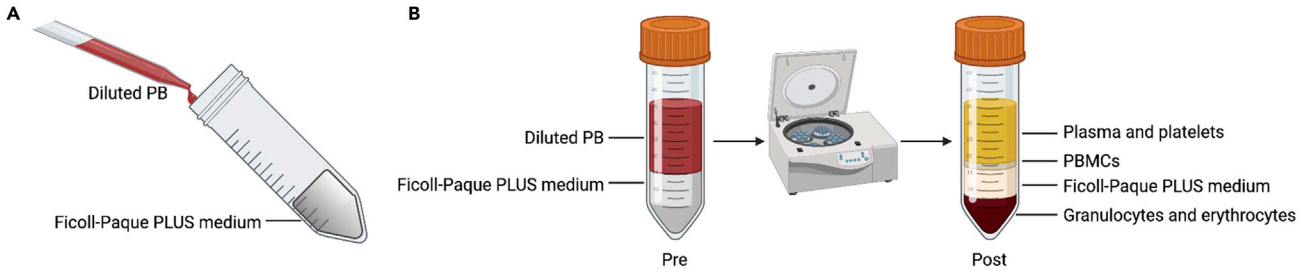


Figure 2. Schematic representation of PBMC isolation

(A) The diluted peripheral blood (PB) should be carefully stratified on top of the Ficoll-Paque™ PLUS medium. Holding the tube at an angle (45°) can help in the process.

(B) Pre-centrifugation sample stratification and post-centrifugation sample separation into four layers.

13. Proceed with the next step "activation of T cells from human PBMCs".

Alternatives: Alternatively, it is possible to freeze the isolated PBMCs for later use.

Activation of T cells from human PBMCs

Timing: ~20 min

In this step at day 1, T cells are activated and expanded from PBMCs by using CTS™ (Cell Therapy Systems) Dynabeads™ CD3/CD28. The goal is to enrich the PBMC culture for T cells. Here, we adapted from CTS™ Dynabeads™ CD3/CD28 user guide on www.thermofisher.com.

14. Dilute the isolated PBMCs to 1 × 10^6 WBCs/mL with TexMACS™ medium.
15. Carefully resuspend CTS™ Dynabeads™ CD3/CD28 by vortexing (more than 30 s).
16. Wash CTS™ Dynabeads™ CD3/CD28.
    a. Take an aliquot of CTS™ Dynabeads™ CD3/CD28 (7.5 µL of beads per million of lymphocytes, 1:3 lymphocyte:bead ratio) and mix it to an equal volume of TexMACS™ medium in a 15 mL Falcon tube. Vortex the 15 mL Falcon tube.
    b. Place the 15 mL Falcon tube on a DynaMag™-15 Magnet for 1 min, aspirate off the supernatant.
    c. Once removed the 15 mL Falcon tube from the magnet, resuspend the washed beads in 7.5 µL of TexMACS™ medium per million of lymphocytes.
17. Add the washed CTS™ Dynabeads™ CD3/CD28 to the isolated PBMCs.
18. Carefully mix the PBMCs with the Dynabeads™ and plate them into a Flask at a concentration of 1 × 10^6 WBCs/mL. We recommend using T25 Flask for 5–15 mL of cell:bead suspension, T75 for 15–30 mL and T175 for 30–50 mL. Gently mix the Flask content for 10 min on a rocker or manually every 2 min.
19. Incubate at 37°C and 5% CO_2.

Note: If the lymphocyte count is not known, add the CTS™ Dynabeads™ CD3/CD28 to the cells at a 1:1 WBC:bead ratio.

Alternatives: CTS™ Dynabeads™ CD3/CD28 (with a concentration of 4 × 10^8 beads/mL) are a GMP-grade product and therefore expensive. Alternatively, research-grade Dynabeads™ Human T-Expander CD3/CD28 (Cat#11141D, Gibco™, Thermo Fisher Scientific) containing less concentrated beads (1 × 10^8 beads/mL) are commercially available. If using the research-grade Dynabeads, please follow the specific manufacturer's recommendations.

Alternatives: For better representation of CAR T cell clinical manufacturing, the PBMCs can be cultured with Dynabeads™ in MACS® GMP Cell Differentiation Bags (Miltenyi Biotec) following the manufacturer's recommendation. However, this culture method is more laborious and time consuming than the flask culture.

Note: See troubleshooting 1.

Lentiviral transduction of T cells

Timing: ~1 h

In this part of the protocol, T cells from human PBMCs are lentivirally transduced to express the CAR construct. Here, we describe the transduction with an anti-CD19 FMC63-based CAR construct, but this same protocol was used to generate other anti-CD19 CAR T cells (Michelozzi I.M. et al., 2020 ASH abstract).

Note: The CAR-expressing lentiviral vector used and the lentiviral particle production are described in (Ghorashian et al., 2019).

20. After 17–24 h (day 2), harvest the cell:bead mixture into two pre-labeled 50 mL Falcon tubes.
21. To improve cell recovery, wash the Flask twice with CliniMACS® PBS/EDTA buffer.
22. Centrifuge the 50 mL Falcon tubes with the cell:bead mixture at 220×*g* for 5 min at room temperature.
23. Discard the supernatant and resuspend the cell:bead mixture in 10 mL of TexMACS™ medium.
24. Count the resuspended cells using the same instrument as day 1 for consistency. Record the WBC and lymphocyte counts.

Note: As the addition of Dynabeads™ to the PBMCs enriches the cell culture for T cells at the expenses of other cell types, the WBC number at day 2 may be similar to or lower than the one measured the day before.

Note: The transduction of 2.5 × 10^6 beads-activated T cells is sufficient for a 4-condition experiment. Moreover, we recommend including a non-CAR control in the experiment (e.g., T cells transduced with an empty or scrambled vector, or untransduced T cells) undergoing the same experimental conditions as the CAR-transduced cells.

25. Resuspend the cell:bead mixture in TexMACS™ medium at a concentration of 1 × 10^6 WBCs/mL.
26. Aliquot 500 μL of cell:bead mixture per well into a 24-well plate.

Alternatives: If CAR T cells are generated by gamma-retrovirus transduction, it is recommended to use RetroNectin®-coated non-treated plates (CytoOne®, Starlab). The coating of the plate is performed following the manufacturer's instruction (Takara Bio Inc.).

27. Add the lentiviral supernatant to the cells at a multiplicity of infection (MOIs) between 1–10 (Ghorashian et al., 2019).
28. Mix well.
29. Incubate the plate at 37°C and 5% CO_2.

Optional: 24 h post-transduction (day 3), top up with TexMACS™ medium each well containing transduced beads-activated T- cells to augment T cell expansion. Alternatively, spin down the plate at 220×*g* for 5 min, aspirate no more than half of the supernatant without perturbing the cells, and add fresh TexMACS™ medium into each well.

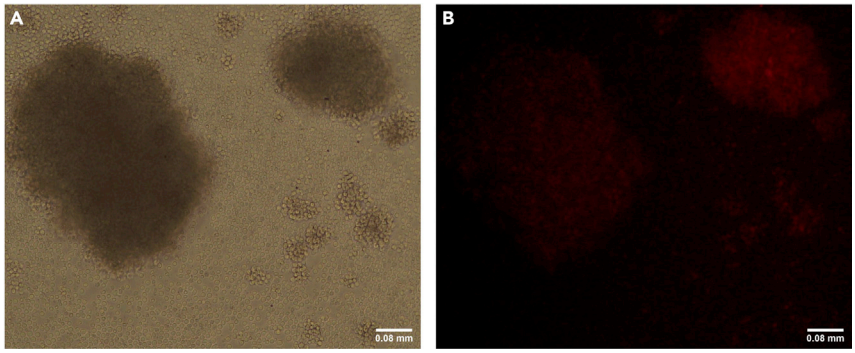


Figure 3. CAR T cell culture at day 8 before antigen stimulation
(A) Activated CAR T cells tend to form clusters in culture.
(B) mCherry fluorescent signal under an inverted fluorescence microscope. Magnification 10×. Scale bars in mm.

Note: The transduction occurs within 16 h. Beads-activated CAR T cells are incubated for longer, until day 6, to allow their expansion.

30. Daily check the cell culture to assess expansion, activation and transduction level.

CAR T cells tend to form clusters when activated, a hallmark of T cell activation, and express mCherry, a fluorescent protein encoded by the reporter gene present in the lentiviral vector construct used by us to transduce T cells. mCherry expression is a proxy for CAR expression (Figure 3).

Note: See troubleshooting 2.

Dynabead removal from CAR T cell culture

Timing: ~1 h

In this section of the protocol, CTS™ Dynabeads™ CD3/CD28 are magnetically removed from the cell culture (day 6).

31. Harvest the cell:bead mixture in a 50 mL Falcon tube.
32. To improve cell recovery, wash the wells with PBS.
33. Top up the 50 mL Falcon tube with PBS.
34. Centrifuge the 50 mL Falcon tube at 220×*g* for 5 min at room temperature.
35. Discard the supernatant and resuspend the cell pellet in 13 mL of TexMACS™ medium.
36. Move the cells into a 15 mL Falcon tube.
37. Remove the Dynabeads™ from the cell culture.
    a. Place the 15 mL Falcon tube on DynaMag™-15 Magnet for 1 min.
    b. Carefully aspirate the cell content without perturbing the beads and transfer it into a new 15 mL Falcon tube.
38. Count the cells using an automated cell counter and record their concentration and viability.
39. Seed the cells in TexMACS™ medium at a concentration of 1×10^6/mL.
40. Incubate at 37°C and 5% CO_2.

Optional: Leave the cells resting for 1 day after bead-removal, before antigen-stimulation, to maximize the CAR-dependent- and minimize the Dynabeads™-driven intracellular signaling response detected by mass cytometry.

Alternatives: At this stage of the protocol, CAR T cells can be live-frozen for later use.

Stimulation of CAR T cells with irradiated antigen-positive target-cells

Timing: ~1 h

Two days later (day 8), CAR T cells are co-cultured with irradiated antigen-positive target-cells (NALM6 cells) to test their functionality. Target-cells are irradiated to impede their overgrowth in culture.

41. Count the NALM6 and the CAR T cells and resuspend each of them to a concentration of 1 million/mL in TexMACS™ medium.
42. Irradiate NALM6 cells (40 Gy) with 137 Cesium radioactive source.

Note: Irradiator performance can vary. The timing required for irradiation has to be specifically calculated based on the properties of the irradiator available.

Alternatives: Instead of irradiating the target cells, it is possible to treat them with Mitomycin C before co-culture (MacLeod et al., 2017).

43. Aliquot CAR T cells in 96-, 24- or 12-well plates according to the number of cells and add NALM6 in 1:1 ratio (in the stimulated condition).
44. Aliquot CAR T cells in 96-, 24- or 12- plates according to the number of cells and add an equal volume of TexMACS™ medium (in the unstimulated condition).

Note: We recommend to aliquot 100 µL, 500 µL, and 1 mL of CAR T cells per well in a 96-, 24-, and 12-well plate, respectively.

45. Incubate at 37°C and 5% CO_2.
46. Proceed with the sample preparation for mass cytometry analysis (see below from step 47).

Note: In order to proceed to the mass cytometry phenotyping, the stimulation of at least 0.2–0.5 × 10^6 CAR T cells in this step is recommended.

Note: Here, we analyze the cells by mass cytometry 24 h post antigen-stimulation. However, the stimulation period may vary according to the user's needs, from few minutes (to assess biochemical changes, as reported in (Fisher et al., 2019)) to days (to assess late effector functions e.g., proliferation, exhaustion and killing potential). If specifically interested in CAR T cell exhaustion, repeated antigen stimulation rounds (antigen stress test) may be a further option (Kiesgen et al., 2021).

Note: If using cell lines genetically engineered to express the target antigen, add the wild-type cell line in the unstimulated condition, to account for non-antigen driven effects of the co-culture.

Brefeldin A treatment, IdU staining and fixation of samples

Timing: ~5 h

At day 9, unstimulated and stimulated samples are treated with Brefeldin A, an inhibitor of the endoplasmic reticulum-Golgi protein transport, to avoid the release of cytokines into the media, improving their intracellular detection. Subsequently, samples are stained with Cell-ID™ 127 IdU,

a thymidine analogue incorporated into the DNA during S-phase of the cell cycle, to specifically identify proliferating cells. Its signal is detected in the 127I channel of CyTOF® mass cytometer.

Finally, samples are fixed to preserve their biochemical and molecular state.

Note: If the readers are not performing cytokine or proliferation analyses, proceed directly to cell fixation (from step 49).

Note: Equilibrate at room temperature Brefeldin A Solution and Cell-ID^{TM} 127 IdU reagents before their use. The reagent dilutions and the timing of incubations are defined according to the manufacturer's recommendations (https://www.biolegend.com/en-us/products/brefeldin-a-solution-1-000x-1499?GroupID=BLG2181 and https://fluidigm.my.salesforce.com/sfc/p/#700000009DAw/a/4u0000019iFX/uPaSg89NU8Nh7ZHqE0rwjNPwEr13MZLbRGhyS0aA0mc).

47. Add Brefeldin A Solution to a final concentration of 1× (1:1,000 dilution) to unstimulated and stimulated samples. Mix gently by pipetting and incubate for 4 h at 37°C and 5% CO_2.
48. After 3 h 30 min, dilute Cell-ID^{TM} 127 IdU 1:1,000 in unstimulated and stimulated samples (final concentration of 50 μM). Mix gently by pipetting and incubate for 30 min at 37°C and 5% CO_2.
49. During the incubation, prepare fresh 1.6% formaldehyde (FA) by diluting 16% FA in PBS (1:10 dilution). For example, dilute 5 mL of 16% FA in 45 mL of PBS. For four experimental conditions, as in this case, prepare 5 mL of 1.6% FA.

Optional: Filter the stock FA before diluting it.

50. At the end of the incubation, harvest the cells into pre-labeled individual 15 mL Falcon tubes for each experimental condition (4 tubes).
51. Wash each well with 200 μL of Maxpar® PBS to collect any residual cell.
52. Centrifuge the 15 mL Falcon tubes at 220×*g* for 5 min at room temperature.

Note: The fixation section of this protocol (steps 49, 53–60) is adapted from "Fix Cells" section of the protocol "Maxpar Cell Surface Staining with Fresh Fix" on https://www.fluidigm.com/binaries/content/documents/fluidigm/marketing/maxpar-cell-surface-staining-400276-pr/maxpar-cell-surface-staining-400276-pr/fluidigm%3Afile.

Differently from Fluidigm's protocol, we fix cells before cell surface staining procedure to preserve the samples, block all biochemical processes and to prepare the samples for the "cell barcoding" step. We also reduce the incubation timing (7 instead of 10 min) for a less harsh fixation and we increase the centrifuge speed (1,200 instead of 800×*g*) to augment cell recovery. Moreover, we wash away the fixative after incubation.

Note: The following volumes are recommended for 1–3 million cells. If staining a larger number of cells, scale up these volumes.

Note: The panel of antibodies provided in the protocol works well with fixed epitopes. If using a different panel of antibodies sensitive to fixation refer to troubleshooting 3 and 4.

53. Aspirate off the supernatant, and aliquot 1 mL of the freshly prepared 1.6% FA into each 15 mL Falcon tube.
54. Gently mix by vortexing.
55. Incubate for 7 min at room temperature.
56. Wash with 2 mL of Maxpar® Cell Staining Buffer (CSB) and centrifuge at 1,200×*g* for 5 min at room temperature.

Note: After fixation, samples are centrifuged at higher speed (1,200×*g* for 5 min) to increase cell recovery.

57. Discard the supernatant and gently pipette or vortex to resuspend the cells in the residual volume.
58. Repeat the wash (step 56) (2 washes in total).
59. After the second wash, aspirate the supernatant and do not resuspend the cell pellet to preserve cell integrity. Leave the pelleted cells in the residual Maxpar® CSB volume (approximately 200 uL).
60. Store the cell pellets in 15 mL Falcon tubes at 4°C.

Pause point: Cells can be stored in FA at 4°C for up to one week (7–10 days).

Note: Longer term-storage in FA can increase conformational changes in surface antigens.

Viability staining with Cell-ID™ cisplatin

Timing: ~30 min

In this part of the protocol, unstimulated and stimulated fixed samples are stained with cisplatin (Cell-ID™ Cisplatin), an agent that preferentially penetrates dead cells with damaged membrane. Its signal, detected in the 195/194Pt channel of CyTOF® mass cytometer, allows distinguishing between live (cisplatin low) and dead (cisplatin high) cells.

Here, we adapted the section of the protocol from Fluidigm's "Cell-ID™ Cisplatin" product information sheet https://www.fluidigm.com/binaries/content/documents/fluidigm/resources/cell-id-cisplatin-prd018/cell-id-cisplatin-prd018/fluidigm%3Afile.

Differently from Fluidigm's protocol, for 0.2×10^5–1×10^6 cells, we use cisplatin at a 10-fold lower concentration (0.5 instead of 5 μM), and we reduce the timing of its incubation (1 instead of 5 min) to resolve the differences between fixed live and fixed dead cells. Centrifugation is performed at higher speed due to the cells being fixed. Moreover, we wash cells in Maxpar® PBS rather than Maxpar® CSB to proceed with the next step of cell barcoding.

Note: Equilibrate at room temperature Cell-ID™ Cisplatin immediately before use.

61. Resuspend the samples in the residual volume left and count them.

Note: Do not use viability dye (e.g., Trypan Blue) while counting as cells are fixed.

62. Centrifuge the cells at 1,200×*g* for 5 min at room temperature.
63. Resuspend the cell pellets in 500 μL of Maxpar® PBS.
64. Dilute Cell-ID™ Cisplatin 1:5,000 in Maxpar® PBS (1 μM cisplatin).
65. Aliquot 500 μL of 1 μM cisplatin into 500 μL of cells (cisplatin final concentration of 0.5 μM) and resuspend carefully.
66. Incubate for 1 min on a rocker at room temperature.
67. Quench the cisplatin by adding 5 mL of Maxpar® CSB to each 15 mL Falcon tube. Centrifuge at 1,200×*g* for 5 min, discard the supernatant and gently vortex to resuspend the cell pellet.
68. Add 4 mL of Maxpar® PBS to wash cells and centrifuge at 1,200×*g* for 5 min.
69. Discard the supernatant and gently vortex to resuspend the cell pellet.
70. Continue with the next step "cell barcoding".

⚠ CRITICAL: Cisplatin is a mutagen and a carcinogen. It is toxic if swallowed, inhaled or if in contact with skin. Handle cisplatin using personal protective equipment.

Table 1. List of barcodes and assigned samples

Barcode code	Isotope combinations	Sample name
1	Pd102, Pd104, Pd105	
2	Pd102, Pd104, Pd106	
3	Pd102, Pd104, Pd108	
4	Pd102, Pd104, Pd110	
5	Pd102, Pd105, Pd106	
6	Pd102, Pd105, Pd108	
7	Pd102, Pd105, Pd110	
8	Pd102, Pd106, Pd108	
9	Pd102, Pd106, Pd110	
10	Pd102, Pd108, Pd110	
11	Pd104, Pd105, Pd106	
12	Pd104, Pd105, Pd108	
13	Pd104, Pd105, Pd110	
14	Pd104, Pd106, Pd108	
15	Pd104, Pd106, Pd110	
16	Pd104, Pd108, Pd110	
17	Pd105, Pd106, Pd108	
18	Pd105, Pd106, Pd110	
19	Pd105, Pd108, Pd110	
20	Pd106, Pd108, Pd110	

Cell barcoding

Timing: ~1 h 20 min

In this step, the different experimental conditions derived from one donor are uniquely barcoded using Cell-ID™ 20-Plex Pd Barcoding Kit. This kit contains 20 different barcodes, each of them characterized by the exclusive combination of 3 Palladium (Pd) isotopes (Table 1). Here, we use 4 barcodes to label each donor's experimental conditions.

Here, we adapted the protocol from Fluidigm's "Cell-ID™ 20-Plex Pd Barcoding Kit" user guide https://www.fluidigm.com/binaries/content/documents/fluidigm/resources/cell-id-20-plex-pd-barcoding-kit-ug-prd023/cell-id-20-plex-pd-barcoding-kit-ug-prd023/fluidigm%3Afile.

Note: The following volumes are recommended for 1–3 million cells. If staining a larger or smaller number of cells, scale up or down these volumes, respectively.

71. Dilute 10× Barcode Perm Buffer 1:10 in Maxpar® PBS (final concentration of 1×).
72. Aliquot 1 mL of 1× Barcode Perm Buffer into each sample and centrifuge them at 1,200×*g* for 5 min.
73. Gently discard the supernatant and repeat the wash, step 72 (2 total washes).
74. Resuspend each pellet in 800 μL of 1× Barcode Perm Buffer.
75. Equilibrate at room temperature one set of 4 barcodes for 10 min.
76. Assign a unique barcode code to each sample and take record (Table 1).
77. Quickly spin (10 s) the barcode tubes using a mini microfuge.
78. Add 100 μL of 1× Barcode Perm Buffer to each barcode, gently resuspend and transfer them (approximately 110 μL per barcode) to the assigned samples.
79. Gently vortex the samples and incubate them for 30 min at room temperature on a rocker.

⚠ CRITICAL: While the samples are incubating, invert the tubes twice (every 10 min). This procedure will prevent cell pelleting and increase the barcoding efficiency.

80. Centrifuge the barcoded samples at 1,200×*g* for 5 min, discard the supernatant and gently vortex.
81. Add 2 mL of Maxpar® CSB to the barcoded samples and centrifuge them at 1,200×*g* for 5 min.
82. Discard the supernatant and repeat step 81 (2 total washes).
83. Resuspend each barcoded cell pellet in 200 μL of Maxpar® CSB.
84. Centrifuge the barcoded samples at 1,200×*g* for 5 min and store them at 4°C as cell pellets (without resuspending the cell pellets and without removing the supernatant).

Pause point: The barcoded samples can be stored at 4°C for up to one month (C.J.T.'s lab confidential). It is important to store them as pellets in minimum volume to prevent loss of barcodes.

Note: If more than one donor is present in the experiment, multiplex conditions per donor pooling together the different conditions per each donor. After the pooling, one single tube per donor will be processed containing all the experimental conditions.

Note: See troubleshooting 3 and 5.

Sample multiplexing and surface staining

Timing: ~ 1 h 10 min

In this part of the protocol, the barcoded samples are pooled together generating one multiplexed sample, which will be stained with metal-labeled antibodies and acquired. Here, we pool together 4 experimental conditions into one 15 mL Falcon tube. This step allows to speed up the staining process while minimizing batch effects and technical/experimental variability.

The multiplexed samples are incubated with Fc receptor blocking solution (Human TruStain FcX™), to reduce unspecific antibody binding, before being stained with an antibody mix for surface antigens. In total, we stain and acquire one 15 mL Falcon tube (for one donor).

85. Pool all the barcoded samples from one single donor into a pre-labeled 15 mL Falcon tube.

Note: If barcoded samples were stored for longer than one month, wash them before pooling to prevent cross-contamination due to barcode leakage.

86. Resuspend the pooled cells into the 15 mL Falcon tube and count them without using a viability dye.

Note: We adapted this section of the protocol from "Stain Cells with Surface Antibodies" section from "Maxpar Cell Surface Staining with Fresh Fix" on https://www.fluidigm.com/binaries/content/documents/fluidigm/marketing/maxpar-cell-surface-staining-400276-pr/maxpar-cell-surface-staining-400276-pr/fluidigm%3Afile.

Note: The following volumes are recommended for the staining of 1–3 million cells. If staining a larger number of cells, scale up these volumes.

87. Centrifuge the 15 mL Falcon tube at 1,200×*g* for 5 min at room temperature.
88. Dilute Human TruStain FcX™ 1:20 in Maxpar® CSB following the manufacturer's recommendations.
89. Resuspend the cell pellet in 50 μL of diluted Human TruStain FcX™, mix well.
90. Incubate for 10 min at room temperature.
91. During the incubation, prepare the surface antibody mix (Table 2).

Table 2. Surface antibody mix

Marker	Metal isotope	Clone	Volume (μL) for 1 sample
CD3	154-Sm	UCHT1	1.00
CD4	145-Nd	RPA-T4	1.00
CD8a	146-Nd	RPA-T8	1.50
CD19	165-Ho	HIB19	1.00
CD22	151-Eu	HIB22	3.00
CD25	169-Tm	2A3	1.00
CD44	111-Cd	IM7	4.00
CD69	115-In	FN50	3.00
CD127	168-Er	A019D5	1.50
HLA-DR	170-Er	L243	1.50
Tim-3	144-Nd	F38-2E2	4.00
CSB	n/a	n/a	27.50
Total	**n/a**	**n/a**	**50.00**

⚠ CRITICAL: Vortex and quickly spin (110×*g* for 30 sec) each antibody before preparing the mix.

92. Aliquot 50 μL of surface antibody mix into the multiplexed sample (total staining volume 100 μL) and gently mix.
93. Incubate for 30 min at room temperature.

⚠ CRITICAL: During the incubation, gently vortex the multiplexed sample every 10 min to prevent cell clumping.

94. Add 2 mL of Maxpar® CSB to the multiplexed sample and centrifuge at 1,000×*g* for 7 min.
95. Proceed with "Cytoplasmic protein staining".

Cytoplasmic protein staining

Timing: ~1 h 45 min

In this part of the protocol, the multiplexed sample is processed for cytoplasmic protein staining using eBioscience™ Foxp3/Transcription Factor Staining Buffer Set, and labeled with cytoplasmic antibody mix.

We adapted this section of the protocol from "BestProtocols: Staining Intracellular Antigens for Flow Cytometry, Protocol B: one step protocol: intracellular (nuclear) proteins". https://www.thermofisher.com/uk/en/home/references/protocols/cell-and-tissue-analysis/protocols/staining-intracellular-antigens-flow-cytometry.html

96. Aspirate off the supernatant and pulse vortex the multiplexed sample to disaggregate the cell pellet.
97. Prepare the Foxp3 Fixation/Permeabilization solution by diluting the Fixation/Permeabilization Concentrate 1:4 into the eBioscience™ Fixation/Permeabilization Diluent.

Note: The following volumes are recommended for the staining of 1–3 million cells. If staining a larger number of cells, scale up these volumes.

98. Aliquot 1 mL of Foxp3 Fixation/Permeabilization solution to the multiplexed sample and pulse vortex.
99. Incubate for 30 min at room temperature in the dark.

Table 3. Cytoplasmic antibody mix

Marker	Metal isotope	Clone	Volume (μL) for 1 sample
GM-CSF	159-Tb	BVD2-21C11	1.50
Granzyme B	173-Yb	GB11	1.50
IFN-γ	158-Gd	B27	1.50
IL-17A	164-Dy	N49-653	1.50
IL-2	166-Er	MQ1-17H12	1.50
mCherry	155-Gd	16D7	1.00
Perforin B	175-Lu	B-D48	1.50
TGF-β	163-Dy	TW4-6H10	1.50
TNF-α	152-Sm	Mab11	1.50
1× Perm Buffer	n/a	n/a	37.00
Total	**n/a**	**n/a**	**50.00**

Note: In case of perm-sensitive isotopes, incubate for 30 min at 4°C in the dark.

100. During the incubation, dilute the 10× Permeabilization Buffer (Perm Buffer) 1:10 into Maxpar® Water (final concentration 1×) and prepare the cytoplasmic antibody mix (Table 3).

⚠ CRITICAL: Vortex and quickly spin (110×*g* for 30 s) each antibody before preparing the mix.

101. Aliquot 2 mL of 1× Perm Buffer to the multiplexed sample and centrifuge at 1,000×*g* for 7 min at room temperature.
102. Aspirate off the supernatant and repeat step 101.
103. Discard the supernatant and resuspend the cell pellet in 50 μL of 1× Perm Buffer.
104. Aliquot 50 μL of cytoplasmic antibody mix into the multiplexed sample (total staining volume 100 μL), gently vortex and incubate for 30 min at room temperature.

⚠ CRITICAL: During the incubation, gently vortex the multiplexed sample every 10 min to prevent cell clumping.

105. Wash the multiplexed sample by adding 2 mL of 1× Perm Buffer and centrifuge it at 1,000×*g* for 7 min at room temperature.
106. During the incubation, prepare 50% Methanol in Maxpar® PBS and store it at −20°C until its use in the step 111. Store an aliquot of Maxpar® CSB at 4°C until its use in step 113.
107. Aspirate off the supernatant and wash the cells with 2 mL of Maxpar® CSB.
108. Centrifuge at 1,000×*g* for 7 min at room temperature, discard the supernatant and resuspend the cells in the residual volume by gently vortexing.
109. Proceed with "Phosphoprotein and nuclear protein staining".

Phosphoprotein and nuclear protein staining

Timing: ~1 h 20 min

In this part of the protocol, the multiplexed sample is fixed in ice-cold 50% Methanol and stained for phosphoprotein and nuclear protein detection.

We adapted this section of the protocol from "Stain Cells with Phosphoprotein Antibodies" section from "Maxpar Phosphoprotein Staining with Fresh Fix" protocol on https://www.fluidigm.com/binaries/content/documents/fluidigm/resources/maxpar-phosphoprotein-staining-with-fresh-fix/maxpar-phosphoprotein-staining-with-fresh-fix/fluidigm%3Afile

Table 4. Phosphoprotein and nuclear antibody mix

Marker	Metal isotope	Clone	Volume (μL) for 1 sample
Cleaved Caspase 3 [D157]	142-Nd	D3E9	1.00
Cyclin B1	153-Eu	GNS-1	1.25
Foxp3	162-Dy	PCH101	1.50
NFAT1	143-Nd	D43B1	1.50
p4E-BP1 [T37/T46]	149-Sm	236B4	1.25
pAMPKa [T172]	160-Gd	40H9	2.50
pBAD [S112]	161-Dy	40A9	1.25
pBTK [Y551]	147-Sm	24a/BTK	3.00
pCREB [S133]	176-Yb	87G3	1.50
pERK1/2 [T202/Y204]	167-Er	20A	0.75
pHistone H2A.X [S139]	209-Bi	JBW301	2.00
pHistone H3 [S28]	89-Y	HTA28	1.50
pMKK3 [S189]/MKK6 [S207]	157-Gd	D8E9	1.00
pp38 [T180/Y182]	156-Gd	D3F9	1.50
pPDPK1 [S241]	141-Pr	J66–653.44.22	2.50
pRB [S807/S811]	150-Nd	J112-906	1.50
pS6 [S235/S236]	172-Yb	N7-548	1.50
pSRC [Y418]	148-Nd	SC1T2M3	2.50
pZAP70 [Y319]	171-Yb	17a	1.50
CSB	n/a	n/a	19.00
Total	**n/a**	**n/a**	**50.00**

Note: In this section of the protocol, use ice-cold reagents and keep the sample on ice.

Note: The following volumes are recommended for the staining of 1–3 million cells. If staining a larger number of cells, scale up these volumes.

110. Incubate the multiplexed sample on ice for 1 min.
111. Aliquot 1 mL of ice-cold 50% Methanol to the multiplexed sample, gently mix and incubate for 10 min on ice.
112. During the incubation, prepare the phosphoprotein and nuclear antibody mix (Table 4).

⚠ CRITICAL: Vortex and quickly spin (110×*g* for 30 sec) each antibody before preparing the mix.

113. Add 2 mL of ice-cold Maxpar® CSB to the multiplexed sample and centrifuge it at 1,000×*g* for 7 min.
114. Discard the supernatant and repeat step 113.
115. Discard the supernatant and gently vortex to resuspend the cells in the residual volume (approximately 50 μL).
116. Add 50 μL of phosphoprotein and nuclear antibody mix to the cells (total staining volume 100 μL). Mix well and incubate for 30 min on ice.

⚠ CRITICAL: During the incubation, gently vortex the multiplexed sample every 10 min to prevent cell clumps.

117. Add 2 mL of ice-cold Maxpar® CSB to the multiplexed sample and centrifuge it at 1,000×*g* for 7 min.
118. Discard the supernatant and repeat step 117.
119. Gently vortex to resuspend the cells in the residual volume.
120. Proceed with "Cell fixation and DNA staining with Cell-ID™ Intercalator-Ir".

Cell fixation and DNA staining with Cell-ID™ Intercalator-Ir

Timing: ~25 min

In this part of the protocol, the multiplexed sample is fixed to ensure that antibodies are firmly attached onto the cells and to allow the subsequent staining with iridium (Cell-ID™ Intercalator-Ir), a nucleic acid intercalator that labels nucleated cells. The intensity of its signal, detected in the 191/193Ir channel of CyTOF, allows to distinguish singlets (with lower DNA content) from doublets/aggregates (with higher DNA content). The following part of the protocol is adapted from "Fix Cells" and "Stain Cells with Cell-ID Intercalator-Ir" sections from "Maxpar Phosphoprotein Staining with Fresh Fix" protocol on https://www.fluidigm.com/binaries/content/documents/fluidigm/resources/maxpar-phosphoprotein-staining-with-fresh-fix/maxpar-phosphoprotein-staining-with-fresh-fix/fluidigm%3Afile

Note: The following volumes are recommended for the staining of 1–3 million cells. If staining a larger number of cells, scale up these volumes.

121. Prepare 1.6% FA by diluting 16% FA in PBS (1:10 dilution). For example, dilute 0.2 mL of 16% FA in 1.98 mL of PBS.
122. Aliquot 1 mL of freshly prepared 1.6% FA into the tube after having vortexed it.
123. Gently vortex to mix well.
124. Incubate for 10 min at room temperature on a rocker.
125. During the incubation, equilibrate at room temperature the Cell-ID™ Intercalator-Ir.
126. Wash the sample with 1 mL of Maxpar® CSB and centrifuge it at 1,000×*g* for 7 min at room temperature.
127. Discard the supernatant and gently vortex to resuspend the cells in the residual volume.
128. Stain the sample with the Cell-ID™ Intercalator-Ir.
    a. Make an intercalator solution by diluting the Cell-ID™ Intercalator-Ir 1:1,000 with Maxpar® Fix and Perm buffer (final concentration 125 nM, e.g., 1 µL of Cell-ID™ Intercalator-Ir 125 µM in 999 µL of Maxpar® Fix and Perm buffer).
    b. Vortex the intercalator solution.
    c. Aliquot 1 mL of intercalator solution into the sample and vortex.
    d. Incubate overnight (16–18 h) at 4°C.

Alternatives: The sample can be incubated with the intercalator solution for 1 h at room temperature.

However, as intercalation is a slow process, short incubation with the intercalator solution can affect the quality of the iridium staining resulting in less tight iridium signals (DNA peaks).

129. The following day, proceed with the "Sample acquisition" step.

Pause point: Samples can be stored at 4°C in intercalator solution for 48 h until their acquisition. For longer storage, after step 127 perform an additional wash with 200 µL of Maxpar® CSB and store pelleted cells at 4°C.

Note: If staining more multiplexed samples, prepare the intercalator solution separately for each sample.

Sample acquisition

Timing: ~ 1h (sample preparation and acquisition)

In this part of the protocol, the multiplexed sample is prepared for acquisition at an Helios™ (WB Injector) mass cytometer equipped with the Super Sampler unit. EQ™ Four Element Calibration Beads are spiked in to control the instrument performance and to ensure inter-experiment comparability.

The following part of the protocol is adapted from "Prepare Cells for Acquisition" section from "Maxpar Phosphoprotein Staining with Fresh Fix" protocol on https://www.fluidigm.com/binaries/content/documents/fluidigm/resources/maxpar-phosphoprotein-staining-with-fresh-fix/maxpar-phosphoprotein-staining-with-fresh-fix/fluidigm%3Afile

Note: Keep the reagent and the cells at this stage of the protocol at 4°C until acquisition.

Note: The following volumes are recommended for 1–3 million cells. If processing a larger number of cells, scale up the volumes.

130. Add 2 mL of Maxpar® Cell Acquisition Solution (CAS) to the multiplexed sample and centrifuge at 1,200×*g* for 5 min.
131. Discard the supernatant and resuspend the cell pellet in the residual volume by vortexing.
132. Repeat step 130, aspirate off the supernatant and resuspend the cells in 1 mL of CAS.
133. Take an aliquot of resuspended cells for the count.
134. Dilute the cells in CAS to a concentration of 0.5–1×10^6/mL, if needed.
135. Carefully mix the EQ™ Four Element Calibration Beads.
136. Add the EQ™ Four Element Calibration Beads to the sample at a 1:5 bead:cell ratio.
137. Filter the sample into a polypropylene FACS tube using a 35 μm cell strainer cap and vortex it immediately before acquisition.
138. Acquire the sample for 10,000 s at a flow rate of < 600 events/s. The acquisition takes 45 min for a sample with less than 1 × 10^6 cells (0.6–0.95 × 10^6 cells).

Note: The acquisition will take longer if acquiring a sample with larger number of cells.

Note: See troubleshooting 5.

Note: If analyzing more multiplexed samples on the same day, process and acquire one multiplexed sample at a time. Samples can be stored as cell pellet in Maxpar® CSB at 4°C before step 130–138.

139. Normalize the acquired FCS files against the EQ™ Four Element Calibration Beads and debarcode them using Fluidigm CyTOF Software.

EXPECTED OUTCOMES

This protocol allows for the molecular characterization of CAR T cell products by mass cytometry.

Here, CAR T cells are identified based on the expression of a reporter gene (mCherry) present in the lentiviral vector construct used to transduce T cells. mCherry expression is a proxy for CAR expression (Figure 4A).

CAR T cell products can be further investigated for the proportions of different cell subsets (e.g., CD4 and CD8 subpopulations) (Figure 4B).

The proportions of CD3, CD4 and CD8 cells detected by mass cytometry are confirmed by parallel flow cytometry analysis (Figure 5).

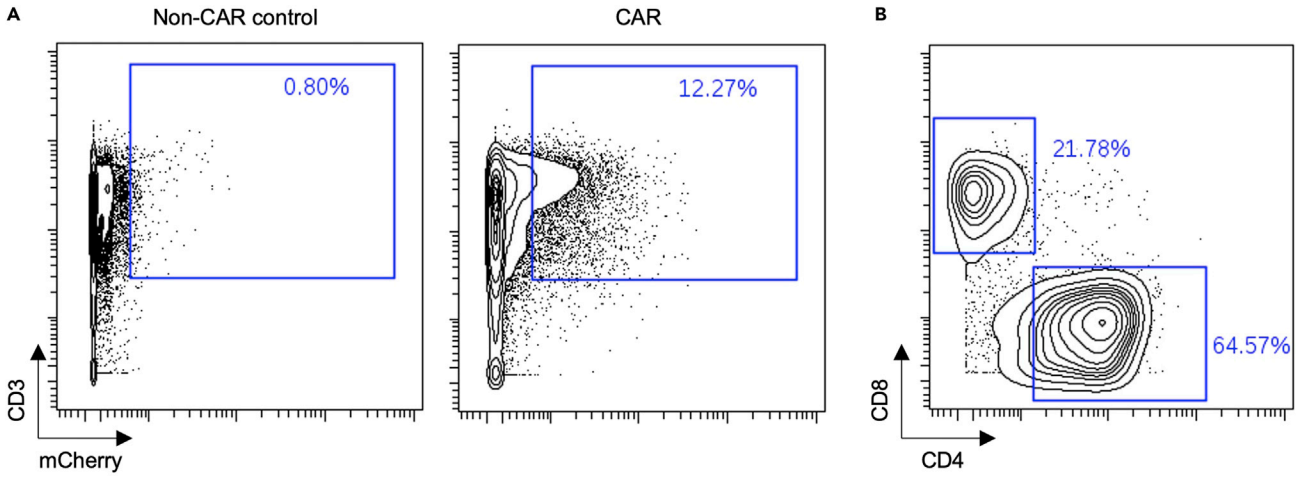


Figure 4. CAR T cell identification and immunophenotyping by mass cytometry

(A) Representative mass cytometry plots for CD3 and mCherry expression in unstimulated non-CAR control and FMC63 CAR T cells. CAR T cells were gated on CD3+mCherry+ live cells.

(B) Representative mass cytometry plots showing CD4 and CD8 composition in unstimulated FMC63 CAR T cells.

In this protocol, CAR T cell activation can be assessed by measuring the expression of early (CD25, CD69) and late (HLA-DR) activation markers.

While NALM6 stimulation does not affect CD25 levels in non-CAR controls, anti-CD19 CAR T cells display a marked increase in the expression of CD25 upon stimulation, as compared to the unstimulated condition (Figure 6).

CAR T cell functional phenotype, including proliferation, cytokine production and cytotoxic potential, can also be assessed using this protocol.

In the proposed antibody panel, we included several indicators of proliferation/mitosis, such as pRB, pHistone H3 and Cyclin B1. Moreover, the measurement of IdU DNA incorporation (performed in step 48) serves as a functional proliferation assay quantifying the proportion of cells in S-phase of the cell cycle. The incorporated IdU can be measured as a single marker (Figure 7 Top) or in combination with Cyclin B1 to dissect the different phases of the cell cycle (Figure 7 Bottom).

Antigen stimulation induces CAR T cell proliferation as shown by the increased proportion of cells having incorporated IdU as compared to the unstimulated condition (Figure 7).

Mass cytometry applied to heterotypic co-cultures allows the simultaneous characterization of effector (CAR T) and target (CD19+ NALM6) cells, distinguished based on the expression of cell type specific markers. For instance, the antibody panel proposed includes both pan T cell (e.g., CD3) and target-cell specific (CD19, CD22) markers.

Although cytotoxic assays are generally performed using non-irradiated target-cells (MacLeod et al., 2017; Kiesgen et al., 2021), in our protocol, the quantification of live target-cells (CD19+) after CAR T cell and non-CAR control co-culture can be a surrogate of CAR T cell killing potential. If using irradiated target-cells, irradiation-derived cell death needs to be accounted for by using the appropriate non-CAR control (Figure 8 Top).

In addition, measuring the production of effector cytokines (e.g., Granzyme B and Perforin B) can provide further evidence for CAR T cell cytotoxicity (Figure 8 Bottom).

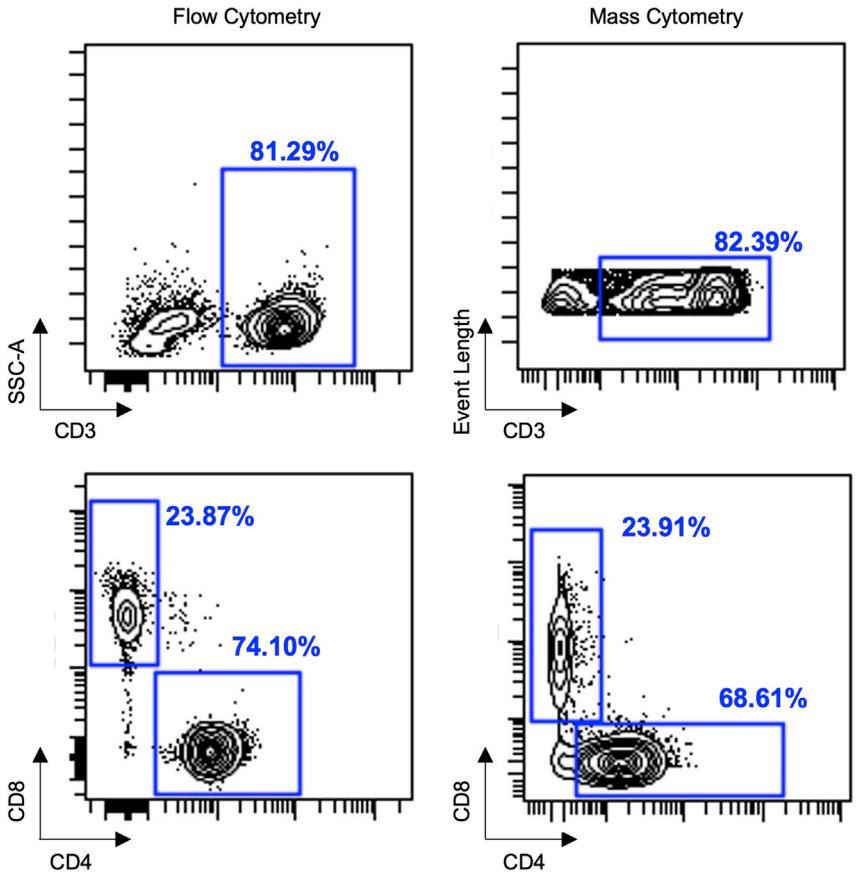


Figure 5. Comparison of flow cytometry and mass cytometry capability to identify cell populations
Representative flow cytometry and mass cytometry plots for CD3, CD4 and CD8 expression in non-CAR controls.

Lastly, this protocol can be applied to comprehensively evaluate the effect that drugs or inhibitors of interest have on CAR T cell signaling and functionality.

For example, upon stimulation, we identified a subset of CAR T cells (CD3+mCherry+) also expressing their target antigen CD19 (Figure 9A). The transfer of CAR target molecules from the surface of the target-cells onto the CAR T cell membrane is a process known as trogocytosis and it is associated with CAR T cell activation and fratricide killing (Hamieh et al., 2019). To demonstrate that the CD19+ CAR T cells identified here were indeed trogocytic cells and not a consequence of a staining artifact, we treated CAR T cells with Latrunculin A, an inhibitor of actin polymerization (required for trogocytosis) (Martínez-Martín et al., 2011).

Upon treatment with Latrunculin A, CD19+ CAR T cell frequencies significantly drop (mean treated vs untreated: 12.74% vs 39.77%, $P < 0.05$), confirming that we are capturing a subset of CAR T cells undergoing trogocytosis (Figure 9B).

QUANTIFICATION AND STATISTICAL ANALYSIS

The debarcoded fcs files generated are analyzed using the Cytobank software. Alternatively, FlowJo™ software (BD Biosciences) can be used.

The first step of mass cytometry data analysis is data clean-up, to manually gate viable single cells based on event length, Gaussian parameters (e.g., Center, Offset and Residual), DNA content (193-Ir DNA) and Cisplatin signal (194-Pt) (Lee and Rahman, 2019; Bagwell et al., 2020).

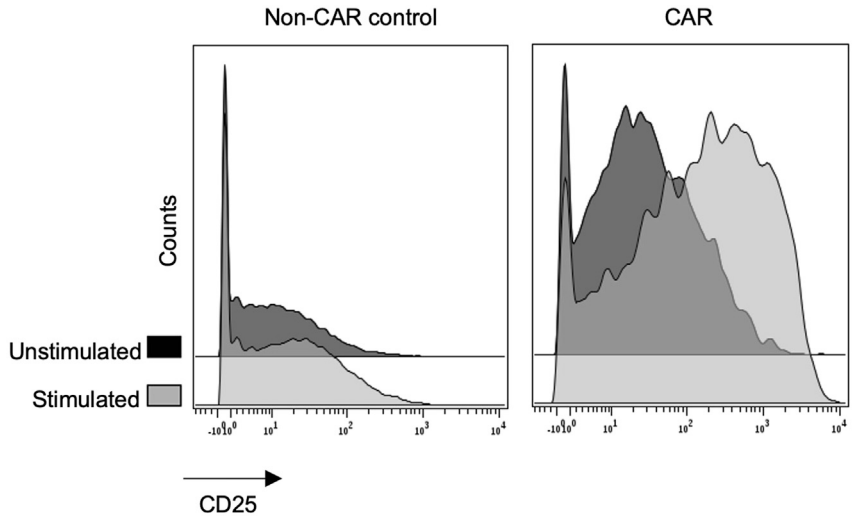


Figure 6. CAR T cell activation

Representative histograms showing CD25 expression in non-CAR control and FMC63 CAR T cells in unstimulated (dark gray) and stimulated (light gray) conditions. Non-CAR control and CAR T cells were gated on live CD3+ and CD3+mCherry+ cells, respectively.

Subsequently, cell subsets can be identified by manual gating strategies or by computational approaches (Chester and Maecker, 2015).

To compare biomarker expression levels between samples (e.g., CAR T cells vs control cells, unstimulated vs stimulated conditions), Earth Mover's Distance (EMD) analysis represents a robust and valid approach (Orlova et al., 2016).

LIMITATIONS

This protocol requires the use of a hematology automated analyzer/automated cell counter and of one Helios™ (WB Injector) mass cytometer equipped with the Super Sampler unit (to minimize line clogs). If these instruments are not available, the duration of the entire protocol may be longer than estimated.

This protocol successfully generates CAR T cells from unfractionated donor-derived PBMCs. However, non T cell PBMCs [e.g., monocytes (Noaks et al., 2021) or natural killer-cells (Singh et al., 2013)] can affect CAR T cell production and/or functionality.

Moreover, inter-individual variability in T cell expansion and transduction efficiency further impacts CAR T cell manufacturing process (Noaks et al., 2021; Brentjens et al., 2011).

In this protocol, a pre-stain Pd barcoding is performed to reduce sample staining and acquisition variability, and reagent/plastic consumption. However, this procedure requires cell fixation and permeabilization steps and can affect the expression of cell surface markers sensitive to these treatments (Amir et al., 2019; Dzangué-Tchoupou et al., 2018).

The preparation of fresh staining mixes for each experiment, as indicated in this protocol, may be time-consuming and associated with batch effects. It is possible to produce large batches of antibody mixes in advance and cryopreserve them into single-use aliquots (Schulz et al., 2019).

TROUBLESHOOTING

Problem 1

Poor T cell activation with anti-CD3/CD28 Dynabeads™ (steps 14–19, 30).

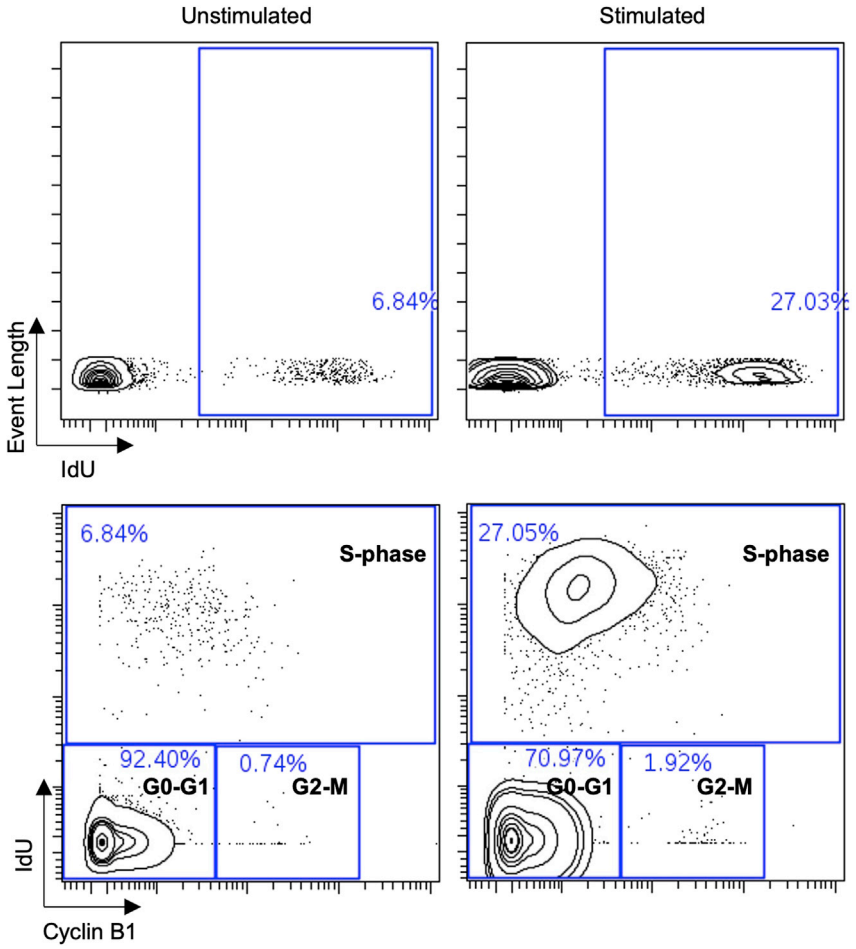


Figure 7. CAR T cell proliferative potential measurement and cell cycle analysis by mass cytometry

Top. Representative mass cytometry plots for proliferating cells (IdU+) in unstimulated and stimulated FMC63 CAR T cells. Bottom. Representative mass cytometry plots of IdU vs Cyclin B1 showing the cell cycle phases (G0-G1, IdU- Cyclin B1-; G2-M, IdU- Cyclin B1+; S-phase, IdU+) in unstimulated and stimulated FMC63 CAR T cells.

Potential solution

It is important to check the Dynabead™ concentration and the cell:bead ratio used in the experiment. Moreover, carefully mix the Dynabeads™ before their use to uniformly resuspend them. Carefully and gently mix the Dynabeads™ with the cell suspension, and gently rotate the plate seeded with cell:bead mixture before starting the incubation. Furthermore, evaluate the percentage of monocytes in the isolated PBMCs as they adhere to/engulf Dynabeads decreasing the T cell:bead contact and consequently T cell activation (Noaks et al., 2021). If it is the case, proceed with monocyte-depletion of the isolated PBMCs before starting CAR T cell production.

Problem 2

Low T cell transduction levels and variability in T cell activation and expansion in culture (step 30).

Potential solution

If the transduction level under the microscope seems low (if a fluorescent reporter is present in the CAR vector), evaluate the level of expression of the CAR or of the reporter protein at FACS before proceeding with mass cytometry sample preparation. If low transduction is confirmed (e.g., less than 10%), restart the experiment.

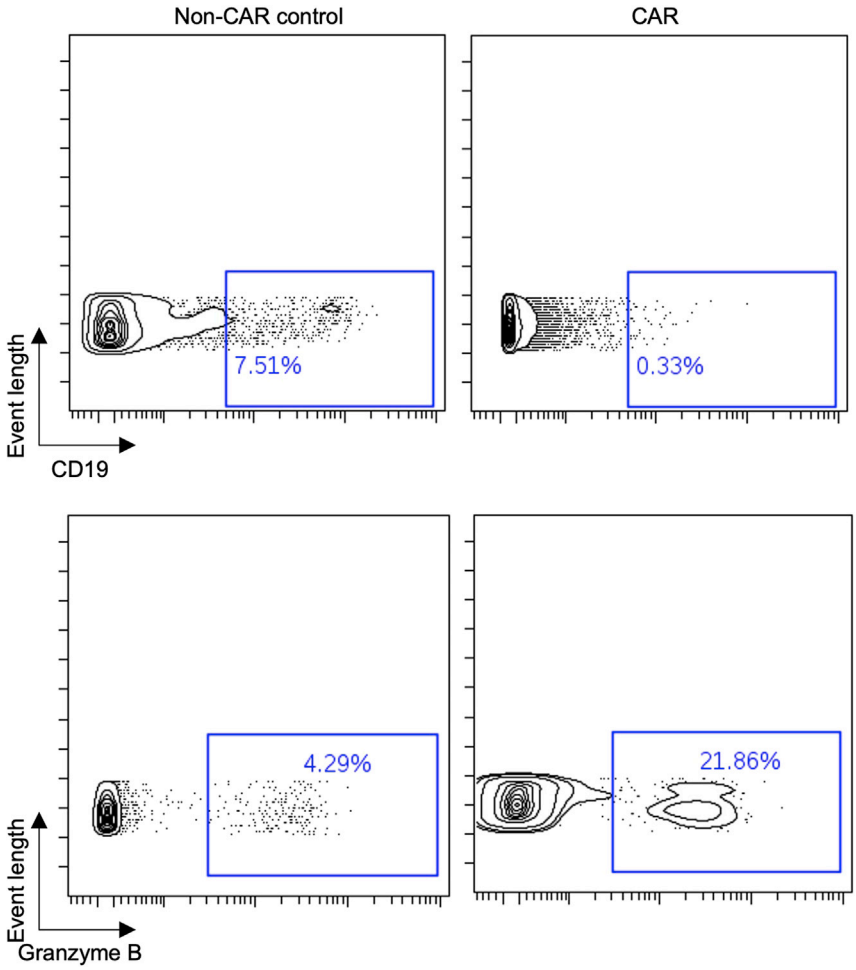


Figure 8. CAR T cell cytotoxic potential assessed by mass cytometry

Top. Representative mass cytometry plots showing the percentage of target cells (CD19+) in stimulated non-CAR control and FMC63 CAR T cell conditions. Bottom. Representative mass cytometry plots showing effector cytokine production (specifically Granzyme B) in stimulated non-CAR control and FMC63 CAR T cells. Non-CAR control and CAR T cells were gated on live CD3+ and CD3+mCherry+ cells, respectively.

Moreover, cell expansion and activation can be affected by inter-donor variability (see the "limitations" section for more information) or by the CAR construct used.

Problem 3

Surface marker expression detection affected by pre-stain Pd barcoding (steps 53–60; 71–84).

Potential solution

If interested in the detection of surface markers sensitive to fixation and permeabilization, it is recommended to proceed with a post-staining Pd barcoding. Alternatively, it is possible to use a fixation and permeabilization-free barcoding system, the Live-cell Cd-CD45 barcoding (Fluidigm) before antibody staining.

Problem 4

Failure of staining post-fixation (steps 53–120).

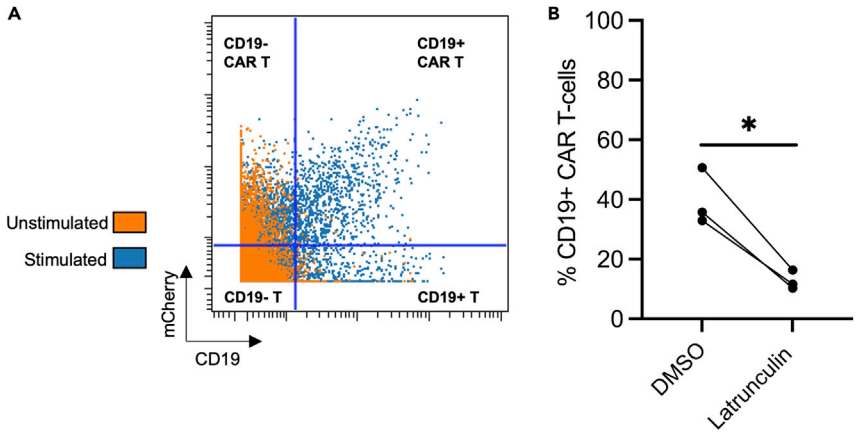


Figure 9. Effect of trogocytosis inhibition on CD19+ CAR T cells assessed by mass cytometry

(A) Representative mass cytometry plot for CD19 staining in unstimulated (orange) and stimulated (blue) CD3+ cells 24 h post-stimulation. mCherry expression discriminates between T- (mCherry-, lower quadrants) and CAR T cells (mCherry +, upper quadrants) in FMC63 condition.

(B) CAR T cells were treated with 1 μM Latrunculin A (Sigma Aldrich) at 37°C for 15 min before co-culture with the target cells, as reported in (Hamieh et al., 2019). Spaghetti plot showing the percentages of CD19+ CAR T cells in untreated (DMSO) and treated (Latrunculin) conditions at 4 h post-stimulation. Statistical significance was calculated by Paired T-test, $p < 0.05$. n = 3 HDs, n = 1 independent experiment.

Potential solution

If performing antibody staining post-fixation, we suggest titrating the antibodies on fixed cells to determine the appropriate dilution of the antibodies against epitopes that had been modified by fixation. Antibody binding efficiencies may be affected by protein conformational changes occurring after fixation, potentially requiring to adjust antibody dose or to switch to a clone binding a different epitope. If staining post-fixation is not successful, the antibody may need to be removed from the panel or used prior to fixation.

Problem 5

Cell loss post sample viability staining and barcoding (CyTOF1 53–71%, CyTOF2 1–56%), and antibody and DNA staining (CyTOF1 71–78%, CyTOF2 46–92%) (steps 61–133).

Potential solution

The mass cytometry sample barcoding and staining protocol proposed here requires several washing steps that can contribute to cell loss. Check the presence of cells in the supernatant. To minimize cell loss, we recommend aspirating and not decanting the cell supernatant after centrifugation, using 15-mL rather than 50 mL tubes (if necessary, try to use V-bottom plates), and centrifuging cells at higher speed or for longer.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr Alice Giustacchini (a.giustacchini@ucl.ac.uk).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate datasets/code.

ACKNOWLEDGMENTS

We thank P.J.A. and C.J.T. for providing reagents. This work is partly funded by the NIHR GOSH BRC. The views expressed are those of the authors and not necessarily those of the NHS, the NIHR, or the Department of Health. Part of this work was supported by the Leukaemia UK John Goldman Fellowship to A.G. (2018/JGF/003), the Rosetrees Trust fund to A.G. (M700), and the Academy of Medical Sciences Springboard Award to A.G. (SBF004\1025). The graphical abstract and Figure 2 have been created in Biorender.com.

AUTHOR CONTRIBUTIONS

Conceptualization, I.M.M and A.G.; methodology, I.M.M., J.S., and T.A.A.; investigation, I.M.M. and A.G.; resources, P.J.A., C.J.T., and A.G.; writing – original draft, I.M.M.; visualization, I.M.M.; supervision, C.J.T. and A.G.; project administration and funding acquisition, A.G.

DECLARATION OF INTERESTS

T.A.A. is currently an employee of Fluidigm.

REFERENCES

Amir, E.D., Lee, B., Badoual, P., Gordon, M., Guo, X.V., Merad, M., and Rahman, A.H. (2019). Development of a comprehensive antibody staining database using a standardized analytics pipeline. Front Immunol. *10*, 1315.

Bagwell, C.B., Inokuma, M., Hunsberger, B., Herbert, D., Bray, C., Hill, B., Stelzer, G., Li, S., Kollipara, A., Ornatsky, O., et al. (2020). Automated data cleanup for mass cytometry. Cytometry A *97*, 184–198.

Brentjens, R.J., Rivière, I., Park, J.H., Davila, M.L., Wang, X., Stefanski, J., Taylor, C., Yeh, R., Bartido, S., Borquez-Ojeda, O., et al. (2011). Safety and persistence of adoptively transferred autologous CD19-targeted T cells in patients with relapsed or chemotherapy refractory B-cell leukemias. Blood *118*, 4817–4828.

Chester, C., and Maecker, H.T. (2015). Algorithmic tools for mining high-dimensional cytometry data. J. Immunol. *195*, 773–779.

Corneau, A., Parizot, C., Cherai, M., Todesco, E., Blanc, C., Litvinova, E., Nguyen, S., Roos-Weil, D., Guihot, A., and Norol, F. (2021). Mass cytometry: a robust platform for the comprehensive immunomonitoring of CAR-T-cell therapies. Br. J. Haematol. *194*, 788–792.

Dzangué-Tchoupou, G., Corneau, A., Blanc, C., Benveniste, O., and Allenbach, Y. (2018). Analysis of cell surface and intranuclear markers on non-stimulated human PBMC using mass cytometry. PLoS ONE *13*, e0194593.

Fisher, J., Sharma, R., Don, D.W., Barisa, M., Hurtado, M.O., Abramowski, P., Porter, L., Day, W., Borea, R., Inglott, S., et al. (2019). Engineering gammadeltaT cells limits tonic signaling associated with chimeric antigen receptors. Sci. Signal *12*, eaax1872.

Ghorashian, S., Kramer, A.M., Onuoha, S., Wright, G., Bartram, J., Richardson, R., Albon, S.J., Casanovas-Company, J., Castro, F., Popova, B., et al. (2019). Enhanced CAR T cell expansion and prolonged persistence in pediatric patients with ALL treated with a low-affinity CD19 CAR. Nat. Med. *25*, 1408–1414.

Hamieh, M., Dobrin, A., Cabriolu, A., van der Stegen, S.J.C., Giavridis, T., Mansilla-Soto, J., Eyquem, J., Zhao, Z., Whitlock, B.M., Miele, M.M., et al. (2019). CAR T cell trogocytosis and cooperative killing regulate tumour antigen escape. Nature *568*, 112–116.

Kiesgen, S., Messinger, J.C., Chintala, N.K., Tano, Z., and Adusumilli, P.S. (2021). Comparative analysis of assays to measure CAR T-cell-mediated cytotoxicity. Nat. Protoc. *16*, 1331–1342.

Lee, B.H., and Rahman, A.H. (2019). Acquisition, processing, and quality control of mass cytometry data. Methods Mol. Biol. *1989*, 13–31.

MacLeod, D.T., Antony, J., Martin, A.J., Moser, R.J., Hekele, A., Wetzel, K.J., Brown, A.E., Triggiano, M.A., Hux, J.A., Pham, C.D., et al. (2017). Integration of a CD19 CAR into the TCR alpha chain locus streamlines production of allogeneic gene-edited CAR T cells. Mol. Ther. *25*, 949–961.

Martínez-Martín, N., Fernández-Arenas, E., Cemerski, S., Delgado, P., Turner, M., Heuser, J., Irvine, D.J., Huang, B., Bustelo, X.R., Shaw, A., et al. (2011). T cell receptor internalization from the immunological synapse is mediated by TC21 and RhoG GTPase-dependent phagocytosis. Immunity *35*, 208–222.

Medvec, A.R., Ecker, C., Kong, H., Winters, E.A., Glover, J., Varela-Rohena, A., and Riley, J.L. (2018). Improved expansion and in vivo function of patient t cells by a serum-free medium. Mol. Ther. Methods Clin. Dev. *8*, 65–74.

Noaks, E., Peticone, C., Kotsopoulou, E., and Bracewell, D.G. (2021). Enriching leukapheresis improves T cell activation and transduction efficiency during CAR T processing. Mol. Ther. Methods Clin. Dev. *20*, 675–687.

Orlova, D.Y., Zimmerman, N., Meehan, S., Meehan, C., Waters, J., Ghosn, E.E.B., Filatenkov, A., Kolyagin, G.A., Gernez, Y., Tsuda, S., et al. (2016). Earth Mover's distance (EMD): a true metric for comparing biomarker expression levels in cell populations. PLoS ONE *11*, e0151859.

Schulz, A.R., Baumgart, S., Schulze, J., Urbicht, M., Grützkau, A., and Mei, H.E. (2019). Stabilizing antibody cocktails for mass cytometry. Cytometry A *95*, 910–916.

Singh, H., Figliola, M.J., Dawson, M.J., Olivares, S., Zhang, L., Yang, G., Maiti, S., Manuri, P., Senyukov, V., Jena, B., et al. (2013). Manufacture of clinical-grade CD19-specific T cells stably expressing chimeric antigen receptor using Sleeping Beauty system and artificial antigen presenting cells. PLoS ONE *8*, e64138.

Vormittag, P., Gunn, R., Ghorashian, S., and Veraitch, F.S. (2018). A guide to manufacturing CAR T cell therapies. Curr. Opin. Biotechnol. *53*, 164–181.