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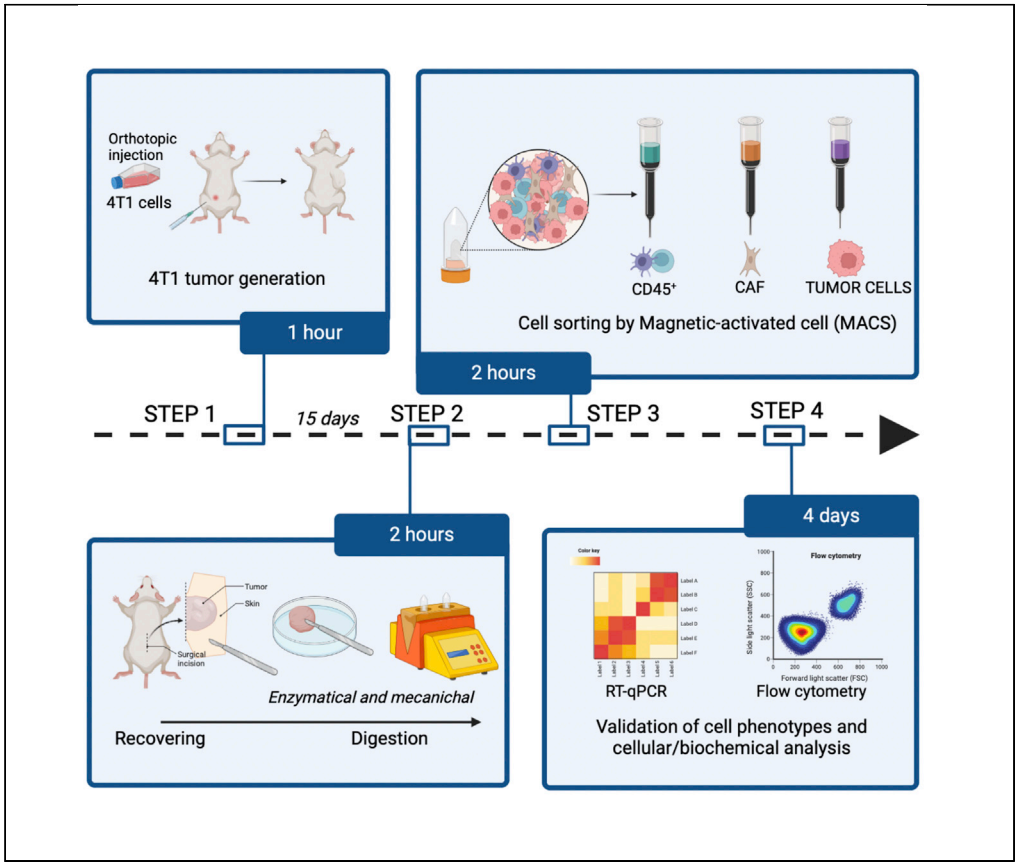
Simultaneous isolation of CD45 tumor-infiltrating lymphocytes, tumor cells, and associated fibroblasts from murine breast tumor model by MACS



The study of the tumor microenvironment (TME) and its interactions with cancer cells is an important issue in cancer research. Here, we present a protocol to sort three important cell populations from murine triple negative breast cancer 4T1 model TME, including CD45$^+$ tumor-infiltrating lymphocytes, cancer-associated fibroblasts, and tumor cells. The protocol includes four steps: generation of 4T1 tumors, tumor collection and digestion, magnetic sorting of the different populations, and phenotypic validation of sorted cells.

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

Laura Kalfeist, Stacy Petit, Loïck Galland, François Ghiringhelli, Sylvain Ladoire, Emeric Limagne

elimagne@cgfl.fr

Highlights

Protocol to isolate CD45$^+$ TILs, CAFs, and tumor cells from a mouse breast tumor model

Steps to generate 4T1 tumors and subsequent tumor collection and digestion

Cell staining and magnetic-activated cell sorting to obtain the three cell populations

Phenotypic validation of sorted cells by flow cytometry and RT-qPCR

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Laura Kalfeist,[1,3,4,5,6] Stacy Petit,[1,3,4,5] Loïck Galland,[1,2,3,4,5] François Ghiringhelli,[1,2,3,4,5] Sylvain Ladoire,[1,2,3,4,5] and Emeric Limagne[1,3,4,5,7,*]

[1]University Bourgogne Franche-Comté, 21000 Dijon, France

[2]Department of Medical Oncology, Centre Georges-François Leclerc, 21000 Dijon, France

[3]Cancer Biology Transfer Platform, Centre Georges-François Leclerc, 1 rue du professeur Marion, 21000 Dijon, France

[4]Centre de Recherche INSERM LNC-UMR1231, 21000 Dijon, France

[5]Genetic and Immunology Medical Institute, Dijon, France

[6]Technical contact: lkalfeist@cgfl.fr

[7]Lead contact

*Correspondence: elimagne@cgfl.fr
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SUMMARY

The study of the tumor microenvironment (TME) and its interactions with cancer cells is an important issue in cancer research. Here, we present a protocol to sort three important cell populations from murine triple negative breast cancer 4T1 model TME, including CD45$^+$ tumor-infiltrating lymphocytes, cancer-associated fibroblasts, and tumor cells. The protocol includes four steps: generation of 4T1 tumors, tumor collection and digestion, magnetic sorting of the different populations, and phenotypic validation of sorted cells.

For complete details on the use and execution of this protocol, please refer to Limagne et al. (2022).[1]

BEFORE YOU BEGIN

Institutional permissions

This protocol needs tumor tissue derived from 4T1 breast cancer model. Ethical approvals are required. All procedures were done according to institutional guidelines under protocols approved by the animal care committee of University of Burgundy (APAFIS #29739-2021021014331042).

Murine tumor models

The murine tumor models used in this protocol are generated by subcutaneous injection of 4T1 tumor cells into the mammary fat pad of female BALB/c syngeneic mice. This protocol will be described in a mouse model of triple negative breast cancer. Nevertheless, this protocol has been tested in part on another tumor model. Indeed, the first step of this protocol : the CD45$^+$ TILs sorting, has been performed also on Lewis LLC1 mouse model representative of lung cancer.[1]

Note: To ensure a competent immune system, we recommend using BALB/c mice between 6 and 8 weeks old.

Cell line preparation

Timing: 4 days

Table 1. Preparation of the 4T1 culture medium

Reagent	Final concentration	Amount
Roswell Park memorial institute 1640 (RPMI 1640)	–	445 mL
Fetal bovin serum (filtered with 0.2 μm filter)	10%	50 mL
penicillin-streptomycin-fungizone (PSA)	1%	5 mL

1. Grow a flask of 4T1 cells in Roswell Park memorial institute 1640 (RPMI 1640) medium with fetal bovine serum (FBS) and penicillin-streptomycin-fungizone (PSA) at moderate density (50/60% of confluence) and ensure a cell viability of at least 90% before injection in mice.

Reagents and buffer preparation

Timing: 20 min

2. Prepare the medium for 4T1 cells by adding Fetal bovin serum (FBS) (10%) and penicillin-streptomycin-fungizone (PSA) (1%) at Roswell Park memorial institute 1640 (RPMI 1640) medium (Table 1).
3. Prepare 1× trypsin solution by adding 5 mL of 10× trypsin solution to 45 mL of Hank's balanced salt solution (HBSS) in falcon tube (Table 2).
4. Put the autoMACS (magnetic-activated cell sorting) buffer at 4°C during the sorting run and keep it on ice throughout the sorting process.
5. Keep the flow cytometry staining buffer at 4°C in the fridge until staining.

Prepare tubes and tools

Timing: 5 min

6. Prepare and annotate 6 falcon tubes (15 mL) with the following annotations:
   a. Falcon 1: Tumor suspension 1#.
   b. Falcon 2: Tumor suspension 2#.
   c. Falcon 3: Fraction A.
   d. Falcon 4: Fraction B.
   e. Falcon 5: Fraction C.
   f. Falcon 6: Fraction D.
7. Prepare and annotate 3 elution tubes:
   a. Tube 1: $CD45^+$ TILs.
   b. Tube 2: CAFs.
   c. Tube 3: Tumor cell.
8. Prepare and annotate 7 Eppendorf tubes:
   a. Tumor suspension.
   b. Fraction A.
   c. Fraction B.
   d. Fraction C.
   e. $CD45^+$ TILs.
   f. CAFs.

Table 2. Preparation of 1× trypsin

Reagent	Final concentration	Amount
Trypsin 10×	1×	5 mL
Hank's balanced salt solution (HBSS)	–	45 mL

g. Tumor cell.

9. Prepare tools and equipment as described below.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
CD45 Antibody, anti-mouse, REAfinity – Vioblue (1:100 dilution)	Miltenyi Biotec	Cat# 130-110-802, RRID: AB_2658222
Brilliant Violet 785™ anti-mouse CD31 Antibody (1:100 dilution)	Biolegend	Cat# 102435, RRID: AB_2810334
CD49f Antibody, anti-human/mouse, REAfinity-PE-vio770 (1:100 dilution)	Miltenyi Biotec	Cat# 130-123-635, RRID: AB_2802050
CD24 Antibody, anti-mouse, REAfinity-PE-vio770 (1:100 dilution)	Miltenyi Biotec	Cat# 130-110-828, RRID: AB_2656546
CD326 (EpCAM) Antibody, anti-mouse, REAfinity-PE-vio770 (1:100 dilution)	Miltenyi Biotec	Cat# 130-117-866, RRID: AB_2728054
Brilliant Violet 605™ anti-mouse CD140a Antibody (1:50 dilution)	Biolegend	Cat# 135916, RRID: AB_2721548
CD140b Antibody, anti-mouse, REAfinity- APC-770 (1:50 dilution)	Miltenyi Biotec	Cat# 130-118-469, RRID: AB_2733095
FAPA Polyclonal Antibody, ALEXA FLUOR® 488 Conjugated (1:50 dilution)	Nordic Biosite	Cat# Bs-5758R-A488
CD26 Antibody, anti-mouse, REAfinity- PE (1:100 dilution)	Miltenyi Biotec	Cat# 130-122-775, RRID: AB_2801934
APC anti-mouse Podoplanin Antibody (1:50 dilution)	Biolegend	Cat# 127410, RRID: AB_10613649
Chemicals, peptides, and recombinant proteins		
Invitrogen™ Réactif TRIzol™	Fisher	Cat# 15596018
Chloroforme	VWR	Cat# 0757-500ML
Isopropanol	Euromedex	Cat# BI-IB0918-500ML
Ethanol absolute	Sigma-Aldrich	Cat#15727
Invitrogen™ Random primers	Fisher	Cat# 10646313
Set of dATP, dCTP, dGTP, dTTP	Promega	Cat# U1420
Invitrogen™ Transcriptase inverse M-MLV (200 U/µL)	Fisher	Cat# 10338842
Invitrogen™ RNaseOUT™	Fisher	Cat# 10154652
SYBR™ Master Mix PCR	Fisher	Cat# 4368708
autoMACS® Running Buffer	Miltenyi Biotec	Cat# 130-091-221
Invitrogen™ eBioscience™ Flow cytometry staining buffer	Fisher	Cat# 50-112-9748
RPMI 1640 with L-glutamine	DUTSCHER	Cat#L0500-500
Fetal bovine serum	DUTSCHER	Cat#500105N1
Penicillin-streptomycin-fungizone	DUTSCHER	Cat#P06-07300
Trypsin EDTA 10×	DUTSCHER	Cat#X0930-100
Hank's balanced salt solution (HBSS)	DUTSCHER	Cat#L0611-500
Critical commercial assays		
Viobility™ Fixable Dyes (1:100 dilution)	Miltenyi Biotec	Cat# 130-109-816
MACS Comp Bead Kit, anti-REA	Miltenyi Biotec	Cat#130-104-693
VersaComp Antibody Capture Kit, LUO	Beckman Coulter	Cat#B22804
Tumor Associated Fibroblast Isolation Kit, mouse	Miltenyi Biotec	Cat# 130-116-474
CD45 (TIL) Microbeads, mouse	Miltenyi Biotec	Cat# 130-110-618
Tumor Cell Isolation Kit, mouse	Miltenyi Biotec	Cat# 130-110-187
Dead Cell Removal Kit, mouse	Miltenyi Biotec	Cat# 130-090-101
Tumor Dissociation Kit, mouse	Miltenyi Biotec	Cat# 130-096-730
Trypan Blue solution	Sigma	Cat#93595-50ML
DAPI Staining Solution	Miltenyi Biotec	Cat# 130-111-570

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental models: Cell lines		
4T1 cells (8–10 passages)	ATCC	Cat# CRL-2539, RRID: CVCL_0125
Experimental models: Organisms/strains		
6 weeks old female BALB/c mice	Charles River	CRL: BALB/cAnNCrl
Oligonucleotides		
Acta2 forward primer	Life Technologies	CCAGCCATCTTTCATTGGGATG
Acta2 reverse primer	Life Technologies	TACCCCCTGACAGGACGTTG
Pdgfra forward primer	Life Technologies	GAATTTGGGGCCTTTGCGTG
Pdgfra reverse primer	Life Technologies	AGGCTGTGCAAATGAGTGGT
Pdgfrb forward primer	Life Technologies	GTTGTACCTTCCGCAGAGAATG
Pdgfrb reverse primer	Life Technologies	GTCACCCAAGGTACGGTTGT
Tgfb forward primer	Life Technologies	CCCAGTCTCCATACATTAACCC
Tgfb reverse primer	Life Technologies	CACCATCACCTTGAACTCTGAC
TgfbR2 forward primer	Life Technologies	ACGTTCCCAAGTCGGATGTG
TgfbR2 reverse primer	Life Technologies	GTTTCAGTGGATGGATGGTCCT
TgfbR3 forward primer	Life Technologies	CTGCCAAGGGAGGTTCACAT
TgfbR3 reverse primer	Life Technologies	AGAACCCTCCGAAACCAGGA
Serpine forward primer	Life Technologies	GTTCATCGCTGCACCCTTTG
Serpine reverse primer	Life Technologies	CTGCTCTTGGTCGGAAAGACT
Fapa forward primer	Life Technologies	GGGAAGCAACTCATGTCCTG
Fapa reverse primer	Life Technologies	CGCCAGAGCTTTGAATAATCACTT
Ptprc forward primer	Life Technologies	GGAAACTTGCTCCCCATCTGA
Ptprc reverse primer	Life Technologies	AAGGCCAGAAGTTTGAGCCA
Cd3e forward primer	Life Technologies	AAGTAATGAGCTGGCTGCGT
Cd3e reverse primer	Life Technologies	ATGTTCTCGGCATCGTCCTG
Cd274 forward primer	Life Technologies	GCTTCTCAATGTGACCAGCA
Cd274 reverse primer	Life Technologies	GAGGAGGACCGTGGACACTA
Arg1 forward primer	Life Technologies	AGAGATTATCGGAGCGCCTT
Arg1 reverse primer	Life Technologies	TTTTTCCAGCAGACCAGCTT
Pcna forward primer	Life Technologies	GAACCTCACCAGCATGTCCA
Pcna reverse primer	Life Technologies	AATTCACCCGACGGCATCTT
Ccnd1 forward primer	Life Technologies	GCTAAACAAGGACCCCCTCC
Ccnd1 reverse primer	Life Technologies	GCTCCCTACTCTCAGGGTGA
Mki67 forward primer	Life Technologies	CAATCCAACTCAAGTAAACGGGG
Mki67 reverse primer	Life Technologies	GGCCCTTGGCATACACAAAA
Il10 forward primer	Life Technologies	TGTCAAATTCATTCATGGCCT
Il10 reverse primer	Life Technologies	ATCGATTTCTCCCCTGTGAA
Software and algorithms		
Cytexpert Software	Beckman Coulter	https://www.beckman.fr/flow-cytometry/instruments/cytoflex/software
Applied Biosystems QuantStudio 6/7 Pro Real-Time PCR Systems	Applied Biosystems	https://www.thermofisher.com/fr/fr/home/global/forms/life-science/quantstudio-6-7-pro-software.html
GraphPad Prism version 7.00	GraphPad Software	https://www.graphpad.com/scientific-software/prism/
Other		
Isoflurane (Vetflurane®)	Virbac	N/A
The MultiMACS™ Cell24 Separator Plus	MIltenyi Biotec	Cat# 130-098-637
gentleMACS Dissociator	MIltenyi Biotec	Cat# 130-093-235
CytoFLEX V5-B5-R3 Flow Cytometer (13 Detectors, 3 Lasers)	Beckman Coulter	Cat#B53000
NanoDrop™ 2000	Thermo Fisher	Cat# ND2000LAPTOP
QuantStudio 3 Real-Time PCR system	Thermo Fisher	Cat# A28567
Multi-24Column Block	MIltenyi Biotec	Cat# 130-095-691
gentle MACS C Tubes	MIltenyi Biotec	Cat# 130-093-237

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
SmartStrainers Filters (70 µm)	MIltenyi Biotec	Cat# 130-095-823
Centrifuge tubes 50 mL	DUTSCHER	Cat# 227261
Centrifuge tubes 15 mL	DUTSCHER	Cat# 188271
Centrifuge tubes 5 mL	DUTSCHER	Cat# 352054
Eppendorf 1, 5 mL	DUTSCHER	Cat# 33290
96-well plate with conical bottom	DUTSCHER	Cat# 20033
MicroAmp™ 96-well optical reaction plate with barcode	Applied Biosystems	Cat# 10407314
MicroAmp™ 96-well optical reaction plate	Applied Biosystems	Cat# 10411785
Forceps	Fischer Scientific	Cat#10470724
Scissors	Fischer Scientific	Cat#15207266
Surgical scalp	Fischer Scientific	Cat#12397999
T75 mm^2 flask CELLSTAR®	Greiner Bio-One	Cat# 391-3146

MATERIALS AND EQUIPMENT

- You will need access to a refrigerator (4°C) and a centrifuge.
- A pair of forceps, scissors and surgical scalps are necessary to carry out the protocol. Make sure they are clean by washing them with soap and ethanol 70%. These utensils do not need to be sterile.
- Access to a magnetic sorting system like LS columns, this protocol was performed with the multi-24Column block and the multiMACS cell 24 separators plus equipment.
- Tumor digestion require the use of a Miltenyi tumor dissociation mouse kit (https://www.miltenyibiotec.com/FR-en/products/tumor-dissociation-kit-mouse.html#gref) containing lyophilized reagents. Their recovery in solution is necessary before the realization of the enzymatic mix. The solutions and buffer to be added are described in Table 3.

STEP-BY-STEP METHOD DETAILS

The following steps describe how to sort three cell populations: $CD45^+$ cells, CAFs and tumor cells from 4T1 tumors generated in BALB/c mice. The first step is to generate 4T1 tumors in mice, for which tumor cells are injected orthotopically into the mammary gland.

Tumor generation in mice by orthotopic injection of 4T1 cells into BALB/c mice

Timing: 15 days

This step describes the culture of 4T1 cells and the injection of tumor cells to generate a tumor in mice.

1. Cultivate the 4T1 cells in RPMI 1640 medium with FBS and PSA in T75 flask.

Note: Their duplication rate is between 12 and 15 h.

Table 3. Dissolution of the dissociation kit

Reagent	Enzyme D	Enzyme R	Enzyme A
RPMI (mL)	3	2.7	–
Buffer A (mL)	–	–	1

Enzyme solutions should be stored at −80°C for up to 6 months.

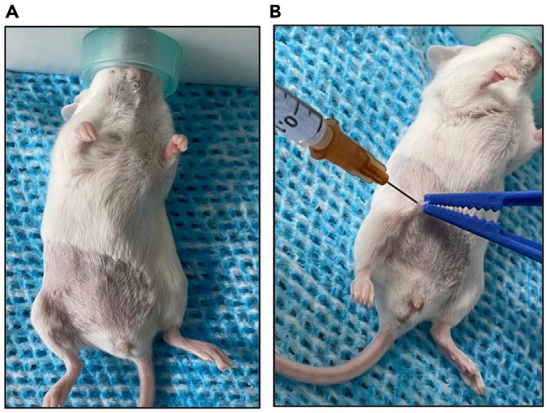

Figure 1. Orthotopic injection of 4T1 cancer cells
(A) The mouse is shaved beforehand and then anesthetized with isoflurane.
(B) Subcutaneous injection of 100μL of tumor cells suspension under the teat of the mouse with a 1 mL syringe and 26G needle.

a. Once the cells reach a subconfluent condition (50%–60%), rinse them with 5 mL of Hank's balanced salt solution (HBSS).
b. Add 3 mL of trypsin to the cells.
c. Incubate for 5 min at 37°C.
d. Add 5 mL of complete RPMI medium to stop trypsin activity.

2. Collect cell suspension and count cells.
   a. Count live cells with trypan blue.

   Note: In a T75 flask, at 50%–60% confluency, it is expected about 1×10^7 cells. We recommend a cell viability of at least 90%.

   b. Then, place the cells in RPMI 1640 medium without FBS and PSA.
3. Adjust cell suspension to a concentration of 1×10^5 cells/100μL RPMI 1640 medium without FBS and PSA. This cell suspension is then subcutaneously injected into the mammary gland of the mouse (Figures 1A and 2B).

   Note: These procedures must be performed under sterile conditions.

4. Monitoring the tumor growth for about 15 days. Tumors are resected the size reaches a minimum volume of 250 mm^3. This is calculated with the following equation:

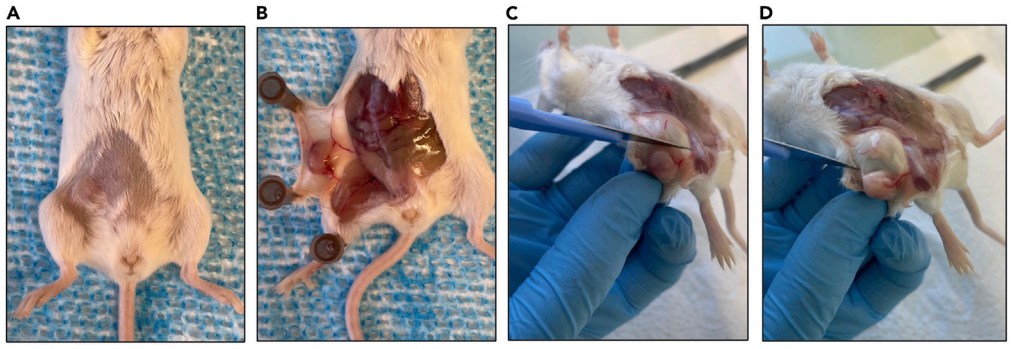

Figure 2. Dissection of the mouse and recovery of the tumor
(A)The tumor is growing in the mammary gland.
(B) Dissection of the tumor. Cut skin up to the diaphragm and then detach from the peritoneum and the skin.
(C and D) Scalpel cutting of the tumor contour.

$$\frac{small\ axis^2 \times major\ axis}{2}$$

⚠ CRITICAL: The size of the tumor would be critical for the number of cells sorted. The maximum volume of the tumor is between 800 and 1000 mm^3 because once this limit is reached, the immune infiltrates are less and the necrosis within the tumor is important. If the tumor has a volume < 250 mm^3, the number of cells sorted is too low to be analyzed.

Tumor removal and dissociation

Timing: 2 h 30 min for 8 mice

This step allows the removal of the tumor from the mouse and the enzymatic and mechanical digestion of the tumor to obtain a cell suspension. The cells are counted in order to continue the protocol with the labeling steps.

Sample harvest

Timing: 30 min for 8 mice

5. Anesthetize the mouse by inhalation with isoflurane (1.5%) and then cull the animal by cervical dislocation.
6. An incision is made in the abdomen and the skin is cut down to the diaphragm (Figures 2A and 2B).
7. The skin of the mouse is gently separated from the peritoneum.

Note: Ensure to preserve the fibrotic capsule surrounding the tumor (Figure 2B).

a. The tumor is removed from the skin using a scalpel, taking care not to remove the capsule over the tumor (Figures 2C and 2D).
b. Then, weigh the tumor and to perform cell sorting immediately after collection to avoid a high percentage of cell dead.

Note: To have a satisfactory number of cells, the adequate tumor size is between 250 and 600 mm^3. For these sizes, the buffer volumes below are appropriate.

Tissue digestion

Timing: 1 h 30 min for 8 mice

8. Reconstitute the miltenyi « Tumor Kit Dissociation » buffer according to Table 3.
9. Prepare an enzyme mix for tumor digestion from the miltenyi « Tumor Kit Dissociation ». The mix should be prepared in RPMI medium without FBS and PSA as described in Table 4.
10. Cut the tumor into 1–2 mm pieces in a petri dish using a scalpel (Figure 3A).
11. Transfer the tumor pieces into C-tubes.
12. Rinse petri dish with 2.5 mL digestion mix and transfer to C-tube.
13. Close the lids of tubes and place them onto the Gentle max. Ensure that the tumor suspension is at the cap (Figure 3B).
14. Turn on the heater to allow the cell suspension to warm up to 37°C.
15. Start the digestion with the program "37C_m_TDK_2".

Table 4. Dissociation mix enzyme

Enzymatic mix dissociation (for between 0.2 g and 1 g of tumor)	
Reagents	Volume (μL)
RPMI 1640	2500
Enzyme D	100
Enzyme R	50
Enzyme A	12.5
Critical: Dissociation enzyme mix must be prepared at the last moment and cannot be stored.	

⚠ CRITICAL: 37C_m_TDK_2 program is suitable for a tumor type with high stiffed tumors such as the 4T1 model. To perform the protocol with other models, their dissociation will have to be adapted according to the product data sheet.

Cell counting

Timing: 30 min

16. Centrifuge the C-tubes for 7 min at 300 × *g* at room temperature (15°C–25°C).
17. Resuspend the pellets in 1 mL of RPMI 1640 medium containing 10% FBS 1% PSA.
18. Pass through a 70 μM Smart Strainers filter on a 50 mL falcon tube.
19. Rinse the C-tubes with 20 mL of complete RPMI 1640 medium.
20. Filter the suspension with the prewashed 70 μM filters.
21. Centrifuge the C-tubes for 5 min at 300 × *g* at room temperature (15°C–25°C).
22. Resuspend the pellets in 1 mL of complete RPMI 1640 medium.
23. Count the cell number in digested tissue sample.
    a. Prepare cell suspension for counting by mixing 10 μL of cell suspension, 89 μL complete RPMI 1640 medium, and 1 μL DAPI.

Note: A control without DAPI is performed to determine more precisely the DAPI negative population.

Note: The complete RPMI 1640 medium must be at room temperature (15°C–25°C).

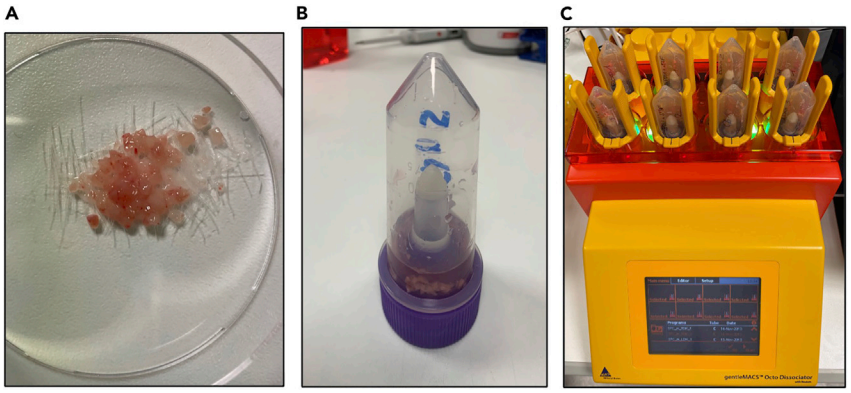

Figure 3. Mechanical and enzymatic digestion of the tumor
(A)The tumor is cut into small pieces into 1–2 mm.
(B)The tumor pieces are placed in a gentle MACS Ctube and added the enzymatic mix. All the tumor pieces are bathed in the liquid at the side of the cap.
(C)The C- tubes are placed on the GentleMax.

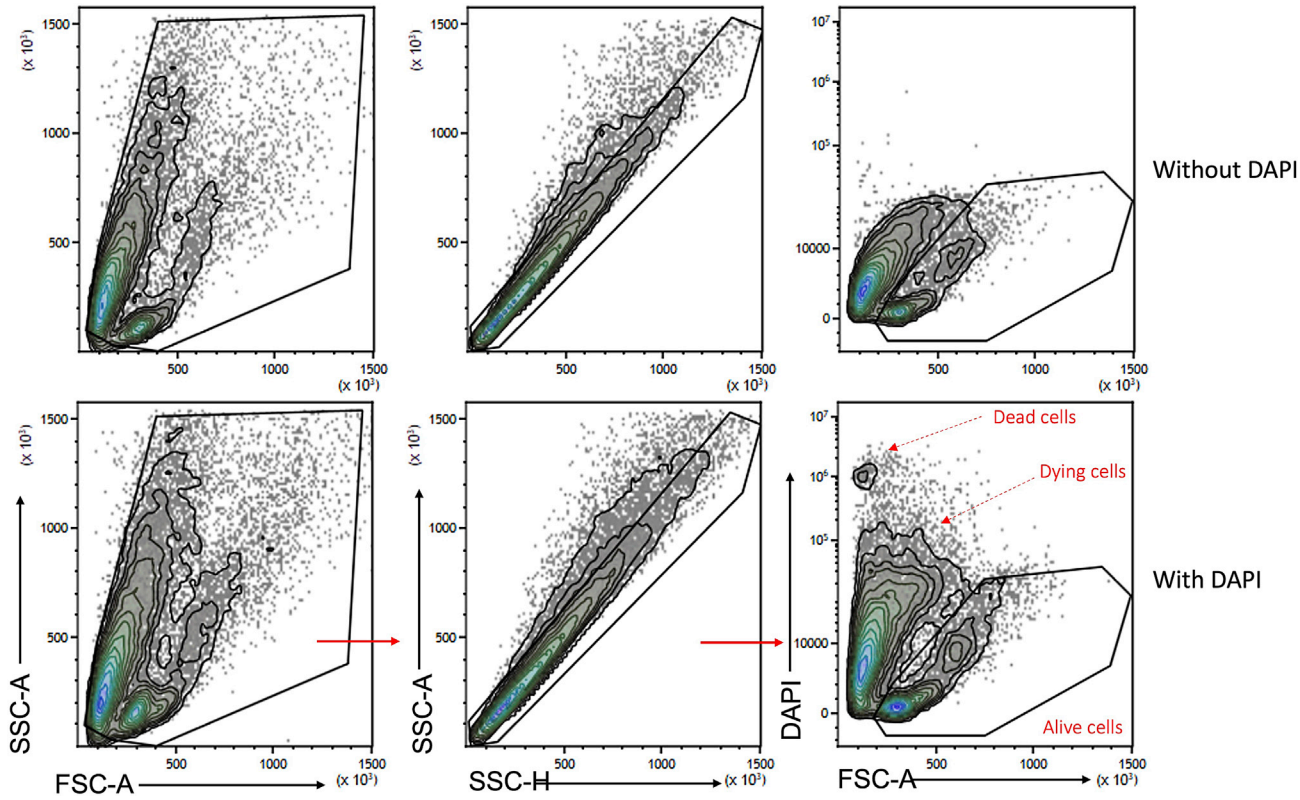

Figure 4. Cell count gating strategy

After removal of debris and doublets, lived cells (DAPI^{-} and FSC$^{int/high}$) were selected to perform cell counting.

b. As shown in Figure 4, flow cytometry analysis gives the estimation of the number of dead and live cells.

Note: Ensure to collect enough number of live cells for further analyses.

Note: The cell count can be done by manual or automatic counting using for example trypan blue.

24. Calculate the number of total cells of the tumor cell suspension and divide them into two parts:
    a. $^{3}/_{4}$ of the tumor cell suspension is used for sorting CD45^{+} TILs and CAFs (suspension #1).
    b. $^{1}/_{4}$ of the suspension for tumor cell sorting (suspension #2).

Note: In order to perform the whole sorting protocol, a minimum of 2 × 10^{7} alive cells is required.

⚠ CRITICAL: In order not to overload the multiMACS columns, do not to load more than 20 × 10^{6} cells per column.

25. From this point in the protocol, a 50 µL sample will be taken from each recovered cell suspension and placed in an annotated Eppendorf (step n°6 of before you begin section) and kept at 4°C.

Collect point will be annotated with a note.

Magnetic-activated cell sorting of the populations

Timing: 4 h

This part of the protocol describes the different steps of cell labeling to perform cell sorting. The sorting steps will also be described. This step allows to obtain the three cell populations: CD45+ cells, CAFs and tumor cells.

Isolation of CD45$^+$ cells

Timing: 45 min

This step describes the staining of CD45$^+$ cells and their isolation.

Cell staining

26. Take the suspension #1.
27. Centrifuge at 300 × *g* for 5 min at room temperature (15°C–25°C).
28. Re-suspend the pellet in 90 µL of autoMACS buffer (https://www.miltenyibiotec.com/FR-en/products/automacs-running-buffer-macs-separation-buffer.html#130-091-221).
29. Add 10 µL of CD45 (TIL) Microbeads (https://www.miltenyibiotec.com/FR-en/products/cd45-til-microbeads-mouse.html#130-110-618).

⚠ CRITICAL: this value is given for 1 × 10^7 alive cells, even if the cell number is fewer than this amount, please keep this value. If there are more cells, the volume of buffer and beads should be adjusted accordingly proportionally.

30. Mix well and incubate for 15 min at 4°C in dark in the fridge.
31. Add autoMACS buffer to have a final volume of 500 µL (keep this volume for a cell amount up to 5 × 10^7).

Magnetic sorting

The separation of the cells will be performed by a magnetic sorting. The CD45$^+$ cells attached to the beads will be collected as a positive fraction. The outgoing fraction will then be used for the sorting for the different populations. This step can be performed on a multiMACS device (Miltenyi Biotech) allowing to perform multiple sorting simultaneously.

Note: The sorting steps can also be performed with a conventional magnet and a single column LS.

32. Place the Multi-24Column Bloc plate on the multiMACS (Figure 6A).
33. Start the "POSSEL" program on the multiMACS and follow the instructions.
    a. Equilibrate the columns with 2 mL of autoMACS buffer.
    b. Load the sample mixed with CD45 microbeads.

Note: Ensure that there is a receptacle below the plate to collect the outgoing fraction. Collect the unlabeled cells that exit the column. This outgoing fraction named as Fraction A (Figure 5).

    c. Wash the column three times with 1 mL of MACS buffer (wait until the columns are empty before adding buffer). Collect the outgoing fraction and pool with fraction A and keep at 4°C or on ice.

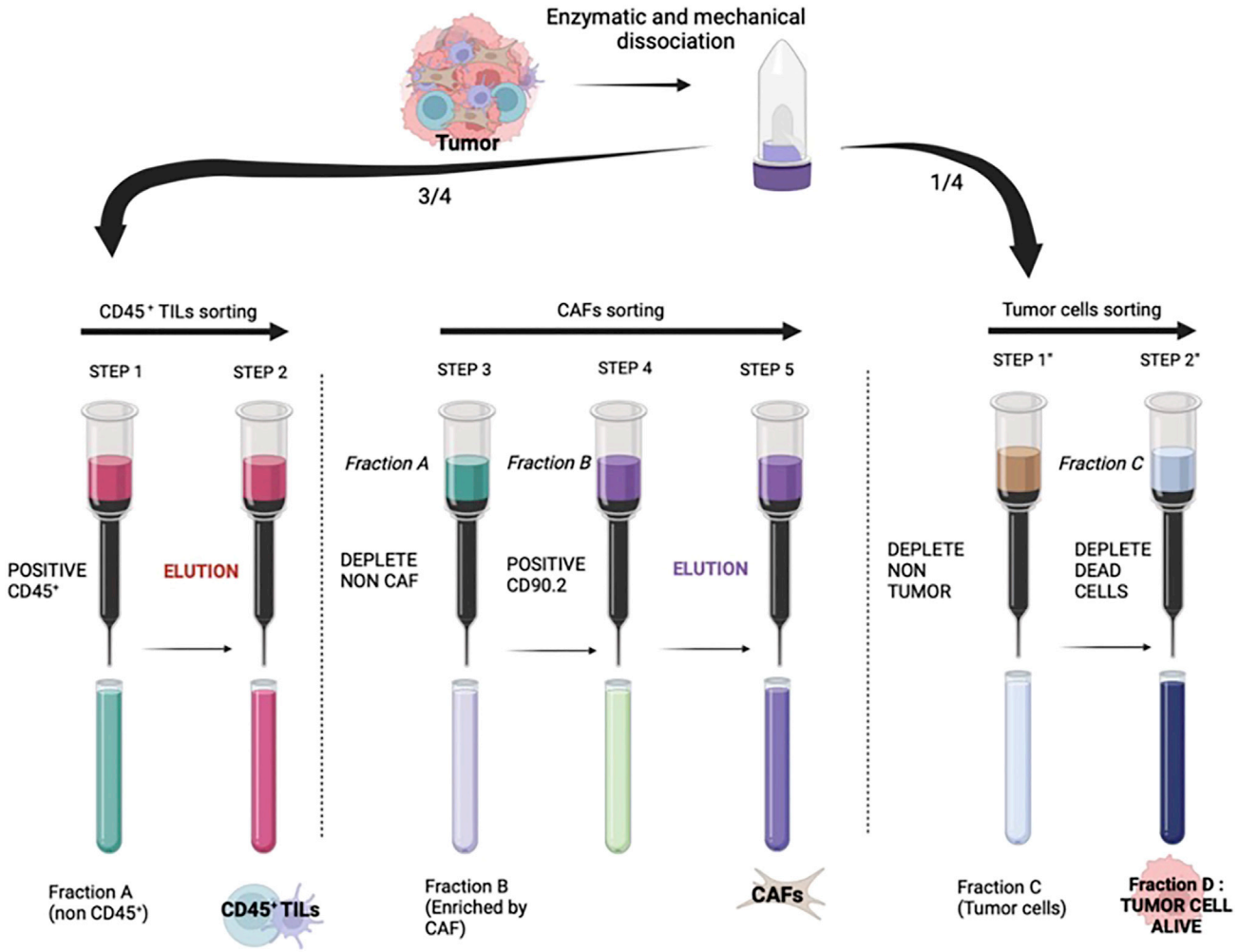

Figure 5. Plan of the different stages of cell sorting

Positive sorting: the sorting is said to be "positive", the cells of interest stained with the microbeads have clung to the column. They are collected in the next step by elution. Deplete sorting: the sorting is called "negative", the cells to be eliminated are stained and cling to the column and the cells of interest are unstained and therefore leave the column (Created with Biorender).

⚠ CRITICAL: Do not forget to preserve fraction A which would be used for the further sorting of CAFs.

Note: Collect sample at this point.

d. Place the plate on the upper part of the elution machine (Figure 6B).
e. Load 1 mL of autoMACS buffer to elute the positive fraction as $CD45^+$ cells in a fresh tube.

34. Centrifuge the $CD45^+$ cells fraction at 300 × *g* for 10 min at 4°C, resuspend the pellet in 100 µL of complete RPMI medium. These cells should be maintained at 4°C for further flow cytometry analysis. If these cells are used for different applications, for example for biochemical analysis, store them at −80°C.

Note: Collect sample at this point.

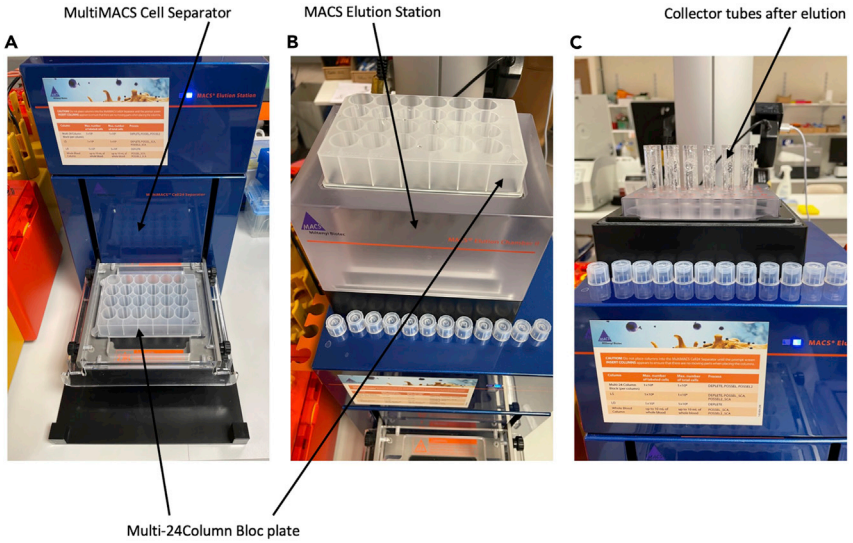

Figure 6. Positive selection of CD45$^+$cells

(A) The Multi-24Column Bloc plate is placed on the lower part of the device; the A fraction is collected in the collector located below.

(B) The Multi-24Column Bloc plate is then placed on the upper part of the device which will allow the elution of the cells in the collection tubes located below each column.

(C) After elution, each tube contains the cells contained in the column.

CAFs isolation (using cancer associated fibroblasts isolation kit)

Timing: 1 h 30 min

This step describes the labeling of "non CAFs" cells and their removal and then the labeling of CAFs and their isolation.

« non CAFs » cells staining:

35. Centrifuge the fraction A at 300 × *g* for 10 min at 4°C.
36. Remove the supernatant with a pipette and then resuspend the pellet in 80 µL of autoMACS buffer.
37. Add 20 µL of the "Non tumor-associated fibroblast" depletion cocktail (https://www.miltenyibiotec.com/FR-en/products/tumor-associated-fibroblast-isolation-kit-mouse.html#130-116-474).

⚠ CRITICAL: this value is indicated for 1 × 10^7 cells at the beginning before the CD45$^+$ sorting, if there are fewer cells, please keep this value. If there are more cells, the volume of buffer and beads should be adjusted.

38. Mix well and incubate for 15 min in dark at 4°C in the fridge.
39. Add buffer up to the final volume of 500 µL (keep this volume for a cell amount up to 5 × 10^7).

Magnetic sorting:

40. Place the Multi-24Column Bloc plate on the multiMACS separator.
41. Start the "DEPLETE" program on the multiMACS and follow the instructions.
    a. Equilibrate the columns with 2 mL of MACS buffer.
    b. Ensure that there is a receptacle below the columns to collect the outgoing fraction.

c. Load samples mixed with non CAFs depletion cocktail to the column.
d. Collect the outgoing fraction (Fraction B), CAFs are enriched in this fraction (Figure 5).
e. Wash the column three times with 1 mL of autoMACS buffer (wait until the columns are empty to add buffer). Collect the outgoing fraction and pool with fraction B.

Note: Collect sample at this point.

Tumor associated fibroblasts staining:

42. Centrifuge fraction B at 300 × *g* for 10 min at 4°C.
43. Remove the supernatant and resuspend the pellet in 80 µL of autoMACS buffer.
44. Add 20 µL of CD90.2-labeled beads allowing for labeling of CAFs.

⚠ CRITICAL: this value is indicated for 1 × 10^7 cells at the beginning, if there are fewer cells, please keep this value. If there are more cells, the volume of buffer and beads should be adjusted.

45. Mix well and then incubate for 15 min in dark at 4°C in the fridge.
46. Add autoMACS buffer to reach a final volume of 500 µL (keep this volume for a cell amount up to 5 × 10^7).

Magnetic sorting:

47. Place the Multi-24Column Bloc plate on the multiMACS separator.
48. Start the "POSSEL" program on the multiMACS and follow the instructions.
    a. Equilibrate the columns with 2 mL of autoMACS buffer.
    b. Ensure that there is a receptacle below the columns.
    c. Load samples mixed with CD90.2 microbeads to the column.
    d. Wash the column three times with 1 mL of MACS buffer.
    e. To collect the CAFs fraction, place the plate with the columns on the upper part of the elution machine. Follow the indications of the machine.
    f. Elute the positive fraction with 1 mL of MACS buffer.
49. Centrifuge the CAFs cells-rich fraction at 300 × *g* for 10 min at 4°C.
50. Resuspend the pellet in 100 µL of complete RPMI medium. Keep at 4°C or on ice until further analysis.

Note: Collect sample at this point.

Isolation of tumor cells

Timing: 45 min

This step describes the labeling of "non tumor cells" and their removal.

Non tumor cell staining:

51. Centrifuge cell suspension #2 (step 24b) at 300 × *g* for 5 min at 4°C.
52. Aspirate the supernatant and resuspend the cell pellet in 80 µL of buffer.
53. Add 20 µL of the "Non tumor cocktail depletion" (https://www.miltenyibiotec.com/FR-en/products/tumor-cell-isolation-kit-mouse.html#130-110-187).

⚠ CRITICAL: this value is indicated for 1 × 10^7 cells at the beginning, if there are fewer cells, please keep this value. If there are more cells, the volume of buffer and beads should be adjusted.

54. Homogenize the well and incubate 15 min at 4°C in the fridge.
55. Adjust the volume to 500 μL with autoMACS buffer (keep this volume for a cell amount up to 5×10^7).

Magnetic sorting:

56. Place the Multi-24Column Bloc plate on the multiMACS separator.
57. Start the "DEPLETE" program on the multiMACS and follow the instructions.
    a. Equilibrate the columns with 2 mL of MACS buffer.
    b. Ensure that there is a receptacle below the columns to collect the outgoing fraction.
    c. Load samples mixed with non tumor depletion cocktail to the column.
    d. Collect the outgoing fraction C which contains the tumor cells.

⚠ CRITICAL: Do not forget to preserve this fraction which contains the tumor cells.

    e. Wash the column three times with 1 mL of MACS buffer. Collect the outgoing fraction and pool with fraction C.

Note: Collect sample at this point.

Elimination of dead cells

Timing: 45 min

This step describes the staining of dead tumor cells and their removal.

Cell staining:

In this protocol, we recommend performing a dead cell removal to eliminate dead tumor cells that can be found in large quantities in 4T1 tumor models that tend to necrotize.

58. Centrifuge fraction C at 300 × *g* for 10 min at 4°C.
59. Remove the supernatant and resuspend the pellet in 100 μL of Dead Cell Removal Microbeads (https://www.miltenyibiotec.com/FR-en/products/dead-cell-removal-kit.html#130-090-101).

⚠ CRITICAL: this value is indicated for 1×10^7 cells at the beginning, if there are fewer cells, please keep this value. If there are more cells, the volume of buffer and beads should be adjusted.

60. Homogenize and incubate 15 min at room temperature (15°C–25°C).

Magnetic sorting:

61. Place the Multi-24Column Bloc plate on the multiMACS separator.
62. Start the "DEPLETE" program on the multiMACS and follow the instructions.
    a. Prepare 1× Buffer from 20× Binding Buffer Stock containing in the kit "Dead cell removal" with sterile H_2O. Store the buffer at 4°C or on ice.
    b. Equilibrate the column with 2 mL of the previously prepared buffer.
    c. Ensure that there is a receptacle below the columns to collect the outgoing fraction.
    d. Load sample mixed with dead cell removal cocktail to the column.
    e. Collect the outgoing fraction D which contains the live tumor cells.

Table 5. Panel for flow cytometry analysis of sorted populations

Populations	Marker	Fluorophore	Final dilution	Volume for 5 samples
Leucocytes	CD45	Vioblue	1:100	5.5 µL
Cancer associated fibroblasts	CD140a (PDGFRa)	BV605	1:50	11 µL
	CD140b (PDGFRb)	APC-vio770	1:50	11 µL
	FAPα	AF488	1:50	11 µL
	Podoplanin	APC	1:50	11 µL
	CD26	PE	1:100	5.5 µL
Endothelial cells	CD31	BV785	1:100	5.5 µL
Tumor cells	CD49f	PE-vio770	1:100	5.5 µL
	CD24	PE-vio770	1:100	5.5 µL
	CD326 (EpCAM)	PE-vio770	1:100	5.5 µL
All cells	Viability (LD Dye)	Viobility 405/452 Fixable Dye	1:100	5.5 µL
Total volume of antibodies				82.5 µL
Volume of staining buffer for 5 samples				467.5 µL

f. Wash the column three times with 1 mL of binding buffer. Collect the outgoing fraction and pool with the fraction D.

Note: It is possible to swap the dead cell removal and non-tumor cell staining steps, but it is recommended to perform these labeling steps in this order to ensure that all dead cells that may be induced during tumor cell sorting are removed.

Note: Collect sample at this point.

Labeling of sorted cells for phenotypic validation by flow cytometry

Timing: 1 h

This step describes the staining of tumor cells, CAFs and CD45$^+$ cells by a panel of antibodies and their analysis by flow cytometer. This step will allow to confirm the phenotype of the sorted populations.

Design of the flow cytometry panel

63. A cytometry panel was used to check the viability and purity of total tumor cell suspension and each purified fraction. This panel is described in Table 5.

Note: For the use of this panel, it is necessary to ensure that your flow cytometry equipment has the appropriate number of lasers and the configuration.

The emission spectra of the fluorochromes used in our flow cytometry panel have been studied and are presented as a spectra analyzer in Figure 7 Care was taken to use fluorochromes with a high spectrum overlap for non-co-expressed markers. For example, the anti-CD31 and anti-CD140b antibody, two non-co-expressed targets, was combined with two fluorochromes with high overlap, BV785 and APC-vio770 respectively.

This part details how to stain tumor-derived cells for flow cytometry analysis. For optimal analysis of your flow cytometry data, we recommend preparing FMO and unstained controls. All the following steps need to be performed in the dark at room temperature (15°C–25°C).

64. Centrifuge the samples in the Eppendorf collected at each step (Fraction A, B and C samples) and the samples of the sorted populations: CD45+ TILs, CAFs and tumor cells (fraction D) at 300 × *g* for 5 min at 4°C.

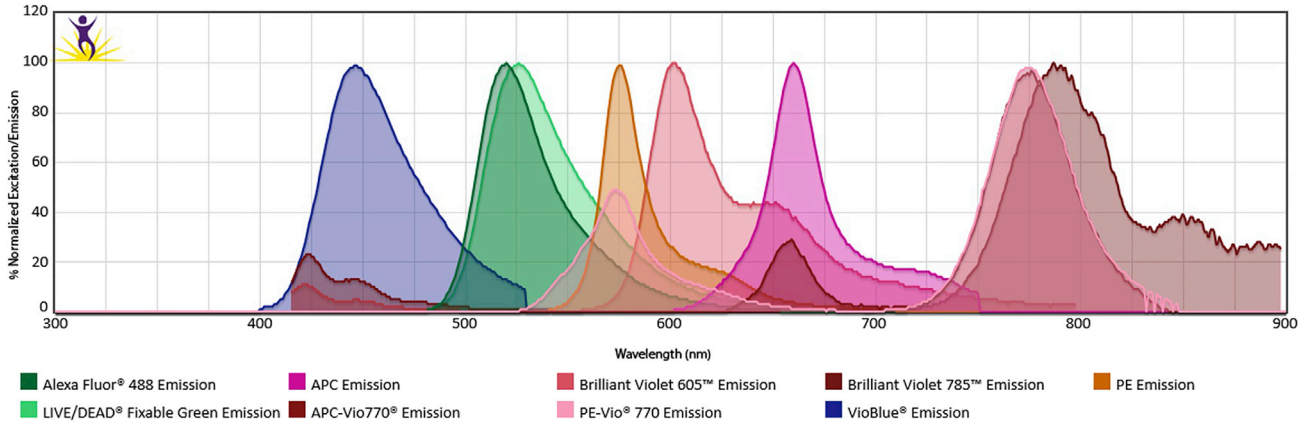

Figure 7. The emission spectra of the fluorochromes

65. Discard the supernatant and resuspend cells in 150 µL of flow cytometry staining buffer (FSB) then transfer to V-bottom 96-well plate.
66. Centrifuge the 96 well plate containing cells at 300 × *g* for 5 min at room temperature (15°C–25°C).
67. Discard the supernatant and resuspend cells in 100 µL of antibody cocktail contained all antibodies prepared in FSB as indicated in Table 3 and incubate the cells at room temperature (15°C–25°C) for 15 min.
68. Centrifuge the 96-well plate at 300 × *g* for 5 min at room temperature (15°C–25°C).
69. Discard supernatant.
70. Wash with 150 µL of FSB per well.
71. Centrifuge the 96-well plate at 300 × *g* for 5 min at room temperature (15°C–25°C).
72. Discard the supernatant and resuspend cells in 150 µL of FSB, the cells are ready to be analyzed by flow cytometer.

Acquisition on the flow cytometer

Timing: 1 h 30 min for sorting and setting samples (17 samples)

This part details the acquisition of cellular fractions on the Cytoflex flow cytometer (Beckman Coulter) using CytExpert software.

73. For compensation setting, prepare unstained and single-stained controls for every fluorochrome-dye used in your panel.

Note: For anti-CD45, anti-CD31, anti-Podoplanin, anti-CD26 and anti-EpCAM antibodies we recommend to used total tumor cell suspension. Due to the low expression of CD140a, CD140b and FAPA markers by the cells, we recommended to used compensation beads for them. Concerning the mortality dye used a mix of stained and unstained tumor suspension (Table 6).

74. Once controls are ready, go to your flow cytometer and create a new experiment.
75. Use unstained and single-stained cells or beads to set appropriate PMT voltage or APD gain and prepare compensation matrix.

Note: $CD45^+$ TILs, CAFs and tumor cells can have different morphological characteristics and consequently can be different in SSC/FSC parameters. FSC/SSC parameters must be checked to correctly identify all cell subsets.

Table 6. Sample detail for fluorescence compensation settings

Type of staining	Sample used for compensation
Unstained	tumor cellular suspension
CD45	tumor cellular suspension
CD31	tumor cellular suspension
Lin (CD49f, CD24 or EpCAM)	tumor cellular suspension
CD26	tumor cellular suspension
Podoplanin	tumor cellular suspension
LD dye	tumor cellular suspension
FAPa	Compensation beads (Versa Comp)
CD140a	Compensation beads (Versa Comp)
CD140b	Compensation beads (for REA antibody)

76. Create the gating strategy as shown in the Figure 8.
77. Load all your sample. For an analysis of CAFs in total tumor suspension recording a minimal of 1×10^6 live singlet cells because these cells are in very low proportion in the total tumor.

Gene expression analysis of sorted fractions

Timing: 5 h

This step describes the extraction of RNA from isolated populations and their reverse transcription into DNA for qPCR. qPCR analysis is performed on the fractions to validate them through the study of genes associated with the sorted subpopulations.

78. Transfer the CD45 TILs, CAFs and tumor cell samples to a 1.5 mL Eppendorf and add 500 µL of Trizol® for a minimum of 15 min to destroy the cells.

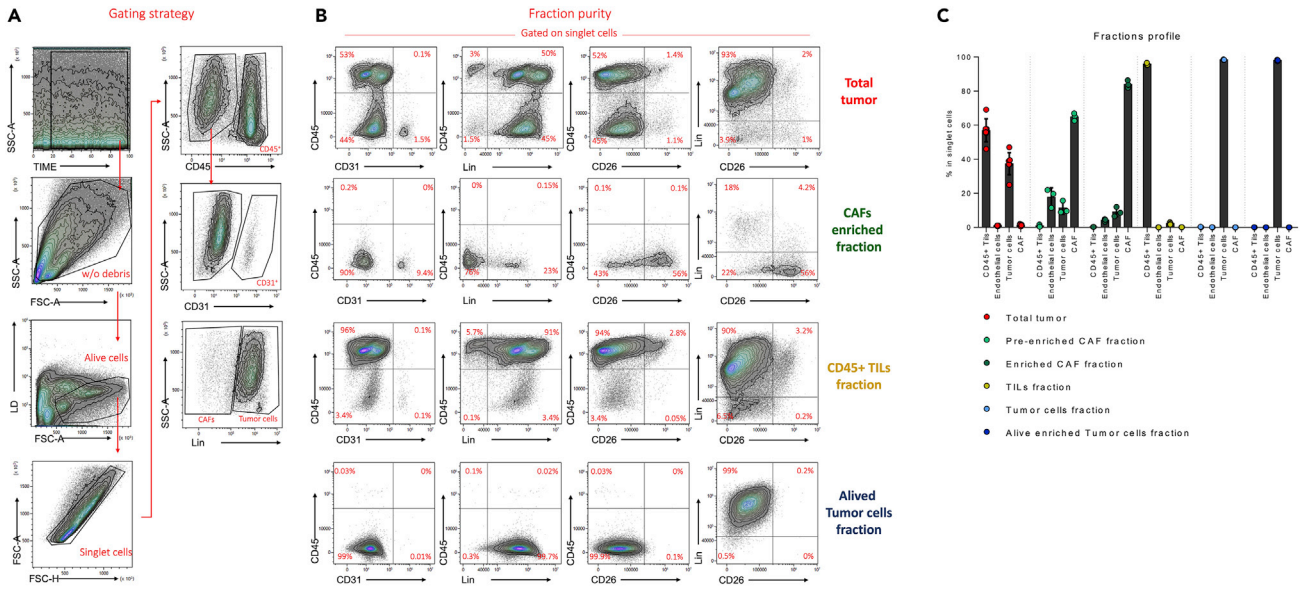

Figure 8. Flow cytometry gating and isolated fraction purity/viability

(A) Gating strategy for cellular subsets identification. Singlet live cells were obtained by using SSC/Time, FSC/SSC and FSC/mortality dye (LD) parameters.

(B) To appreciate each fraction purity, dot plot with CD45, CD31, Lin and CD26 (CAFs marker) staining were shown in CD45+ TILs, tumor cells and CAFs in total tumor suspension.

(C) Proportion of CD45+ TILs, Endothelial cells, Tumor cells and CAFs in tumor cell suspension and purity of each cell subsets in isolated fractions.

Table 7. Reverse transcriptase mix (per sample)	
Solution	Volume
Mix 1	
Random Primer (100 ng/μL)	1
dNTP (25 mM)	0.4 μL
Total RNA (100 ng/ μL)	3 μL
H_2O	11.6 μL
Mix 2	
5× first-stand buffer	2 μL
DTT	1 μL
M-MLV	1 μL
RNAse OUT	1 μL
These mixes must be prepared at the last moment and cannot be stored.	

Pause point: Samples in Trizol® can be frozen at −20°C or −80°C for long-term preservation to continue the experiment later.

79. Add 100 μL of chloroform to the Trizol®, vortex the samples vigorously and then centrifuge at 12000 g for 15 min at 4°C.
80. Collect 250 μL of the upper phase in a new Eppendorf tube and add 250 μL of isopropanol to allow precipitation of the RNA. The mixture should be homogenized and then allowed to precipitate for 10 min at room temperature (15°C–25°C).
81. Centrifuge the RNAs for 15 min at 12,000 × *g* at 4°C.
82. Empty the tube without losing the RNA pellet then fill it with 1 mL of 70% ethanol and vortex it vigorously.
83. Centrifuge for 5 min at 7,500 × *g* at 4°C.
84. Repeat steps 82 and 83 a second time.
85. Dry the RNAs at room temperature (15°C–25°C) for 1 h until the pellet is completely dry.
86. Recover the pellets in 10 μL ultra-pure water. Measure the RNA concentration and purity in each sample with a Nanodrop spectrophotometer.

Note: During the assay, we checked the absorption spectra, and we validated the different ratio 260/280 and 260/230. The ratio 260/230 must be higher than 2 and the ratio 260/280 must be between 2 and 2.2.

Pause point: RNAs in ultra-pure water can be stored at −20°C or−80°C for a better long-term preservation.

Note: Avoid multiple thaw/freezing cycles.

87. Dilute the RNAs to the final concentration of 100 ng/ μL.
88. Transform these RNAs into cDNA by reverse transcription with the addition of mix 1 presented in Table 7.
89. Incubate at 65°C for 5 min.
90. Add the mixture 2 presented in Table 7 and the following program is launched in the thermocycler:
    a. 10 min at 25°C.
    b. 50 min at 37°C.
    c. 15 min at 70°C.
    d. ∞ at 4°C.

Pause point: cDNA can be stored at 4°C for few weeks or −20°C for long-term.

Table 8. qPCR mix (per sample)

Solution	Volume
SYBR Green	7 µL
Forward primer	0.53 µL
Reverse primer	0.53 µL
H_2O	3.5 µL
cDNA	3.5 µL

This mix must be prepared at the last moment and cannot be stored.

91. Dilute the cDNAs by adding 180 µL of ultra-pure water in the Eppendorf tube.
92. Amplify the cDNA by qPCR through adding the mix presented in Table 8 and the primers of the genes of interest presented in the Table 9.
93. Perform the following qPCR program using the QuantStudio™ 3 Real-Time PCR equipment:
    a. 10 min at 95°C.
    b. 15 s at 95°C (40 cycles).
    c. 1 min at 60°C (40 cycles).

Note: This program can also be run on others equipment such as AriaMx Real-Time PCR (qPCR) Instrument (Agilent) or CFX Opus 96 Real-Time PCR System (BioRad).

EXPECTED OUTCOMES

This part provides guidelines for analyzing total tumor cell suspensions and isolated fractions: CAFs, $CD45^+$ TILs and cancer cells.

Data shown in the Figures 8, 9, and 10 were obtained using the 4T1 breast cancer model.

The selection strategy is presented in Figure 8A. Among all live singlet cells, total TILs are identified as $CD45^+$ cells. Among $CD45^-$ cells, endothelial cells were identified as $CD45^-CD31^+$. Then, a lineage : a staining composed of 3 markers characteristic of 4T1 tumor cells (CD49f, EpCAM, CD24) is applied to distinguish tumor cells and cancer associated fibroblast (CAF).[2] Tumor cells are identified as $CD31^-$ Lin^+ (EpCAM, CD49f, CD24) and $CD45^-CD31^-Lin^-$ cells were defined as CAFs (Figures 8A and 8B). The purity of the different fractions was analyzed (Figures 8B and 8C).

Table 9. Primers of the genes of interest for each population

	Genes	Forward	Reverse
Cancer associated fibroblasts (CAFs)	*Acta2*	CCAGCCATCTTTCATTGGGATG	TACCCCCTGACAGGACGTTG
	Pdgfra	GAATTTGGGGCCTTTGCGTG	AGGCTGTGCAAATGAGTGGT
	Pdgfrb	GTTGTACCTTCCGCAGAGAATG	GTCACCCAAGGTACGGTTGT
	Tgfb	CCCAGTCTCCATACATTAACCC	CACCATCACCTTGAACTCTGAC
	TgfbR2	ACGTTCCCAAGTCGGATGTG	GTTTCAGTGGATGGATGGTCCT
	TgfbR3	CTGCCAAGGGAGGTTCACAT	AGAACCCTCCGAAACCAGGA
	Serpine	GTTCATCGCTGCACCCTTTG	CTGCTCTTGGTCGGAAAGACT
	Fapa	GGGAAGCAACTCATGTCCTG	CGCCAGAGCTTTGAATAATCACTT
CD45 TILs	*Ptprc*	GGAAACTTGCTCCCCATCTGA	AAGGCCAGAAGTTTGAGCCA
	Cd3e	AAGTAATGAGCTGGCTGCGT	ATGTTCTCGGCATCGTCCTG
	Cd274	GCTTCTCAATGTGACCAGCA	GAGGAGGACCGTGGACACTA
	Arg1	AGAGATTATCGGAGCGCCTT	TTTTTCCAGCAGACCAGCTT
	Il10	TGTCAAATTCATTCATGGCCT	ATCGATTTCTCCCCTGTGAA
Tumor cells	*Pcna*	GAACCTCACCAGCATGTCCA	AATTCACCCGACGGCATCTT
	Ccnd1	GCTAAACAAGGACCCCCTCC	GCTCCCTACTCTCAGGGTGA
	Mki67	CAATCCAACTCAAGTAAACGGGG	GGCCCTTGGCATACACAAAA

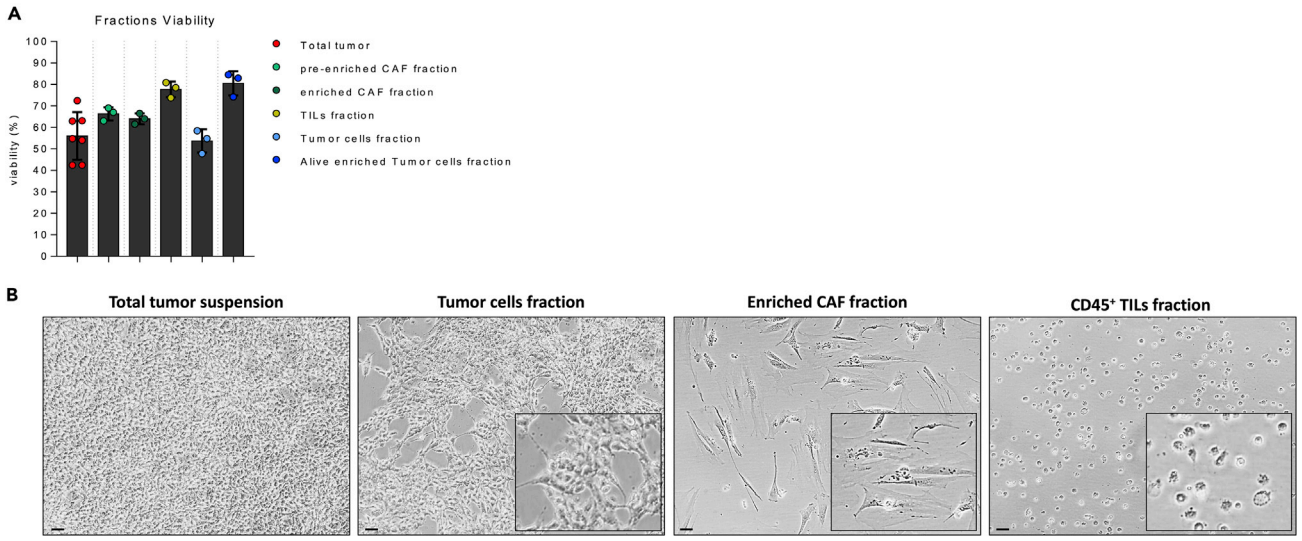

Figure 9. Isolated fractions viability and morphology

(A) Viability of each cell subsets in isolated fractions.

(B) Morphology of each cell subsets in isolated fractions (scale bare = 50 μm).

Viability of each isolated fractions is higher than 65% and they are able to proliferate *in vitro* and have a morphology typical of tumor cells, fibroblasts or immune cells (Figures 9A and 9B).

The phenotype of CAFs was analyzed by using a panel composed of different markers characteristic of CAFs. For this, we were inspired by the work of Madsen's team.[3] The markers chosen to study CAFs are: podoplanin (PDPN), dipeptidyl petidease-4 (DPP4/CD26), CD140a (PDGFRα), CD140b (PDGFRβ), FAPα.[4,5] The phenotypic characterization of each fractions indicated that isolated CAFs fraction over-expressed typical markers as compared to tumor cells such as CD26, FAPa, Podoplanin (PDPN) and PDGFRa (Figures 10A and 10B).

Concerning gene expression profile, CAFs fraction overexpressed TGFβ-related genes such as *Tgfbr2/3*, *Tgfb*, *Serpine1* and *Acta2* as compared to tumor cells and CD45$^+$ TILs. *Pdgfra/b* and *Fapa* were also over-expressed. CD45+ TILs fraction expressed typical genes associated to immune response. We analyzed *Ptprc* (CD45) *Cd3e*, *Il10*, *Cd274* and *Arg1*. All were over-expressed as compared to CAFs or tumor cells. Concerning the characteristic of tumor cells, we decided to evaluate proliferation-associated genes and analyzed Pcna, Ccnd1 and Mki67. All were over-expressed as compared to TILs or CAFs (Figure 10C). This simple transcriptomic analysis reveals that each fraction was correctly isolated and had a distinct phenotype. Consequently, this strategy could be applied in different tumor models or after different treatment (chemotherapies, targeted therapies and others).

The purpose of this protocol is to sort several cell populations from a mouse tumor to study the tumor microenvironment. The subpopulations of interest were CD45+ TILs, tumor cells and CAFs.

The results presented in Figure 8 show that the three fractions have a purity of more than 80%. In addition, viability analysis (Figure 9) shows a viability of more than 65% for each fraction. The morphology study presented in Figure 9 confirms the two previous points by morphological phenotypes corresponding to the desired populations as well as their viability and their capacity of proliferation *in vitro*.

This information was confirmed by the phenotypic study of fractions (Figure 10).

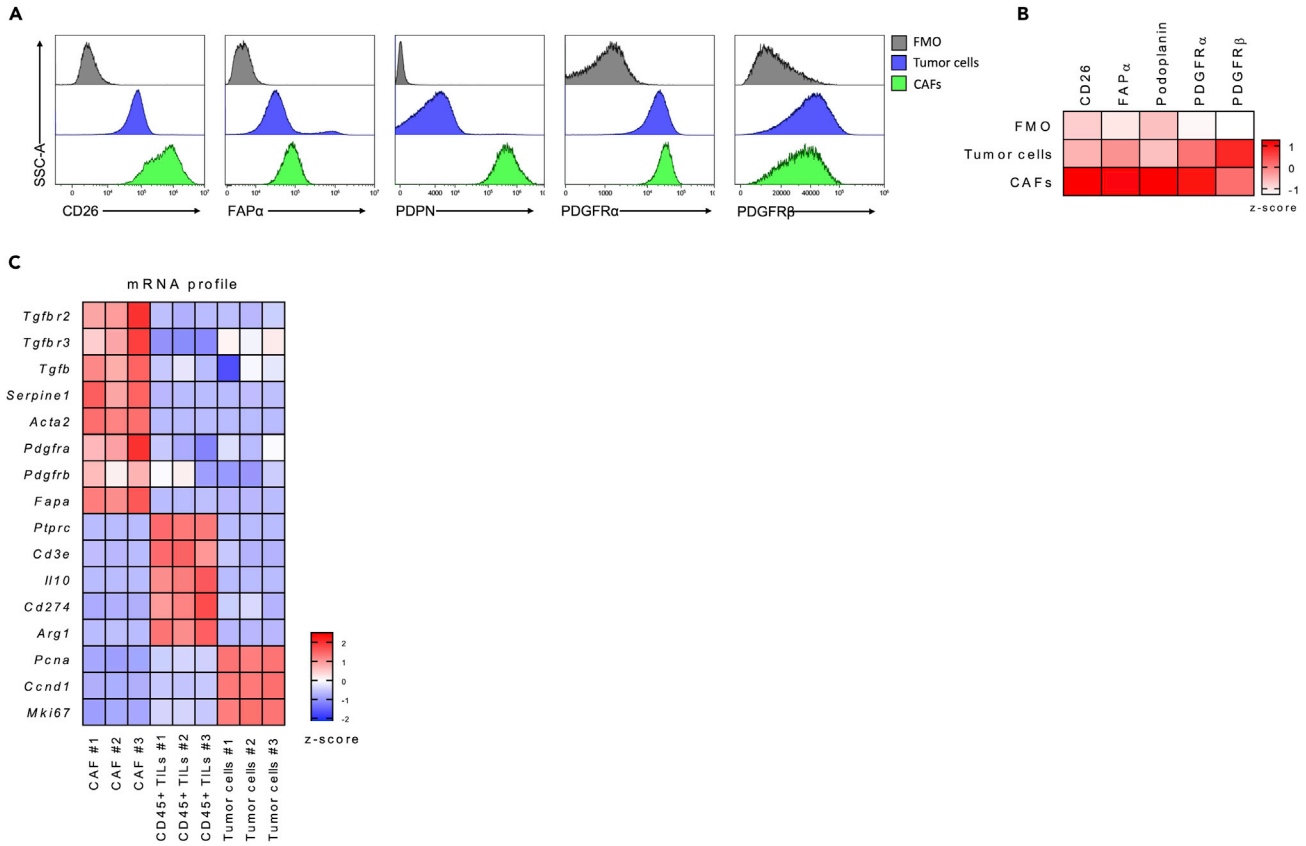

Figure 10. Phenotypic and transcriptomic analysis

(A and B) Representative flow cytometry analysis of CAFs markers (CD26, FAPa, PDPN, PDGFRa, PDGFRb) on tumor cells and CAFs fractions (A). Normalized expression of each marker (B).

(C) Analysis of CAFs, CD45+ TILs and tumor associated genes expression by RT-qPCR in each isolated fraction (n = 3 samples/fraction).

LIMITATIONS

This protocol has been validated on the 4T1 model, which is a fibroblast rich model, but we have not validated this protocol on other tumor types. The proportions of cells and their distribution may vary. Indeed, this protocol must be revalidated on other types of tumors which could have different cell proportions.

TROUBLESHOOTING

Problem 1

Steps 33, 41, 48, 57 and 62: during cell sorting, the successive passage of cells on columns leads to an increase in cell mortality.

Potential solution

Keeping the cells at 4°C during the waiting times and incubation times is very important.

Problem 2

Steps 48 and 62: The number of cells per population obtained after all the sorting is too low.

Potential solution

Inject several mice and removal 2 or 3 tumors per sample then pool the cell suspensions to increase the number of initial cells. This could allow to increase the number of cells per population after sorting. In this case, do not forget to adapt the volumes of microbeads and buffer.

Problem 3

Step 48: The number of CAFs sorted is very low.

Potential solution

The filtration of the cell suspension is recommended by the instructions of the isolation kit, this filtration must be done on a 30 μm filter. Nevertheless, a fibroblast can be up to 30 μm long. The filtration of the suspension can therefore lead to the loss of some of these cells. We therefore advise not to perform this filtration but instead to perform on 70 μm filter.

Problem 4

Step 67: The markers used in the lineage cytometry panel to select tumor cells are not expressed by all tumor lines. Indeed, another murine breast cancer line: the EMT6 line does not express these markers, so the use of this panel is not possible.

Potential solution

Adapt the cytometry panel by changing the lineage antibodies to target the markers expressed by the tumor line of interest.

Problem 5

Steps 33 and 48: during positive sorting, it is possible for unlabeled cells to contaminate the labeled cells of interest and become magnetized in the column.

Potential solution

To avoid this, it is necessary to perform the column washes correctly as described in the protocol.

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 Limagne Emeric] (elimagne@cgfl.fr).

Materials availability

Not concerned. Data sharing not applicable.

Data and code availability

Not concerned. Data sharing not applicable.

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AUTHOR CONTRIBUTIONS

Writing review and editing, L.K., S.P., L.G, S.L., F.G.; Supervision, E.L. All authors have read and agreed to the published version of the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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