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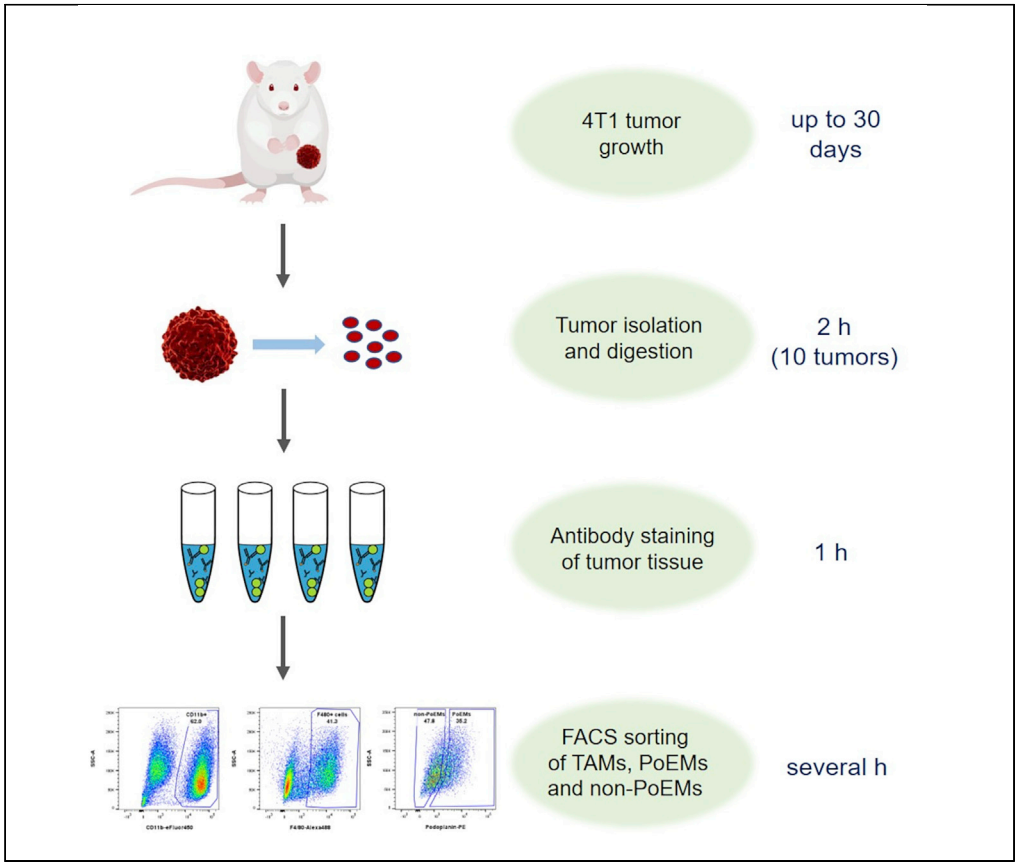
Protocol

Isolation and separation of murine tumor-associated macrophages (TAMs) subpopulations from orthotopic 4T1 breast tumors



Tumor-associated macrophages (TAMs) are highly heterogenous regarding their intratumoral localization, surface marker expression, and molecular properties. This protocol describes the complete procedure for isolation and digestion of murine breast cancer samples and FACS sorting of TAMs from murine orthotopic 4T1 breast tumors. This includes steps to separate PoEMs (podoplanin-expressing macrophages) and non-PoEMs (podoplanin-negative macrophages). Our FACS separation approach could also be used for other tumor types with TAM infiltration.

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HIGHLIGHTS

Procedure to isolate and digest murine 4T1 breast cancer samples

Protocol for FACS sorting of TAMs from murine breast tumors

Steps to separate TAMs into "PoEMs" and "non-PoEMs" subpopulations by FACS

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Isolation and separation of murine tumor-associated macrophages (TAMs) subpopulations from orthotopic 4T1 breast tumors

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SUMMARY

Tumor-associated macrophages (TAMs) are highly heterogenous regarding their intratumoral localization, surface marker expression, and molecular properties. This protocol describes the complete procedure for isolation and digestion of murine breast cancer samples and fluorescence-activated cell sorting (FACS) of TAMs from murine orthotopic 4T1 breast tumors. This includes steps to separate PoEMs (podoplanin-expressing macrophages) and non-PoEMs (podoplanin-negative macrophages). Our FACS separation approach could also be used for other tumor types with TAM infiltration.

For complete details on the use and execution of this protocol, please refer to Bieniasz-Krzywiec et al. (2019).

BEFORE YOU BEGIN

1. Set up a flow cytometry staining panel for your populations of interest. The panel used in this protocol is shown in Table 1.
2. This protocol is optimized for breast tumors (4T1 model) in mice with BALB/c background but has also been tested on mice with C57BL/6 background (E0771 model). It is important to note that other tumor types might produce different isolation outcomes. Make sure you are familiar with the growth kinetics of the tumor model used to be able to accurately schedule the TAM isolation day. Of note, TAMs have been detected in 4T1 tumors as early as on day 4 post-cancer inoculation (Makela et al., 2017).
3. If the isolated cells are to be put in culture, make sure that all procedures are performed under sterile conditions.

Note: Titration of all antibodies and dyes is highly recommended if other types of sorters or software are used, to ensure the best stain index for each isolated population.

Table 1. Flow cytometry panel for the analysis and sorting of murine TAMs, PoEMs and Non-PoEMs

Marker	Fluorophore	Final dilution
Viability	eFluor506	1:400
CD11b	eFluor450	1:400
F4/80	AlexaFluor488	1:100
PDPN	PE	1:200

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-mouse CD11b-eFluor450	eBioscience	48-0112-82
Anti-mouse CD16/CD32	BD Pharmingen	553142
Anti-mouse F4/80-AlexaFluor 488	eBioscience	53-4801-82
Anti-mouse Podoplanin-PE	BioLegend	127407
Chemicals, peptides, and recombinant proteins		
Collagenase I	Thermo Fisher Scientific	17100017
Dispase	Gibco	17105-041
DNAse I	Sigma-Aldrich	11284932001
Critical commercial assays		
Invitrogen™ UltraComp eBeads™ Compensation Beads	Thermo Fisher Scientific	501129040
Red Blood Cell Lysing Buffer - Hybri-Max™	Sigma-Aldrich	R7757
RLT lysis buffer	QIAGEN	79216
Fixable Viability Dye eFluor506	eBioscience	65-0866-14
Deposited data		
RNA-sequencing data	(Bieniasz-Krzywiec et al., 2019)	GSE126722: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE126722
Experimental models: cell lines		
4T1 Cell line	ATCC	CRL-2539
Experimental models: organisms/strains		
Mouse: WT BALB/c	Animalium Gasthuisberg, Leuven, Belgium	n/a
Software and algorithms		
BD FACSDiva™ software, version 8.0	BD Biosciences	23-14523-00
FlowJo™, version 10.4.2	BD Biosciences	n/a
Other		
BD FACSAria™ III Cell Sorter *Details regarding sorter configuration are shown in* Table 2.	BD Biosciences	23-11585-02
Dulbecco's modified Eagle's medium (DMEM)	Gibco	41965039
Dulbecco's phosphate-buffered saline (PBS)	Thermo Fisher Scientific	14190144
EDTA (0.5 M), pH 8.0, RNase-free	Thermo Fisher Scientific	AM9261
Falcon Round-Bottom Polystyrene Test Tubes with Cell Strainer Snap Cap, 5 mL	Thermo Fisher Scientific	352235
Falcon 15 mL Conical Centrifuge Tubes	Thermo Fisher Scientific	14-959-53A
Falcon 50 mL Conical Centrifuge Tubes	Thermo Fisher Scientific	14-432-22
Falcon 40 µm Cell Strainer	Corning	352340
Falcon 70 µm Cell Strainer	Corning	352350
FBS (fetal bovine serum)	Biochrom GmbH	S0115
gentleMACS C Tubes	Miltenyi Biotec	130-093-237
gentleMACS Octo Dissociator with Heaters	Miltenyi Biotec	130-096-427
Gibco Penicillin-Streptomycin (10.000 U/mL)	Thermo Fisher Scientific	15140122
Petri dishes, polystyrene, 60 mm × 15 mm	Merck	P5481-500EA
RPMI-1640 Medium	Thermo Fisher Scientific	11875093
Sodium chloride solution 0.15 M	Sigma-Aldrich	7647-14-5
Sterilin 30 mm Petri Dishes	Thermo Fisher Scientific	121V
Trypsin-EDTA (0.25%)	Thermo Fisher Scientific	25200056
1,5 mL Eppendorf Safe-Lock Tubes	Eppendorf	0030 120.086
96-Well cell culture plates	Thermo Fisher Scientific	8404

Table 2. BD FACSAria™ III cell sorter configuration

Laser	Detector	Filter Setup		Fluorochromes
Violet 407	F		450/40 BP	DAPI / Pacific Blue / V450 / eFluor450 / BV421 / eBFP / Hoechst33258
	E	502 LP	510/50 BP	AmCyan / V450 / eFluor506 / BV510 / Pac Green
	D	600 LP	610/20 BP	BV605 / Pac Orange
	C	630 LP	660/20 BP	Qdot 655
	B	685 LP	710/50 BP	Qdot 705
	A	735 LP	780/60 BP	Qdot 800
Blue 488	C		488/10 BP	SSC
	B	502 LP	530/30 BP	FITC / AlexaFluor 488 / GFP / CFSE / YFP
	A	655 LP	695/40 BP	PerCP-Cy5-5 / PerCP–eFluor 710 / PI
Yellow-Green 561	E		582/15 BP	PE / Td Tomato
	D	600 LP	610/20 BP	PE-Texas Red / PE-eFluor610 / mCherry
	C	630 LP	670/14 BP	PE-Cy5 / PI
	B	685 LP	710/50 BP	PE-Cy5-5
	A	735 LP	780/60 BP	PE-Cy7
Red 633	C		660/20 BP	APC / eFluor660 / Alexa Fluor 647
	B	690 LP	730/45 BP	Alexa Fluor 700 / APC-Cy5-5 / eFluor710
	A	755 LP	780/60 BP	APC-Cy7 / APC - H7 / APC -Alexa750 / eFluor780

MATERIALS AND EQUIPMENT

Complete DMEM medium

Working under sterile conditions, supplement basic DMEM medium with 10% of fetal bovine serum (FBS), 100 units/mL penicillin and 100 μg/mL streptomycin. The complete DMEM medium can be stored at 4°C for up to 4 weeks.

Complete RPMI medium

Working under sterile conditions, supplement basic RPMI medium with 10% of FBS, 100 units/mL penicillin and 100 μg/mL streptomycin. The complete RPMI medium can be stored at 4°C for up to 4 weeks.

DNAse I stock

Working under sterile conditions, dissolve DNAse I powder in DNAse-free water to obtain a clear 100× stock solution of 20 mg/mL. Aliquot and store at −20°C for up to 3 years. Avoid freeze and thaw cycles as this can compromise the DNAse activity.

FACS buffer

Supplement PBS with 2% of FBS and 2 mM of EDTA. Sterile FACS buffer can be stored at 4°C for up to 6 months.

Fc blocking solution

Dilute the Fc block CD16/CD32 antibody in the FACS buffer in the ratio 1:25, i.e., 4 μL of antibody in 96 μL of FACS buffer. The solution should be prepared fresh each time.

Tumor digestion buffer

Supplement complete RPMI medium with 0.1% collagenase type I, 0.2% dispase type I and 0.2 mg/mL DNAse I (dilute the stock 1:100). The powders can be weighed in advance; however, dissolve the powders, add the DNase I stock and pass through a 0.22 μm filter on the day of tumor isolation (the complete buffer can be stored on ice for a few hours).

STEP-BY-STEP METHOD DETAILS

4T1 tumor inoculation and growth

Timing: 30 days if end-stage 4T1 tumors (i.e., on day 24 post-cancer inoculation) are utilized

1. Mouse preparation:
   a. Use healthy, 9–12-weeks-old female BALB/c mice.
   b. Remove the fur around and below the right nipple of the 4th mammary gland of each mouse (Eriksson et al., 2014) by depilation or shaving, to expose the injection site. Cancer cells can be inoculated on the following day.

2. Cancer cell inoculation:
   a. Culture *Mus musculus* 4T1 mammary gland cancer cells in complete DMEM media in a humidified incubator in 5% CO_2 at 37°C, under sterile conditions. The cells should not be allowed to become confluent and should be subcultured at 80% confluence. A subcultivation ratio of 1:5 is recommended. After thawing, passage at least twice before injecting the cells in mice.
   b. Remove the media from cell culture flasks, quickly rinse the cells with PBS and incubate them with 0.25% Trypsin-EDTA solution at 37°C until they detach. Next, add fresh culture medium and collect the cells into 50 mL falcons. Spin down for 5 min at 300 *g* and wash the cell pellets twice in PBS by means of centrifugation (5 min, 300 *g*) to completely wash away the cell culture medium.
   c. Resuspend the pellets in PBS at a concentration of 1 × 10^6 cells per 50 μL. Keep the cellular suspensions on ice at all times.

   Note: It is recommended to prepare an excess volume of cell suspension (10%–20%) to ensure there is sufficient volume for injections.
   d. Inject 1 × 10^6 cells into the right nipple of the 4th mammary gland.

Hold the syringe with the needle tip turned upwards to enter the skin subcutaneously at 10 mm from the inoculation nipple, between the 4th and 5th mammary gland. When inserted, move the needle along the skin towards the inoculation nipple until the tip of the needle is located right under that nipple. While still holding the needle parallel to the skin, move it slightly in all directions around the nipple (within a 3 mm radius). Carefully move the needle tip up into the mammary fat pad till the needle eye comes under the nipple tip. Inject the cells at once, but slowly, so that a visible flat thickening forms around the nipple. After injection, turn the needle 45 degrees to the right and slowly pull it out, while still pressing on the plunger.

3. Tumor growth:
   a. Measure tumor growth with a caliper, 3 times per week for up to 24 days. An exemplary tumor growth curve is shown in Figure 1.

Tumor collection and digestion

Timing: 2 h

Proceed in the following way when tumors reach the growth endpoint or a desired size.

Note: It is recommended to always use tumors of the same stage in order to ensure consistency and comparability of experimental outcomes.

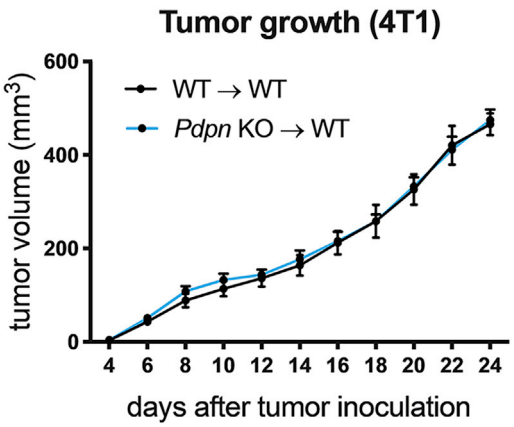

Figure 1. Exemplary tumor growth curve of 4T1 breast cancer cells implanted orthotopically in WT→WT or Pdpn KO→WT bone marrow chimeras
Figure reprinted with permission from (Bieniasz-Krzywiec et al., 2019).

4. Sacrifice the tumor-bearing mice using a method approved by an ethical committee of your institution (e.g., cervical dislocation, CO_2 inhalation) and immediately collect the tumors in cold PBS.

   Note: The draining lymph node can often be found in close proximity to the tumor. Thus, ensure that it is appropriately separated from the tumor.

5. Place each tumor in a plastic dish and chop it with a scalpel into 2–4 mm long pieces.
6. Transfer the tissue into a gentleMACS C Tube containing 5 mL of sterile digestion buffer (RPMI medium containing 0.1% collagenase type I, 0.2% dispase type I and 1% DNAse I stock).

   Note: The powders can be weighed in advance. However, dissolve the powders, add the DNase I stock and pass through a 0.22 μm filter immediately before use.

7. Tightly close the C Tube and attach it upside down onto the sleeve of the gentleMACS Octo Dissociator.

   Note: Ensure that the sample is located in the area of the rotor/stator, covered with digestion buffer, and that there are no tumor pieces on the tube walls.

8. Run the gentleMACS program with the heating function suitable for hard tumors, i.e., '37C_m_TDK_2'.

   Note: If softer tumors are used (e.g., E0771 mammary tumors), the '37C_m_TDK_1' program should be used for tissue dissociation.

Alternatively, when no tissue dissociator is available, mince tumors in 5 mL RPMI medium containing 0.1% collagenase type I and 0.2% dispase type I to very small pieces using a scalpel. Tumor pieces should be then maintained in the same solution for the following 30 min at 37°C, and vortexed every 5 min.

⚠ CRITICAL: *If multiple samples are to be processed, make sure they are all digested for the same amount of time, i.e., transfer each sample to ice exactly after 30 min.*

9. Add 10 mL of FACS buffer (PBS containing 2% FBS and 2 mM EDTA) into the gentleMACS C Tube containing the sample in tumor digestion solution and apply the cell suspension to a 70 μm cell strainer placed on a 50 mL tube. Filter the digested tissue using the strainer.

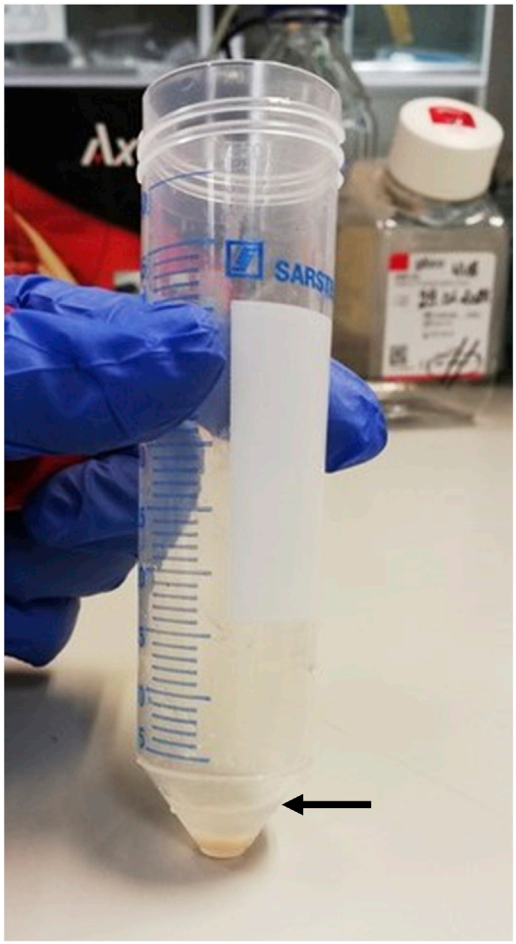

Figure 2. A representative photo of a 4T1 tumor pellet following digestion and straining through a 70 μm strainer, where the volume of the red blood cell lysis buffer can be appreciated (marked with a black arrow).

⚠ CRITICAL: *Wash the strainer with FACS buffer to a uniform final volume of 30–40 mL in each tube.*

10. Centrifuge the cell suspension for 5 min at 350 *g*. Discard the supernatant.

⚠ CRITICAL: *At this point the pellets are very fragile and do not form a firm mass. Therefore, the supernatants should be removed with extreme caution in order not to disrupt and accidentally discard the pellets, i.e., through pipetting rather than aspirating.*

11. Incubate the resulting pellets in 1 mL of the red blood cell lysis buffer for 3 min at 37°C. Immediately place the samples on ice and resuspend them in 10 mL of FACS buffer in order to neutralize the red blood cell lysis buffer. Pipet up and down and pass through a 40 μm cell strainer placed on a 50 mL tube.

Note: Make sure that pellets are well covered by the red blood cell lysis buffer. For 4T1 tumors up to 1 g, 1 mL of the buffer is sufficient, however, for other tumor types this volume might need to be optimized.

An exemplary picture of the pellet covered in red blood cell lysis buffer is shown in Figure 2.

12. Centrifuge the cell suspension for 5 min at 350 *g*. Discard the supernatant. Resuspend the cell pellet in 5 mL FACS buffer and calculate the number of viable cells in each sample using a counting chamber and a light microscope.

⚠ CRITICAL: *Manual cell counting is recommended, as it is crucial to distinguish cells from debris present abundantly in the sample. If an automatic cell counter is used, adjust settings to ensure a precise cell count.*

Flow cytometry antibody staining

Timing: 2 h

The cell suspensions are stained with indicated antibodies for flow cytometric analysis.

13. Resuspend each sample in the blocking solution (dilute the Fc block CD16/CD32 antibody in the FACS buffer in the ratio 1:25, i.e., 4 μL of antibody in 96 μL of FACS buffer) in the following ratio: 1×10^6 cells per 10 μL of the blocking solution.
14. Transfer the samples to FACS tubes.
15. Incubate for 15 min at 4°C.

16. From each sample, collect a small equal volume (e.g., 10 μL) and pool in FACS tubes for the following controls (as detailed in point 18). Ensure to note down the final volumes of your controls:
    a. Unstained
    b. Fluorescence Minus One (FMO) controls - the experimental cells stained with all the fluorophores except for one fluorophore.

⚠ CRITICAL: *FMO controls should be done for all the fluorophores in your panel when starting a new multicolor experiment. This will allow you to assess the spread of all the fluorophores into your missing channel and set your gates accordingly. For detailed information on the FMO controls in the context of tumor microenvironment, please refer to (Young et al., 2016).*

    c. Live cells compensation controls.

17. Cell staining:
    Prepare a 2× concentrated master mix of the appropriate antibodies/dyes in an Eppendorf tube. To each sample (cells in the blocking solution) of a given volume, add the same volume of the 2× concentrated master mix. In this way, the final concentration of the staining mix in each sample is 1× (Table 1). Vortex briefly and incubate for 20 min at 4°C (protected from light). Centrifuge (5 min, 350 *g*) and resuspend the pellets in 3 mL PBS, then centrifuge again (5 min, 350 *g*) to wash the samples. Resuspend the pellets in the FACS buffer, at a concertation of 20 million cells per 1000 μL. Keep at 4°C protected from light until detection and sorting.
18. Control stainings:
    a. Unstained: add the volume of FACS buffer equal to the volume of that sample. Vortex briefly and incubate for 20 min at 4°C (protected from light). Keep at 4°C protected from light until detection.
    b. FMOs: Prepare a 2× concentrated staining mix for each FMO control (containing all the antibodies except for one). Add the volume of 2× mix to each FMO control equal to the volume of that FMO control sample, so that the final concentration of the staining mix in each sample is 1×. Vortex briefly and incubate for 20 min at 4°C protected from light. Centrifuge (5 min, *g* and resuspend the pellets in 3 mL PBS, then centrifuge again (5 min, 350 *g*) to wash the samples. Resuspend the pellets in 100–200 μL of the FACS buffer. Keep at 4°C protected from light until detection.

19. Prepare single stained compensation controls:
    a. For fluorochrome-conjugated antibodies, use UltraComp beads or equivalent beads following the manufacturer's instructions.
    b. For the viability dye, use either ArC™ amine reactive beads (following the manufacturer's instructions) or use any cultured cells as follows:
        i. Detach any cultured cells (e.g., 4T1 cells) with Trypsin-EDTA (0.25%);
        ii. Take an aliquot and incubate at 65°C for 1 min in order to kill the cells and place them on ice immediately afterward;
        iii. Mix an equal volume of viable and heat-killed cells;
        iv. Spin down and wash once with FACS buffer;
        v. Incubate the cell mixture with a viability dye diluted in FACS buffer (the same concentration as your cells) for 20 min on ice protected from light;
        vi. Wash the cells twice with FACS buffer to ensure a complete removal of excessive viability dye.
20. Prepare cell collection tubes (15 mL Falcon tubes containing 4 mL of sterile complete RPMI medium) and store them on ice.

Note: The collection tubes can be prepared a day in advance.

Cell collection and data acquisition

Timing: approximately 30 min per sample

The cells are collected using the BD FACSAria™ III Cell Sorter with the BD FACSDiva™ software.

21. Select parameters including FSC-A, FSC-W, FSC-H, SSC-A, SSC-W, SSC-H, eFluor-506 (viability), eFluor-450 (CD11b), AlexaFluor488 (F4/80), PE (PDPN).
22. Use the unstained control to set appropriate photomultiplier tubes voltages for FSC-A and SSC-A parameters.

Note: given inherent differences between instruments, we encourage to test the panel in advance with your instrument to ensure that all the populations are inside of the axis and/or titrate the antibodies to ensure that the panel is optimal. We strongly discourage the use of the unstained control to adjust the voltages of the fluorochrome detectors.

23. Calculate the compensation matrix with the single stained compensation controls.
24. Use the unstained or the appropriate FMO control to create gates for various populations of interest, e.g., total TAMs (single, viable, $CD11b^+$, $F4/80^+$), PoEMs (single, viable, $CD11b^+$, $F4/80^+$, $PDPN^+$) and non-PoEMs (single, viable, $CD11b^+$, $F4/80^+$, $PDPN^-$) (Figure 3).
25. Sort different TAM populations into corresponding collection tubes, i.e., 15 mL Falcon tubes containing 4 mL of sterile complete RPMI medium (see point 20). Store isolated cells on ice for up to 2 h. If possible, the sorting and collection tubes should be kept at 4°C during the sorting. Use a 100 μm nozzle and sort at a 1-3 flow rate.

⚠ CRITICAL: *It is important to gradually process the already sorted cells withing a 2 h time frame, as after that time, live cells are progressively being lost. Therefore, the processing should be done in parallel to the sorting of remaining samples.*

26. Analyze the data with the FlowJo software (optional).

Processing of isolated cells

27. Wash the collected cells with PBS and put them in culture in DMEM complete in Sterilin 30 mm Petri Dishes (0.5 million cells per dish) or in 96-well cell culture plates (up to 3×10^4 cells per

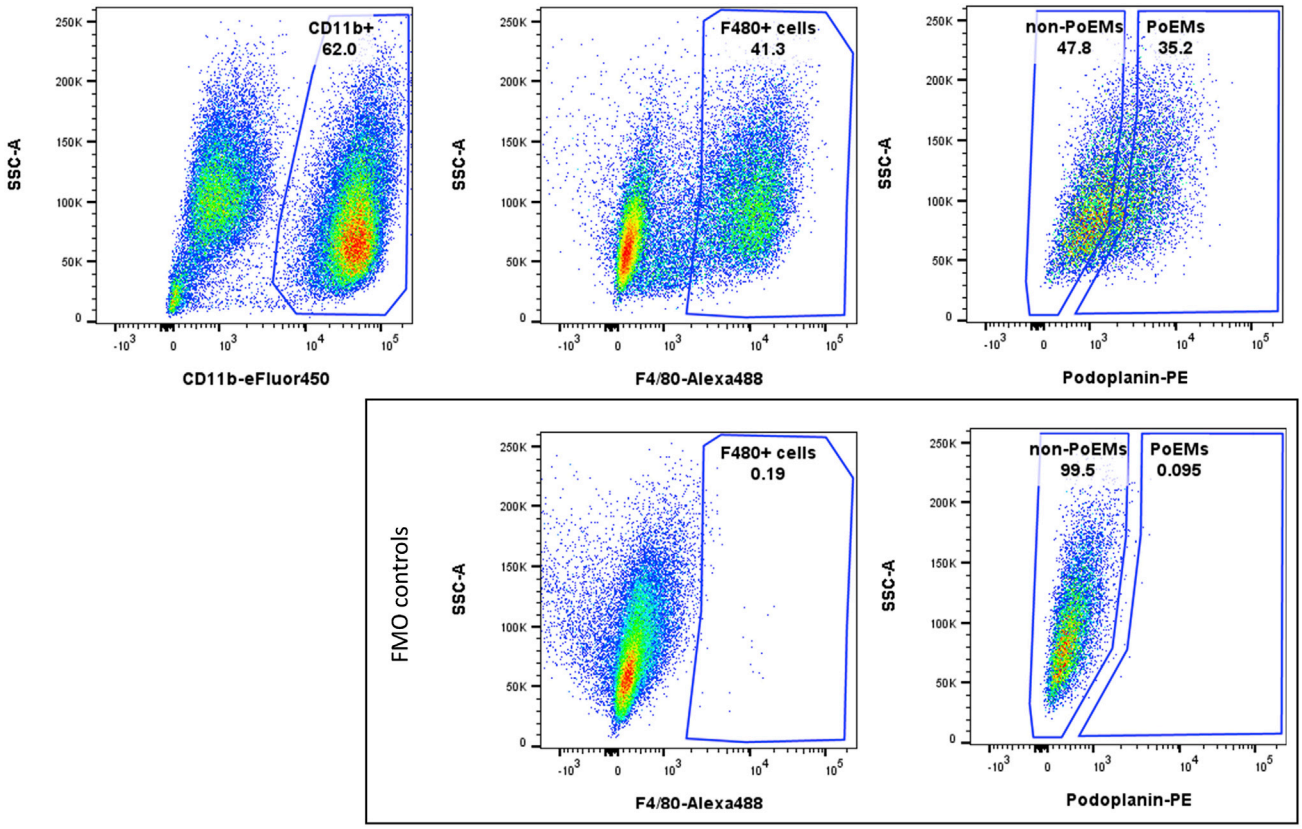

Figure3. Gating strategy for the isolation of various populations of 4T1 tumor-infiltrating TAMs, including total TAMs (single, viable, CD11b$^+$, F4/80$^+$), PoEMs (single, viable, CD11b$^+$, F4/80$^+$, PDPN$^+$) and non-PoEMs (single, viable, CD11b$^+$, F4/80$^+$, PDPN$^-$).

well). Alternatively, spin the cells down (5 min, 300 *g*), wash them in PBS (5 min, 300 *g*), aspirate the supernatant and collect the cells in RLT lysis buffer (according to the manufacturer's instructions) and immediately store at −80°C until the RNA isolation.

Note: An alternative lysis buffer for RNA extraction can be used.

EXPECTED OUTCOMES

PoEMs constitute 30% of all TAMs infiltrating end-stage (e.g., on day 24 post-cancer inoculation) 4T1 tumors. The maximum number of total TAMs isolated from end-stage 4T1 tumors is 1×10^6.

Sorted populations may be cultured, used for various types of cellular assays or their RNA/protein can be directly isolated.

LIMITATIONS

The flow cytometry panel shown in this protocol can only be used for the isolation of total TAMs, PoEMs and non-PoEMs. This panel, however, can be extended to other immune and non-immune (e.g., endothelial) cell types present in 4T1 tumors. For such extended panels, please refer to (Bieniasz-Krzywiec et al., 2019). Of note, when sorting immune cells that are also found in the blood and are indistinguishable from those infiltrating the tissue, mice must be perfused prior to tumor dissection in order to exclude such blood-derived populations.

TROUBLESHOOTING

Problem 1

Larger pieces of the tumor tissue may remain after digestion with the gentleMACS Octo Dissociator.

Potential solution

To further increase the cell yield allow the remaining tissue to settle and remove 1.5 mL of the supernatant to a fresh tube. Insert the C Tube with the remaining tissue pieces onto the sleeve of the gentleMACS Octo Dissociator and run the following program: m_imptumor_01. Combine the resulting cell suspension with the previously removed supernatant.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Massimiliano Mazzone (massimiliano.mazzone@kuleuven.vib.be)

Materials availability

This study did not generate new unique reagents.

Data and code availability

The RNA sequencing data of 4T1 PoEMs and non-PoEMS are deposited in the GEO database under the accession number: GSE126722 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE126722).

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

P.B.-K., R.M.-P., and C.R.-D. performed the experiments, data acquisition, and data interpretation and wrote this protocol. M.M. supervised the study and wrote this protocol.

DECLARATION OF INTERESTS

M.M. and P.B.-K. are co-inventors of the patent "Podoplanin-positive macrophages" (WO2019081714A1).

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