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1. Description

1.1 Background information

Single-cell suspensions are a prerequisite for many experiments, for example to achieve the highest possible purity and recovery during cell separations with MACS® Technology. The gentleMACS™ Dissociator provides optimized programs to attain single-cell suspensions from various tissues, for example, from implanted mouse tumors. In combination with C Tubes, the gentleMACS Dissociator allows the automated tissue dissociation in a closed system, enabling sterile sample handling. A single tube or two tubes can be processed in parallel.

This protocol has been developed for the preparation of TILs (refer to 2.1) or TILs and tumor cells (refer to 2.2) from mouse B16 melanomas, CT26 colon carcinomas, or mPAC pancreatic tumors.

1.2 Reagent and instrument requirements

- gentleMACS Dissociator
- gentleMACS C Tubes (# 130-093-237)
- MACSmix™ Tube Rotator (# 130-090-753)
- Cell strainer (70 µm mesh size)
- (Optional) ART® 1000 REACH™ pipet tips (Molecular BioProducts, Inc.) for removal of dissociated material from the closed C Tubes.
- PEB buffer: Prepare a solution containing phosphate-buffered saline (PBS), pH 7.2, 0.5% bovine serum albumin (BSA), and 2 mM EDTA by diluting MACS BSA Stock Solution (# 130-091-376) 1:20 with autoMACS® Rinsing Solution (# 130-091-222). Keep buffer cold (2–8 °C).
 - ▲ **Note:** EDTA can be replaced by other supplements such as anticoagulant citrate dextrose formula-A (ACD-A) or citrate phosphate dextrose (CPD). BSA can be replaced by other proteins such as gelatine, mouse serum, or fetal bovine serum (FBS).

1.2.1 Reagent requirements for the protocols in section 2.2

- RPMI 1640 (# 130-091-440)
- Collagenase I solution: Prepare a solution containing 10,000 Collagenase Digenstion Units (CDU)/mL Collagenase I (e.g. Collagenase I, Sigma-Aldrich, # C0130) in PBS.
- Dispase II solution: Prepare a solution containing 32 mg/mL Dispase II (e.g. Dispase II, Roche, # 04942078001) in PBS.
- DNase I solution: Prepare a solution containing 5 MU/mL DNase I (e.g. DNase I, Calbiochem, # 260913) in buffer according to the manufacturer's recommendation.

2. Protocol for the preparation of single-cell suspensions from implanted mouse tumors

▲ For details on the use of the gentleMACS Dissociator, refer to the gentleMACS Dissociator user manual.

▲ For cell culture experiments subsequent to tissue dissociation, all steps should be performed under sterile conditions.

▲ When using the MACSmix™ Tube Rotator, operate it on permanent run at a speed of approximately 12 rpm. Alternatively turn tube every 5 minutes to resuspend settled tissue fragments.

▲ Tumors were induced by subcutaneous injection of 10⁶ cells of the tumor cell lines into the upper side of the left and right hind paws of anesthetized C57/Bl6 mice (B16, progressive mPAC), or BALB/c mice (CT26).

▲ The protocols below have been developed to process mouse tumor samples in the range of 0.2–3.5 g per C Tube.

2.1 Protocol for the preparation of tumor-infiltrating lymphocytes (TILs) from mouse B16 melanomas

1. Resect the tumor and cut it into small pieces of approximately 5 mm.
2. Transfer the tissue into the gentleMACS C Tube containing 5 mL of PEB buffer.
3. Tightly close C Tube and attach it upside down onto the sleeve of the gentleMACS Dissociator.
 - ▲ **Note:** It has to be ensured that the sample material is located in the area of the rotor/stator.
4. Switch on the gentleMACS Dissociator and choose the gentleMACS Program **m_impTumor_01**.
5. Run the gentleMACS Program **m_impTumor_01**.
6. After termination of the program, detach C Tube from the gentleMACS Dissociator.

7. (Optional) Perform a short centrifugation step to collect the sample material at the tube bottom.
 8. Resuspend sample and apply the cell suspension to a cell strainer (70 µm mesh size) placed on a 50 mL tube.
▲ **Note:** Dissociated tissue can be removed from the closed C Tube by pipetting through the septum-sealed opening in the center of the cap of the C Tube. Use ART 1000 REACH 1000 µL pipette tips.
 9. Wash cell strainer with 5 mL of PEB buffer.
 10. Discard cell strainer and add PEB buffer to a final volume of 50 mL.
 11. Centrifuge cell suspension at 300×g for 10 minutes. Aspirate supernatant completely.
 12. Resuspend cells and add PEB buffer to a final volume of 50 mL.
 13. Centrifuge cell suspension at 300×g for 10 minutes. Aspirate supernatant completely.
 14. Resuspend cells with PEB buffer to the required volume for further applications.
- 2.2 Protocol for the preparation of TILs and tumor cells from mouse B16 melanomas, CT26 colon carcinomas, or mPAC pancreatic tumors**
- 2.2.1 Preparation of single-cell suspensions from mouse B16 melanomas or mouse CT26 colon carcinomas**
1. Resect the tumor and cut it into small pieces of approximately 5 mm.
 2. Transfer the tissue into the gentleMACS C Tube containing 5 mL of RPMI 1640.
 3. Tightly close C Tube and attach it upside down onto the sleeve of the gentleMACS Dissociator.
▲ **Note:** It has to be ensured that the sample material is located in the area of the rotor/stator.
 4. Switch on the gentleMACS Dissociator and choose the gentleMACS Program **m_impTumor_02**.
 5. Run the gentleMACS Program **m_impTumor_02**.
 6. After termination of the program, detach C Tube from the gentleMACS Dissociator.
 7. Add 150 µL of Collagenase I solution and 150 µL of Dispase II solution.
 8. Incubate sample for 40 minutes at 37 °C using the MACSmix™ Tube Rotator.
 9. Add 2 µL of DNase I solution into the C Tube.
 10. Attach C Tube upside down onto the sleeve of the gentleMACS Dissociator.
▲ **Note:** It has to be ensured that the sample material is located in the area of the rotor/stator.
 11. Using mouse B16 melanomas choose and run the gentleMACS Program **m_impTumor_02**.
Using mouse CT26 carcinomas choose and run the gentleMACS Program **m_impTumor_03**.
 12. After termination of the program, detach C Tube from the gentleMACS Dissociator.
13. (Optional) Perform a short centrifugation step to collect the sample material at the tube bottom.
 14. Resuspend sample and apply the cell suspension to a cell strainer (70 µm mesh size) placed on a 50 mL tube.
▲ **Note:** Dissociated tissue can be removed from the closed C Tube by pipetting through the septum-sealed opening in the center of the cap of the C Tube. Use ART 1000 REACH 1000 µL pipette tips.
 15. Wash cell strainer with 5 mL of PEB buffer.
 16. Discard cell strainer and add PEB buffer to a final volume of 50 mL.
 17. Centrifuge cell suspension at 300×g for 10 minutes. Aspirate supernatant completely.
 18. Resuspend cells with PEB buffer to the required volume for further applications.
- 2.2.2 Preparation of single-cell suspensions from mouse mPAC pancreatic tumors**
1. Resect the tumor and cut it into small pieces of approximately 5 mm.
 2. Transfer the tissue into the gentleMACS C Tube containing 5 mL of RPMI 1640.
 3. Add 150 µL of Collagenase I solution and 150 µL of Dispase II solution.
 4. Tightly close C Tube and incubate sample for 20 minutes at 37 °C using the MACSmix Tube Rotator.
 5. Attach C Tube upside down onto the sleeve of the gentleMACS Dissociator.
▲ **Note:** It has to be ensured that the sample material is located in the area of the rotor/stator.
 6. Switch on the gentleMACS Dissociator and choose the gentleMACS Program **m_impTumor_04**.
 7. Run the gentleMACS Program **m_impTumor_04**.
 8. After termination of the program, detach C Tube from the gentleMACS Dissociator.
 9. Incubate sample for 20 minutes at 37 °C using the MACSmix Tube Rotator.
 10. Add 2 µL of DNase I solution into the C Tube.
 11. Attach C Tube upside down onto the sleeve of the gentleMACS Dissociator.
▲ **Note:** It has to be ensured that the sample material is located in the area of the rotor/stator.
 12. Choose and run the gentleMACS Program **m_impTumor_04**.
 13. After termination of the program, detach C Tube from the gentleMACS Dissociator.
 14. (Optional) Perform a short centrifugation step to collect the sample material at the tube bottom.
 15. Resuspend sample and apply the cell suspension to a cell strainer (70 µm mesh size) placed on a 50 mL tube.
▲ **Note:** Dissociated tissue can be removed from the closed C Tube by pipetting through the septum-sealed opening in the center of the cap of the C Tube. Use ART 1000 REACH 1000 µL pipette tips.
 16. Wash cell strainer with 5 mL of PEB buffer.

17. Discard cell strainer and add PEB buffer to a final volume of 50 mL.
18. Centrifuge cell suspension at 300×g for 10 minutes. Aspirate supernatant completely.
19. Resuspend cells with PEB buffer to the required volume for further applications.

All gentleMACS Protocols are available at www.miltenyibiotec.com.

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