PBMC thawing

Notes

- Frozen cells should be thawed quickly but diluted slowly to remove DMSO.
- Thawed cells are fragile and must be handled gently. Gentle procedures include **slow inversions of tubes for cell homogenization (instead of flushing cells), gentle suspension of cell pellets with a 5 mL pipette (instead of a p1000 pipette, see see Figure 1), adding the medium on the side of the tubes (instead of on the top of the cells), and centrifugation at 200 x*g* (1000 rpm on a Heraeus Megafuge 1.0R).**
- According to some publications, a nuclease treatment step in the thawing procedure may be useful to avoid cell clump formation as a result of dying cells. For nuclease treatment, prepare 24.5 ml of pre-warmed complete medium with 25 U/ml final benzonase per frozen vial (containing up to 50 million PBMCs) and perform steps 5, 6, and 9 with this medium followed by a washing step in 10 ml complete medium (no benzonase) prior to counting the PBMCs. However, in our hands, PBMC thawing with benzonase at 25 U/mL or 50 U/mL had no effect on 24 h post-thaw cell recovery (automated cell count) and viability (assessment of annexin V and propidium iodide binding by flow cytometry) - see experiments winter 2016.
- A 16 to 24 h recovery period after thawing may be beneficial. Dead cells can be visualized better on the next day, in addition freshly thawed cells secrete high levels of cytokines such as IFN-gamma (as published by several investigators and observed in our experiments). The concentration and medium of the PBMCs during this period may affect the overall recovery.

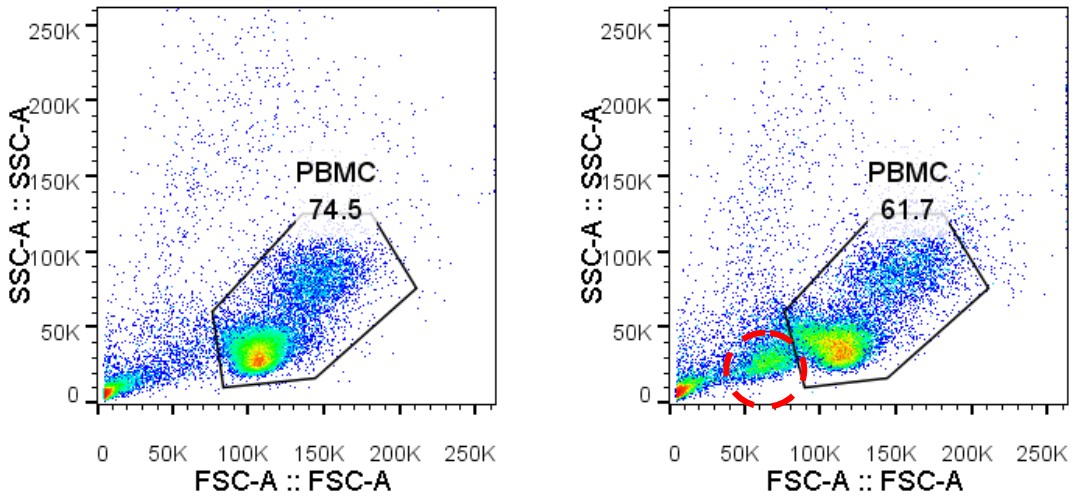

FIGURE 1:
Left: PBMC from donor 298 after thawing and gentle suspension of cell pellets with a 5 mL pipette. Frozen cell recovery was 89.6%.
Right: PBMC from the same vial after gentle suspension of cell pellets with a p1000 pipette. Frozen cell recovery was 60.9%. Note the increased cell debris outside of the gated PBMC.

Reagents

- Complete PBMC medium (e.g. RPMI-1640 with 5 to 10% heat-inactivated FBS, 100 U/ml penicillin, 100 microg/mL streptomycin, 1 mM sodium pyruvate, 2 mM L-glutamine; X-vivo 15 medium (Lonza))

Protocol

1. **Warm complete medium to 37°C** in a water bath.
2. Remove vials from liquid nitrogen and transport them to the lab on dry ice or liquid nitrogen.
3. Thaw frozen vials, only 3 vials at a time, in a 37°C water bath or in the cell incubator. **When cells are nearly completely thawed**, carry the vials to the hood and swipe them with 70% EtOH.
4. Invert the cryovial for cell homogenization and take out gently PBMCs and transfer the cells into a **50 mL tube**.
5. Add 9 mL of warm complete medium **dropwise** i.e. one drop per second while mixing gently the cells in the 50 mL tube. Use 1 mL to rinse out the vials.

6. Wash 1: Spin the cells at 200 x*g* (1000 rpm on a Heraeus Megafuge 1.0R) for 8 min at room temperature.
7. Optional: Wash 2: Suspend the cell pellet with a 5 mL pipette (not a p1000). Spin the cells at 200 x*g* (1000 rpm) for 8 min at room temperature.
8. Count cells and determine viability
    8.1 Suspend the cell pellet with a 5 mL pipette (not a p1000) and add complete medium, suspend slowly the cell pellet using the 1 ml pipet and add medium to a cell concentration of approximately **3 to 4x10^6 cells/mL (e.g. if the vial contained 20x10^6 fresh cells, add approximately 4 mL medium since some cell loss is expected after thawing)**.
    8.2 Perform the counting on 20 microL cell aliquots in duplicate or triplicate. Just prior to pipetting out the cells for the counting, invert gently the tube 5-6 times in order to homogenize the cell suspension.
    8.3 Cell counting with the Cellometer 2000 (linearity: 2 to 12x10^6 cells/mL):
        8.3.1 Principle: Cells are stained with acridine orange, AO, and propidium iodide, PI. AO is a nuclear staining (nucleic acid binding) dye permeable to both live and dead cells. It stains all nucleated cells to generate green fluorescence. PI can only enter dead cells with compromised membranes. PI stains all dead nucleated cells to generate red fluorescence. Cells stained with both AO and PI fluoresce red due to quenching, so all live nucleated cells fluoresce green and all dead nucleated cells fluoresce red.
        8.3.2 Protocol: Remove the 2 plastic covers of the counting slide (each slide has 2 counting chambers). Mix 20 microL cells with 20 microL AO/PI. **Mix 5 times and load 20 microL into the chamber**. Adjust the focus (perform an image preview) and read **immediately** and **only once** the cell staining (perform a cell count using the "immune cells, low RBC" program). Record the live cell concentration and the percentage of live cells.
9. Seed PBMCs at app. 3.0x10^6 cells/ml in a flask or plate:
    9.1 P24 well (2 cm^2): 0.5 to 1 mL
    9.2 P12 well (4 cm^2): 1 to 2 mL
    9.3 P6 well (10 cm^2): 2.5 to 5 mL
    9.4 T25 cm^2 plates: 6 to 9 mL
    9.5 T75 cm^2 plates: 18 to 18 mL
10. Leave PBMCs for recovery (37°C; 5% CO_2 for 16 to 24 h).
11. Harvest PBMCs (monocytes should detach after gentle pipetting of the wells or scraping of the plates).
12. Spin PBMCs: 300x*g* (in general 1200 rpm) for 8 min at room temperature (this step is important to remove all factors secreted post-thaw).
13. Proceed to assay(s).