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 5mL pipette (instead of a p1000 pipette, **Figure 1**), adding the medium on the side of the tubes (instead of on the top of the cells), and low speed centrifugation at 200*xg* (1000rpm on a Heraeus Megafuge 1.0R)
- A 4 to 24h recovery period after thawing may be beneficial. For instance, freshly thawed cells secrete cytokines such as IFN-gamma (as published by several investigators and observed in our experiments). FSC/SSC profile evolves during the recovery with an optimal profile 4 at post thaw (**Figure 2**)
- 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. 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.

FIGURE 1: Left: PBMC from RFS donor 298 after thawing and gentle suspension of cell pellets with a 5mL 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.

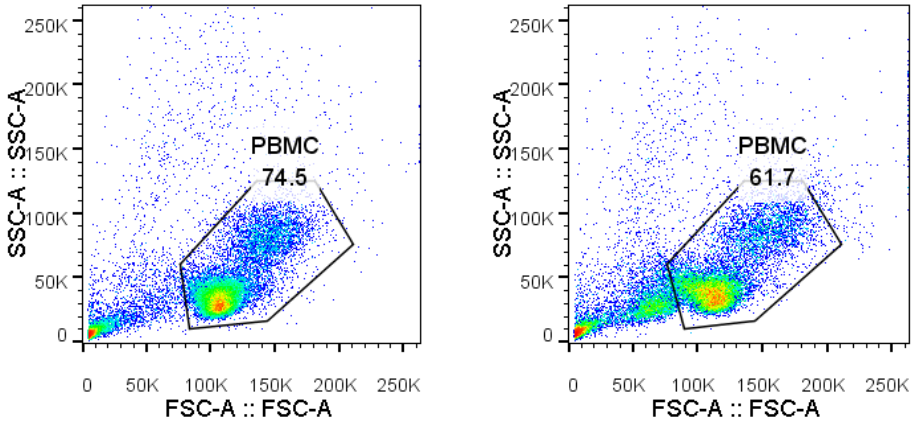
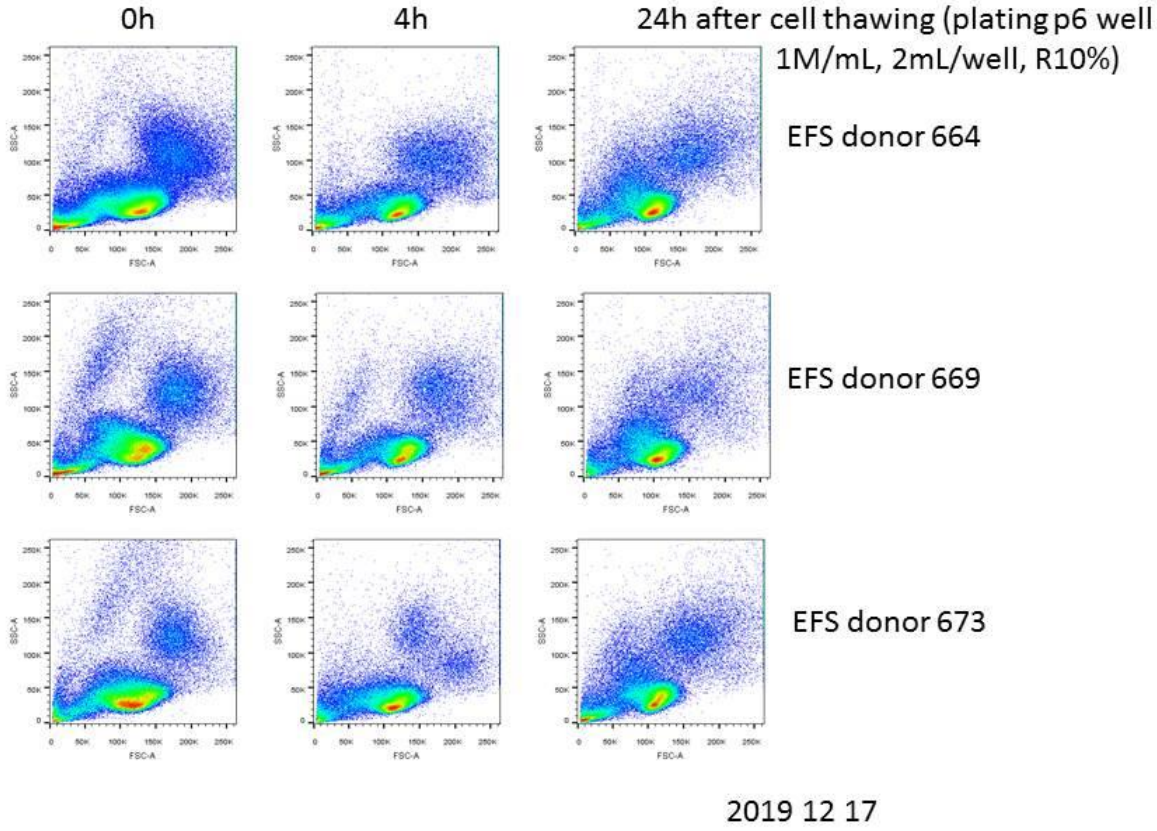


FIGURE 2: FSC/SSC profile 0, 4 and 24h post thaw. Note the optimal profile at 4h post thaw.



Reagents

- Complete PBMC medium (e.g. RPMI-1640 with 5 to 10% heat-inactivated FBS, 100U/ml penicillin, 100microg/mL streptomycin, 1mM sodium pyruvate, 2mM 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 **50mL tube**.
5. Add 9mL of warm complete medium **dropwise** i.e. one drop per second while mixing gently the cells in the 50mL tube. Use 1mL to rinse out the vials.
6. Wash 1: Spin the cells at 200x*g* (1000 rpm on a Heraeus Megafuge 1.0R) for 8min at room temperature.
7. Optional: Wash 2: Suspend the cell pellet with a 5mL pipette (not a p1000). Spin the cells at 200x*g* (1000rpm) for 8min at room temperature.
8. Count cells and determine viability
   - 8.1 Suspend slowly the cell pellet with a 5mL pipette to an estimated cell concentration of 2 to $5x10^6$ cells/mL.
   - 8.2 Perform the counting on 20microL 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: 0.5 to $10x10^6$ 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 20microL cells with 20microL AO/PI. Mix 5 times and load 20microL 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. Suspend PBMCs at $1.0x10^6$ cells/ml R10% or R5% medium. Optimal plating is into a T25cm² or a T75cm² flask standing up (max 10 or 20mL respectively). Cells can also be cultured into a p6 well plate (2-3mL per well), a p12 well plate (1-2mL per well), or a 50mL tube inclined 5% with a nearly unscrewed cap to allow gas exchanges see picture below (max 10mL cells)

10. Leave PBMCs for recovery (37°C; 5% CO_2 for 4 to 24h).
11. Harvest PBMCs: detach monocytes by gentle pipetting of the wells/plates or tube inversion. Remove cell aggregates by passing cells on a 100microns cell strainer (essential for cell sorting or ELISPOT assays)
12. Spin PBMCs: 300x*g* (in general 1200rpm) for 8min at room temperature (this step is important to remove all factors secreted post-thaw).
13. Proceed to assay(s).