FR-AgEncode: a French pilot project to enrich the annotation of livestock genomes

Sorting of swine $CD3^+$ $CD4^+$ $CD8^{+/-}$, $CD3^+$ $CD4^-$ $CD8^+$ and $CD3^+$ $CD8^-$ cells protocol

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Sorting of swine $CD3^+$ $CD4^+$ $CD8^{+/-}$, $CD3^+$ $CD4^-$ $CD8^+$ and $CD3^-$ $CD8^-$ cells

Required reagents
- polystyrene 5 ml FACS tubes (12 x 75 mm, BD Falcon Cat#352058)
- anti-pig CD3 (clone PPT3, Southern Biotech Cat#4510-01)
- anti-pig CD4 (clone 74-12-4, Southern Biotech Cat#4515-01)
- anti-pig CD8a (clone 76-2-11, Southern Biotech Cat#4520-01)
- goat anti-mouse IgG1-FITC (Southern Biotech Cat#1070-02)
- rat anti-mouse IgG2a-PerCP-eFluor710 (eBioscience Cat#46-4210)
- goat anti-mouse IgG2b-PE (Southern Biotech Cat#1090-09)
- LIVE/DEAD® Fixable Far Red Dead Cell Stain Kit (Life Technologies Cat#L10120)
- cell strainer cap, 35 µm (BD Falcon Cat#352235)
- PBS without Ca2+ /mg2+ (Euroio, Cat#CSPBS01-01)
- Fetal Calf Serum (Eurobio, Cat#CVFSVF06-01)
- EDTA

Before starting:
Prepare PBS containing 5% FCS and 0.5mM EDTA.
Reconstitute vials of LIVE/DEAD® Fixable Far Red Dead Cell Stain Kit (Life Technologies) with 100µL of the provided DMSO.
Prepare collection tubes by coating polypropylene 5 ml FACS tubes overnight with 4mL of FCS at 4°C.

Important note:
PBMCs must be prepared before according to the "Separation of PBMCs from swine blood sample" protocol and samples must have very few red blood cells. If necessary, another step of red blood cells lysis must be performed by incubating cells during 10 minutes with 5ml of NH4CL NH4Cl 1X (prepared from NH4Cl 10X: 74.7 g de NH4Cl, 85 ml Tris HCl ph:7.5, qsp H2O 1L). Stop the reaction by adding 5ml of RPMI+10%FCS. Add PBS qsp 50mL. Spin at 1300 rpm for 10 min.

Controls for compensation matrix:
$FITC^+$, PE^+, PerCP-eFluor710^+ and far red live/dead$^+$ controls are prepared by labeling 5.10^5 PBMCs per condition with each primary antibody at once and its corresponding secondary antibody. 5.10^5 unlabelled cells are also needed to set up the compensation matrix on the cytometer.

Preparation of cells

1. A maximum of 40.10^6 PBMCs from each swine blood samples are transferred in polystyrene 5 ml FACS tubes and centrifuged at 1500rpm for 5min at 20°C. Supernatant is then discarded by inverting the tube.
2. PBMCs are incubated 15min at 4°C with primary antibodies: anti-pig CD3 (1/200, clone PPT3, mouse IgG1), anti-pig CD4 (1/200, clone 74-12-4, mouse IgG2b) and anti-pig CD8a (1/200, clone 76-2-11, mouse IgG2a) diluted in 1mL of PBS containing 5% FCS and 0.5mM EDTA.
3. Cells are washed by adding 3mL of PBS containing 5% FCS and 0.5mM EDTA and centrifuged at 1500rpm for 5min at 20°C. Supernatant is then discarded by inverting the tube.
4. The pellet is suspended with secondary antibodies: goat anti-mouse IgG1-FITC (1/500), rat anti-mouse IgG2a-PerCP-eFluor710 (1/200) and goat anti-mouse IgG2b-PE (1/500) diluted in 1mL of PBS containing 5% FCS and 0.5mM EDTA and incubated 15min at 4°C.
5. Cells are washed by adding 3mL of PBS and centrifuged at 1500rpm for 5min at 20°C. Supernatant is then discarded by inverting the tube.
6. The pellet is suspended with LIVE/DEAD® Fixable Far Red Dead Cell Stain Kit, 10µL per tube diluted in 2mL of PBS and incubated 20min at 4°C.

7. Cells are washed by adding 3mL of PBS containing 5% FCS and 0.5mM EDTA and centrifuged at 1500rpm for 5min at 20°C. Supernatant is then discarded by inverting the tube.
8. The pellet is finally suspended in PBS containing 5% FCS and 0.5mM EDTA at 10.10^6 cells/mL. Cells are filtered with cell strainer cap, 35 µm.

Flow cytometry acquisition and sorting of cells

9. Automated compensation matrix is realized.
10. Viable $CD3^+$ $CD4^+$, $CD3^+$ $CD4^-$ $CD8^+$ and $CD3^-$ $CD8^+$ cells are sorted on a BD FACSAria™ (100µm nozzle, 4-way purity sort). The sorted cells are collected in polypropylene 5 ml FACS tubes pre-coated overnight with FCS at 4°C.
11. For each pig, PBMC are analyzed as described in Figure 1, 10 000 events are acquired.
12. For each tube of sorted cells, purity of cells is assessed by analyzing 2000 events with the same gating strategy.

Note: The minimum of purity of sorted cells obtained was 96.3%. Purity of sorted cells was comprised between 98.8 and 99.6% (lower and upper CI of mean, n=28).

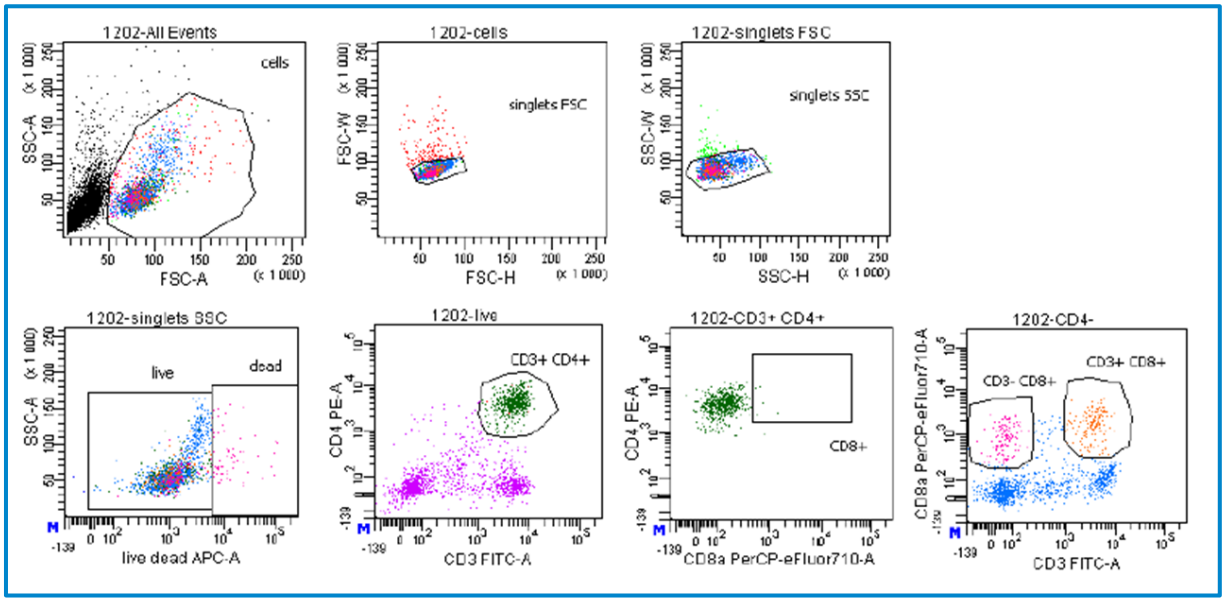

Figure 1: Gating strategy of cells. Cells are first plotted on FSC *vs* SSC and "cells" gate is adjusted. Then "cells" are plotted on FSC-H *vs* FSC-W and SSC-H *vs* SSC-W to remove doublets. Live and dead cells are identified in a live/dead *vs* SSC-A plot. "live cells" are not stained by live/dead reagent. "Live cells" are then plotted on CD3-FITC *vs* CD4-PE and $CD3^+$ $CD4^+$ cells are gated and sorted. Among those cells some are $CD8^-$ and correspond to $CD4^+$ $CD8^-$ T cells and others are $CD8^+$ and correspond to double positive $CD4^+$ $CD8^+$ T cells. NOT($CD3^+$ $CD4^+$) cells are then plotted on CD3-FITC *vs* CD8a-PerCP-eFluor710 and "$CD3^-$ $CD8^{+"}$ cells corresponding to NK cells and "$CD3^+$ $CD8^{+"}$ cells corresponding to CD8 cytotoxic T cells are sorted.

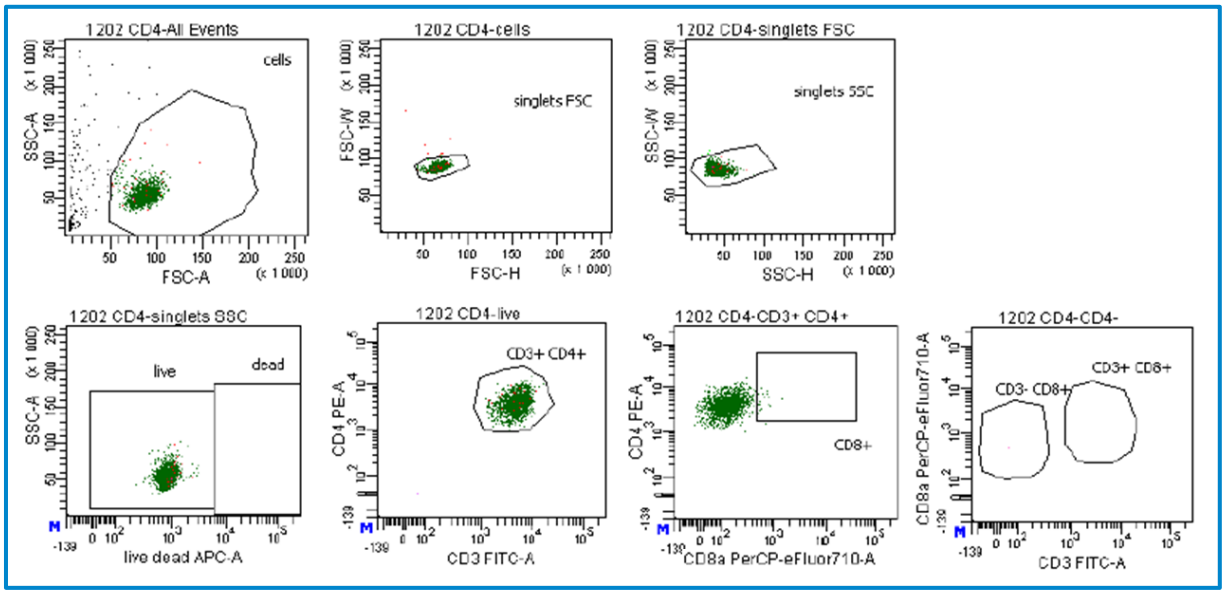

Figure 2: Evaluation of the purity of sorted cells. One example of $CD3^+$ $CD4^+$ cells is shown.