

Harvest and Staining Protocol Red Pulp Macrophages/CD8+ T cells (Spleen)

Pool if possible 3 female mice before sorting

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- 1) Take spleen
- 2) Smash spleen over 100µm (or 70µm) strainer using syringe plunger, wash with 10-20ml DMEM/10% FCS/P/S
- 3) Centrifuge 5min, 500g, 4°C
- 4) Resuspend pellet in 1ml Red Blood Cell Lysis Solution (Promega, Z3141), incubate 5min on ice, stop reaction by adding 1xPBS up to 50ml
- 5) Centrifuge 5min, 500g, 4°C
- 6) Discard supernatant→resuspend in 1ml PBS/2%BSA
- 7) Filter through 70µm strainer and wash the strainer with another 1ml of PBS/BSA
- 8) Split sample→ e.g. unstained (10% of sample), Panel (see below for Antibodies) → transfer aliquots into FACS tubes
- 9) Centrifuge 5min, 500g, 4°C
- 10) Resuspend in 100µl PBS/2%BSA+blocking AB (1:500), incubate for 10-15min on ice
- 11) Directly add viability dye (1:4000)+ staining mix
! NO washing inbetween the Viability dye and the staining mix → staining mix is calculated per mouse, so double the amount if two mice are pooled, triple the amount if three mice are pooled,
...
- 12) Incubate 30min, 4°C, dark
- 13) Add 1ml PBS/2% BSA→ Centrifuge 5min, 500g, 4°C
- 14) Resuspend in 300µl PBS/2% BSA→ filter over 40µm strainer (5ml Polystyrene round bottom tubes with Cell Strainer) immediately before sorting and rinse the filter with 1ml PBS/BSA

Staining mix:

Marker	Fluorochrome	Laser	Company	Dilution
Live/Dead	APC-eF780	R	eBioscience, 65-0865-14	1:1,000
Ter119	APC-Cy7	R	Biolegend, 116223	1:100
CD8	APC	R	Biolegend, 100711	1:200
F4/80	FITC	B	Biolegend, 123108	1:100
CD19	PerCPCy5.5	B	Biolegend, 115533	1:100
CD3	PE	B (Vet Med) or Y/G	Biolegend, 100205	1:200
Ly-6C	PECy7	B	Biolegend, 128017	1:200
Ly-6G	PECy7	B	Biolegend, 127617	1:200
NK1.1	PECy7	B	Biolegend, 108713	1:100
CD45	AF700	R	Biolegend, 103128	1:100

Viability Dye: Fixable Viability Dye eBioscience, 65-0865-14

Blocking AB: Anti-mouse CD16/CD32 purified, eBioscience 14-0161-82

FACS tubes (sterile): Falcon #352058

Sorting (FACS Aria Version 2 or higher):

-use 70µm nozzle, bi-exponential setting for all axis (except SSC and FSC)

FACS tube sorting:

-sort around 20.000 events/sec

-Precision mode: Purity (or 4-way purity if sort 4 populations)

-if sort 4 populations: rare populations outer position (to prevent cross-contamination)

-if sort 2 populations: use inner positions (more stable)

- Sheath fluid to use: FACS FLOW

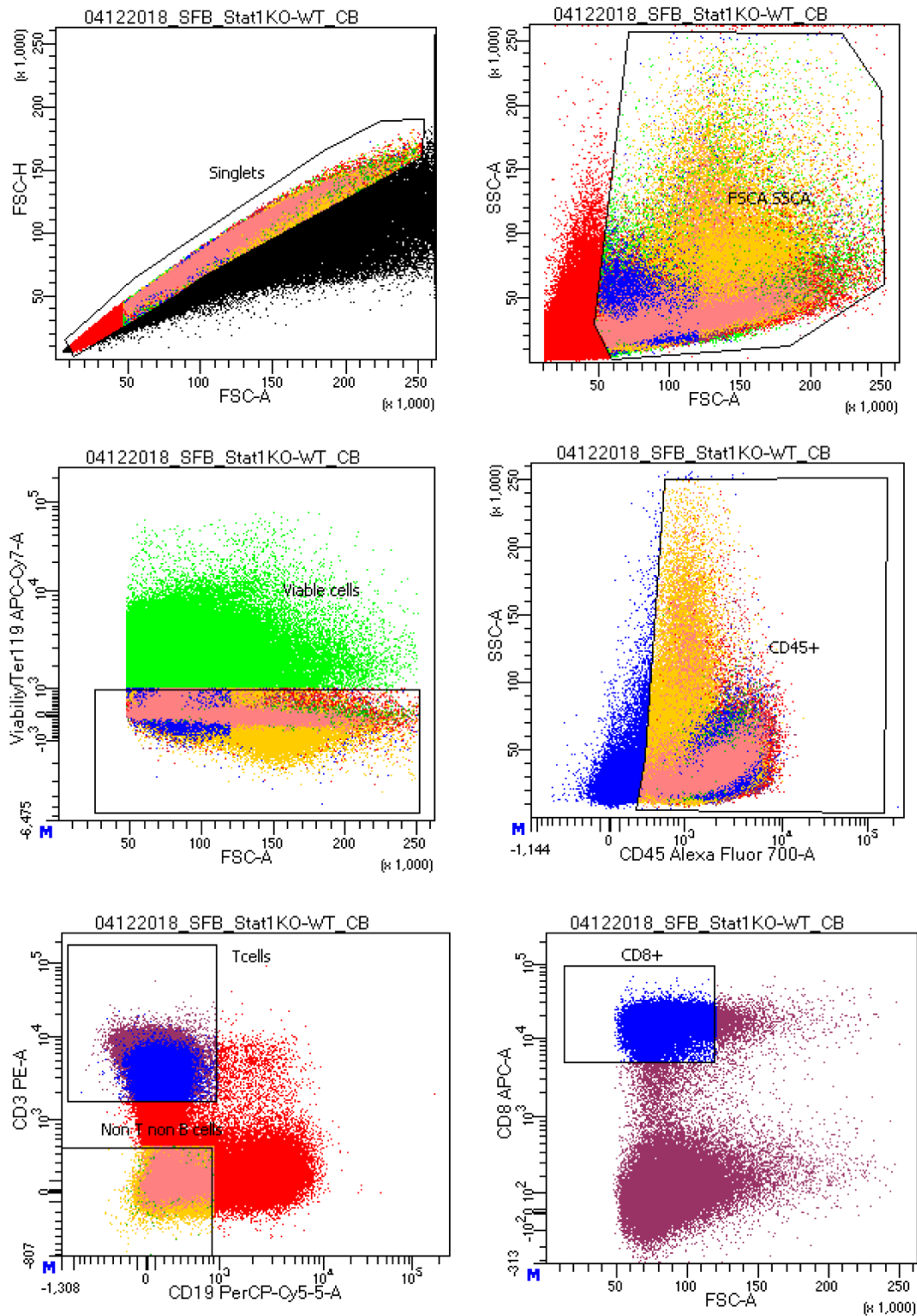
Expected Yield per spleen of an 8-12 week old mouse:

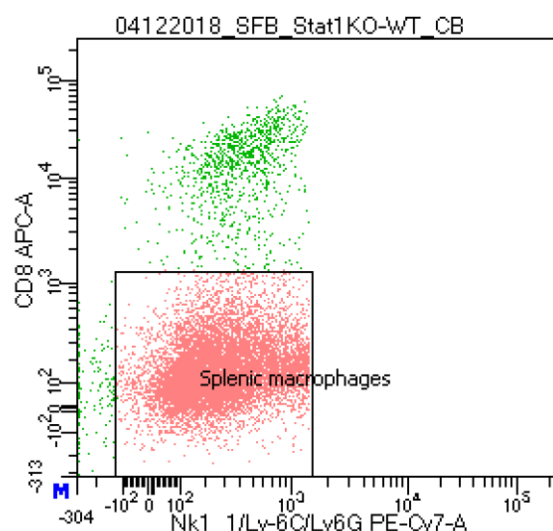
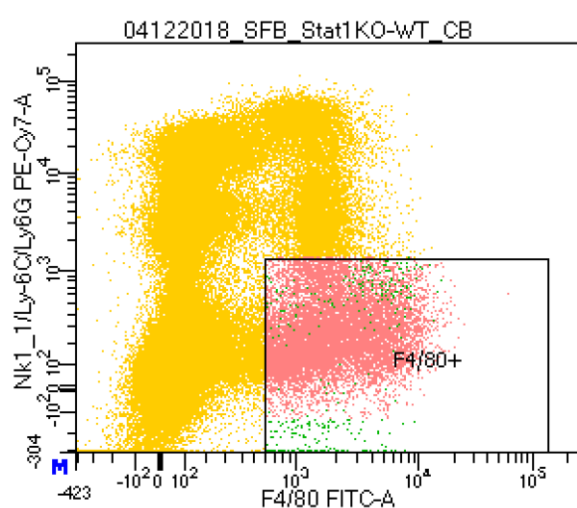
Red pulp Macrophages: ~100.000 – 300.000 cells

CD8+ T-cells: >1.000.000 cells

Gating strategy for splenic red pulp MΦ and CD8+ T cells (FACS-Aria version 2 or higher):

Stained Pool:





Tube: WT_CB

Population	#Events	%Parent	%Total
All Events	1,165,509	###	100.0
Singlets	1,000,000	85.8	85.8
FSCA SSCA	622,688	62.3	53.4
Viable cells	478,303	76.8	41.0
CD45+	428,208	89.5	36.7
Tcells	129,213	30.2	11.1
CD8+	42,240	32.7	3.6
Non T non B cells	184,112	43.0	15.8
F4/80+	12,110	6.6	1.0
Splenic macroph	10,976	90.6	0.9

Experiment Name:	SFB Sorting_extraNK staining			
Specimen Name:	04122018_SFB_Stat1KO			
Tube Name:	WT_CB			
Record Date:	Dec 4, 2018 1:29:40 PM			
\$OP:	CeMM			
GUID:	7eef90da-dcc1-440c-ab17-e4567daffb90			
Population	#Events	%Parent	FSC-A Mean	FSC-H Mean
All Events	1,165,509	###	76,017	57,377
Singlets	1,000,000	85.8	66,528	55,180
FSCA SSCA	622,688	62.3	87,341	71,286
Viable cells	478,303	76.8	88,386	72,696
CD45+	428,208	89.5	90,355	74,207
Tcells	129,213	30.2	83,333	69,704
CD8+	42,240	32.7	81,407	68,305
Non T non B cells	184,112	43.0	88,070	72,066
F4/80+	12,110	6.6	116,784	90,210
Splenic macrophages	10,976	90.6	114,021	88,686