FACS HRWL_IMM_001

Purpose

This test differentiates immune cell sub-populations via Fluorescence-Activated Cell Sorting.

Description: increased CD4-positive T cell number (MP:0008074), decreased CD4-positive T cell number (MP:0008075), etc., …

This protocol refers to data generated at Harwell up until the IMPC Toronto meeting of October 2013.

Experimental Design

- **Minimum number of animals :** 4M + 4F
- **Age at test:** Week 16
- **Sex:** We would expect the results of this test to show sexual dimorphism

Equipment

· Scissors and forceps for biopsy

· Precision balance

· Calibrated single and multichannel pipettes

· GentleMACS tissue dissociator

· Plate shaker

· Moxi-z Cell counter

· Refrigerated centrifuge

· Flow Cytometer (FACS Canto II)

Supplies

· Microplate 96 U Well PS (SLS #MIC9020)

· Petrie dishes (BD Falcon #353004)

· Dispensing troughs

· Extra long 10µl pipette tips for antibody solutions

· GentleMACS C Tubes (Miltenyi Biotec, #130-096-334)

· 50ml Falcon tubes

· 70m cell strainers that fit 50ml Falcon tubes (BD Falcon, #352350)

· Moxy-Z Cassettes

· Select Microplate 96 U Well PS (SLS #MIC9020)

· RPMI (SIGMA, #R8758 - 100ml bottles)

· FCS (PAA, #A15-151)

· PBS 10X (SIGMA, #D1408)

· EDTA 0.5M (Invitrogen, #15576-028)

· Collagenase II (Serlabo, #CLS2LS004176), stock solution: 70 mg/mL, aliquoted and stored at –20°c

· DNAse I (SIGMA, #DN25), stock solution: 10 mg/mL, aliquoted and stored at –2 0°c

· 10x RBC lysis solution (eBiosciences, #00-4300-54)

Procedure

Reagent preparation

· **RPMI 2% FCS buffer:** for spleen processing steps

· **FACS buffer**: PBS 1X; EDTA 5 mM; 0.5%FCS (v/v). Stable for up to 1 month in the fridge.

· **RBC Lysis buffer**: Prepare a 1X solution of 10X eBiosciences solution.

· **Stopping buffer**: PBS 1X/EDTA 0.1M (37.5g in one litre). Require 300ul per sample.

· **Buffer for organ collection:** RPMI for organ collection

· **Enzyme stock solutions**:

DNAse I (10 mg/ml), 10mg in 10ml RPMI/ 2% FSC and freeze into 500 L aliquots

500 L Collagenase II (70 mg/ml) 1mg in 14ml RPMI/ 2% FSC and freeze into 500 L aliquots

- **Antibody cocktails for Panels 1 & 2**

Protect antibodies and prepared cocktails from direct light.

Each sample will require 50µl of diluted antibody cocktail.

Prepare a minimum volume of 600µl.

Antibody cocktails should be vortexed to ensure homogeneity of the solutions.

MRC Harwell Spleen Flow Cytometry Panels 1 & 2

Panel 1:

Marker / Antigen	Fluorochrome	Specificity	Supplier	Catalogue number	Size	Final concentration in cocktail
Live/dead	SytoxBlue	Live / dead	Invitrogen	S11348	250 ul	1:10000
CD5	BV421	T-cells, highest on helper T-cells, Not NK (also includes B1 Bcells)	BD Pharmingen	562739	50 µg	1/400
CD4	FITC	Helper T cells	BD Pharmingen	557307	0.1 mg	1:3200
CD8	PE-CF594	Cytotoxic T cells	Invitrogen /Life Technologies	MCD0824	1 ml	1:3200
CD25	APC	Regulatory T cells	BD Pharmingen	557192	0.1 mg	1:800

CD62L	APC-Cy7	Level of expression distinguishes naive, effector, and memory T cells	BD Pharmingen	560514	50 µg	1:100
CD44	PE	Activated CD4+ and CD8+ T cells	BD Pharmingen	553134	0.1 mg	1:400
CD161	PECy7	NK cells (as well as NK-T cells)	BD Pharmingen	552878	0.1 mg	1:100

Panel 2:

Marker / Antigen	**Fluorochrome**	**Specificity**	**Supplier**	**Catalogue number**	**Size**	**Final concentration in cocktail**
Live/dead	SytoxBlue	Live / dead	Invitrogen	S11348	250 ul	1:10000
F4/80	PE	Mature macrophages	eBiosciences	12-4801-82	100 ug	1:50
CD19	BV510	Overall B Cells	BD Horizon	562956	50 µg	1:800
IgD	APC	Mature B cells	BD Pharmingen	560868	50 µg	1:200
Ly6C	FITC	Monocytes / Macrophages (also neutrophils and some T-subsets)	BD Pharmingen	553104	0.5 mg	1:200

Ly6G	BV421	Granulocytes	BD Pharmingen	562737	50 µg	1/200	
CD5	BV421	T-cells, highest on helper T-cells, Not NK (also includes B1 Bcells)	BD Pharmingen	562739	50 µg	1/400	
CD11b	PE-CF594	Monocytes, dendritic cells	BD Pharmingen	562287	0.1 mg	1:800	
CD11c	PECy7	Dendritic cells (has also been detected on mouse splenic NK cells)	BD Pharmingen	558079	0.1 mg	1:100	
MHCII (anti-Mouse I-A/I-E)	APC-Cy7	Activated Dendritic cells	Insight Biotechnology	107628	100 ug	1:400	

<u>MRC Harwell Gating Strategy Version 1</u>

Mix	Population	Subset	Parameter 1	Parameter 2	Parameter 3	Parameter 4	Parameter 5	Monitor	
A	NK total		CD5-	CD161+	CD44+			%	Innate
	T cells, NKT cells, B1		CD5+					%	
		NKT total	CD5+	CD161+	CD44+			%	
		NKT Effector	CD5+	CD161+	CD44+	CD62L-		%	Innate like
		NKT Resting	CD5+	CD161+	CD44+	CD62L+		%	

	iNKT	CD5+	CD161+	CD44+	CD4+		%		
	CD4 T cells total	CD5+	CD4+				%		
	CD4 Effector	CD5+	CD4+	CD25-	CD44+	CD62L-	%	Helper	
	CD4 Resting /Naive	CD5+	CD4+	CD25-	CD44+	CD62L+	%		
	Tregs	CD5+	CD4+	CD25+			%		
	Tregs Effector	CD5+	CD4+	CD25+	CD44+	CD62L-	%	Regulatory	
	Tregs Resting	CD5+	CD4+	CD25+	CD44+	CD62L+	%		
	CD8 T cells total	CD5+	CD8+				%		
	CD8 Effector	CD5+	CD8+	CD44 high	CD62L-		%	Cytotoxic	
	CD8 Resting	CD5+	CD8+	CD44 high	CD62L+		%		
	CD8 naive	CD5+	CD8+	CD44 low	CD62L+		%		**Gated on live cells**
	gd + B1	CD5+	CD4- CD8-				%	Innate like	
B									
	Neutrophils	CD11b+	Ly6G high				%		

	Monocytes		CD11b+	Ly6G-	Ly6C high			%	Myeloid	
	Macrophage		CD11b+	Ly6G-	Ly6C-	F4/80+	MHCII-low	%		
	Eosinophils		CD11b+	Ly6G-	Ly6C-	F4/80-	SSC High	%		
	B cell total		CD11b-	CD19+	MHCII+			%	B cells	
	B2 total		CD11b-	CD19+	MHCII+	CD5-		%		
	B2 mature	Trans. 2+ Trans. 3+ Mature + GC	CD11b-	CD19+	MHCII+	CD5-	IgD+	%		
	B2 immature + MZB	Trans. 1 + MZB	CD11b-	CD19+	MHCII+	CD5-	IgD-	%		
	B1 Total		CD11b-	CD5+	CD19+	MHCII+		%		
DCs	DC total		CD11c+	MHCII+				%	DCs	
	pDCs		CD11c+	MHCII low				%		
	cDC CD11b type		CD11c+	MHCII+	CD11b+			%		
	cDC CD8a type		CD11c+	MHCII+	CD11b-			%		

- **Read buffer / dead cell exclusion dye**

SytoxBlue at 1:10000 concentration.

Require 200ul per well (i.e. 400ul for each spleen).

· **Enzyme cocktail (7X):** 500 L of DNAse I (10 mg/ml), 500 L Collagenase II (70 mg/ml) into 4 ml RPMI, 2% FCS (v/v). Amount is sufficient for 12 spleens.

Other preparations on the day

· Bring Stop solution and FACS buffer to room temperature.

· Prepare wet ice box.

· Number Falcon tubes, C-Tubes & Eppendorfs for dilutions and set out in racks.

· Place open C-tubes on wet ice and add 2.6ml RPMI with 2%FCS.

Note all centrifuge steps are: 5 min, 290 x g at 4°C

Spleen collection

· Collect the spleen from euthanized mice.

· Remove all fat from the spleen and weight the organ on a petri dish (do not hydrate the organ before weighing it as it would lead to substantial errors in measurement).

· Place the spleen in a 1.5ml eppendorf tube with 1mL of RPMI on ice.

· Once in the lab, transfer each spleen to a GentleMacs C-tube containing 2.6ml RPMI with 2%FCS on ice. Note that if the spleen weight exceeds the recommended value of 250 mg of tissue, transfer only part of the spleen (100 mg).

Spleen dissociation

· Add 400 L of 7X enzyme cocktail to the GentleMACS C tube already containing 2.6 mL of RPMI/2% FCS and the spleen.

· Clip the tube on GentleMACS dissociator and run the IMPC program located in the Favourites folder (this takes roughly 20 mins).

· Add 300 L of stopping reaction to block enzymatic digestion and dissociate T/DC interactions.

· Filter through 70um Nylon mesh filter to a 50 mL Falcon tube.

· Wash the GentleMACS C tube with 5ml FACS buffer, filter and pool with flow through from previous step.

· Centrifuge and discard supernatant.

· Resuspend total splenocytes in 1 mL FACS buffer.

Cell counting

· Prepare a 1:300 (3ul in 897) diluted aliquot from 1ml splenocyte suspension gently vortex.

· Perform count using the Moxy-Z cell counter.

· Note down the cell count in a spreadsheet that corrects for dilution and calculates the number of cells per µl.

· Pipette the volume containing exactly 2 million cells to a 96 well plate in horizontal fashion starting from A1 onwards for panel 1 staining.

· Do the same for panel 2 staining in separate wells leaving a few empty rows between the panels to avoid cross contamination.

· Top up to final volume of 100ul using FACS buffer, centrifuge and discard supernatant.

Red blood cell lysis, blocking & staining

· Add the 100ul of lysis buffer to the cell pellet from the previous step.

· Pipette up and down 2-3 times to break up the pellet and ensure complete lysis.

· Incubate on ice for 1 minute.

· Centrifuge, discard supernatant and resuspend in 200ul FACS buffer.

· Again centrifuge and discard S/N and resuspend in 50ul of 1:100 FC block and incubate on ice for 15 mins. Top up to 200ul using FACS buffer after incubation.

· Centrifuge, discard supernatant and resuspend in 200ul FACS buffer.

· In order to eliminate aggregated antibodies from your mix, centrifuge each antibody cocktail for 8 mins at 20,000 g and 4°C.

· Centrifuge, discard supernatant and resuspend in 50ul mAB mix in appropriate wells for individual panels followed by incubation on ice and in the dark for 20 minutes. Top up to 200ul with FACS buffer after incubation.

· Wash twice as in step two and at the end of second cycle resuspend the pellet in 200ul of read buffer (Sytox Blue diluted 1:10000 in FACS buffer).

Analysis

Set up the analyser to acquire 300,000 events in the SSC-W Vs SSC-H singlets gate (P1).

Parameters and Metadata

Spleen weight HRWL_IMM_001_001 | v1.2

simpleParameter

Req. Analysis: false **Req. Upload:** true **Is Annotated:** false

Unit Measured: g

Number of live cells acquired Panel 1 HRWL_IMM_002_001 | v1.0

simpleParameter

Req. Analysis: false **Req. Upload:** true **Is Annotated:** false

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CD4 Effector HRWL_IMM_003_001 | v1.2

simpleParameter

Req. Analysis: false **Req. Upload:** true **Is Annotated:** false

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CD4 Resting/Naive HRWL_IMM_004_001 | v1.2

simpleParameter

Req. Analysis: false **Req. Upload:** true **Is Annotated:** false

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CD4 T cells total HRWL_IMM_005_001 | v1.2

simpleParameter

Req. Analysis: false **Req. Upload:** true **Is Annotated:** false

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CD8 Effector HRWL_IMM_006_001 | v1.2

simpleParameter

Req. Analysis: false **Req. Upload:** true **Is Annotated:** false

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CD8 Naive HRWL_IMM_007_001 | v1.3

simpleParameter

Req. Analysis: false **Req. Upload:** true **Is Annotated:** false

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CD8 Resting HRWL_IMM_008_001 | v1.4

simpleParameter

Req. Analysis: false **Req. Upload:** true **Is Annotated:** false

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CD8 T cells total HRWL_IMM_009_001 | v1.2

simpleParameter

Req. Analysis: false **Req. Upload:** true **Is Annotated:** false

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gd + B1 HRWL_IMM_010_001 | v1.2

simpleParameter

Req. Analysis: false **Req. Upload:** true **Is Annotated:** false

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iNKT HRWL_IMM_011_001 | v1.2

simpleParameter

Req. Analysis: false **Req. Upload:** true **Is Annotated:** false

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NK Total HRWL_IMM_012_001 | v1.2

simpleParameter

Req. Analysis: false **Req. Upload:** true **Is Annotated:** false

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NKT Effector HRWL_IMM_013_001 | v1.2

simpleParameter

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NKT Resting HRWL_IMM_014_001 | v1.2

simpleParameter

Req. Analysis: false **Req. Upload:** true **Is Annotated:** false

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NKT Total HRWL_IMM_015_001 | v1.2

simpleParameter

Req. Analysis: false **Req. Upload:** true **Is Annotated:** false

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T/NKT/B1 HRWL_IMM_016_001 | v1.2

simpleParameter

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Tregs HRWL_IMM_017_001 | v1.2

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Tregs Effector HRWL_IMM_018_001 | v1.2

simpleParameter

Req. Analysis: false **Req. Upload:** true **Is Annotated:** false

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Tregs Resting HRWL_IMM_019_001 | v1.2

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Number of live cells acquired Panel 2 HRWL_IMM_020_001 | v1.0

simpleParameter

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B cell total HRWL_IMM_021_001 | v1.2

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B1 Total HRWL_IMM_022_001 | v1.3

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Req. Analysis: false **Req. Upload:** true **Is Annotated:** false

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B2 Total HRWL_IMM_023_001 | v1.2

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Req. Analysis: false **Req. Upload:** true **Is Annotated:** false

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B2 Immature + MZB HRWL_IMM_024_001 | v1.2

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Req. Analysis: false **Req. Upload:** true **Is Annotated:** false

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B2 Mature HRWL_IMM_025_001 | v1.2

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cDC CD8a type HRWL_IMM_026_001 | v1.2

simpleParameter

Req. Analysis: false **Req. Upload:** true **Is Annotated:** false

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cDC CD11b type HRWL_IMM_027_001 | v1.2

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DC Total HRWL_IMM_028_001 | v1.2

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Eosinophils HRWL_IMM_029_001 | v1.2

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Neutrophils HRWL_IMM_030_001 | v1.3

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Macrophages HRWL_IMM_031_001 | v1.3

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Monocytes HRWL_IMM_032_001 | v1.2

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Req. Analysis: false **Req. Upload:** true **Is Annotated:** false

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pDCs HRWL_IMM_033_001 | v1.2

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Req. Analysis: false **Req. Upload:** true **Is Annotated:** false

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Total cell count in spleen HRWL_IMM_034_001 | v1.2

simpleParameter

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Dilution Applied HRWL_IMM_035_001 | v1.0

procedureMetadata

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Options: 1:10, 1:300, 1:500,

FACS Equipment ID HRWL_IMM_036_001 | v1.0

procedureMetadata

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Options: FACS Analyzer,

FACS Equipment model HRWL_IMM_037_001 | v1.0

procedureMetadata

Req. Analysis: false **Req. Upload:** false **Is Annotated:** false

Options: CANTO-II,

FACS Equipment manufacturer HRWL_IMM_038_001 | v1.0

procedureMetadata

Req. Analysis: false **Req. Upload:** false **Is Annotated:** false

Options: Becton Dickinson,

Sample on ice after collection? HRWL_IMM_039_001 | v1.0

procedureMetadata

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Options: Yes, No,

Well Location Panel 1 HRWL_IMM_040_001 | v1.0

procedureMetadata

Req. Analysis: false **Req. Upload:** false **Is Annotated:** false

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Well Location Panel 2 HRWL_IMM_041_001 | v1.0

procedureMetadata

Req. Analysis: false **Req. Upload:** false **Is Annotated:** false

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Batch comments HRWL_IMM_042_001 | v1.0

procedureMetadata

Req. Analysis: false **Req. Upload:** false **Is Annotated:** false

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Sample analysis date HRWL_IMM_043_001 | v1.0

procedureMetadata

Req. Analysis: false **Req. Upload:** false **Is Annotated:** false

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Sample collection date HRWL_IMM_044_001 | v1.0

procedureMetadata

Req. Analysis: false **Req. Upload:** false **Is Annotated:** false

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Moxy Fit HRWL_IMM_045_001 | v1.0

procedureMetadata

Req. Analysis: false **Req. Upload:** false **Is Annotated:** false

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Sample comments HRWL_IMM_046_001 | v1.0

procedureMetadata

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