FACS analysis using splenocytes

1. Introduction

Fluorescence activated cell sorter (FACS) is a powerful tool to measure and analyze cell surface molecules of single cells which flow in fluid stream through a beam of light to detect the fluorescences of the cells. Animal experiments for biomedical research are required using the animals of assured genetic quality. FACS can be applied to determine immunological profiling of the various mouse strains such as standard inbred and immunodeficient strains by using antibodies specific for leukocytes and other immune cells. Leukocyte alloantigens such as CD45, CD90, IgM , and major histocompatibility complex (MHC) molecules are polymorphic among mouse strains and can be good candidates as marker molecules for genetic monitoring. In order to detect these marker molecules, we stained the splenocytes by fluophor-conjugated antibodies specific to alloantigens, and analyzed by FACS.

2. Materials

FACSCalibur (BD Biosciences)
Cell Strainer 70 μm (BD FALCON_#352350)
Plunger from 2.5 ml syringe (TERUMO_#SYR002)
50 ml tube (BD FALCON_#352070)
5 ml FACS tube (BD FALCON_#352008)
0.5~1.5 ml microfuge tubes or 96-well plates (V- or U-bottom)
Nylon mesh
Surgical scissors and forceps
Centrifuge (TOMY_EX-136)
Microcentrifuge (Eppendorf_5415R)

<10 X PBS>

NaCl	80 g
KCl	2 g
KH_2PO_4	2 g
Na_2HPO_4	11.5 g
Fill up to	1000 ml

<FACS buffer>

Fetal Calf Serum	20 ml
10% NaN_3	10 ml
10 X PBS	100 ml
H2O	870 ml

<10 X ACK lysing buffer>

NH_4Cl	82.9 g
$KHCO_3$	10 g
EDTA-2Na	0.372 g
Fill up to	1000 ml

3. Methods

<Preparation of the single cell suspension from spleen>

1. Place the Cell Strainer on 50 ml tube
2. Put spleen on the Cell Strainer
3. Add 0.5 ml 1 X ACK lysing buffer to remove red blood cells
4. Tamp spleen with the rubber end of a plunger from a 2.5 ml syringe against the cell strainer to make single splenocytes.
5. Add 0.5 ml 1 X ACK lysing buffer
6. Repeat 4 and 5
7. Leave the cell suspension for 3 min at room temperature
8. Add 5-10 ml FACS buffer
9. Cetrifuge at 1500 rpm, 10min, 4 ^{o}C (TOMY_EX-136)
10. Aspirate the supernatant
11. Re-suspend the cells in 5-10 ml FACS buffer
12. Count cell number by hematocytometer
13. Cetrifuge at 1500 rpm, 10min, 4 ^{o}C
14. Aspirate the supernatant
15. Re-suspend the cells in FACS buffer (10^8 cells/ml)
16. Cells are ready for staining

<Cell Staining>

1. Transfer 10 μl of the cell suspension to 0.5-1.5 ml tube or 96-well plate
2. Prepare antibody mixture containing optimal concentration of a fluorescent monoclonal antibody specific for cell surface antigen. Please refer to dilution ratio* as described in below table.
3. Add 10 μl antibody mixture
4. Incubate for 20 min at 4 °C
5. Add 200-500 μl FACS buffer to wash cells
6. Microfuge at 3000 rpm, 5 min, 4 °C (Eppendorf_5415R)
7. Aspirate the supernatant
8. Re-suspend the cells in 200-500 μl FACS buffer
9. Microfuge at 3000 rpm, 5 min, 4 °C
10. Aspirate the supernatant
11. For indirect staining, add 10 μl SAv-PerCP-Cy5.5 solution and repeat 4 to 10.
12. Re-suspend the cells in 200-500 μl FACS buffer
13. Transfer to 5 ml tube by passing through a nylon mesh
14. Cells are ready for analysis by FACSCalibur

List of primary antibodies

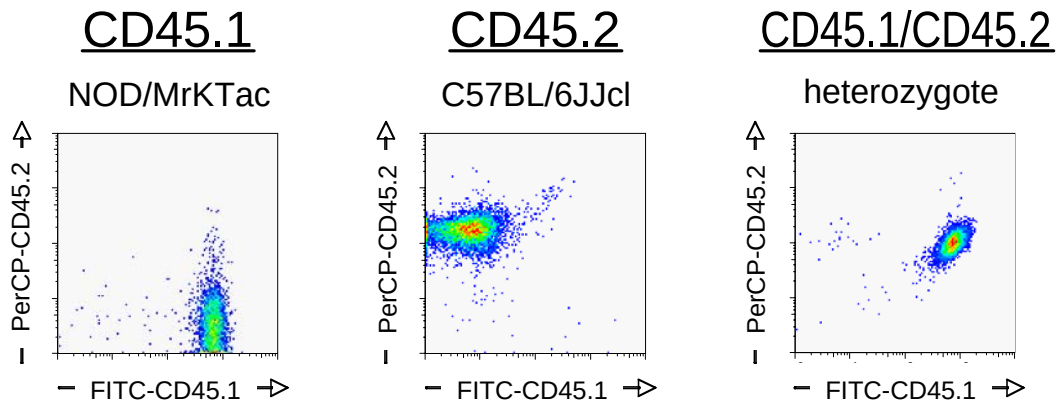
ANTIGEN	CLONE	CONJUGATE	VENDOR	CAT#	DILUTION*
CD45.1	A20	FITC	BD Pharmingen	553775	1:200
CD45.2	104	PerCP-Cy5.5	BD Pharmingen	553772	1:400
CD90.1	OX-7	FITC	BD Pharmingen	554897	1:400
CD90.2	30-H12	PE	BD Pharmingen	553014	1:800
IgMa	DS-1	FITC	BD Pharmingen	553516	1:200
IgMb	AF6-78	PE	BD Pharmingen	553521	1:400
H-2Kb	AF6-88.5	FITC	BD Pharmingen	553569	1:200
H-2Kd	SF1-1.1	PE	BD Pharmingen	553566	1:400
H-2Kk	36-7-5	PE	BD Pharmingen	553593	1:400
H-2Kq	KH114	FITC	BD Pharmingen	553597	1:200
H-2Db	KH95	FITC	BD Pharmingen	553573	1:100
H-2Dd	34-2-12	PE	BD Pharmingen	553580	1:200
H-2Dk	15-5-5	FITC	BD Pharmingen	553585	1:100
H-2Dq/H-2Lq**	6-27.5	Biotin	BD Pharmingen	558969	1:200
TCRβ	H57-597	PE	BD Pharmingen	553172	1:200
IgM	polyclonal	FITC	Southern Biotech	1021-02	1:200

**Indirect staining: Use PerCP-Cy5.5-conjugated streptavidin

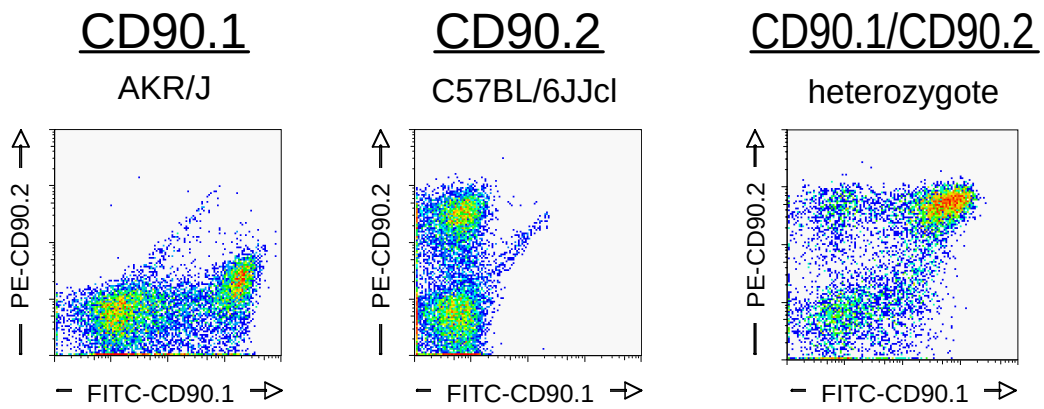
SAv-PerCP-Cy5.5	streptavidin	PerCP-Cy5.5	BD Pharmingen	551419	1:400

4. Genetic background monitoring

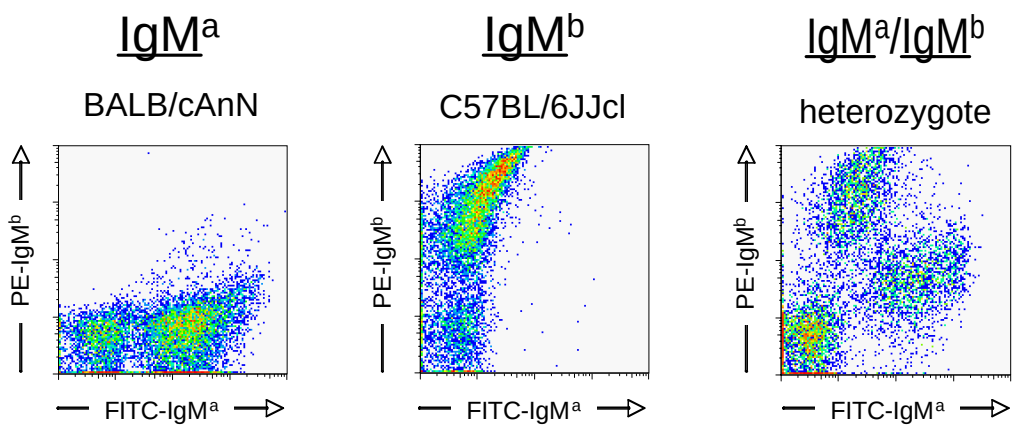
4-1. CD45 (Chr. 1)



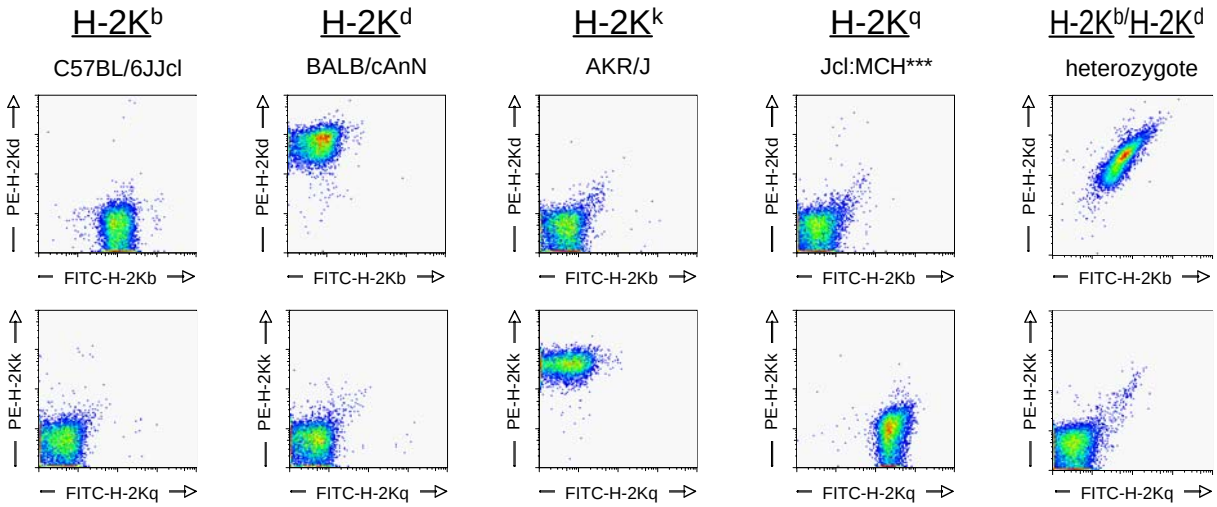
4-2. CD90 (Chr. 9)



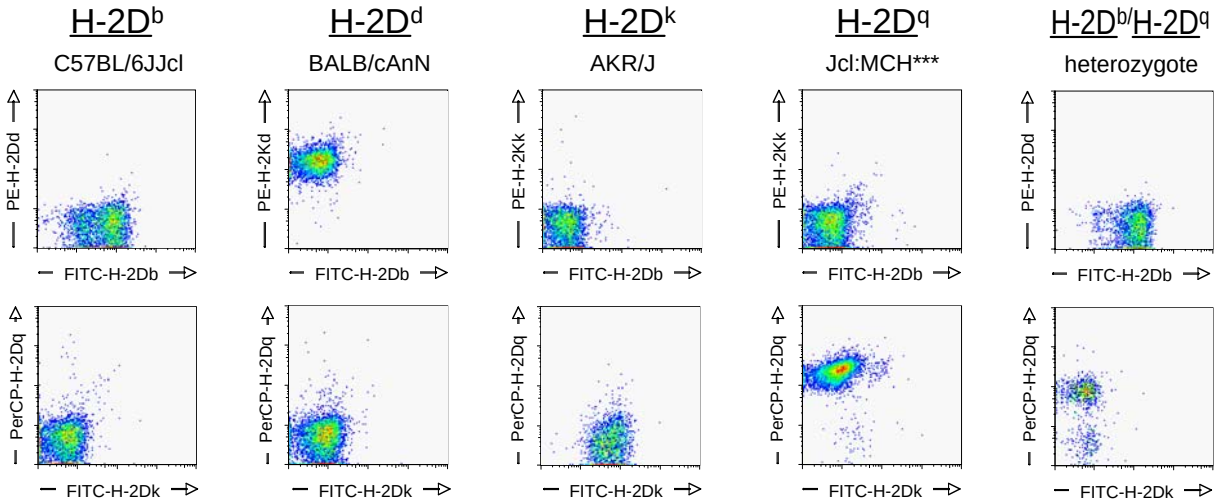
4-3. IgM (Chr. 12)



4-4. H-2K (Chr. 17)

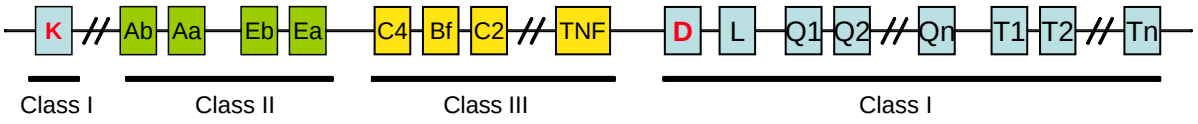


4-5. H-2D (Chr. 17)



***Jcl:MCH were produced by four-way cross of Jcl:ICR-derived inbred strains IAI/Jcl, ICT/Jcl, IPI/Jcl, and IQI/Jcl

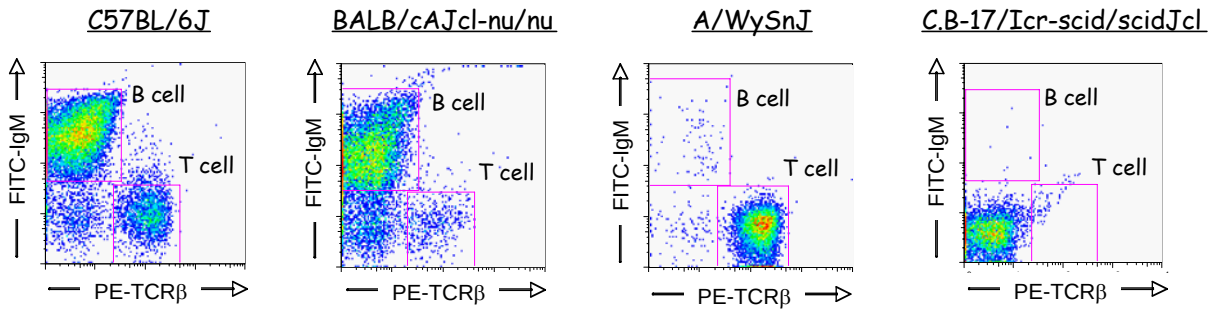
Mouse H-2 complex (MHC) genomic map (Chr. 17)



Alloantigens Chart

Mouse Strains	Chr.1 CD45	Chr,9 CD90	Chr.12 IgM	Chr.17 H2D	Chr.17 H2K
C57BL/6J	2	2	b	b	b
129/	2	2	a	b	b
BALB/c	2	2	a	d	d
C3H	2	2	a	k	k
CBA	2	2	a	k	k
DBA/1	1	1	a	q	q
DBA/2	2	2	a	d	d
NOD	1	2	b	b	d

5. Immunological profiling of immunodeficient strains



C57BL/6JJcl: Standard inbred mouse strain

BALB/cJcl-nu/nu: Athymic mouse

A/WySnJ: B-cell deficient mouse

C.B-17/Icr-scid/scidJcl: Severe Combined Immunodeficient mouse

List of mouse strains used

Strain Name	Source
NOD/MrKTac	Taconic
C57BL/6JJcl	Japan CLEA
BALB/cAnN	RIKEN BRC (#00641)
AKR/J	Jackson Lab (#000648)
Jcl:MCH (ICR)	Japan CLEA
BALB/cAJcl-nu/nu	Japan CLEA
A/WySnJ	Jackson Lab (#000647)
C.B-17/Icr-scid/scidJcl	Japan CLEA

Reference

1. Techniques for Immune Function Analysis, Application Hand book 1st Edition, BD Biosciences

2. Introduction to Flow Cytometry: A Learning Guide, Manual Part Number 11-11032-01, April, 2000, BD Biosciences

3. BD Biosciences Phermingen 2005/06 catalogue

4. Microbial Status and Genetic Evaluation of Mice and Rats, proceeding of the 1999 US/Japan conference

5. Koizumi, T., Maeda, H., and Hioki, K. Sleep-time variation for ethanol and the hypnotic drugs tribromoethanol, urethane, pentobarbital, and propofol within outbred ICR mice. *Exp Anim* 51:119-124 (2002)

6. Nehls, M. , Pfeifer, D. , Schorpp, M. , Hedrich, H. and Boehm, T. New member of the winged-helix protein family disrupted in mouse and rat nude mutations. *Nature* 372: 103–107 (1994)

7. Miller, D. J. and Hayes, C. E. Phenotypic and genetic characterization of a unique B lymphocyte deficiency in strain A/WySnJ mice. *Eur J Immunol* 21:1123-1130 (1991)

8. Bosma, G. C., Custer, R. P., and Bosma, M. J. A severe combined immunodeficiency mutation in the mouse. *Nature* 301: 527-530 (1983)

RIKEN BRC