Flow cytometry

謝長奇

中西醫結合研究所

流式細胞儀應用

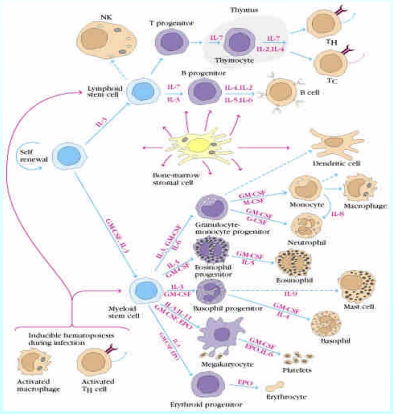
- A brief list of applications that use flow cytometers includes:
  - Disease diagnosis
  - Chromosome karyotyping
  - Cell function analysis
  - Cancer therapy monitoring
  - Detecting fetal cells
  - Cell kinetics
  - Identifying tumor cells
  - Cytogenetics
  - Fundamental cell biology

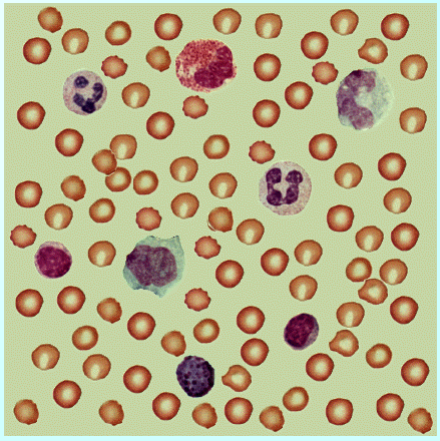
流式細胞儀應用

- 免疫細胞分析
  - Cell surface marker analysis
  - Intracellular cytokines analysis
  - Platelet analysis
- 細胞凋亡
- 上樣與資料分析

免疫細胞表面標記分析

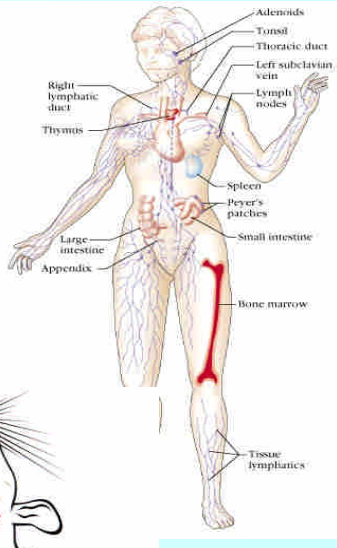
參與免疫反應的細胞

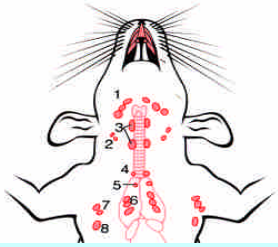




參與免疫反應的細胞

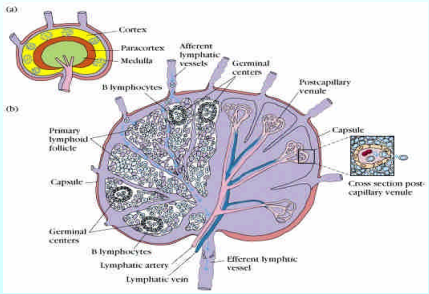
- Primary organs
  - bone marrow
  - thymus
- Secondary organs and tissues
  - spleen
  - lymph nodes
  - Peyer's patches



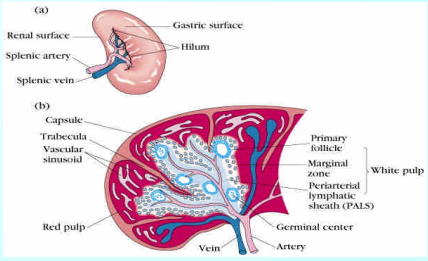


參與免疫反應的組織

lymph node



spleen



LYMPHOCYTE SURFACE ANTIGENS

- **IMMUNOGLOBULINS**: "Antibodies" secreted by or found on B-Cells.
  - Has a huge range of specificities achieved by DNA rearrangement.
  - Five general types (see below): IgG, IgA, IgE, IgD, IgM

LYMPHOCYTE SURFACE ANTIGENS

- **T-CELL (TcR) RECEPTORS**: They bind to the Antigen-Presenting Cell.
  - **VARIABLE REGIONS** are on the T-Cell Receptor. They allow us to develop variability and diversity in the immune response.
- **"CD" ANTIGENS**: Systematic classification of surface-antigens with diverse functions. Cell-surface markers.

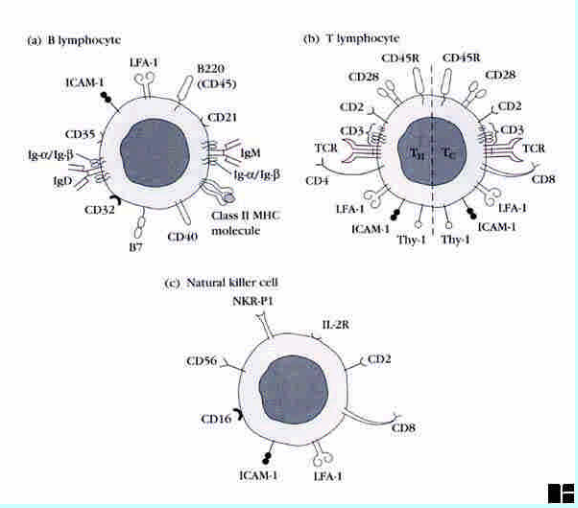
Cluster of Differentiation (CD) Antigens

- Leukocytes express distinct assortments of molecules on their cell surfaces
- many of which reflect either different stages of their lineage-specific differentiation
- different states of activation or inactivation

Cell surface immunophenotypes

- different **leukocyte subpopulations**, including
  - the functionally distinct mature lymphocyte subpopulations
  - B-cells
  - helper T-cells (TH)
  - cytotoxic T-cells (TC)
  - natural killer (NK) cells

Antigen markers on mature lymphocyte populations



Conventions for Naming Leukocyte Surface Molecules

- Named according to a particular function affected by an anti-leukocyte mAb
  - the lymphocyte function-associated antigen 1**,** or LFA-1, was so named because antibodies recognizing this structure interfere with lymphocyte cell adhesion events and optimal lymphocyte function.
- According to individual laboratory preferences.
  - B7 and B220, except that the leading "B" reminds us that these antigens are typically expressed on B lymphocytes.
- Named systematically by assigning them a cluster of differentiation (CD) antigen number
  - identical unique reactivity pattern with different leukocyte populations.

CD antigens have also been named by one of the other conventions

- CD54 = LFA-1
  - is widely expressed on a variety of haematopoietic cells
- CD80 = B7 (or now B7-1)
- CD45 = B220
- CD4 = L3T4; W3/25
  - expressed almost exclusively on T helper (TH) lymphocytes and cells of the monocyte**/**macrophage lineage

Table of CD Antigens by the NIH

PROW Protein Reviews On The Web NCI NCB

Index of information available from PROW

Current guides: expanded format including Summary Sentence and Abstract
Past guides: older guides with excellent information, some data may be dated

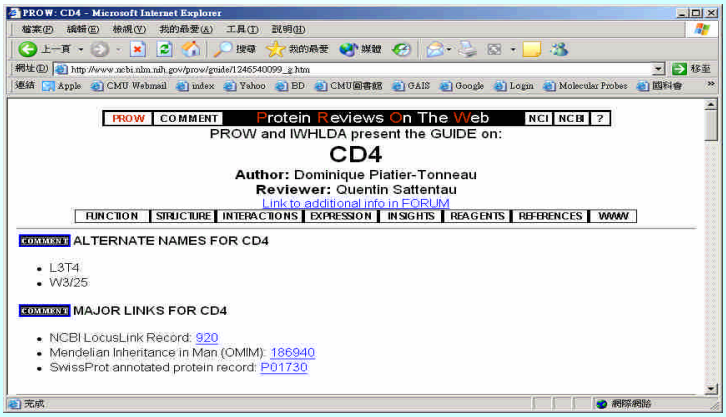
CD molecule	Alternate Names	Current Guides	Past Guides	Entrez Gene	Assigning Workshop
CD1a	R4; HTA1		CD1a	909	
CD1b	R1		CD1b	910	
CD1c	M241; R7		CD1c	911	
CD1d	R3		CD1d	912	
CD1e	R2		CD1e	913	
CD2	CD2R; E-rosette receptor; T11; LFA-2		CD2	914	
CD3delta	CD3d			915	
CD3epsilon	CD3e			916	
CD3gamma	CD3g			917	
CD4	L3T4; W3/25		CD4	920	
CD5	Leu-1; Ly-1; T1; Tp67		CD5	921	
CD6	T12		CD6	923	
CD7	gp40			924	
CD8alpha	Leu2; Lyt2; T cell co-receptor; T8			925	
CD8beta	Leu2; CD8; Lyt3			926	
CD9	DRAP-27; MRP-1; p24		CD9	928	
CD10	EC 3.4.24.11; neprilysin; CALLA; enkephalinase; gp100; NEP			4311	
CD11a	AlphaL integrin chain; LFA-1alpha		CD11a	3683	
CD11b	AlphaM integrin chain; AlphaM-beta2; C3biR; CR3; Mac-1; Mo1		CD11b	3684	
CD11c	AlphaX integrin chain; Axb2; CR4; leukocyte surface antigen p150,95		CD11c	3687	

http://mpr.nci.nih.gov/prow/

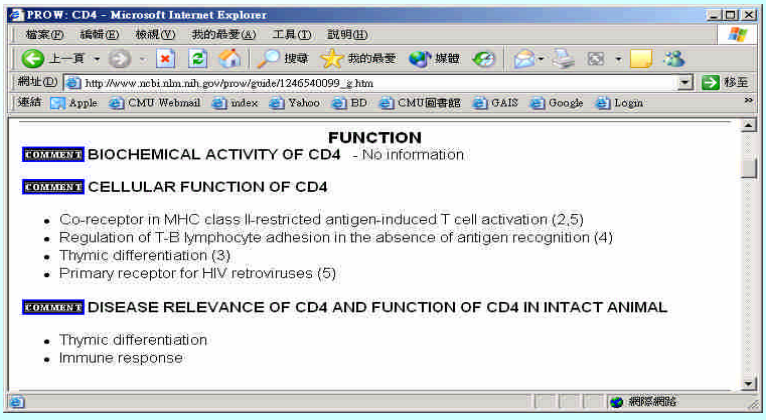
Cluster of Differentiation information

- PROW:
  - Protein Reviews On the Web is an online resource that features PROW Guides
- IWHLDA:
  - International Workshops on Human Leukocyte Differentiation Antigens
- 8th International Conference on Human Leucocyte Differentiation Antigens
  - Adelaide, South Australia 12-16 December 2004

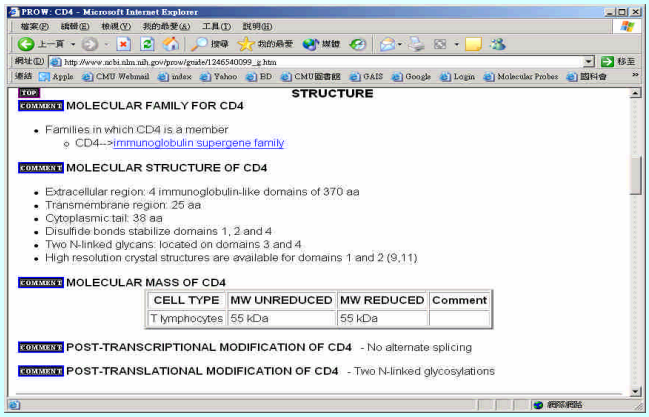
The GUIDE of PROW and IWHLDA



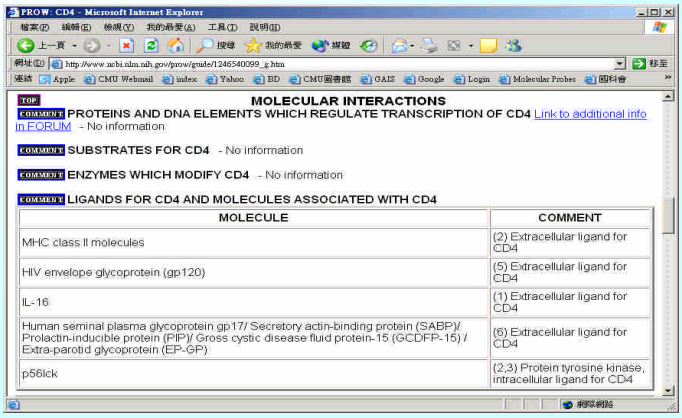
CD antigen - Function



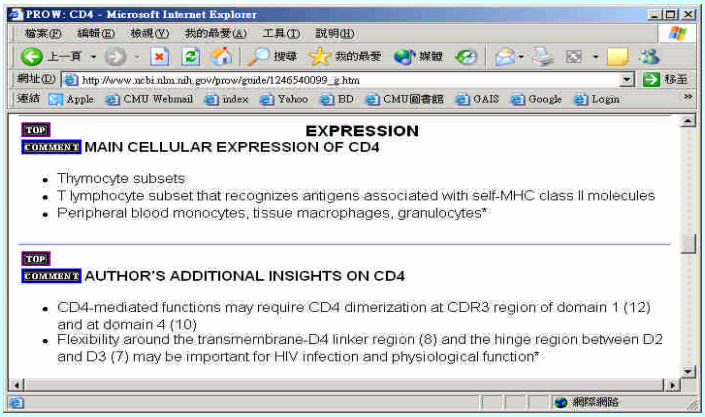
CD antigen - Structure



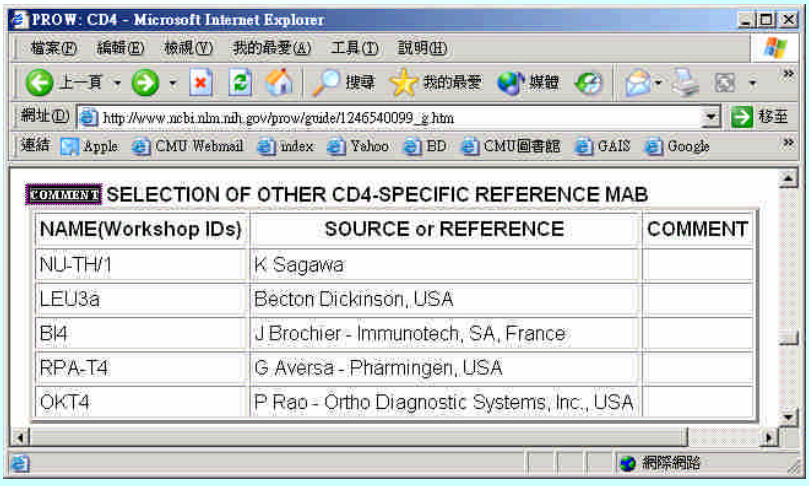
CD antigen – Molecular interaction



CD antigen – Expression



CD antigen – Reagent



Leukocyte subpopulation

- T lymphocyte
  - CD1~8, CD27, CD28, CD38, CD39, CDw60, CD45, CD45RA, CD45RB, CD45RO, CD98, CD99, CD99R, CD100, CDw101
- B lymphocyte
  - Cd10, CD19~24, CD37, CD40, CD53, CD72~75, CDw76, CD77, CD78, CD79a, CD79b, CD80~83, CDw84, CD85, CD86
- Dendritic cells
  - CD4, CD8, CD11c, CD13, CD80, CD86, CD123, CD205, CD209, B7-DC, TLR3

Leukocyte subpopulation

- Monocyte/Macrophage
  - CD11b, CD13, CD14, CD80, CD86, CD115, Mac-3, TLR2, TLR4
- Myeloid cells
  - CDw12, CD13~w17, CD32~35, CD64, CDw65, CD66a~68, CD87~93
- NK
  - CD11b, CD56, CD57, CD59, CD94, NK1.1, PanNK
- Platelet
  - CD9, CD31, CD36, CD41a, CD41b, CD42a~42d, CD61, CD63, CD107a, CD107b

Leukocyte subpopulation

- Activated antigen
  - CD25, CD26, CD30, CD69~71, CD95~97
- Adhesion molecular
  - CD11a~11c, CD15s, CD18, CD29, CD43~44R, CD48, CD49a~49f, CD50, CD51/61, CD54~55, CD59, CD62E, CD62L, CD62P, CD102~104, CDw108
- Endothelial cells
  - CD105, CD106, CDw109

Leukocyte subpopulation

- Epithelial cells
  - CD104, CD133
- Cytokine receptor
  - CD25, CD115, CDw116, CD117, CDw119, CD120a, CD120b, CDw121a, CDw121b, CD122, CDw124, CD126, CDw127, CDw128, CDw130
- Toll-like receptors
  - TLR1~10

Anti-CD marker w/w Fluorochrome

- **Fluorochrome**
  - **Excitation**: UV, Argon-ion laser, Diode
    - 340 nm, 488 nm, 635 nm
  - **Emission**:
    - FL1: 530/30 nm, FITC, GFP
    - FL2: 585/42 nm, PE, PI
    - FL3: 650 nm, 7-AAD, PerCP, PE-Cy5
    - FL4: 661/16 nm, APC, APC-Cy7, TOTO-3

Table 6.5.1 Two-Color Monoclonal Antibody Panel Recommended by the U.S. Centers for Disease Control

Tube	Green fluorescence	Red fluorescence	Purpose of admixture
1	CD45	CD14	Gating on lympohocytes[a]
2	Isotype	Isotype	Determine background fluorescence
3	CD3[b]	CD4	Count $CD3^+/CD4^+$ T cells
4	CD3[b]	CD8	Count $CD3^+/CD8^+$ T cells
5	CD3[b]	CD19	Count total T (CD3) and B (CD19) cells
6	CD3[b]	CD16/56	Count total T (CD3) and NK (CD16/56) cells

[a] Lymphocyte gating on FS and SS should yield >98% $CD45^{++}$ and <2% $CD14^+$ cells. This approach assumes that the efficiency of the lysing system will remain constant for the rest of the tubes in the panel.
[b] The repeated use of CD3 in four tubes serves as a control for tube-to-tube variability; the values of all four tubes should be within 3% of each other.

Table 6.5.2 Three-Color Monoclonal Antibody Panels Recommended by the CDC

Panel	Antibodies	Purpose of admixture
A[a]	CD3/CD4/CD45[b]	Gate on $CD45^{++}$ and side scatter, count CD3/CD4 cells
	CD3/CD8/CD45[b]	Gate on $CD45^{++}$ and side scatter, count CD3/CD8 cells
	CD3/CD19/CD45[b]	Gate on $CD45^{++}$ and side scatter, count CD3 and CD19 cells
B[c]	CD3/CD19/CD16-56	Count T, B, and NK cells
	CD3/CD4/CD8	Count total T (CD3), CD3/CD4, and CD3/CD8 cells

[a]Panel A is recommended for instruments incapable of yielding absolute cell numbers directly, and isotype control is not needed, for CD45 identifies leukocyte subpopulations based on fluorescence intensity.
[b]The repeated use of CD3 serves as a control for tube-to-tube variability; the values of all tubes should be within 3% of each other.
[c]Panel B is recommended for systems capable of counting absolute cell numbers directly from the flow cytometer.

Table 6.5.3 Four-Color Monoclonal Antibody Panel Recommended by the CDC

	Antibodies	Purpose of admixture
Tube 1[a]	CD3/CD4/CD8/CD45	Gate on $CD45^{++}$ and side scatter, count total, $CD3^+/CD4^+$, and $CD3^+/CD8^+$ T cells
Tube 2[b]	CD3/CD19/CD56/CD45	Gate on $CD45^{++}$ and side scatter, count total T, B, and NK cells

[a]The repeated use of CD3 serves as a control for tube-to-tube variability; the values obtained from all tubes should be within 3% of each other.
[b]CD56 can be replaced by CD16, or both antibodies might be used simultaneously in a single color.

Table 6.3.1 Typical Concentration of a Selection of Widely Used Immunological Markers, Compared with Cytokine Receptors

Marker	Cell type[a]	Concentration (molecules/cell)	Reference
Commonly used markers			
CD2	T cells (blood)	40,000	Martin et al. (1983)
CD3	T cells (blood)	57,000	Bikoue et al. (1996)
CD4	T cell subset (blood)	47,000	Bikoue et al. (1996)
CD8	T cell subset (blood)	145,000	Bikoue et al. (1996)
CD5	T cells (blood)	50,000	Bikoue et al. (1996)
CD19	B cells (blood)	27,000	Bikoue et al. (1996)
CD45	Lymphocytes	217,000	Bikoue et al. (1996)
sIg	Chronic lymphocytic leukemia	6,500-22,500	Dighiero et al. (1980)
Cytokine receptors			
CD121a	Lymphocytes	<100	Dower et al. (1985)
CD25	T cells (blood)	<500	Le Mauff et al. (1987)
CD25	In vitro-activated T cells	>30,000	Le Mauff et al. (1987)
CD122	T cells (blood)	700	Ben Aribia et al. (1989)
CD124	Resting B lymphocytes (mouse)	400	Lowenthal et al. (1988)
CD126	Activated B cells	300	Kishimoto (1989)

[a]Human, unless indicated otherwise.

Table 6.3.2 Fluorochrome Properties

Fluorochrome	Absorption maximum wavelength (nm)	Emission maximum wavelength (nm)	Extinction coefficient ($mol^{-1}cm^{-1}$)	Quantum yield
Fluorescein	495	520	8.2×10^4	0.3
R-Phycoerythrin	546	580	2×10^6	0.8
R-PE/Cy5 tandem	546	667	2×10^6	<0.8
Cy3	552	565	1.3×10^5	>0.15
PerCP	478	677	3.2×10^5	NA[a]

[a]NA, not available.

Handling, Storage, and Preparation of Human Blood Cells

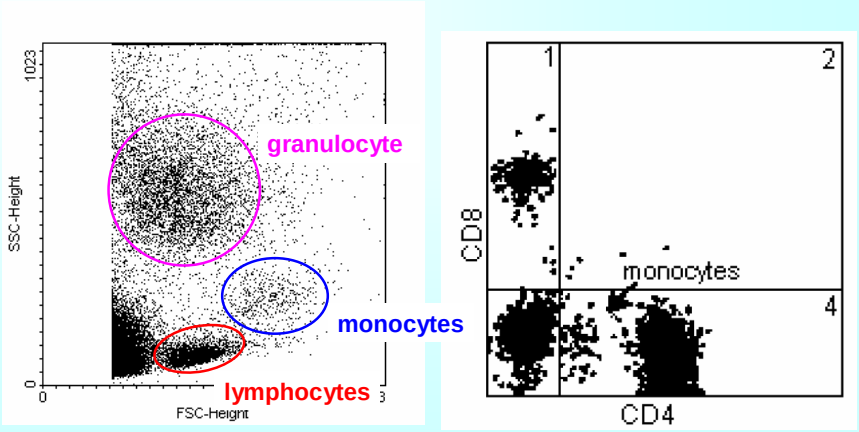
Table 5.1.1 Recommended Anticoagulants and Storage Times for Commonly Performed Assays

Assay	Anticoagulant	Time limitation
Lymphocyte immunophenotyping	Sodium heparin or EDTA	Store ≤72 hr
Myeloid immunophenotyping	EDTA	Use immediately
Neutrophil function	Sodium heparin or EDTA	Use immediately
Platelet activation	EDTA	Use immediately
Platelet markers	EDTA	Use immediately
Reticulocyte enumeration	EDTA	Store ≤72 hr at 4°C
DNA analysis	Sodium heparin or EDTA	Use immediate for cell-cycle analysis; store ≤72 hr for ploidy analysis

Whole blood analysis

- RBC lysis (50 μl whole blood)
  - Hypotonic shock-1
    - [9]: H_2O→[1]: 10x DPBS
    - [5]: 0.1x HBSS→[5]: 2x HBSS
  - 10x ammonium chloride lysis solution
    - 89.9 g NH_4Cl
    - 10.0 g $KHCO_3$
    - 370.0 mg tetra-sodium EDTA
    - Adjust to pH 7.3. Store at 2 to 8 deg. C in a tightly closed bottle
  - 1000~1500 events/second in Hi speed (60 μl/min)

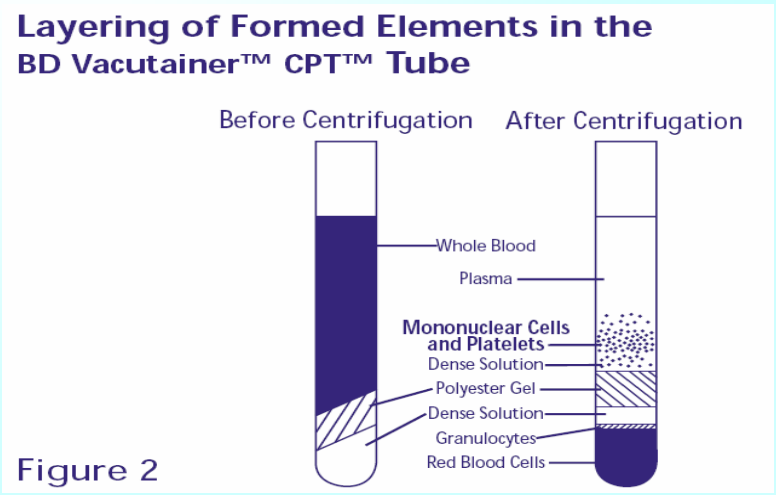
Whole blood analysis



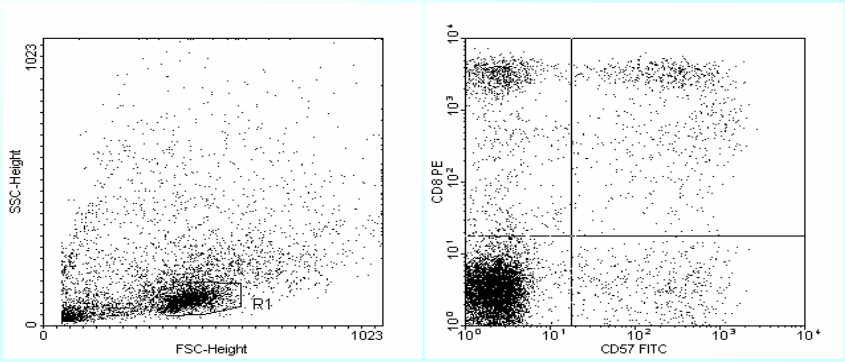
Purified mononuclear cells

- Ficoll-Paque™ PLUS
  - Dilute whole blood with HBSS (PBS, DPBS)
  - Ficoll-Paque [3] + dilute blood [4]
  - 600~800 xg in RT (25~18°C) for 30 min w/o brake
- VACUTAINER CPT (Cell Preparation Tubes, BD Cat. No. **362753, 362761**)
- HISTOPAQUE (Sigma)
  - 1077 for human
  - 1083 for rat, mouse
  - 1119 for separate MNC and neutrophils

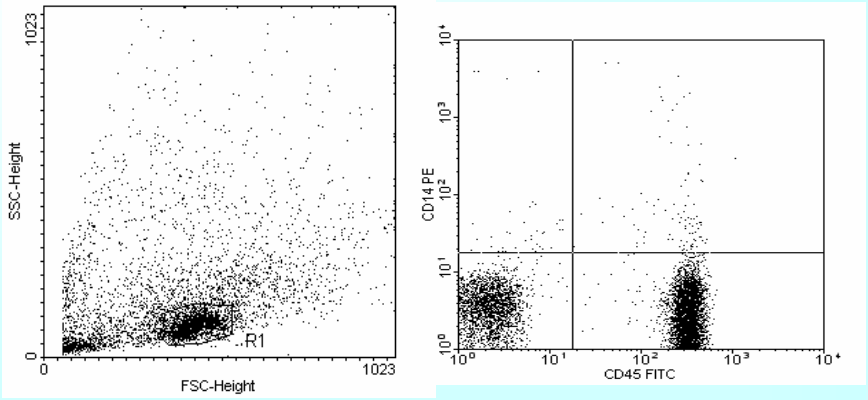
VACUTAINER CPT, 1500~1800 x*g*



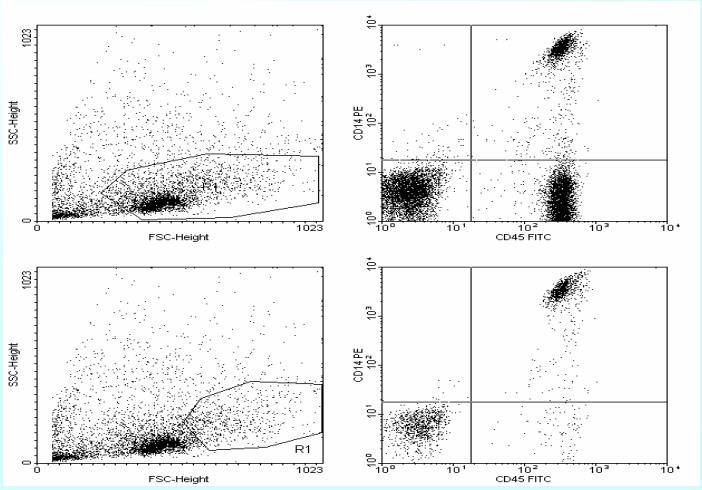
Purified mononuclear cells lymphocytes



Purified mononuclear cells monocvtes for CD14



Purified mononuclear cells lymphocytes/monocytes

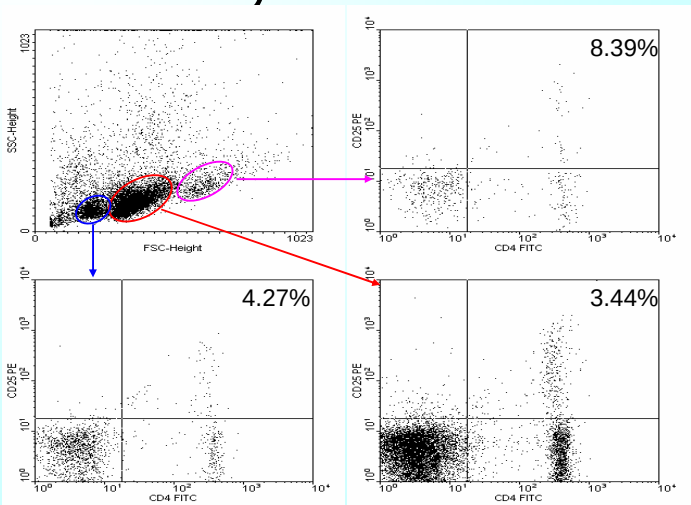


Spleen analysis

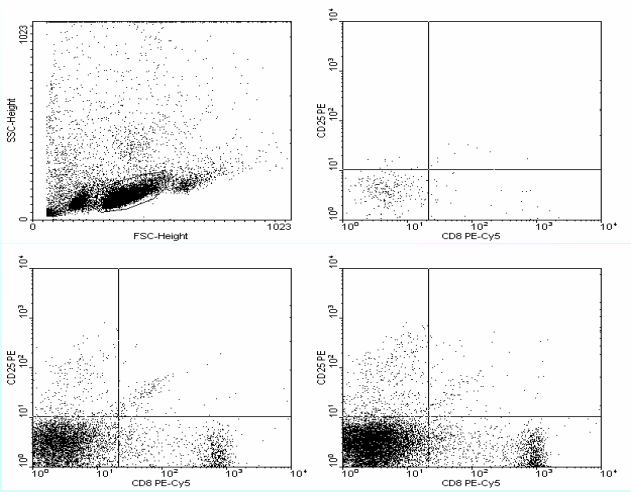
Spleen analysis

- Hypotonic shock
  - Shaking time
  - For NK activity analysis
- Cell surface marker staining
  - $1x10^7$/ml → take 50 μl ($5x10^5$ cells)
  - w/o fix cells
  - Resuspend in HBSS containing NaN_3 and 2% FBS
  - 800~1000 events/sec in Hi speed (60 μl/min)

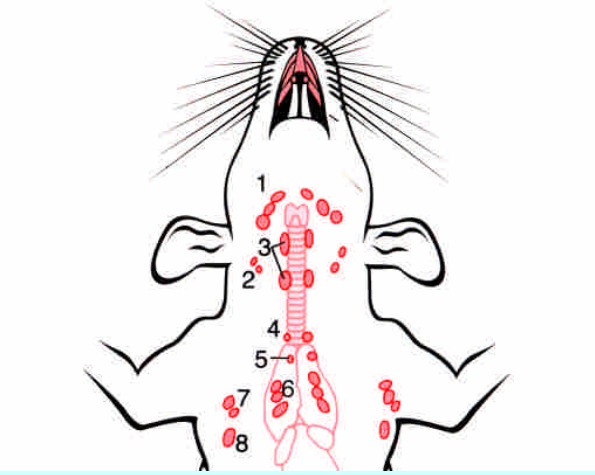
Spleen analysis-CD4/CD25



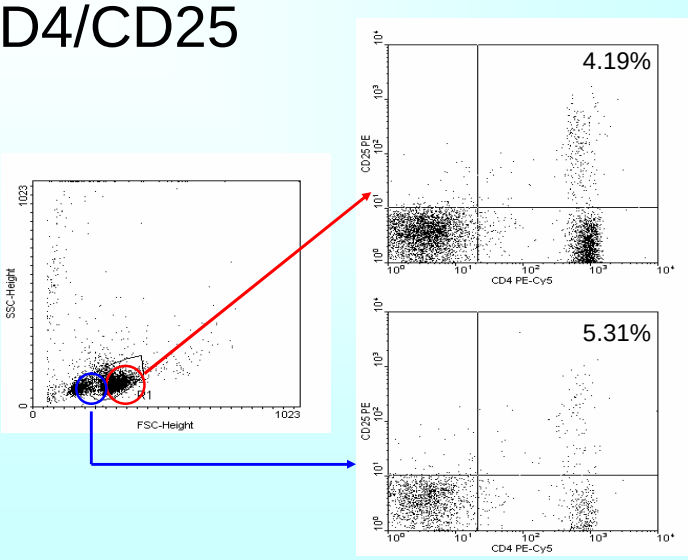
Spleen analysis-CD8/CD25



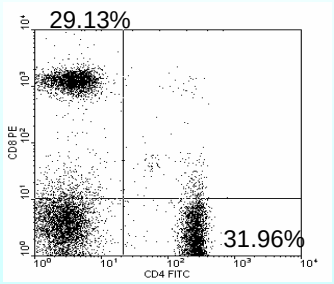
Lymph node analysis

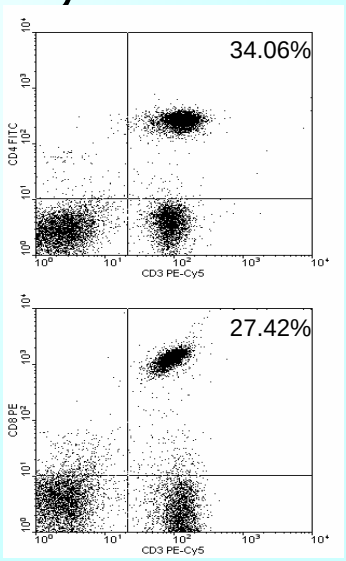


Lymph node analysis- CD4/CD25

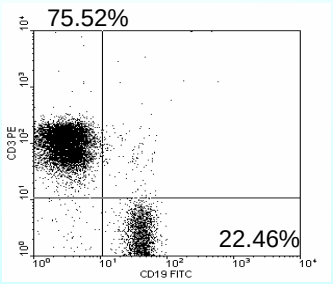


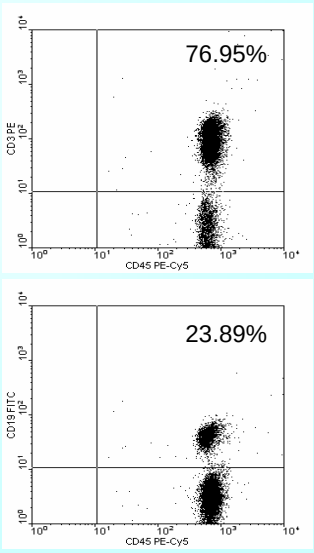
Lymph node analysis-CD3/4/8



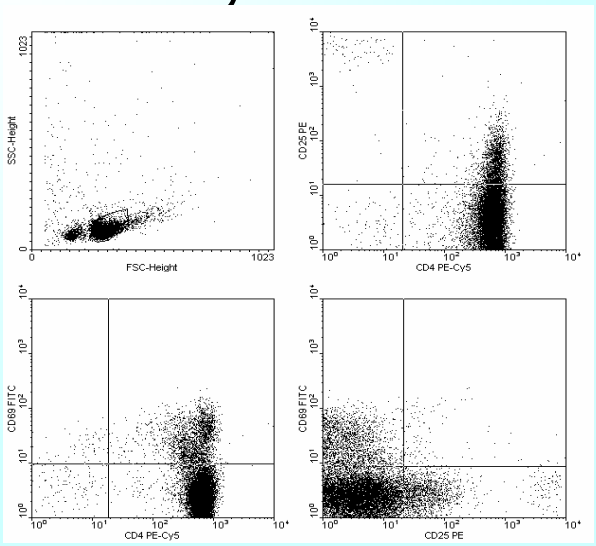


Lymph node analysis-CD3/19/45

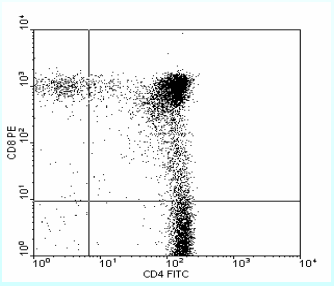


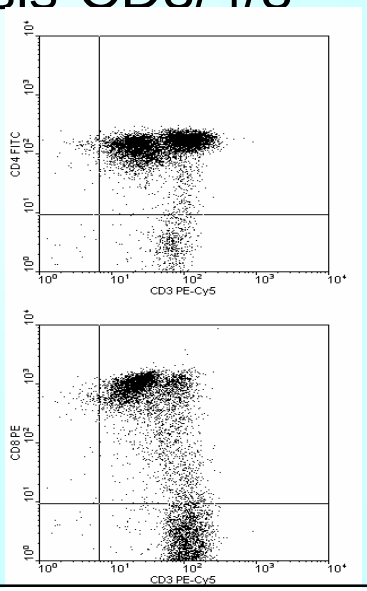


Thymus analysis-CD4/25/69

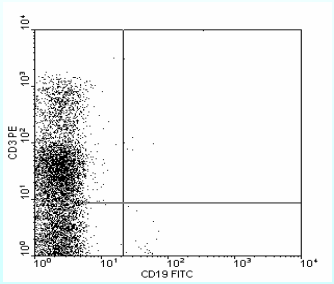


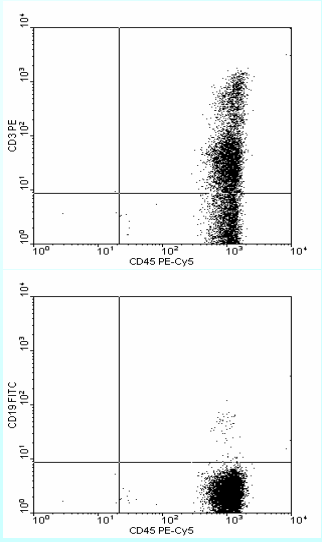
Thymus analysis-CD3/4/8





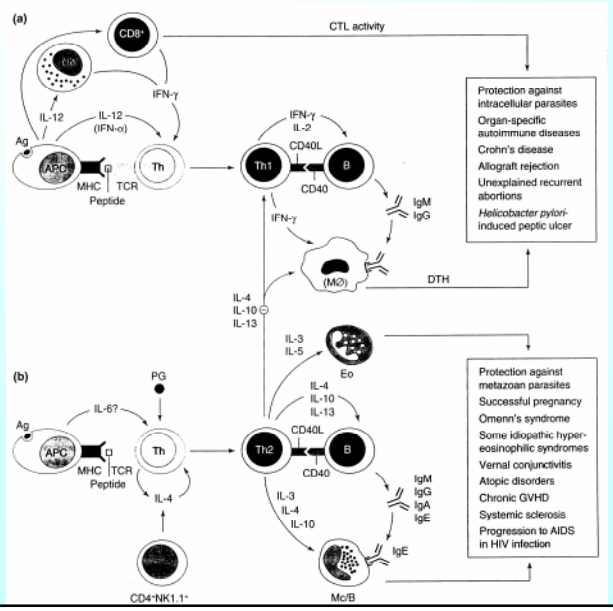
Thymus analysis-CD3/19/45





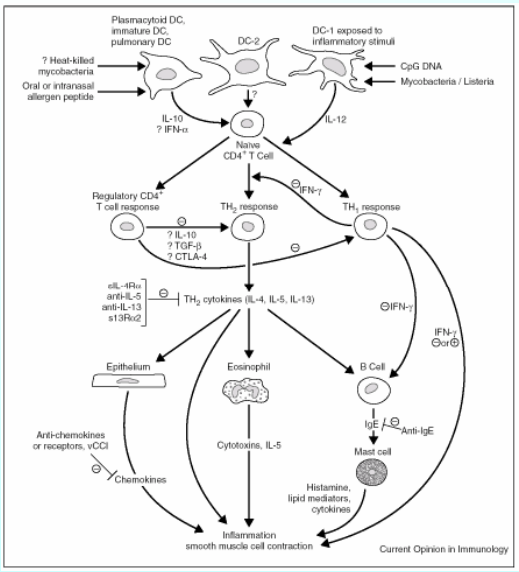
Intracellular cytokines analysis

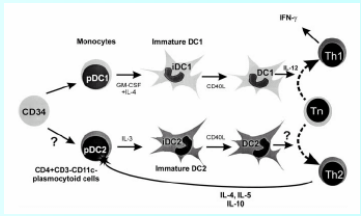
The Th1/Th2 paradigm



Sergio Romagnani. Immunology Today. 18: 263-266. 1997.

Th2 cell in allergic disease

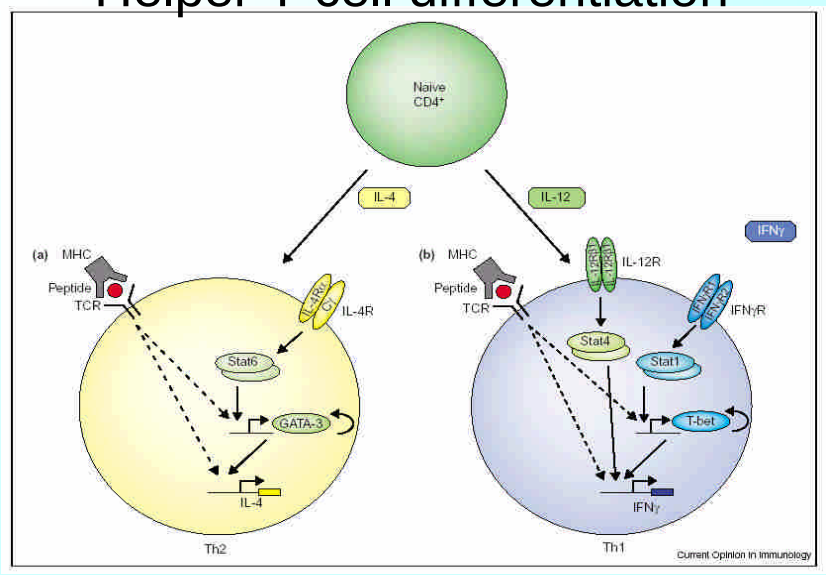




N Novak. et al., Allergy. 54: 792-803. 1999

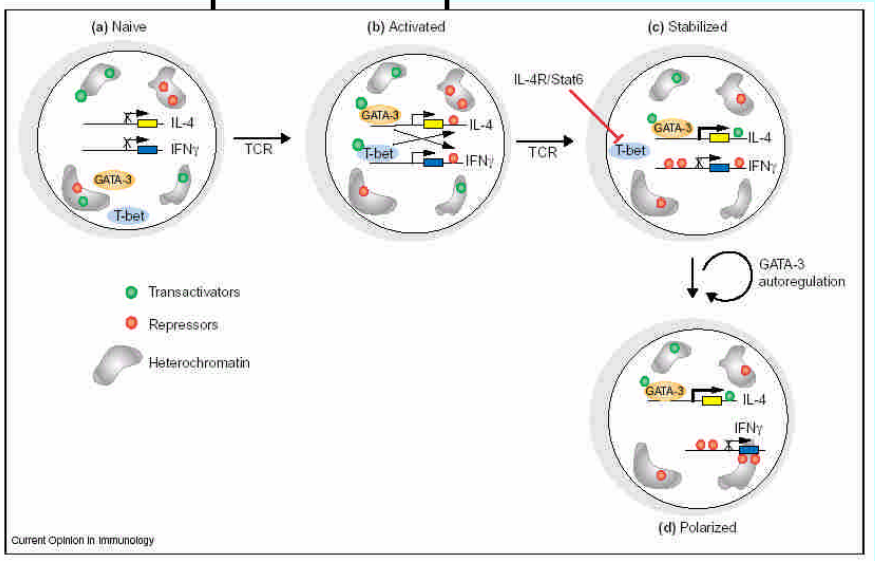
Lewis, David B. Curr. Opin. Immunol. 14: 644-651. 2002

Helper T cell differentiation



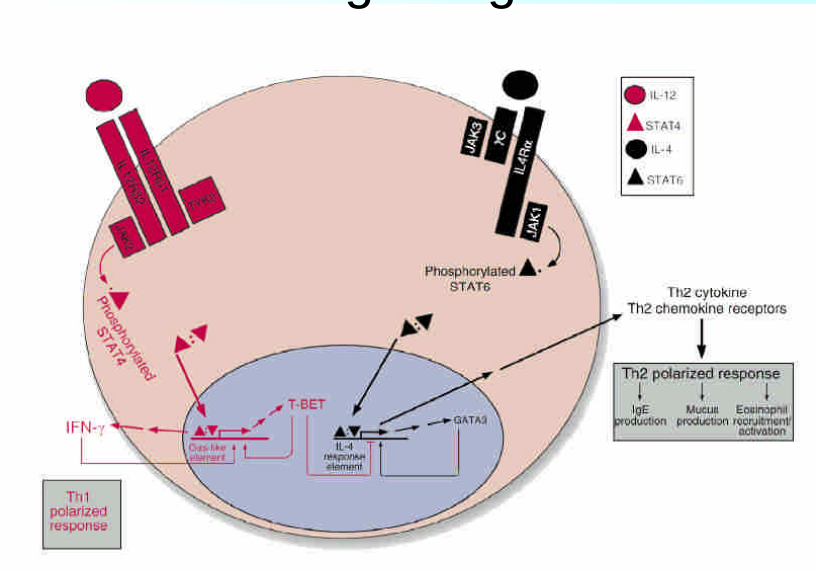
Jane L Grogan and Richard M Locksley. Curr. Opin. Immunol. 14: 366-372. 2002.

Helper T cell polarization



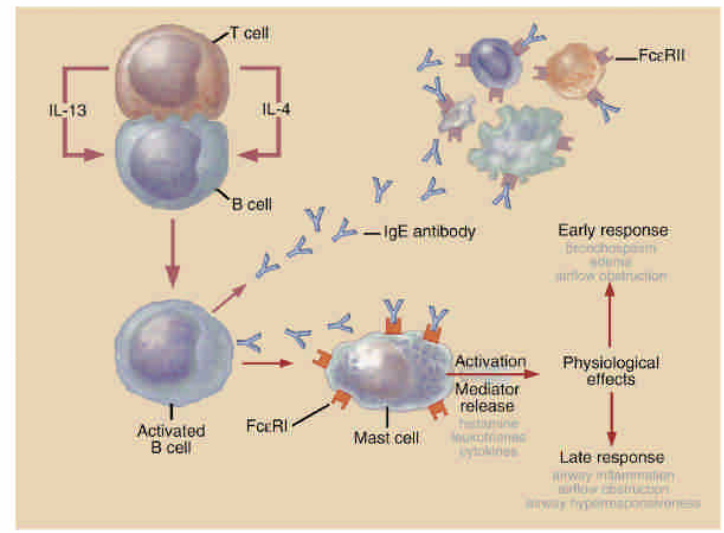
Jane L Grogan and Richard M Locksley. Curr. Opin. Immunol. 14: 366-372. 2002.

JAK-STAT signaling in asthma



Alessandra B. Pernis and Paul B. Rothman. J. Clin. Invest. 109:1279–1283 (2002).

Th2 activate B cell



Alessandra B. Pernis and Paul B. Rothman. J. Clin. Invest. 109:1279–1283 (2002).

Analysis approach

- Environments
  - Ag stimulation → ELISA
- Signaling gene expression
  - Northern
  - Western
  - PCR, Real Time-PCR
- Intracellular cytokine detection
  - FACS

Allergic disorder

- Asthma
- Dermatitis
- Autoimmune disease

Stimulation of Cells

- Activation
  - 50 ng/ml of PMA (Phorbol-12-myristate-13-acetate), + 1 μM of ionomycin, or 250 ng/ml calcium ionophore A23187
  - ConA (3-5 μg/ml)
  - 6-48 hr
- Re-stimulation
  - anti-CD3 (10ug/ml immobilized)+anti-CD28 (2ug/ml soluble) 5hr
- Inhibit intracellular cytokine transport
  - 1-3 μM monensin, or 1-5 μg/ml brefeldin A
  - 4-6 hr

Mouse Cytokine Intracellular Staining Quick Guide

Mouse Cytokine	Cell Source	Activation	Incubation Time	Restimulation	Intracellular Block	Antibody
IL-1a	mouse PEC	mINFg (100ng/ml)(2h4)/LPS (100ng/ml)(22hr)	2hr/22hr	-	Monensin	ALF-161
IL-1b	mouse PEC	mINFg (100ng/ml)(2h4)/LPS (100ng/ml)(22hr)	2hr/22hr	-	Monensin	B122
IL-2	mouse spleen	ConA (3ug/ml) (2d)/IL-2 (20ng/ml)+IL-4 (20ng/ml) (3d)	2d/3d	anti-CD3 (10ug/ml immobilized)+anti-CD28 (2ug/ml soluble) 5hr	Monensin	JES6-5H4
IL-4	mouse spleen	ConA (3ug/ml) (2d)/IL-2 (20ng/ml)+IL-4 (20ng/ml) (3d)	2d/3d	anti-CD3 (10ug/ml immobilized)+anti-CD28 (2ug/ml soluble) 5hr	Monensin	BVD6-24G2
IL-6	mouse spleen	ConA (3ug/ml) (2d)/IL-2 (20ng/ml)+IL-4 (20ng/ml) (3d)	2d/3d	anti-CD3 (10ug/ml immobilized)+anti-CD28 (2ug/ml soluble) 5hr	Monensin	MP5-20F3
IL-10	mouse spleen	ConA (3ug/ml) (2d)/IL-2 (20ng/ml)+IL-4 (20ng/ml) (3d)	2d/3d	anti-CD3 (10ug/ml immobilized)+anti-CD28 (2ug/ml soluble) 5hr	Monensin	JES5-16E3
IL-12	mouse PEC	mIFNg (100ng/ml) (2hr)/LPS (100ng/ml) (22hr)	2hr/22hr	-	Monensin	C17.8
GM-CSF	mouse spleen	ConA (3ug/ml) (2d)/IL-2 (20ng/ml)+IL-4 (20ng/ml) (3d)	2d/3d	anti-CD3 (10ug/ml immobilized)+anti-CD28 (2ug/ml soluble) 5hr	Monensin	MP1-22E9
IFN-g	mouse spleen	ConA (3ug/ml) (2d)/IL-2 (20ng/ml)+IL-4 (20ng/ml) (3d)	2d/3d	anti-CD3 (10ug/ml immobilized)+anti-CD28 (2ug/ml soluble) 5hr	Monensin	XMG1.2
TNF-a	mouse spleen	ConA (3ug/ml) (2d)/IL-2 (20ng/ml)+IL-4 (20ng/ml) (3d)	2d/3d	anti-CD3 (10ug/ml immobilized)+anti-CD28 (2ug/ml soluble) 5hr	Monensin	MP6-XT22
TNF-a	mouse spleen	ConA (3ug/ml) (2d)/IL-2 (20ng/ml)+IL-4 (20ng/ml) (3d)	2d/3d	anti-CD3 (10ug/ml immobilized)+anti-CD28 (2ug/ml soluble) 5hr	Monensin	TN3-19.12

Annotations: mouse PEC=mouse thioglycolate-elicited peritoneal macrophages; ConA=Concanavalin A; Iono=Ionomycin; LPS=Lipopolysaccharide; PMA=Phorbol Myristate Acetate; 2d=2 day culture; 5hr=5 hour culture

Human Cytokine Intracellular Staining Quick Guide						
Human Cytokine	Cell Source	Activation	Incubation Time	Restimulation	Intracellular Block	Antibody
IL-1a	PBMC	LPS 100ng/ml	24hr	-	Monensin	CRM8
IL-1b	PBMC	LPS 100ng/ml	24hr	-	Monensin	CRM56
IL-2	PBMC	PMA (30-50ng/ml)/Iono (1ug/ml)	5hr	-	Monensin	MQ1-17H12
IL-4	PBMC	anti-CD3 (10µg/ml, immobilized) + anti-CD28 (2µg/ml, soluble) + IL-2 (10ng/ml) + IL-4 (20ng/ml) (2d); IL-2 (10ng/ml) + IL-4 (20ng/ml) (3d)	2d/3d	PMA (5ng/ml) + Ionomycin (500ng/ml) (4hr)	Monensin	MP4-25D2
IL-6	PBMC	LPS 100ng/ml	5hr	-	Monensin	MQ2-13A5
IL-10	PBMC	LPS 100ng/ml	24hr	-	Monensin	JES3-9D7
IL-12	PBMC	hIFNg (100ng/ml) (2hr)/LPS (100ng/ml) (22hr)	2hr/22hr	-	Monensin	C8.6
IFN-g	PBMC	PMA (30-50ng/ml)/Iono (1ug/ml)	5hr	-	Monensin	4S.B3
TNF-a	PBMC	PMA (30-50ng/ml)/Iono (1ug/ml)	5hr	-	Monensin	MAb11
Annotations: Iono=Ionomycin; PMA=Phorbol Myristate Acetate; LPS=Lipopolysaccharide; 2d=2 day culture; 5hr=5 hour culture; LPS for activation of human PBMC obtained from Sigma (#L-8274)						

Flurochrome-conjugated staining surface antigen for T helper cells

- 10^6 cells in 100 µl of staining buffer
- mAb CD3ε-PerCP → T cell
- CD4-FITC → T helper cell
- 30 min, 4 ˚C in dark

- Staining buffer
  - DPBS without Mg2+ or Ca2+
  - 1 % FBS
  - 0.1 % NaN3
  - Adjust pH 7.4-7.6, filter, store at 4 ˚C

Fix the cells

- 4 % (w/v) paraformaldehyde in DPBS with 0.54 % glucose
- 20 min. at 4˚C
- Cell can be kept overnight in fixation buffer at 4˚C in dark

Permeabilize cells

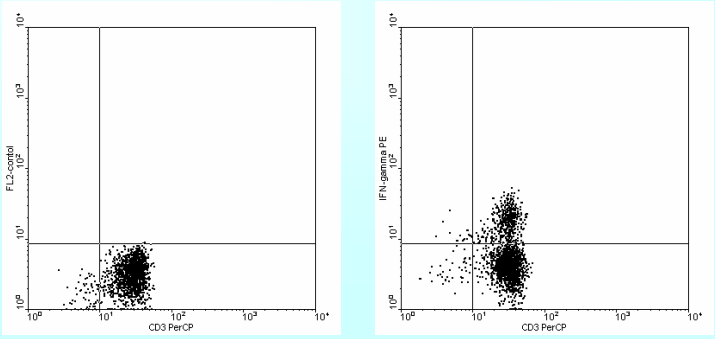
- Wash cells 2 times in permeabilization buffer and pellet
- permeabilization buffer:
  - DPBS
  - 1 % FBS
  - 0.1 % (w/v) NaN_3
  - 0.1 % (w/v) saponin
  - 0.1 % glucose
  - 0.01 M HEPES
  - 0.035 % $NaHCO_3$
  - Adjust buffer pH to 7.4-7.6 and filter

Stain intracellular cytokines

- Resuspend fixed cells in 100 μl of permeabilization buffer
- mAb anti-IFN-γ, anti-IL-4, anti-TNF-α
- Incubate at 4˚C for 30 min. in dark

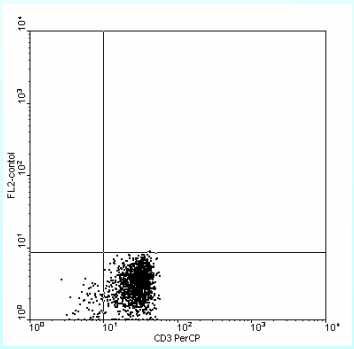
Analysis

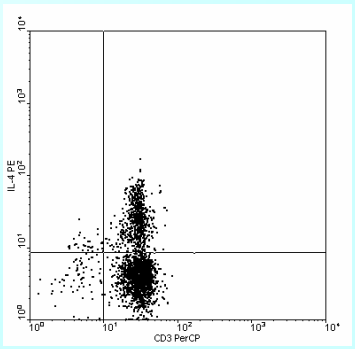
- Resuspense cells in staining buffer
- Set PMT voltage and compensation
- IFN-γ



Analysis

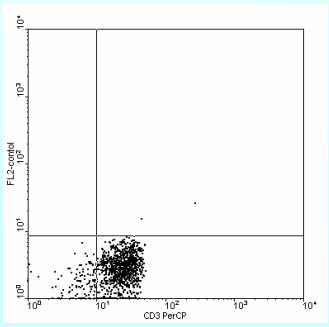
- Resuspension cells in staining buffer
- Set PMT voltage and compensation
- IL-4

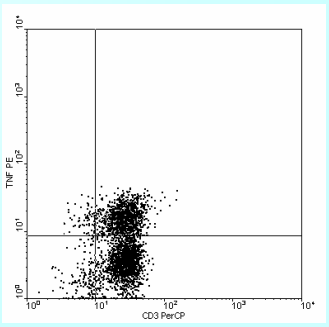




Analysis

- Resuspension cells in staining buffer
- Set PMT voltage and compensation
- TNF-α





Analysis

- Resuspension cells in staining buffer
- Set PMT voltage and compensation
- IL-4 and IFN-γ double stain

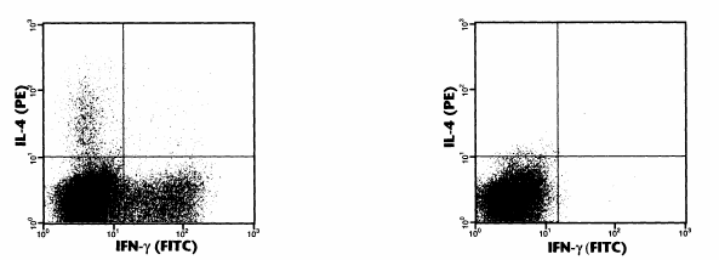
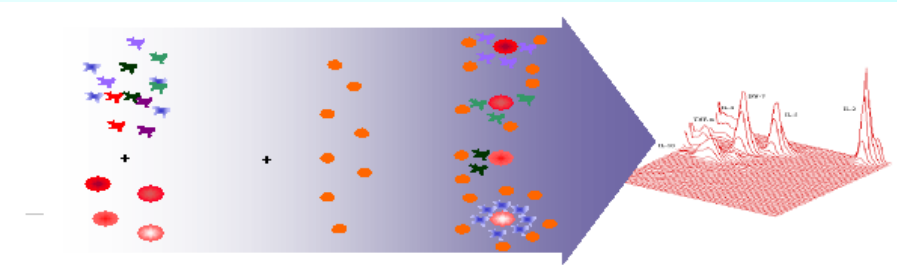


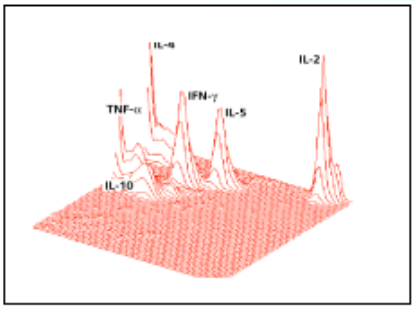
Figure 1: Three color flow cytometric analysis of activated human peripheral blood mononuclear cells (PBMCs) for CD4, IFN-γ, and IL-4. Discrimination of Th1, Th2, and Th0 populations.

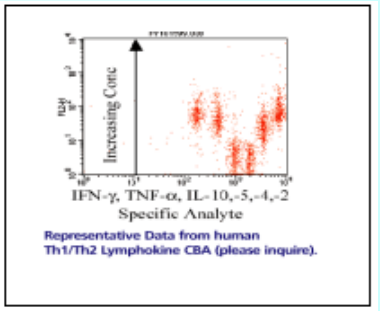
流式細胞多重分析技術
Cytometric Bead Array, CBA

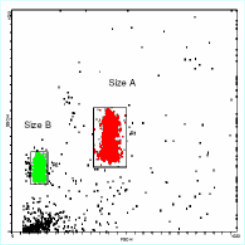
- 細胞激素分析的方法乃是利用抗體標定微球吸附細胞培養上清液中之細胞激素，在利用流式細胞儀分析細胞激素所呈現出來之不同螢光讀值，再與標準曲線對照而得其分泌量

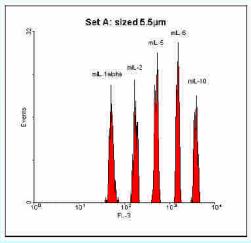


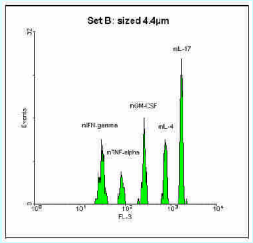
(1)Add unknowns to capture bead array (2)Add detection reagents (3)Acquire samples (4)Batch analysis using CBA software

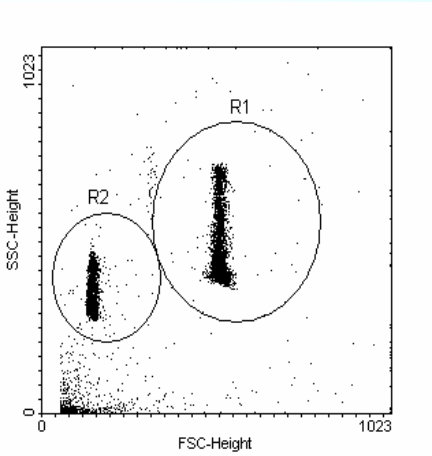

IL-6
IL-2
TNF-α
IFN-γ
IL-5
IL-10

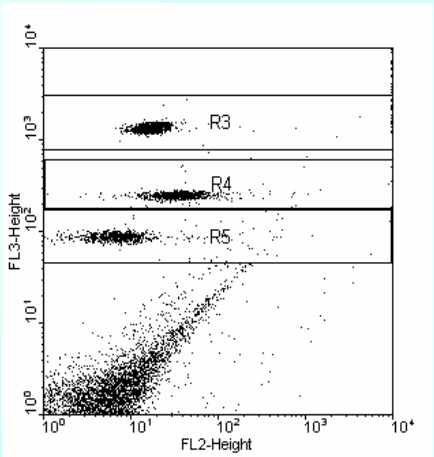

Increasing Conc
IFN-γ, TNF-α, IL-10,-5,-4,-2
Specific Analyte
Representative Data from human
Th1/Th2 Lymphokine CBA (please inquire).


Size A
Size B


Set A: sized 5.5µm
IL-6
IL-5
IL-2
IL-10
Events

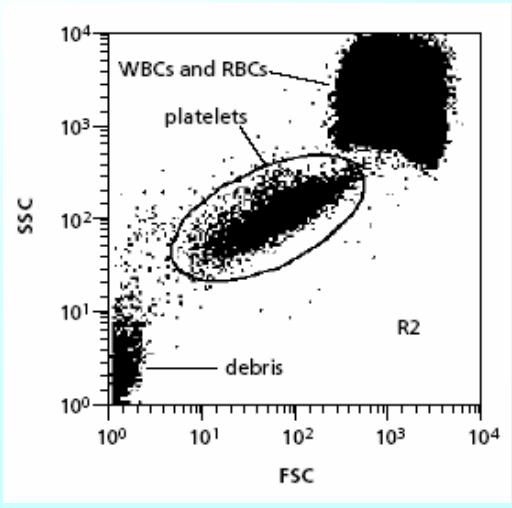

Set B: sized 4.4µm
IL-17
IL-4
Events


R1
R2
SSC-Height
FSC-Height
0
1023

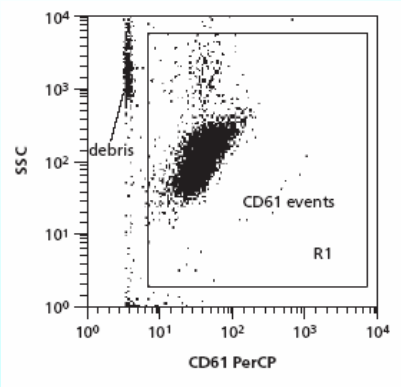

R3
R4
R5
FL3-Height
FL2-Height

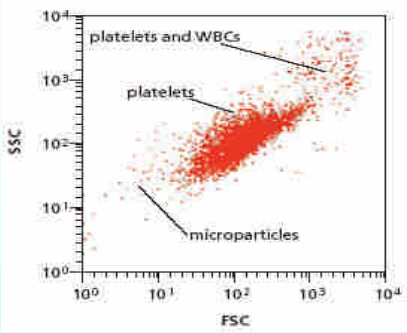
Platelet analysis

Platelet analysis-**Scatter Gating**

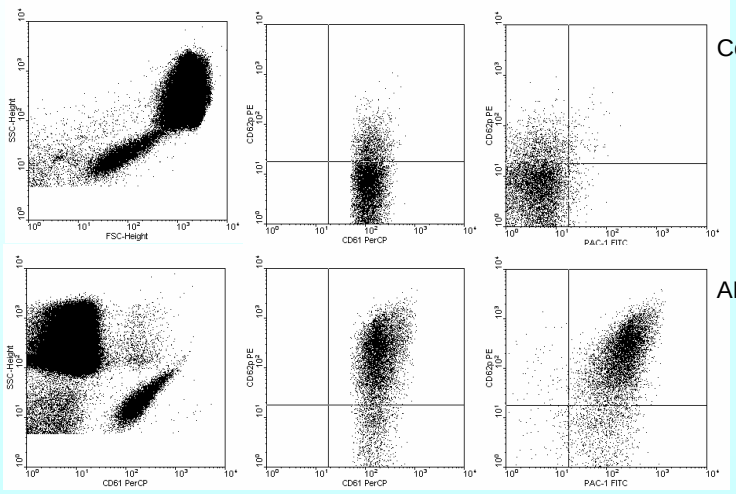


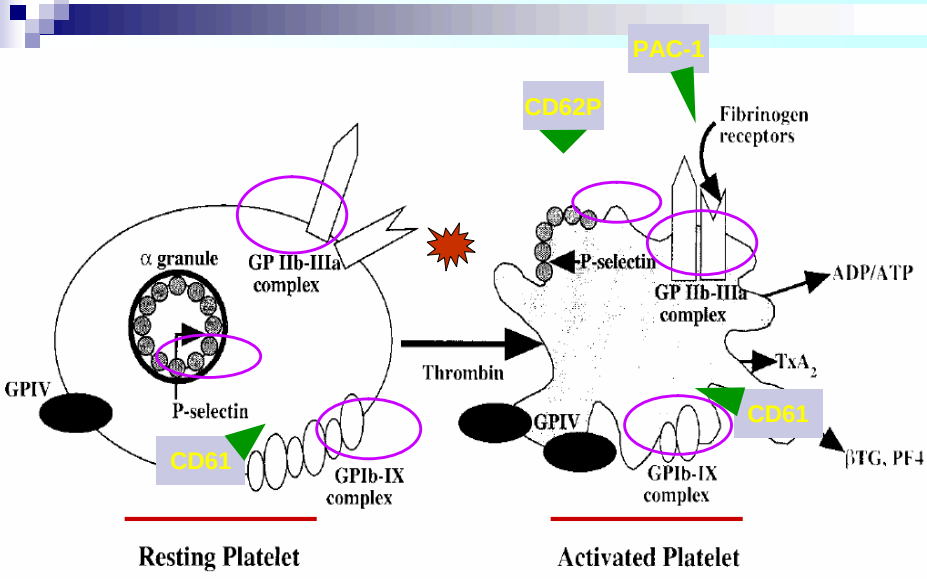
Platelet analysis-**Fluorescence Gating**





Platelet analysis



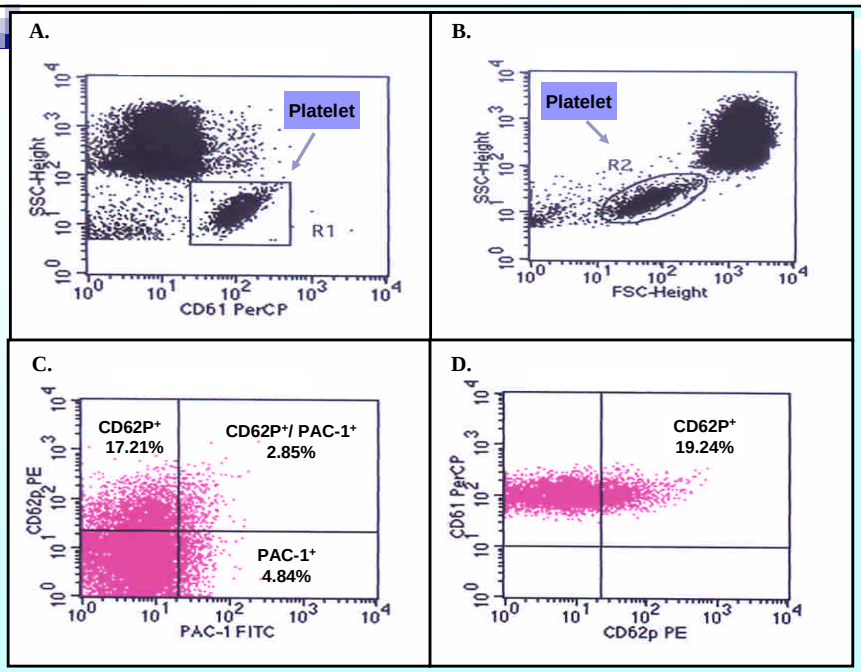


Surface markers of platelet of platelet activation.

Modified from: Kestin et al. Circulation (1993) 88(4 Pt 1): 1502-1511.

活化血小板測定意義

- 抗體之選用:
  - PAC-1 (結合活化血小板膜上之GPIIb/IIIa)
  - CD62P (結合活化血小板膜上之P-selectin)
  - CD61 (結合靜止及活化血小板膜上之GPIb/IX)
- PAC-1 / CD61 :
  - 活化血小板數目/ 所有血小板數目
  - 觀察抗氧化補充劑對血小板膜蛋白之影響
- CD62P / CD61:
  - 活化血小板數目/ 所有血小板數目
  - 觀察抗氧化補充劑對血小板α- granule膜蛋白之影響



Flow Cytometry Human Platelet Activation Analysis

Table 6.10.3 Activation-Dependent Changes in Platelet Surface Labeling of Monoclonal Antibodies and Annexin V[a]

Activation-dependent platelet surface change	Resting platelet	Activated platelet
Changes in surface receptor expression		
CD36	+	++
GPIb-IX	++	+
GPIIb-IIIa	++	+++
Conformational changes in GPIIb-IIIa (integrin $\alpha_{IIb}\beta_3$)		
Ligand-induced binding sites (LIBS)	–	+++
PAC1	–	+++
Receptor-induced binding sites on fibrinogen (RIBS)	–	+++
Development of a procoagulant surface		
Factor VIII binding	–	+++
Factor V/Va binding	–	+++
Factor X/Xa binding	–	+++
Phosphatidylserine expression (detected by annexin V)	–	+++
Exposure of granule membrane proteins		
CD40L (or CD154)	–	+
CD63 (lysosomes)	–	++
LAMP-1 (lysosomes)	–	++
LAMP-2 (lysosomes)	–	++
Lectin-like oxidized LDL receptor-1 (LOX-1)	–	+
P-selectin (CD62P, α-granules)	–	+++
Platelet surface binding of secreted platelet proteins		
Multimerin	–	+
Thrombospondin	–	+

[a]Annexin V is a 35 to 36 kDa protein that binds to phosphatidylserine in the presence of Ca^{2+}.

PREPARATION OF PLATELET-ENRICHED PLASMA

For many platelet assays, the platelets do not need to be purified by density-gradient separation. Platelet-enriched plasma, prepared by enrichment of platelets from peripheral blood (Ault, 1988), is often an acceptable specimen.

Materials

Peripheral blood in EDTA or appropriate anticoagulant
Tyrode's buffer (see recipe)
15-ml conical centrifuge tube

1. Centrifuge 7 ml blood (in collection tube) 10 min at 200 × *g*, 25°C.
2. With a sterile pipet, transfer the plasma layer to a 15-ml conical centrifuge tube. Centrifuge 10 min at 1600 × *g*, 25°C.
3. Remove and discard supernatant. Resuspend pellet containing platelets in Tyrode's buffer or a buffer containing EDTA.

Table 6.10.2 Anticoagulants Used in the Study of Platelets

Anticoagulant	Mechanism of action
Acid citrate dextrose (ACD)	Weak Ca^{2+} chelator
Citrate theophylline adenosine dipyridimole (CTAD)	Chelates Ca^{2+} and increases intracellular cAMP, keeping platelets "quiet"
Corn trypsin inhibitor	Activated coagulation factor XII inhibitor
EDTA[a]	Strong Ca^{2+} chelator, dissociates GPIIb-IIIa complex
Heparin[a]	Combines with anti-thrombin III to inhibit thrombin activity
Hirudin	Direct thrombin inhibitor
D-Phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (P-PACK)	Direct thrombin inhibitor
Sodium citrate	Weak Ca^{2+} chelator

[a]These anticoagulants should be avoided for evaluation of platelet function studies by flow cytometry (see Strategic Planning).

Table 6.10.1 Applications of Flow Cytometry to the Study of Platelets[a]

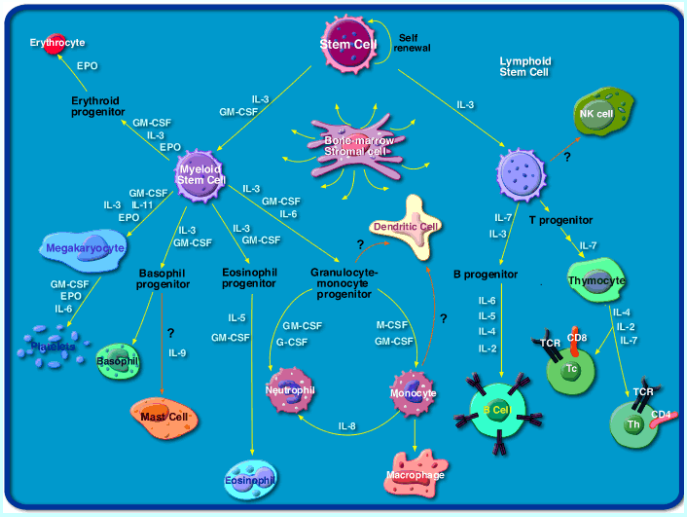
Measurement of platelet activation[b]
Activation-dependent monoclonal antibodies/reagents
Modulation of constitutively expressed surface receptors
Procoagulant platelet-derived microparticles
Leukocyte-platelet aggregates
Platelet-platelet aggregates
Diagnosis of specific disorders
Bernard-Soulier syndrome
Glanzmann thrombasthenia
Storage pool disease
Heparin-induced thrombocytopenia
Immune thrombocytopenias
Monitoring of antiplatelet agents
GPIIb-IIIa antagonists
Thienopyridines
Monitoring of thrombopoiesis
Reticulated platelets
Blood bank applications
Quality control of platelet concentrates
Identification of leukocyte contamination in platelet concentrates
Immunophenotyping of platelet HPA-1a
Detection of maternal and fetal anti-HPA-1a antibodies
Platelet cross-matching

Platelet counting

Research applications

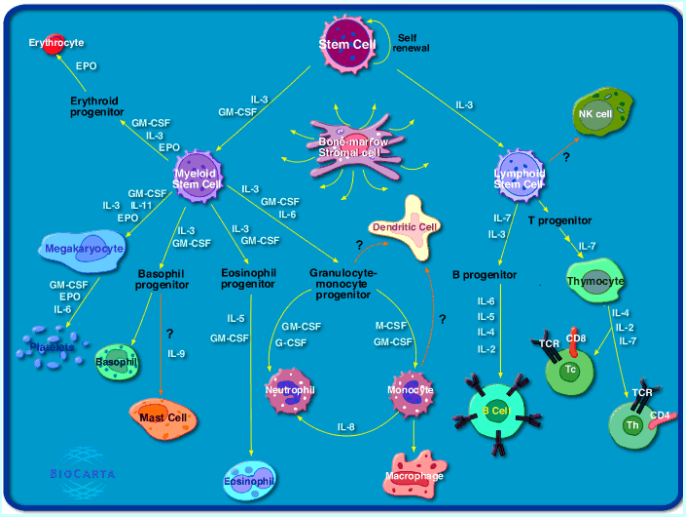
- Platelet survival, tracking, and function in vivo
- Platelet recruitment
- Bacteria-platelet interactions
- Calcium flux
- Cytoskeletal rearrangement
- Fluorescence resonance energy transfer
- Signal transduction

Regulation of Hematopoiesis by Cytokines - Human



http://cgap.nci.nih.gov/Pathways/BioCarta/stemPathway

Regulation of Hematopoiesis by Cytokines - Mouse



http://cgap.nci.nih.gov/Pathways/BioCarta/m_stemPathway

Apoptosis analysis

細胞凋亡

- 細胞凋亡又稱為程序化細胞死亡，是某些生理或病理條件下，細胞接受某種信號的觸發後主動發生一連串連續性細胞變化，最終導致細胞死亡而不引起炎症的細胞死亡過程。細胞凋亡現象是由 Kerr Whyuie 等人於1972 年首次提出，1980 年他們在對細胞凋亡進行長期觀察和分析後提出細胞凋亡不同於細胞壞死。

細胞凋亡的生物學意義

- 細胞凋亡被認為是與細胞增生相反的方式，來調節細胞群體，它不僅對胚胎發生、器官發育、分化作用、及保持機體的平衡穩定等過程至為重要，而且對控制細胞的增殖、腫瘤發生和發展極為重要。通過細胞凋亡，機體及時清除受損及危險的細胞，因此細胞凋亡對機體的正常發展具有十分重要的生物學意義。

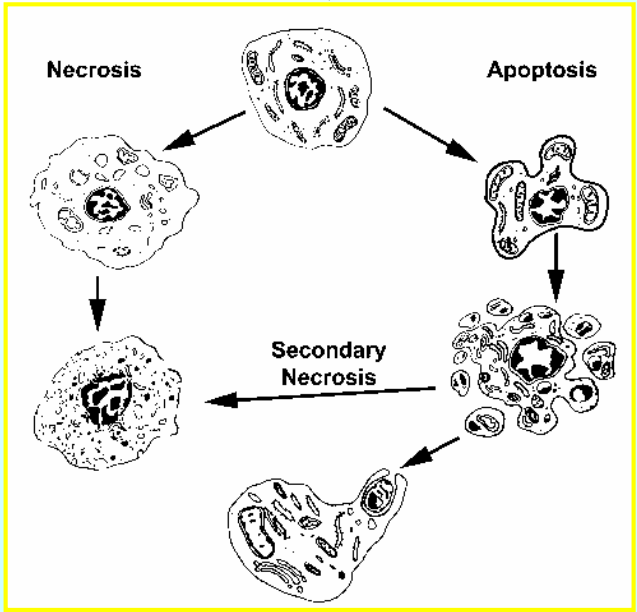
細胞凋亡的生物學意義

- 細胞凋亡可經由
  - Deprivation of growth factors
  - γ-irradiation
  - Oxygen free radical (OFR) production
  - Receptor-ligand interaction
  - Inhibition protein kinase
  - …

凋亡細胞的特徵

- 細胞會喪失微絨毛、偽足等胞膜結構，隨後，細胞會皺縮、核質濃縮，細胞密度增大。
- 細胞核離散，呈現月牙形凝集在核膜下。
- 隨著細胞膜和細胞核分離，會裂解形成凋亡小體 (Apoptotic body)。
- 凋亡過程的最後階段發生細胞核的 DNA 降解，降解後產生的 DNA 片段由185-200 bp 多聚體組成。在瓊脂凝膠上呈現特徵性凋亡“梯型”。

凋亡細胞的特徵



程序性死亡-凋亡的活化

- 粒線體
  - 膜電位的失衡
  - Apaf-1/cyt. c/AIF release
  - Bcl family regulation
- TNFR/FAS superfamily
- P53/Rb cell cycle checkpoint pathway
- Toll-Like receptor pathway
- Nicotinic acetylcholine receptors pathway
- Cell-cell interaction
- Growth factor/cytokine pathway
- …

程序性死亡-凋亡程序訊息傳遞

- Caspase pathway (Mit., TNFR, P53…)
- PI3K-Akt pathway
- Activate cytokine/growth factor release

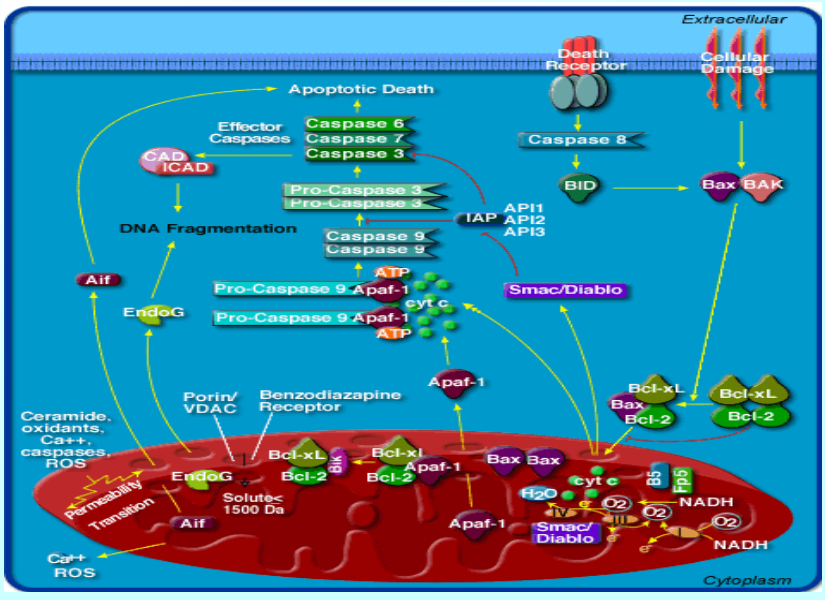
程序性死亡-DNA fragmentation

- Endo G
- CAD/DFF
- Lamin B1/Lamin degradation

粒腺體在凋亡程序中所扮演的角色

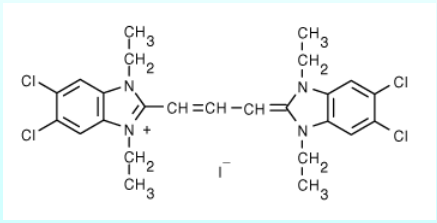
- 能量代謝失衡
- Produce OFR
- Alter the redox state of the cell
- Cause cycling of Ca^{2+} ions
- Release cyt c, Apfa-1, AIF
- Regulate protooncogene Bcl-2 family
- Release endonuclease

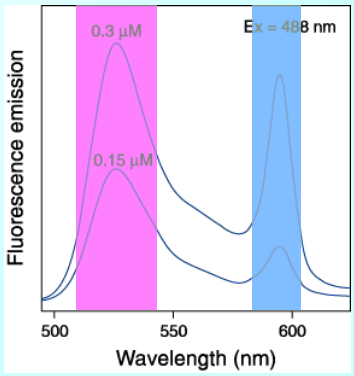
粒腺體在凋亡程序中所扮演的角色



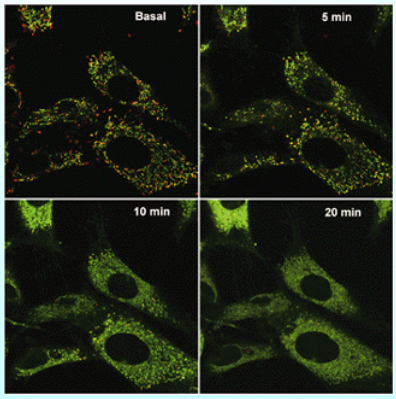
粒腺體膜電位的偵測

- 5,5',6,6'-tetrachloro-1,1', 3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1; $CBIC_2(3)$)



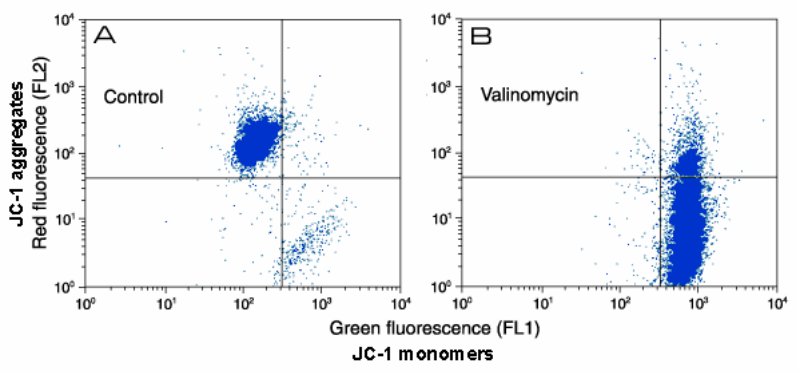


粒腺體膜電位的偵測



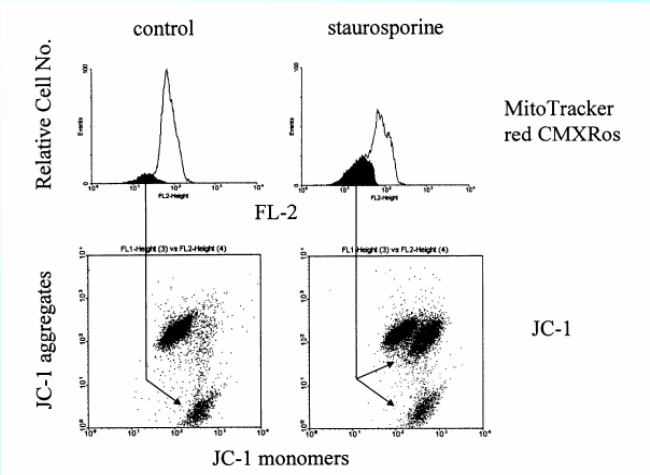
NIH 3T3 fibroblasts stained with JC-1 showing the progressive loss of red J-aggregate fluorescence and cytoplasmic diffusion of green monomer fluorescence following exposure to hydrogen peroxide. Images show the same field of cells viewed before H_2O_2 treatment, and 5, 10 and 20 minutes after treatment.
(Images contributed by Ildo Nicoletti, Perugia University Medical School.)

粒腺體膜電位的偵測



Bivariate JC-1 analysis of mitochondrial membrane potential in HL60 cells by flow cytometry. The sensitivity of this technique is demonstrated by the response to valinomycin-induced depolarization for two hours. Figure courtesy of Dr Andrea Cossarizza, University of Modena and Reggio Emilia.

粒腺體膜電位的偵測



粒腺體能量代謝異常-PS presentation

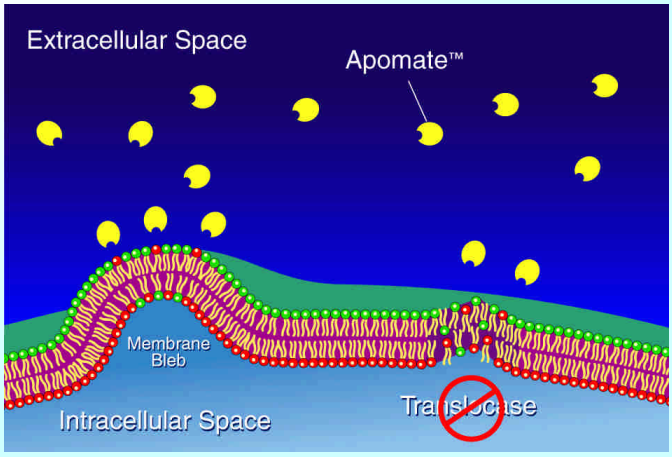
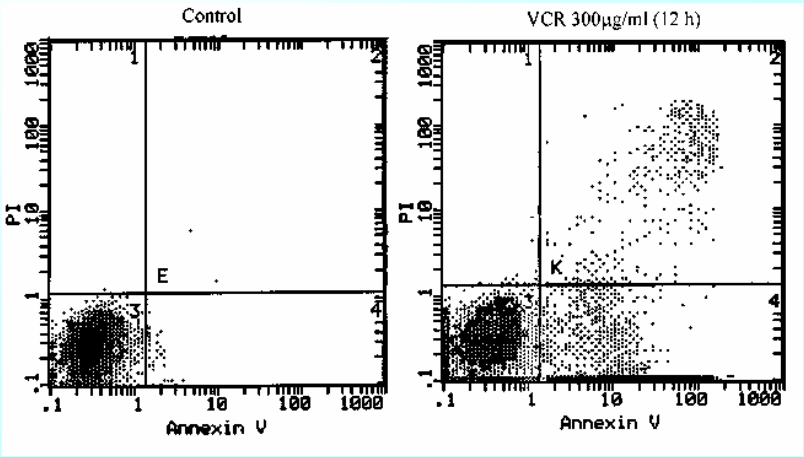


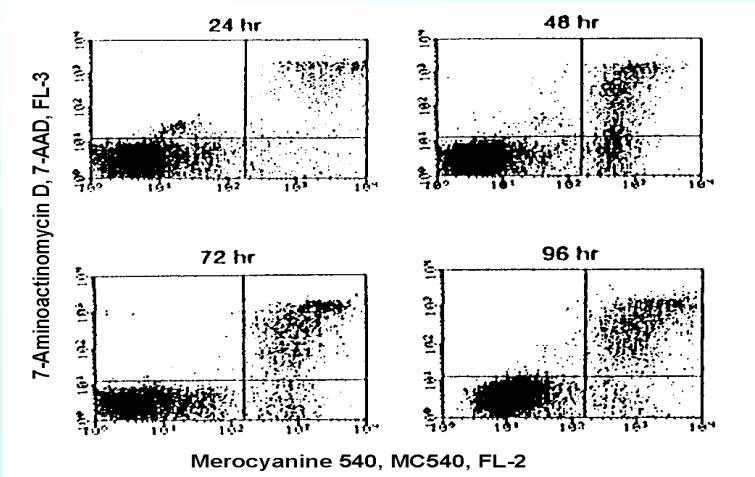
Figure from North American Scientific, Inc.
http://www.nasi.net/ProductCenter/mi/mi_howapoptosis.html

粒腺體能量代謝異常
-PS presentation/Annexin V-FITC/PI

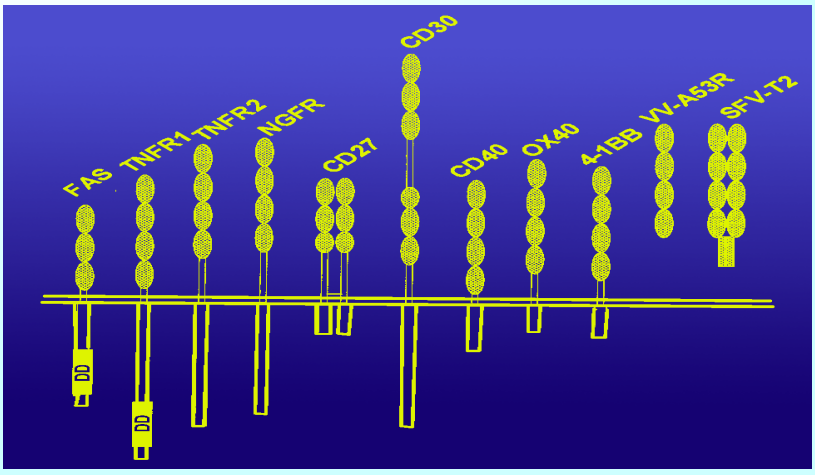


Biotechnol. Appl. Biochem. 2001, **33**: 127–132

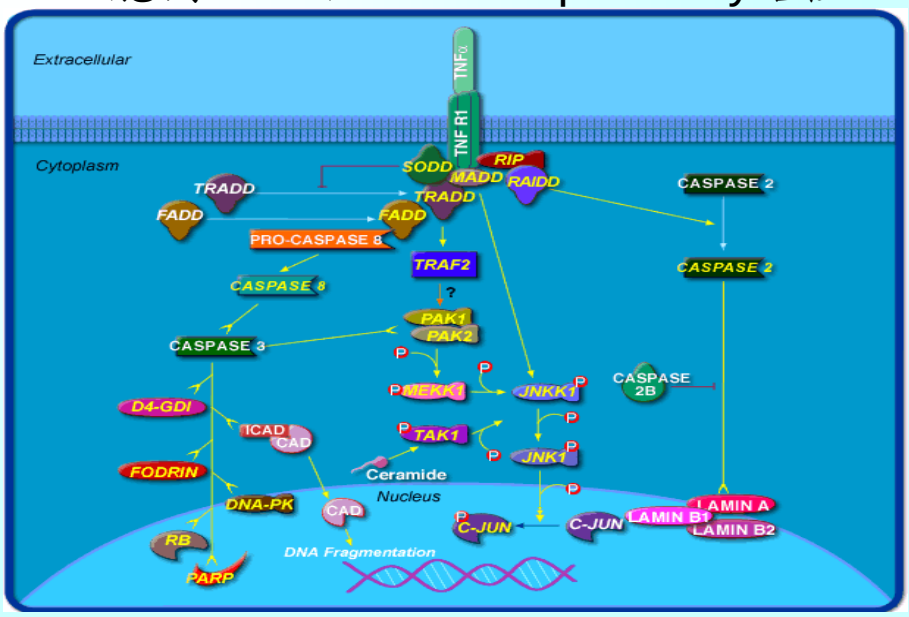
粒腺體能量代謝異常
-PS presentation/Merocyanine 540/7-AAD



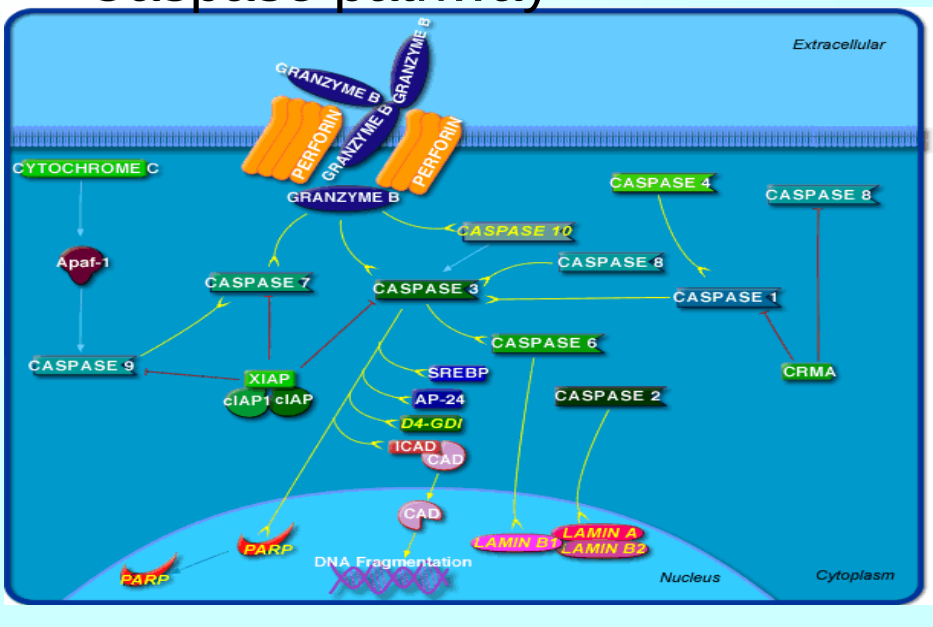
細胞凋亡-活化TNFR superfamily路徑



細胞凋亡-活化TNFR superfamily路徑



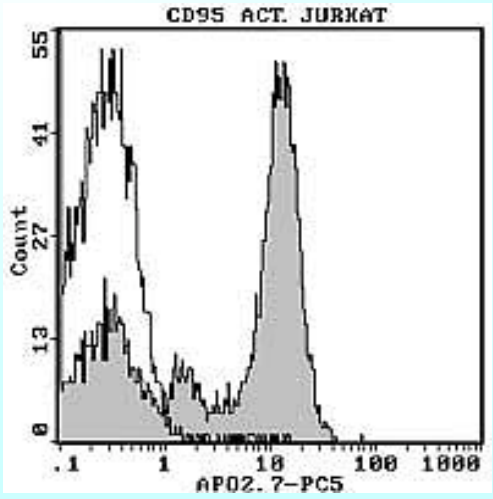
Caspase pathway



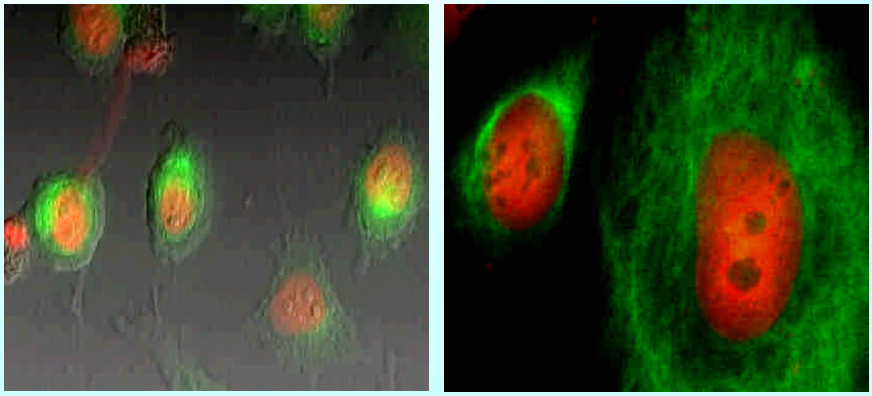
Caspase pathway-reagents

Caspase	Colorimetric	Fluorimetric-1	Fluorimetric-2	Fluorimetric-3
Caspase 3	Ac-DEVD-pNA (405)	Ac-DEVD-AMC (E/M=360/460)	FAM-DEVD-FMK (E/M=490/520)	Ac-DEVD-AFC (E/M=400/505)
Caspase 6	Ac-VEID-pNA	Ac-VEID-AMC	FAM-VEID-FMK	Ac-VEID-AFC
Caspase 1	Ac-YVAD-pNA	Ac-YVAD-AMC	FAM-YVAD-FMK	Ac-YVAD-AFC
Caspase 8	Ac-LETD-pNA	Ac-LETD-AMC	FAM-LETD-FMK	Ac-LETD-AFC
Caspase 9	Ac-LEHD-pNA	Ac-LEHD-AMC	FAM-LEHD-FMK	Ac-LEHD-AFC
	Sigma	Sigma	Gentaur	Roche

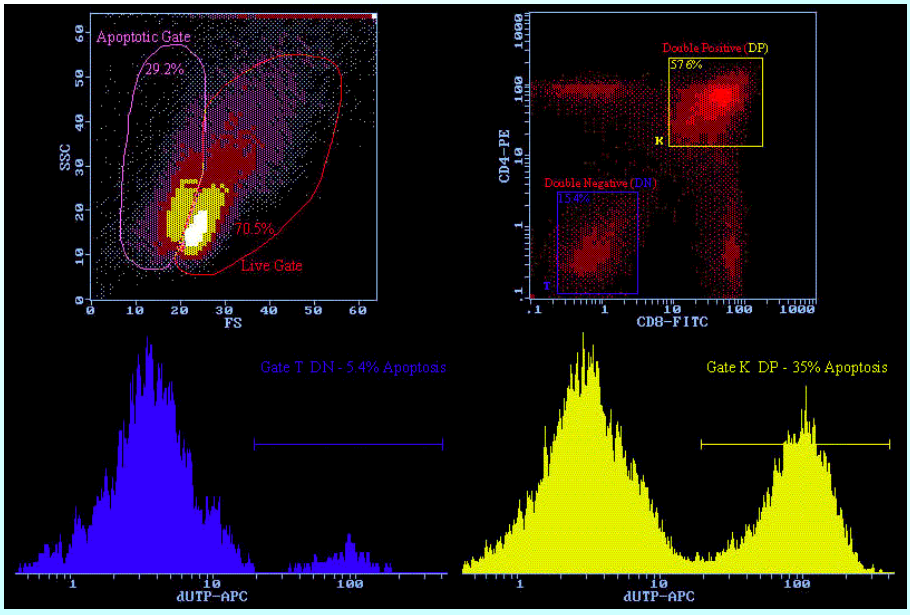
APO 2.7 detection



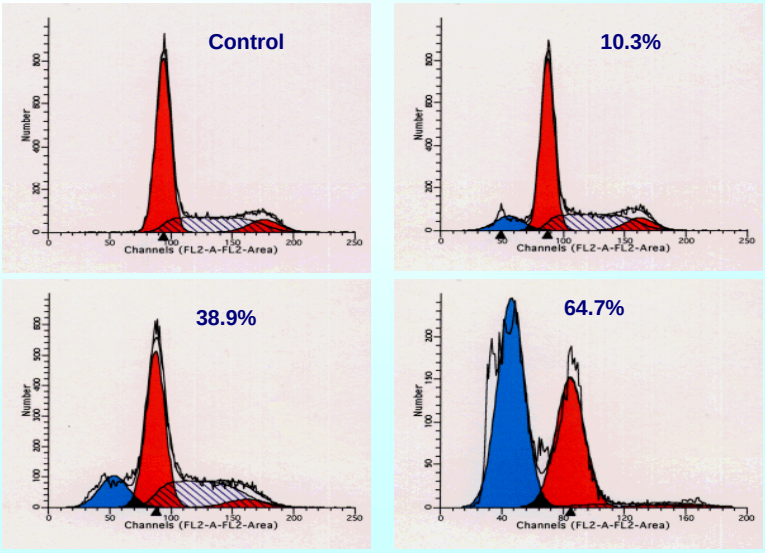
Tubulin/PI stain



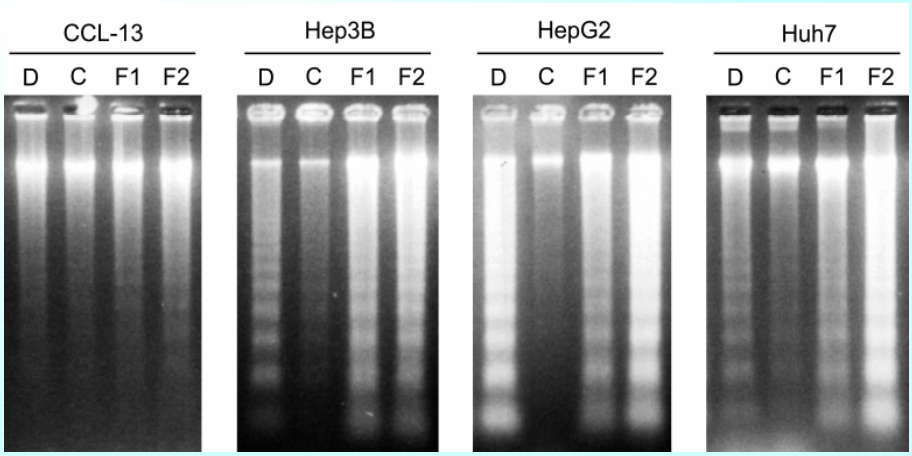
TUNEL Assay



PI Stain for analysis pre-G0 peak

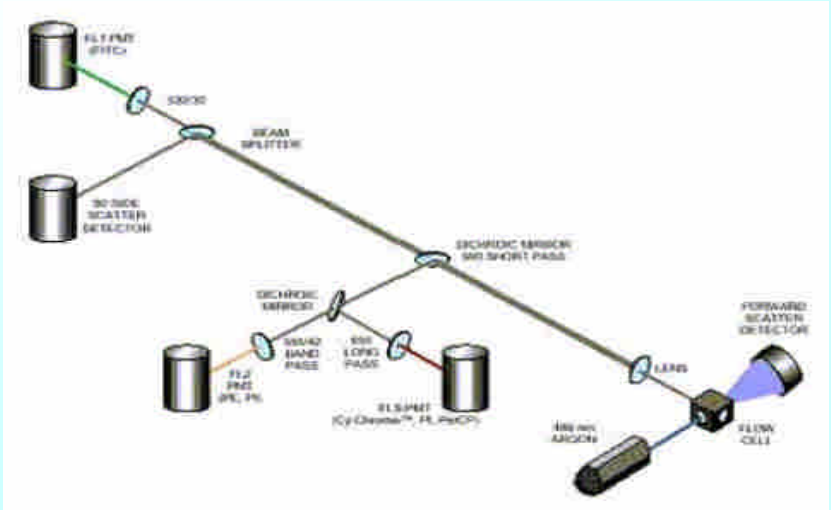


DNA fragmentation

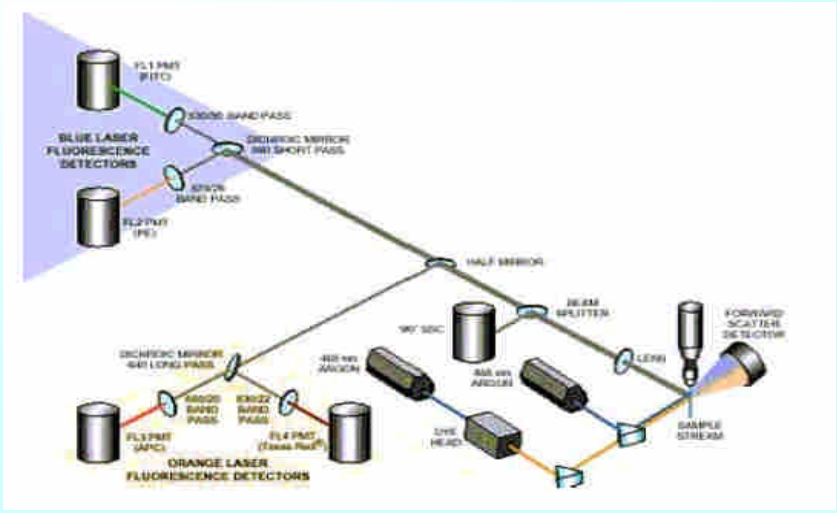


上樣與資料分析

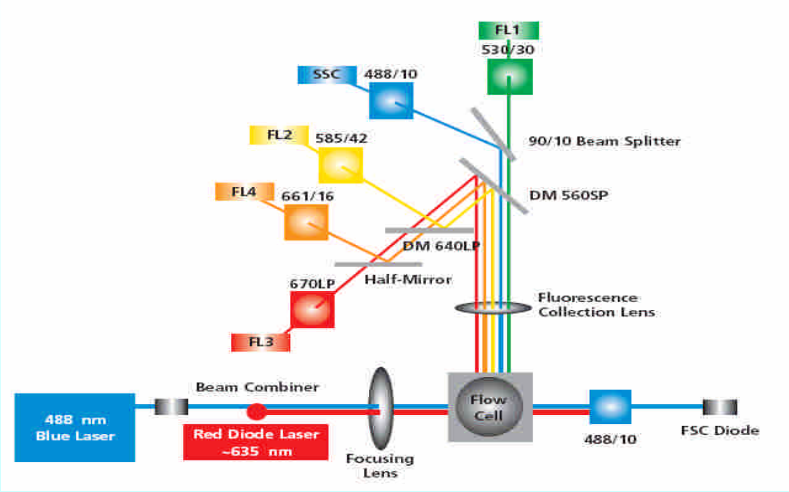
A single laser flow cytometer



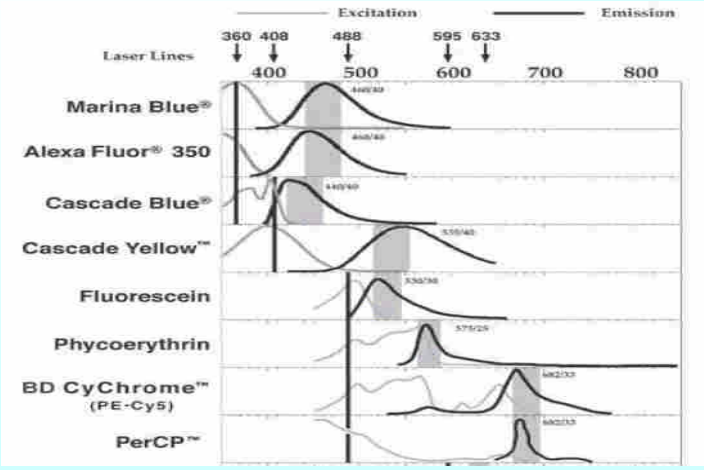
A dual laser flow cytometer



A dual laser flow cytometer



Fluorochromes



Fluorochromes

Fluorochrome	Laser Excitation Wavelength (nm)	FACScan™ FACSCalibur™ (1 laser)	FACSCalibur™ (2 lasers)	FACStar™ FACStarPlus™ FACSVantage™ (1 laser)	FACSVantage™SE FACStarPlus™ (2lasers)
Fluorescein	488	YES	YES	YES	YES
Phycoerythrin (PE)	488	YES	YES	YES	YES
PE-Texas Red	488	YES	YES	YES	YES
BD Cy-Chrome (PE-Cy5)	488	YES	YES	YES	NO[§]
Propidium Iodide	488 & 595	YES	YES	YES	YES
Peridinin Chlorophyl Protein (PerCP)	488	YES	YES	YES[-]	YES[-]
Texas Red	595	NO	NO	NO	YES[--]
Allophycocyanin (APC)	595 & 633	NO	YES	NO	YES[§]
APC-Cy7	595 & 633	NO	YES	NO	YES

FACS 日常操作

- 儀器本體，及Macintosh電腦。
  - a. 電源：電源在儀器右側下方，操作時要先啟動儀器本體再打開電腦及印表機。
  - b. 暖機時間：儀器需5 ~10分鐘的暖機時間
  - c. 儀器面板：
    - 流速控制鍵(LO/MED /HI)
    - 功能控制鍵(BACKFLUSH/RUN/STANDBY/ PRIME-DRAIN/FILL) 。

FACS 日常操作

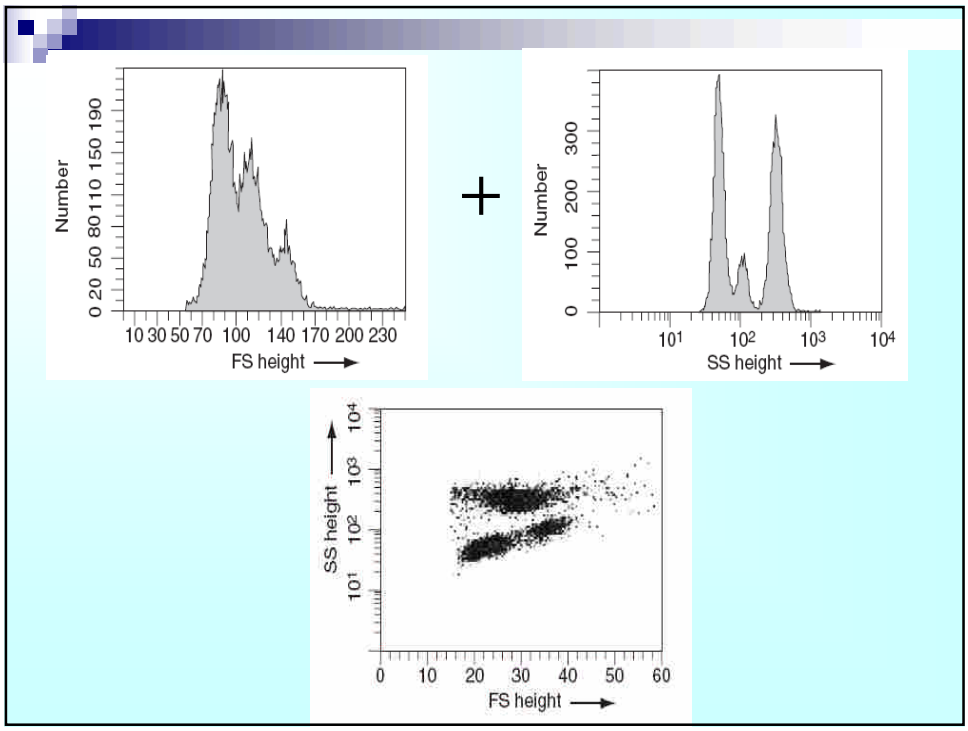
- 流速控制：
  - LO： 樣品流速：12 µl /min
  - MED： 樣品流速：35 µl /min
  - HI： 樣品流速：60 µl /min
- 功能控制：
  - RUN： 綠色時表示樣品開始輸注。(黃色時表示儀器不正常，請檢查是否漏氣)
  - STANDBY： 無樣品或暖機時之正常位置，此時雷射功率會自動降低以延長雷射壽命。
  - PRIME： 自動沖洗進樣針並將PBS 注滿Flow Cell，使用時機如：裝機開機、更換PBS、清洗儀器或清洗進樣針等。

FACS 開機

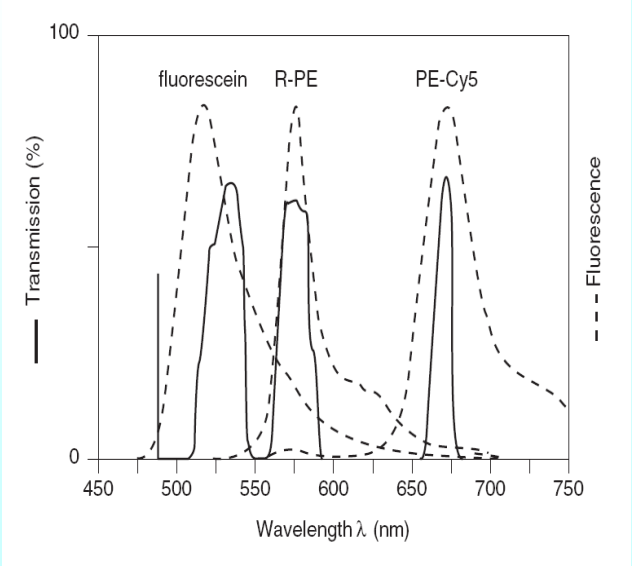
- 開啟細胞儀電源。
- 開啟其他周邊配備電源，如印表機及M.O.。
- 開啟電腦。
- 確認鞘流液筒有八分滿的FACS FLOW，確實旋緊。
- 將廢液倒掉，並在廢液筒中加入100 ml 家用漂白水。
- 將氣壓閥方向調在加壓(Pressurize)位置。
- 排除液流過濾氣中的氣泡。
- 使用1 ml PBS 為樣品，執行PRIME 功能兩次。
- HIGH RUN 兩分鐘，即可開始分析樣品。

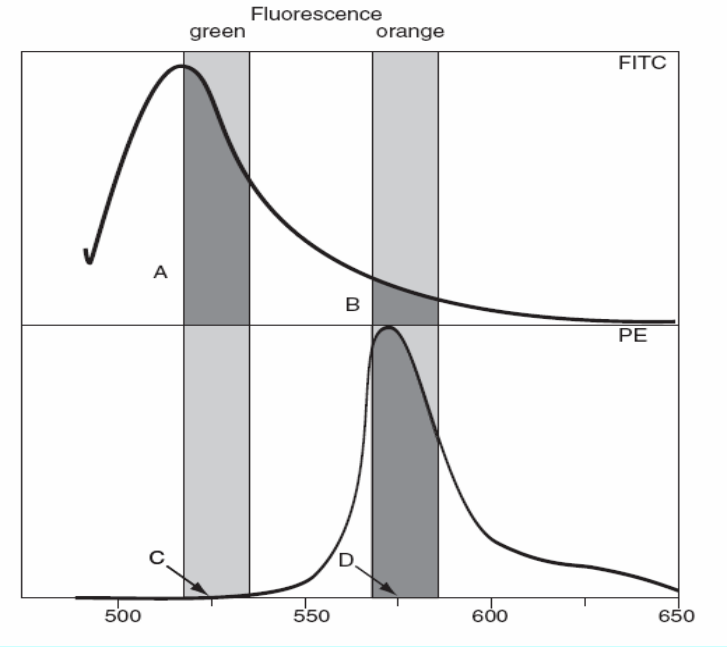
檢品上機之確認事項

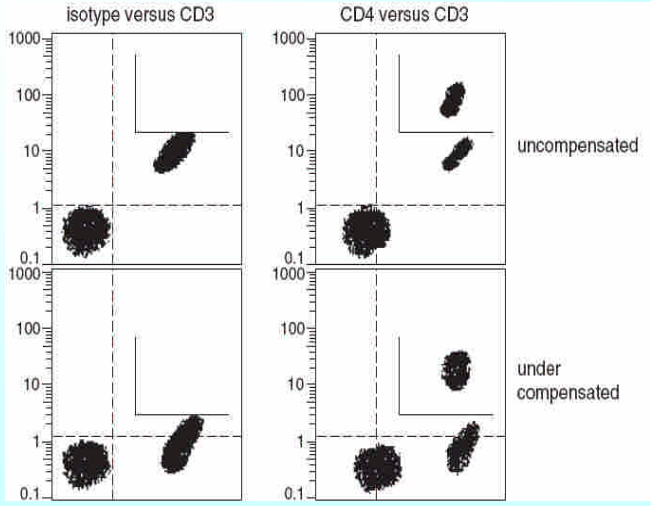
- 是否已將檢品濃度調至1X10^6 cells/ml？
- 是否已去除檢品中之細胞團塊，以防止管路堵塞？可使用附濾網FALCON試管(Cat. No.2235) 或30-50 μm 的尼龍篩網。
- 是否已將檢品放至FALCON 2052 試管中？試管是否有裂痕？
- 是否已將專用鞘流液筒充填至八分滿？
- 是否已將廢液倒掉，並在廢液筒中加入100 ml 漂白水？
- 是否已將液流過濾器中之氣泡排空？
- 是否已將所有管線及管路裝置妥善？並將氣壓閥方向調至正確定位？
- 是否已執行Prime 兩次以將管路及Flow Cell 中之氣泡排空？
- 填寫使用登記表。

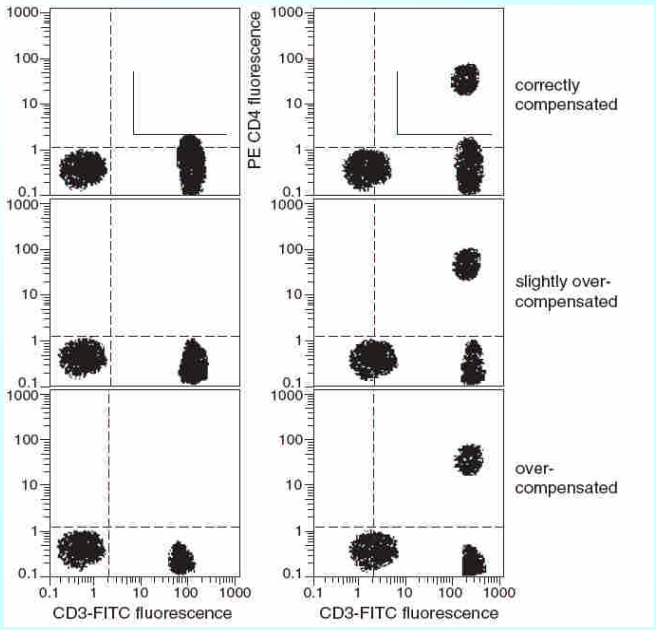


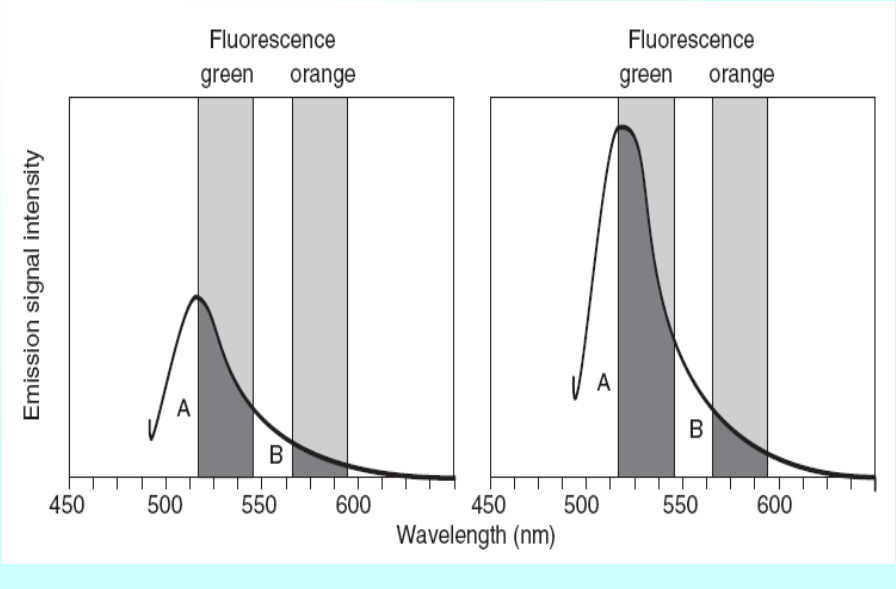
Compensation

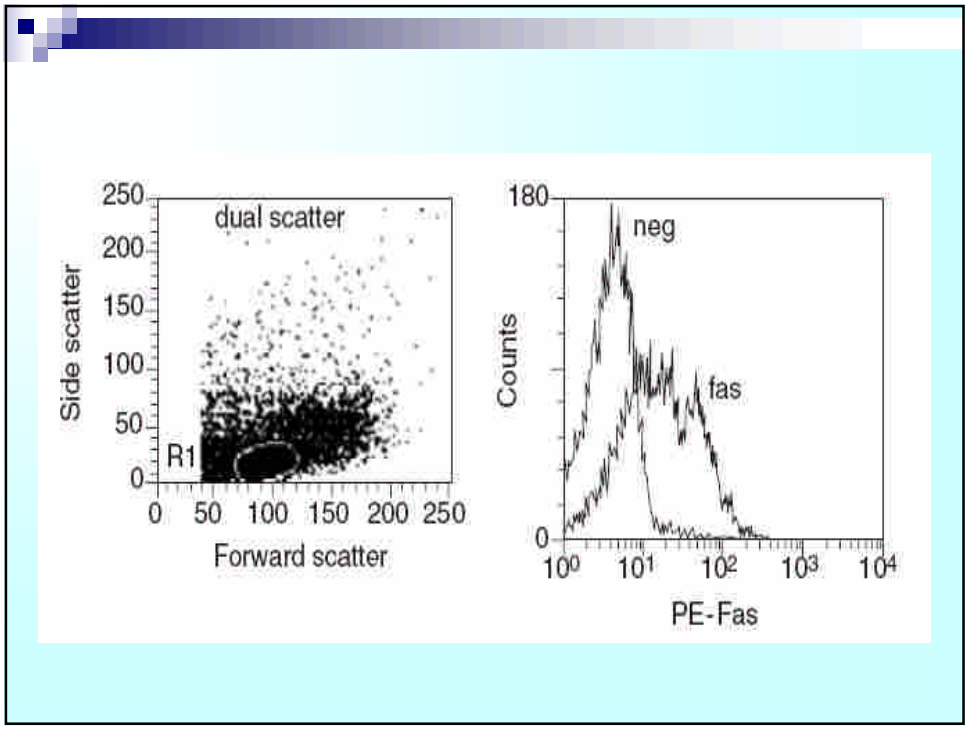


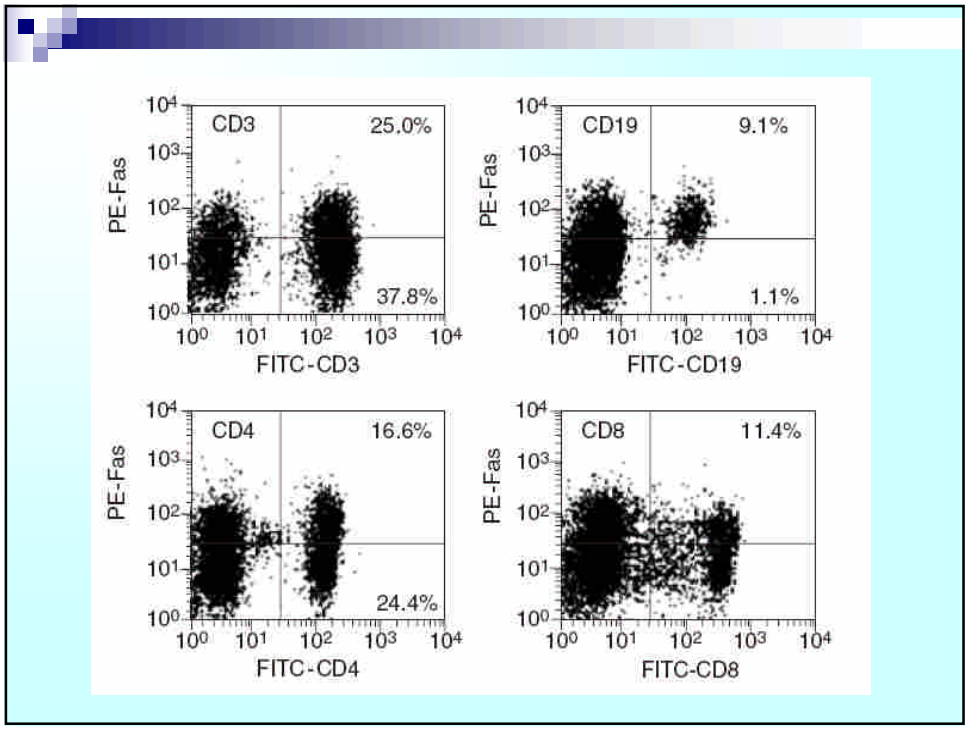

Fluorescence
green
orange
FITC
A
B
PE
C
D
500
550
600
650


isotype versus CD3
CD4 versus CD3
1000
100
10
1
0.1
uncompensated
under
compensated


PE CD4 fluorescence
correctly compensated
slightly over-compensated
over-compensated
CD3-FITC fluorescence
CD3-FITC fluorescence


Fluorescence
green
orange
Fluorescence
green
orange
Emission signal intensity
A
B
A
B
450
500
550
600
450
500
550
600
Wavelength (nm)


dual scatter
Side scatter
Forward scatter
R1
250
200
150
100
50
0
0 50 100 150 200 250
neg
fas
Counts
PE-Fas
180
0
10^0 10^1 10^2 10^3 10^4


CD3
25.0%
37.8%
CD19
9.1%
1.1%
CD4
16.6%
24.4%
CD8
11.4%
PE-Fas
FITC-CD3
FITC-CD19
FITC-CD4
FITC-CD8
10^0 10^1 10^2 10^3 10^4

FACS 關機

- 執行「日常除污」與「日常清洗」時機：
  - 如上機樣品含特殊染劑(如DNA/RNA核酸染料)，需執行「日常除污」與「日常清洗」。
  - 全血樣品如進行Lyse w/o Wash分析(如CD34 Stem Cell)，需執行「日常除污」與「日常清洗」
  - 一般細胞株、或全血樣品進行Lyse-Wash分析，只需執行「日常清洗」。

“日常除污”程序 (FACS Rinse)

- 將樣品支持架左移。
- 取2 ml FACS Rinse 上樣品，讓儀器的真空系統抽取約1 ml 的液體。
- 將樣品支持架回正，執行PRIME 功能兩次，按HI RUN，然後讓FACS Rinse 清洗管路5分鐘。
- 取2 ml Milli-Q 上樣品，重覆上述步驟1-3。

FACS Rinse: 0.5 % Triton X-100 in Milli-Q

“日常清洗”程序 (FACS Clean)

- 將樣品支持架左移。
- 取2 ml FACS Clean 上樣品，讓儀器的真空系統抽取約1 ml 的液體。
- 將樣品支持架回正，執行PRIME 功能兩次，按HI RUN，然後讓FACS Rinse 清洗管路5分鐘。
- 取2 ml Milli-Q ，重覆上述步驟1-3。
- 注意最後只留約1 ml Milli-Q 在試管中。

FACS Clean: 10 % (5 %)漂白水

FACS Calibur 關機

- 按Standby 以冷卻雷射，Standby五分鐘後關閉細胞儀。(務必等五-十分鐘後再關FACSCalibur電源，以延長雷射光源壽命。)
- 倒掉廢液，並回填100 c.c.漂白水。
- 將氣壓閥放在「漏氣」位置。
- 確認退出電腦中BD應用軟體，數據資料已儲存備份。關程式“File”-“Quit”(選擇“Don‘t save”)
- 關閉蘋果電腦。“Special”-“Shutdown”。

FACSCalibur

FACSAria

- Argon-Ion Laser 488nm、He-Ne Laser 633nm，可分析7色螢光
- 多色螢光分析 分選
- 細胞功能性分析
- 稀有細胞分析 分選
- 造血幹細胞分析 分選
- 依據核酸含量分選腫瘤細胞

FACSAria

相關資訊

- 流式實驗指南
- 流式分析技術 (臨床診斷應用、基礎醫學研究、生技製藥應用)
- 螢光染劑與多色流式細胞分析
- 流式細胞應用--細胞存活之檢測
- BD FACSAria Sorting注意事項
- BD FACSAria清洗與滅菌事宜
- Current protocol in cytometry, CP search
- Protocol-online
- Google-Scholar, Scirus

謝謝

謝長奇
http://mail.cmu.edu.tw/~cchsieh/
cchsieh@mail.cmu.edu.tw
TEL: 04-22053366#3508, 3509