实用流式细胞技术及其样品处理
Practical flow cytometry and sample preparation

胡茂志

扬州大学测试中心

二、应用

细胞周期分析(cell cycle)

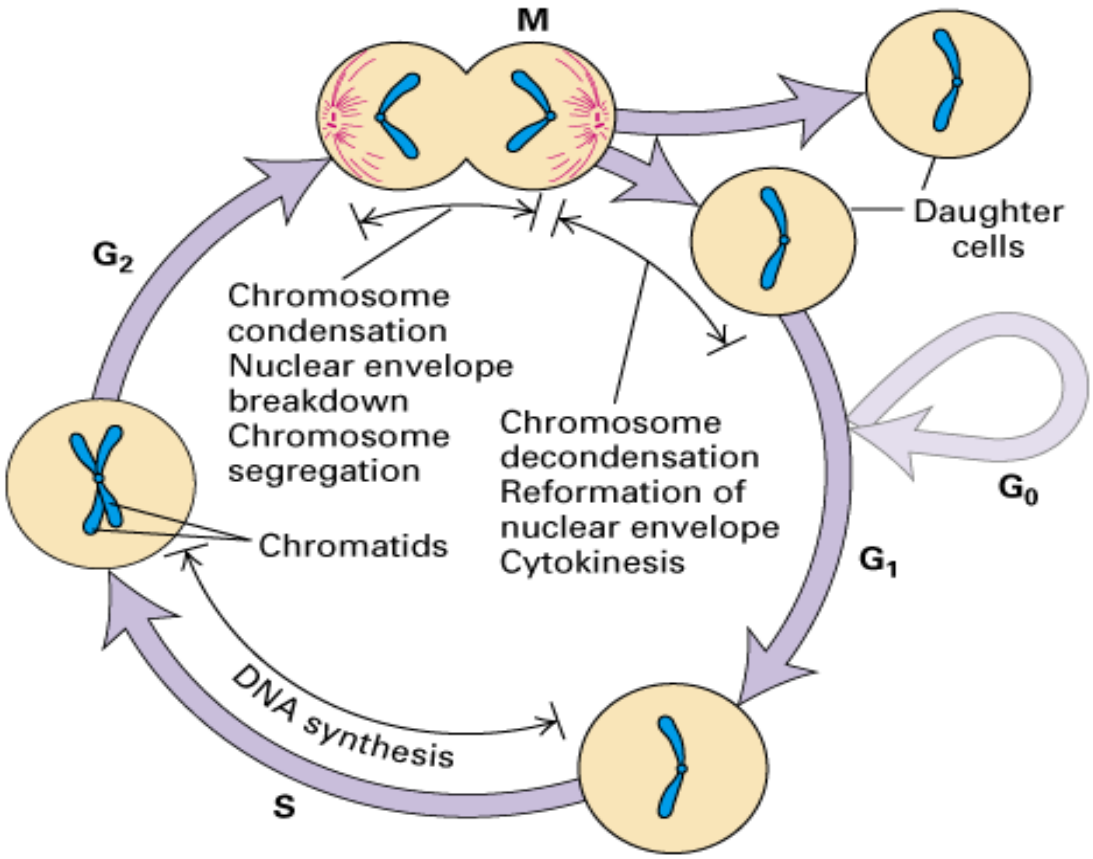
细胞功能分析(cell function)

细胞凋亡分析(apoptosis)

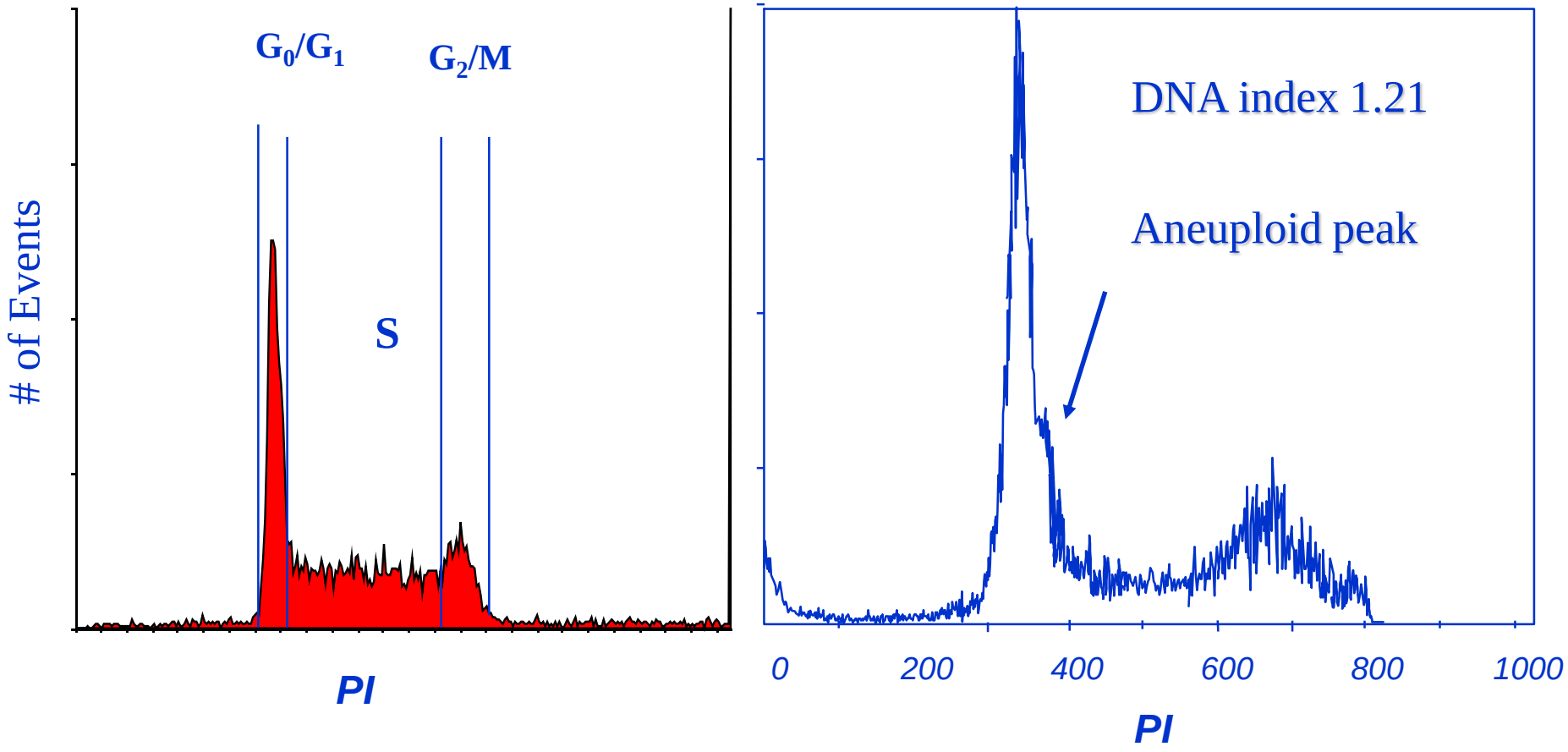
免疫学领域(Immunology)

其他领域(Other)

（一）细胞周期分析

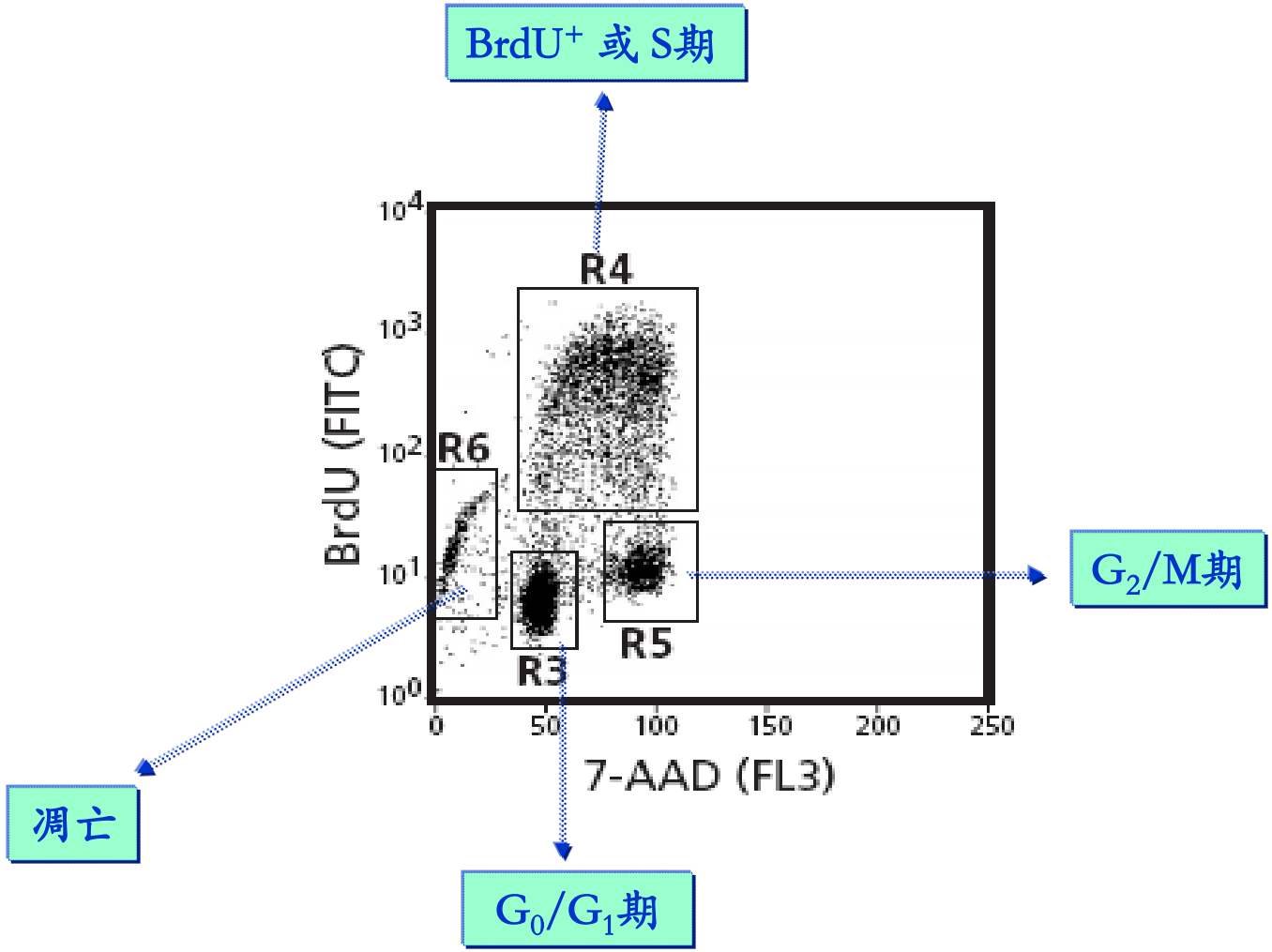

M
Daughter cells
G2
Chromosome condensation
Nuclear envelope breakdown
Chromosome segregation
Chromosome decondensation
Reformation of nuclear envelope
Cytokinesis
G0
G1
Chromatids
DNA synthesis
S

Cellular DNA content

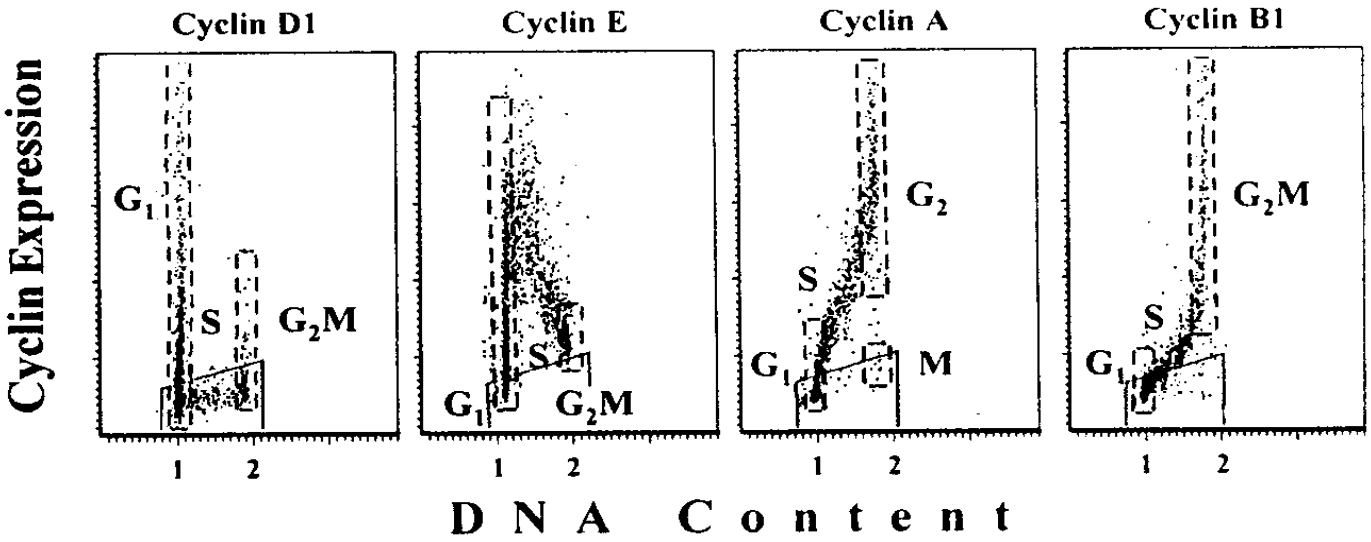


必须用RNase去除RNA

DNA合成---BrdU Incorporation



- 增殖细胞核抗原（Proliferating cell nuclear antigen，PCNA）：参与DNA复制和核酸的切补修复（excision repair ）的一种蛋白
- 增殖相关抗原（proliferation related antigen）：Ki-67，Ki-S1
- 细胞周期蛋白：Cyclin D1, E, A, B1

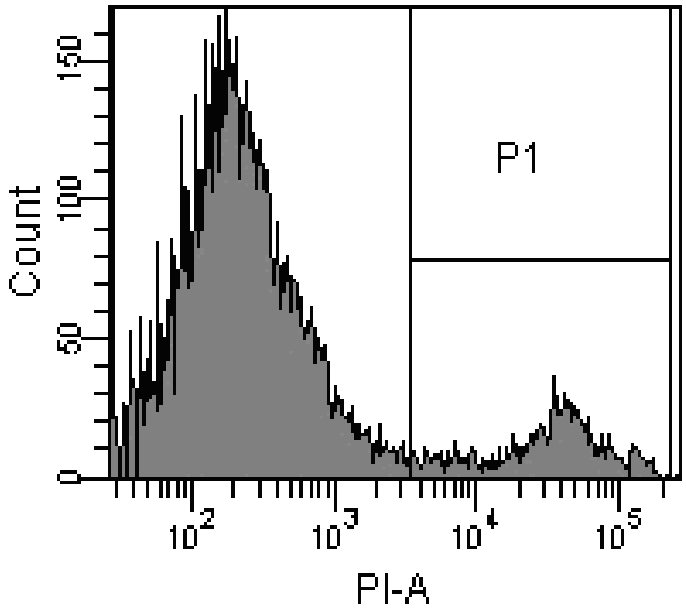


（二）细胞功能分析

- 细胞活性(viability)
- 细胞膜电位(membrane potential)
- 线粒体膜电位(mitochondria membrane potential)、膜蛋白抗原7A6
- 细胞内Ca^{2+}(Intracellular calcium ion)
- 胞浆内pH
- 活性氧（Reactive Oxygen Species）

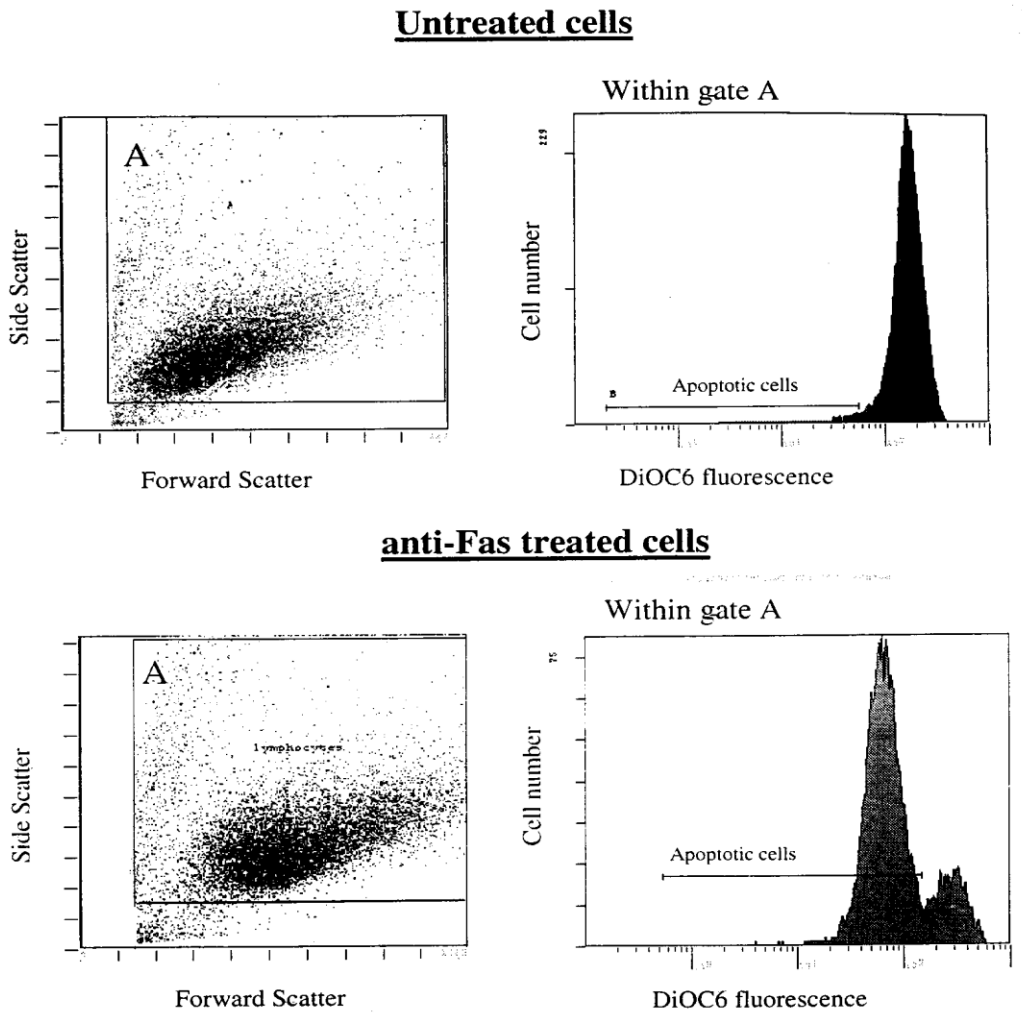
1、细胞活性分析

根据对细胞膜的渗透性不同来区分活/死/凋亡细胞： PI，EB，7-AAD and Hoechst 33342



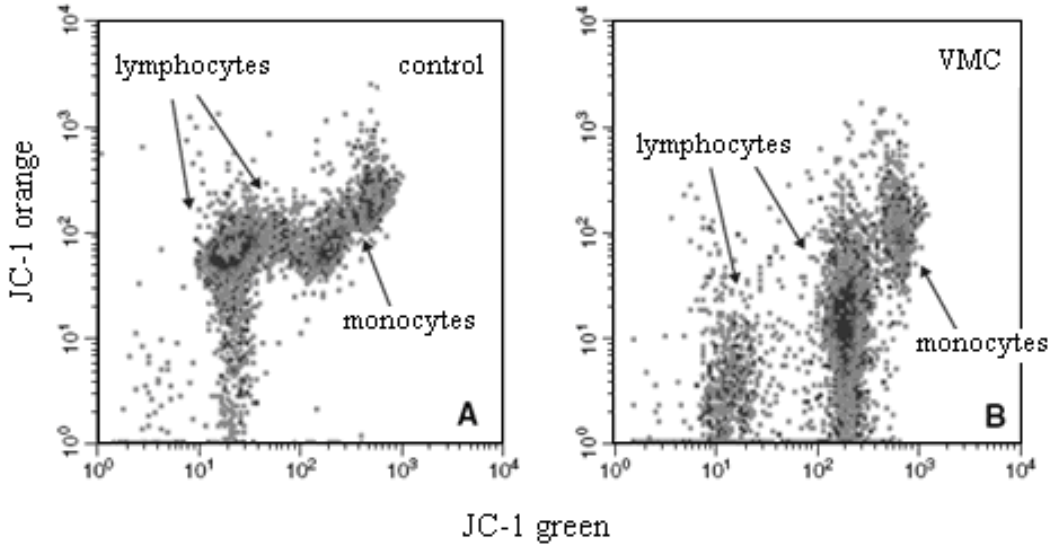
2、细胞膜电位

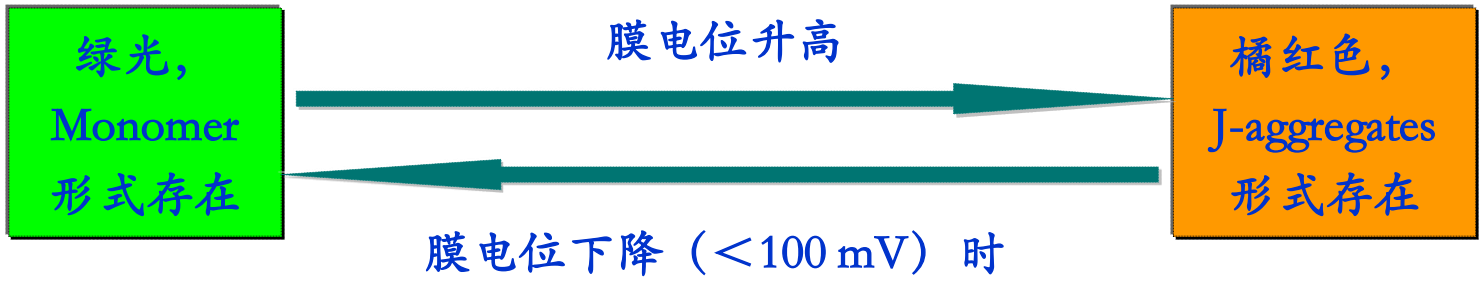
由于细胞膜上各种泵的作用，如钠钾泵、钙泵等，使细胞膜内外维持不同离子的浓度梯度，造成细胞膜电位



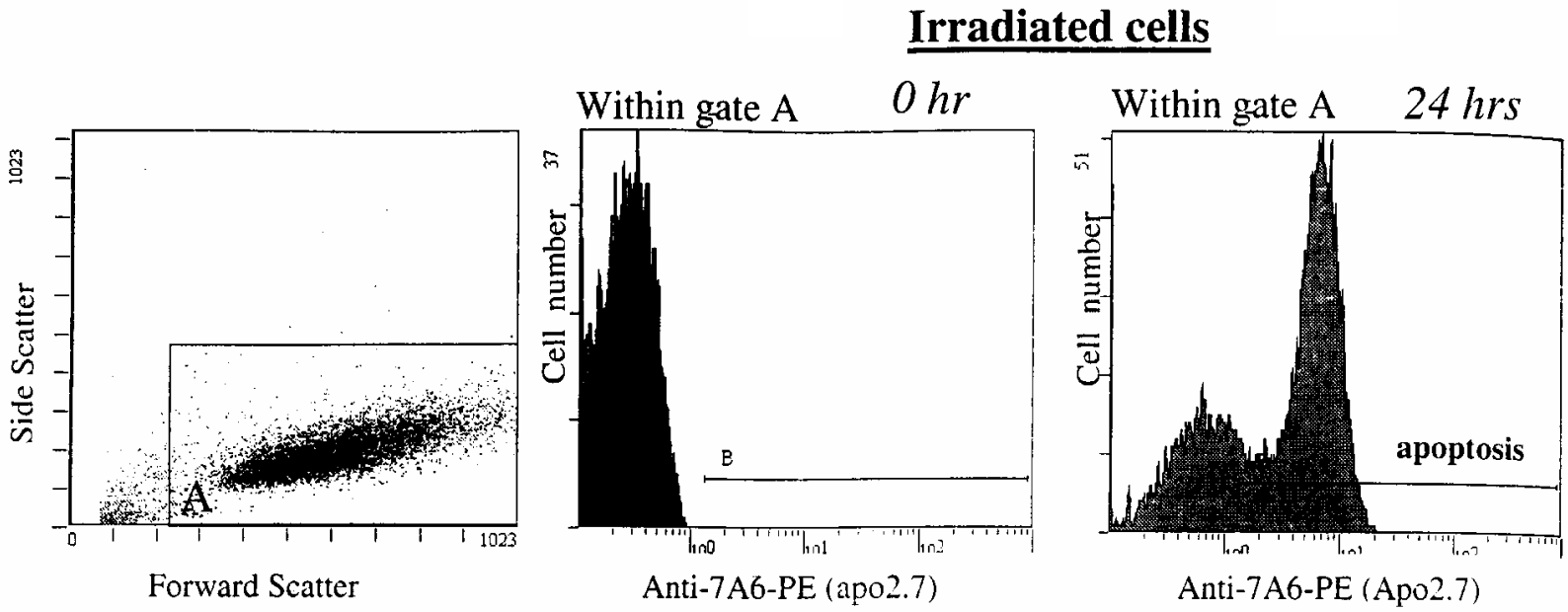
3、线粒体膜电位（MMP）

线粒体在呼吸氧化过程中，将所产生的能量以电化学位能储存于线粒体内膜。JC-1可进入细胞内，特异地与线粒体内膜结合，其聚集程度随MMP升高而增加。





4、线粒体膜蛋白抗原7A6 （38kDa ）



凋亡早期的反应，早于形态学、光学特征变化及台盼蓝染色

5、细胞内Ca^{2+}

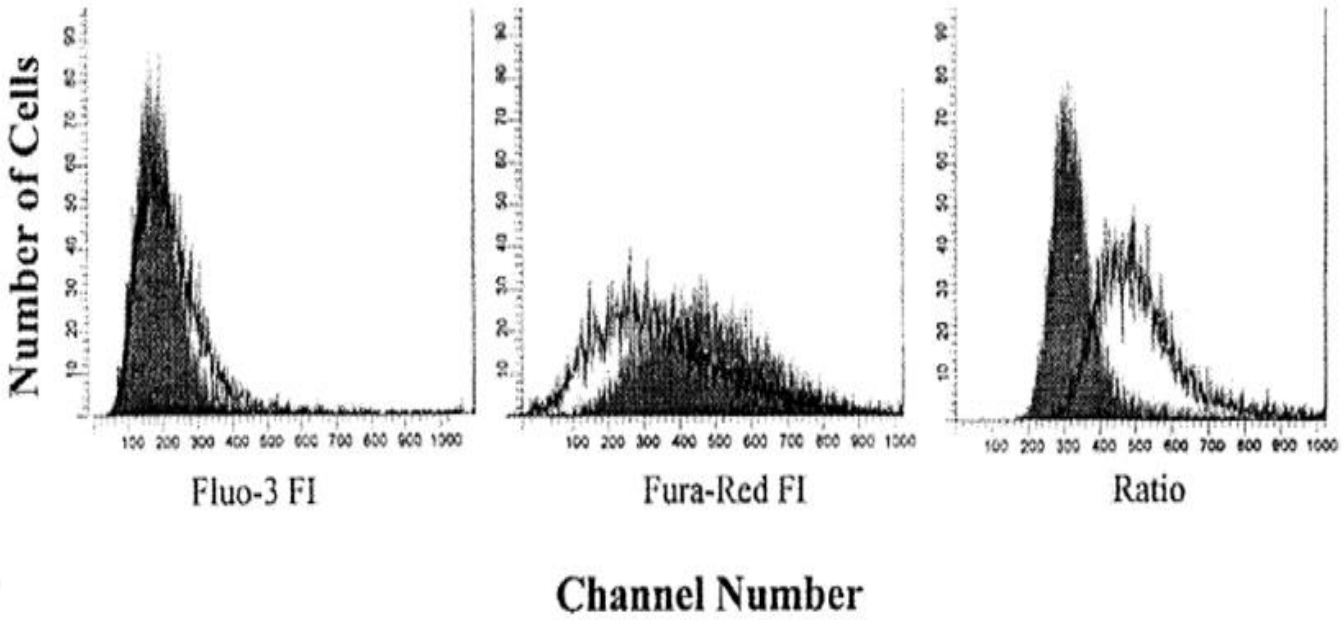
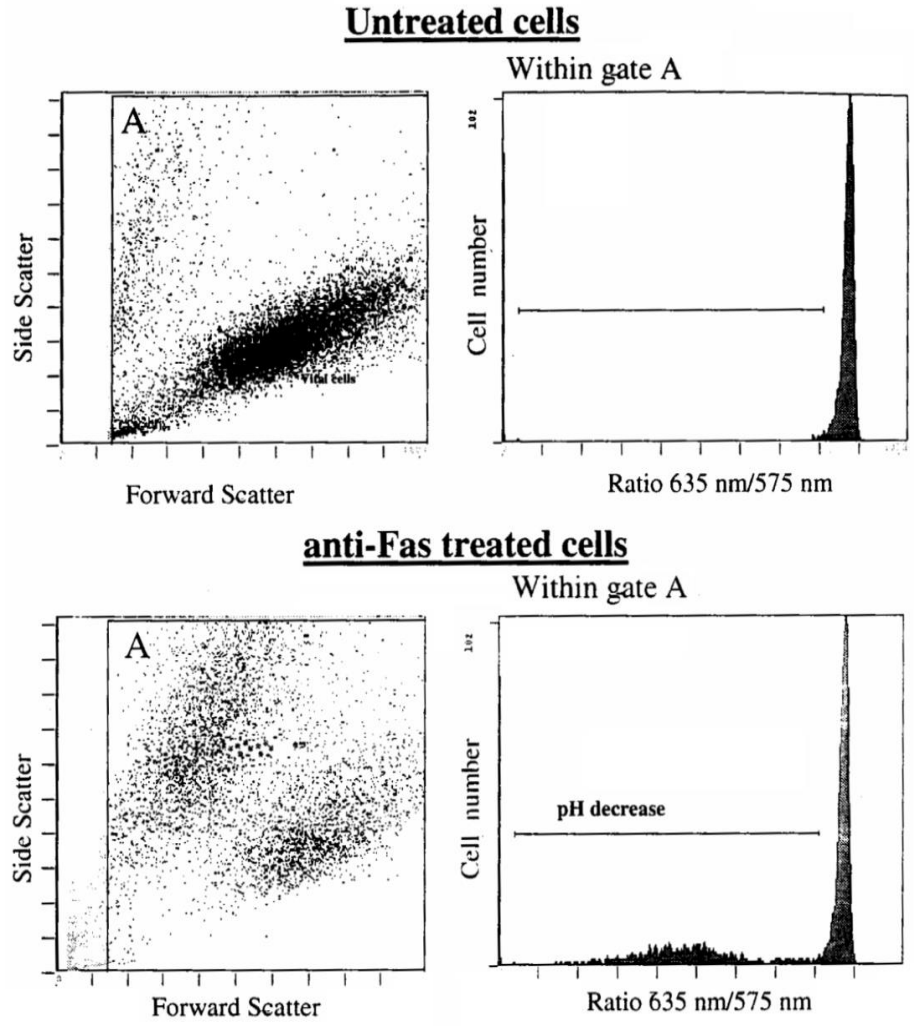


FIG. 1. Flow cytometric detection of free intracellular Ca^{2+} using calcium-chelating fluorescent probes. Representative histograms demonstrating the changes in Fluo-3 and Fura-Red fluorescence intensity (FI) and the Fluo-3/Fura-Red fluorescence ratio induced by BaP-7,8-diol (black line) compared with DMSO control (gray fill) following an 18-h incubation in Daudi human B cells, as described in the text. FI data are expressed as channel number using a linear scale. Reproduced, with permission, from Mounho and Burchiel (17).

浓度上升，与两种情况有关：(1)细胞活化，(2)细胞膜完整性遭到破坏，可作为细胞凋亡或坏死的标志。

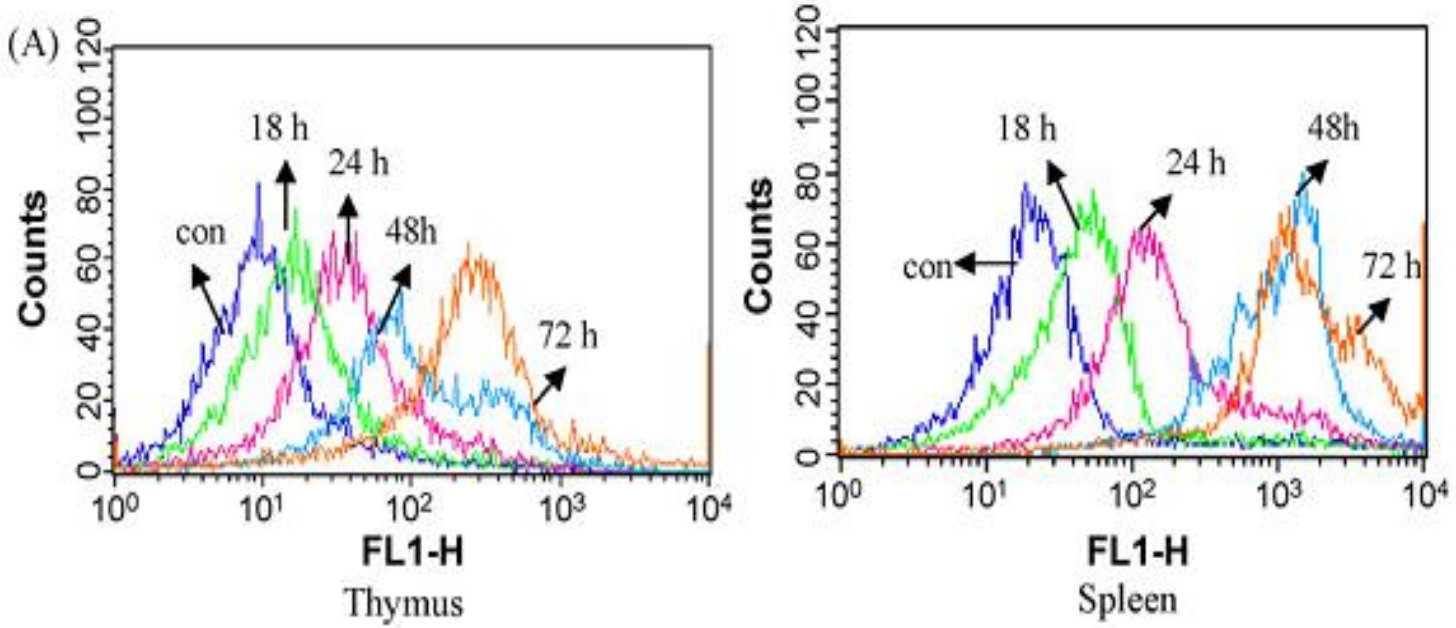
6、胞浆内pH

- 活细胞内pH变化范围较小。
- 某些生物学过程(如细胞分裂及对细胞信号的应答)会出现pHi的变化。
- 增殖细胞比静止期细胞偏碱性。



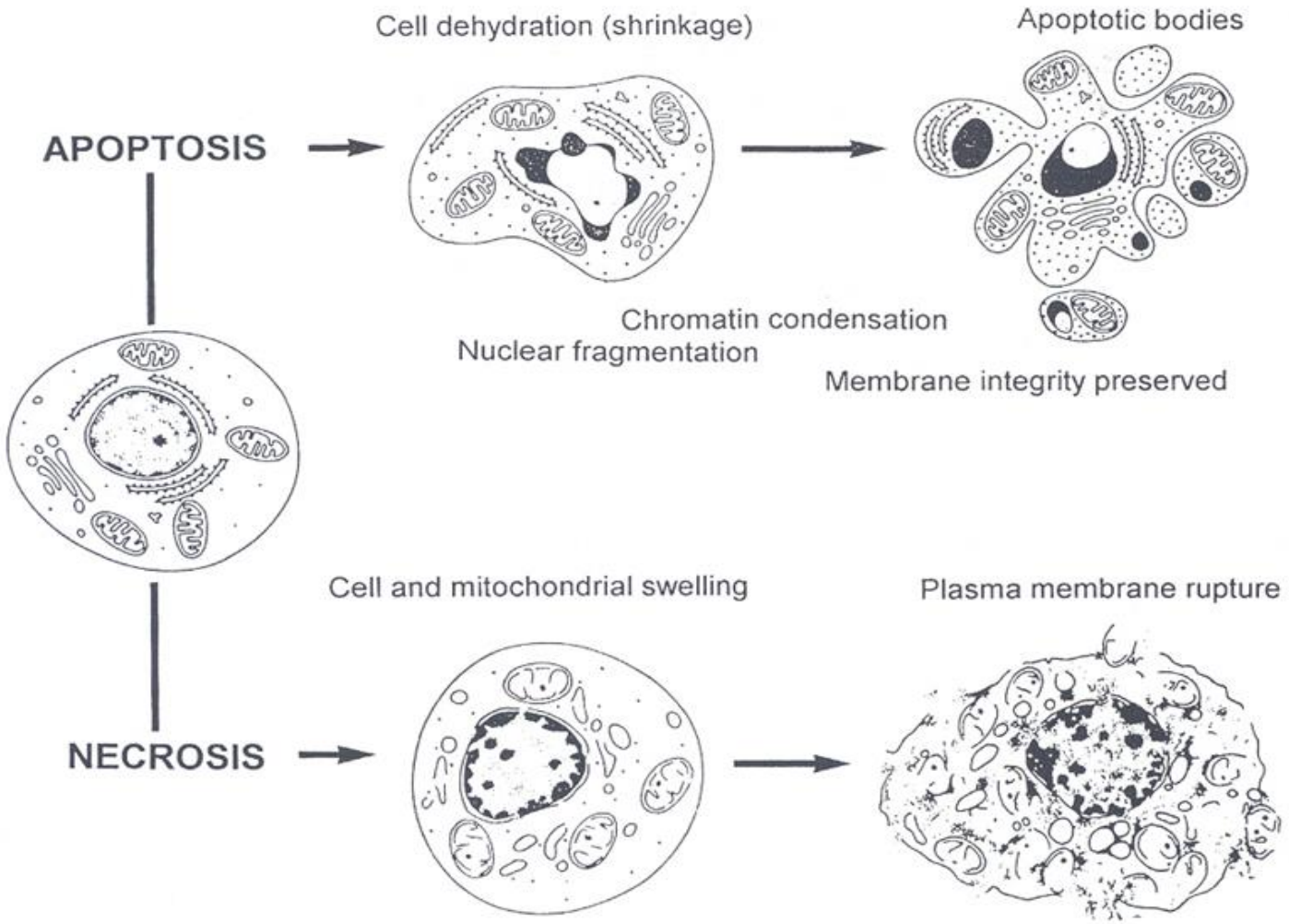
7、活性氧（ROS）

参与细胞生长、发育、衰老和凋亡以及许多生理和病理过程。

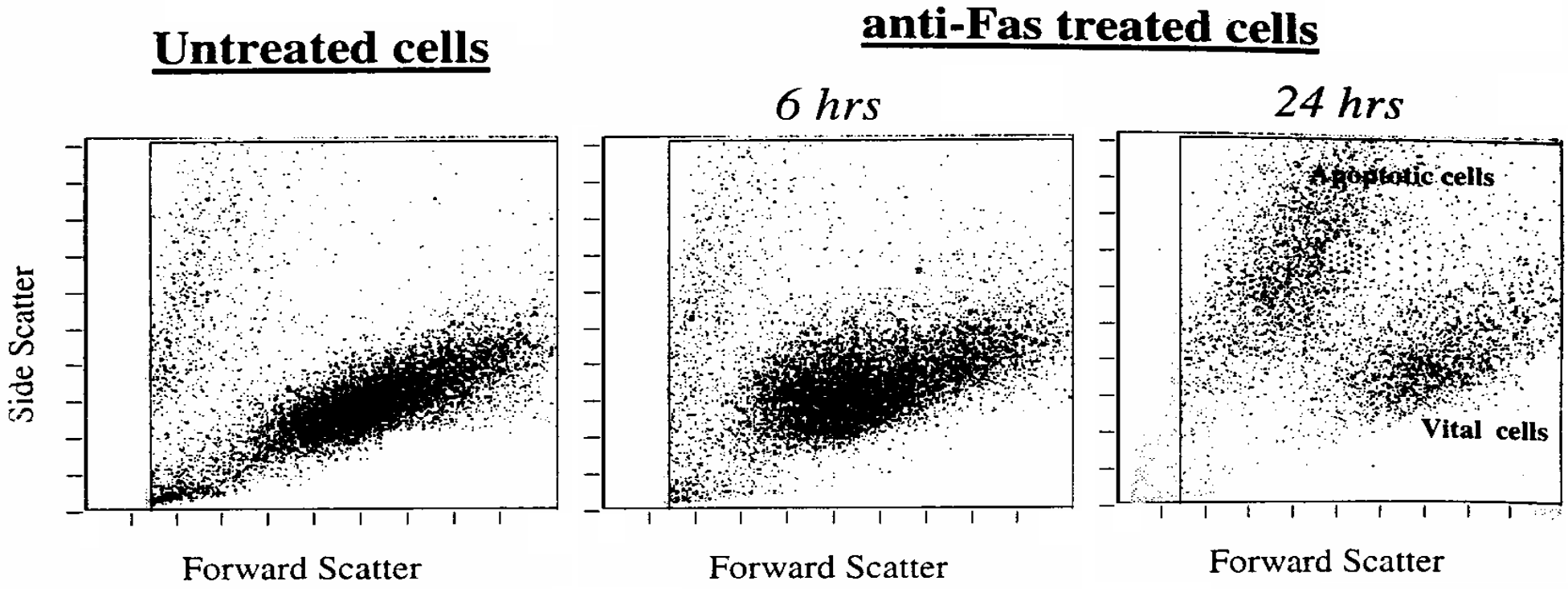


DCFH-DA本身没有荧光，在细胞内转化成DCFH。在ROS作用下，氧化为绿色荧光物质DCF。其荧光强度与细胞内ROS水平成正比。

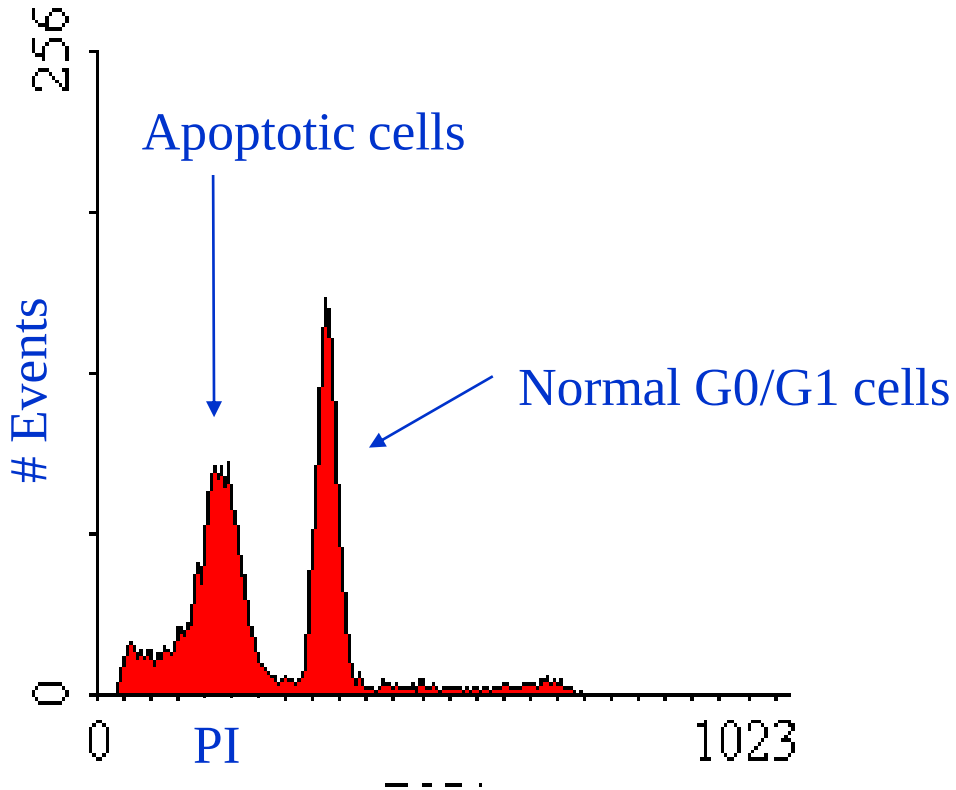
（三）细胞凋亡分析


Cell dehydration (shrinkage)
Apoptotic bodies
APOPTOSIS
Chromatin condensation
Nuclear fragmentation
Membrane integrity preserved
Cell and mitochondrial swelling
Plasma membrane rupture
NECROSIS

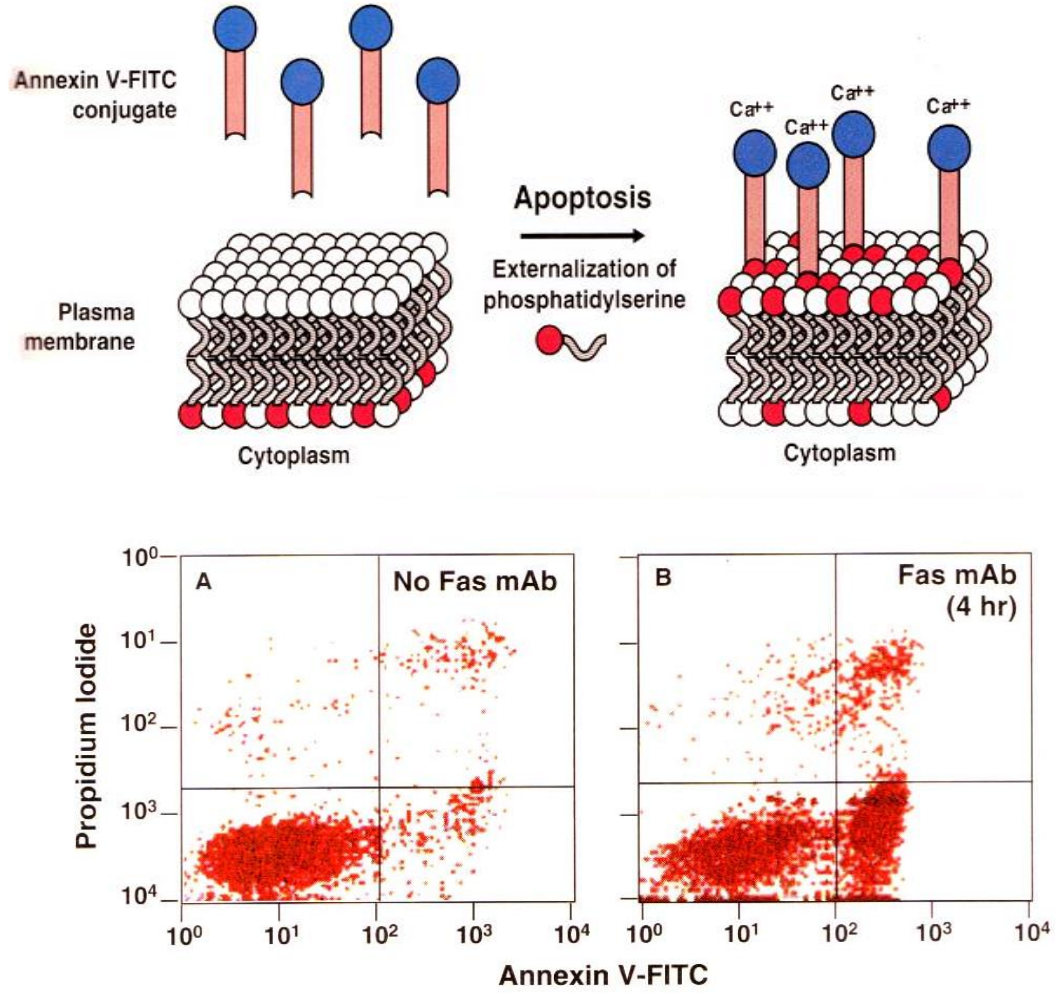
1、散射光信号的变化


Untreated cells
anti-Fas treated cells
6 hrs
24 hrs
Side Scatter
Apoptotic cells
Vital cells
Forward Scatter
Forward Scatter
Forward Scatter

2. DNA含量



3、磷脂酰丝氨酸外翻（Phospholipid redistribution）



4、Caspases-3的激活

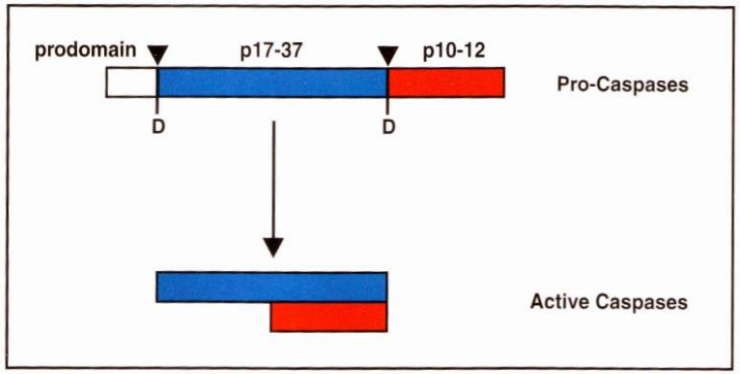


Figure 2. Activation of Caspases. Caspases are synthesized as inactive proenzymes that are processed in cells undergoing apoptosis by self-proteolysis and/or cleavage by another protease. The processed forms consist of large (17-37 kD) and small (10-12 kD) subunits which associate to form an active enzyme.

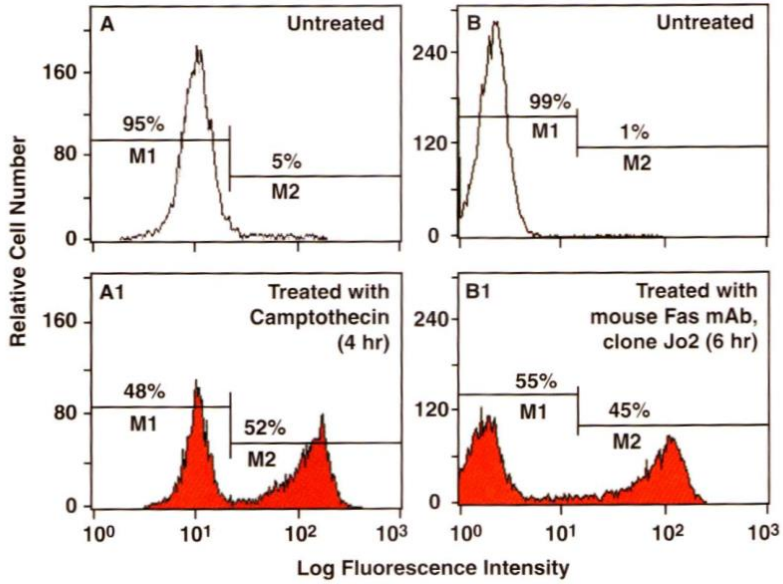


Figure 5. Flow Cytometric Analysis of Apoptotic and Non-Apoptotic Populations Using Anti-Active Caspase-3 Antibodies. Jurkat T cells (A, A1) or mouse thymocytes (B, B1) were left untreated (A, B) or treated for 4 hr with camptothecin (A1) or a mouse Fas monoclonal antibody, clone Jo2 (Cat. No. 15400D) (B1) to induce apoptosis. Cells were stained with PE-conjugated active caspase-3 antibodies (Cat. No. 67345X). Untreated cells were primarily negative for the presence of active-caspase-3, whereas about half of each population of cells induced to undergo apoptosis had detectable active caspase-3.

5. TUNEL

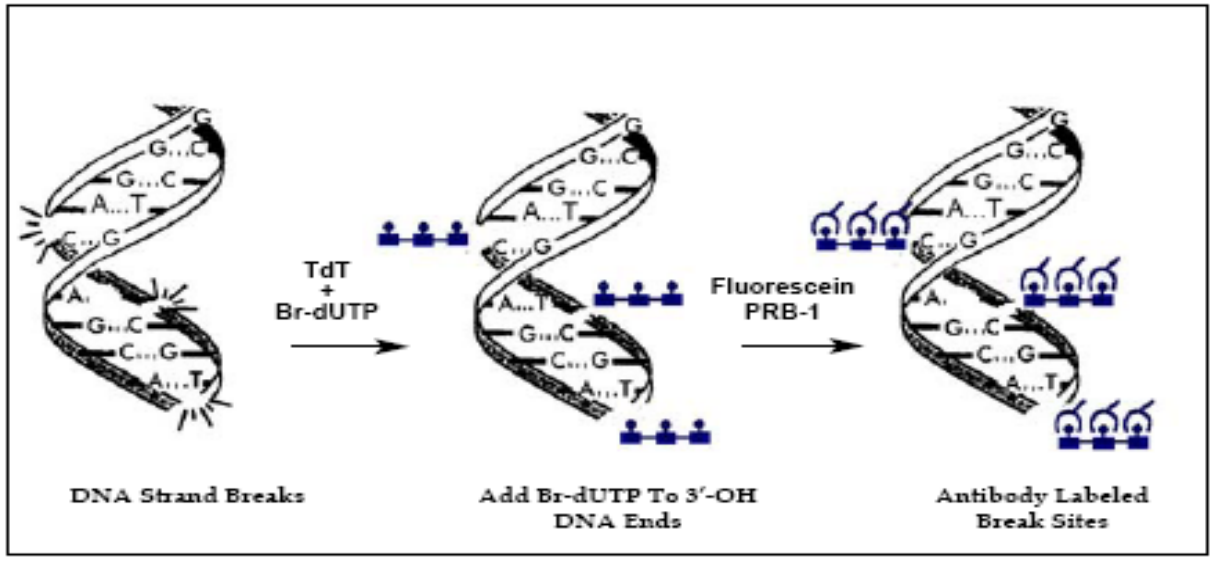


Figure 1: Diagrammatic representation of the addition of bromodeoxyuridine triphosphate (Br-dUTP) catalyzed by terminal deoxynucleotidyl transferase (TdT) to the 3′-OH sites of DNA strand breaks induced in the genome of apoptotic cells.

- 凋亡晚期，核酸内切酶活化，双链DNA会出现许多不对称断点，产生一系列的3’末端。TdT酶能够催化外源性荧光素-dUTP连接到DNA的3’末端。
- 坏死细胞的细胞核DNA 也降解，末端也增多。但这种随机降解所产生的末端较难与荧光素-dUTP结合。因而，坏死的细胞观察不到荧光。

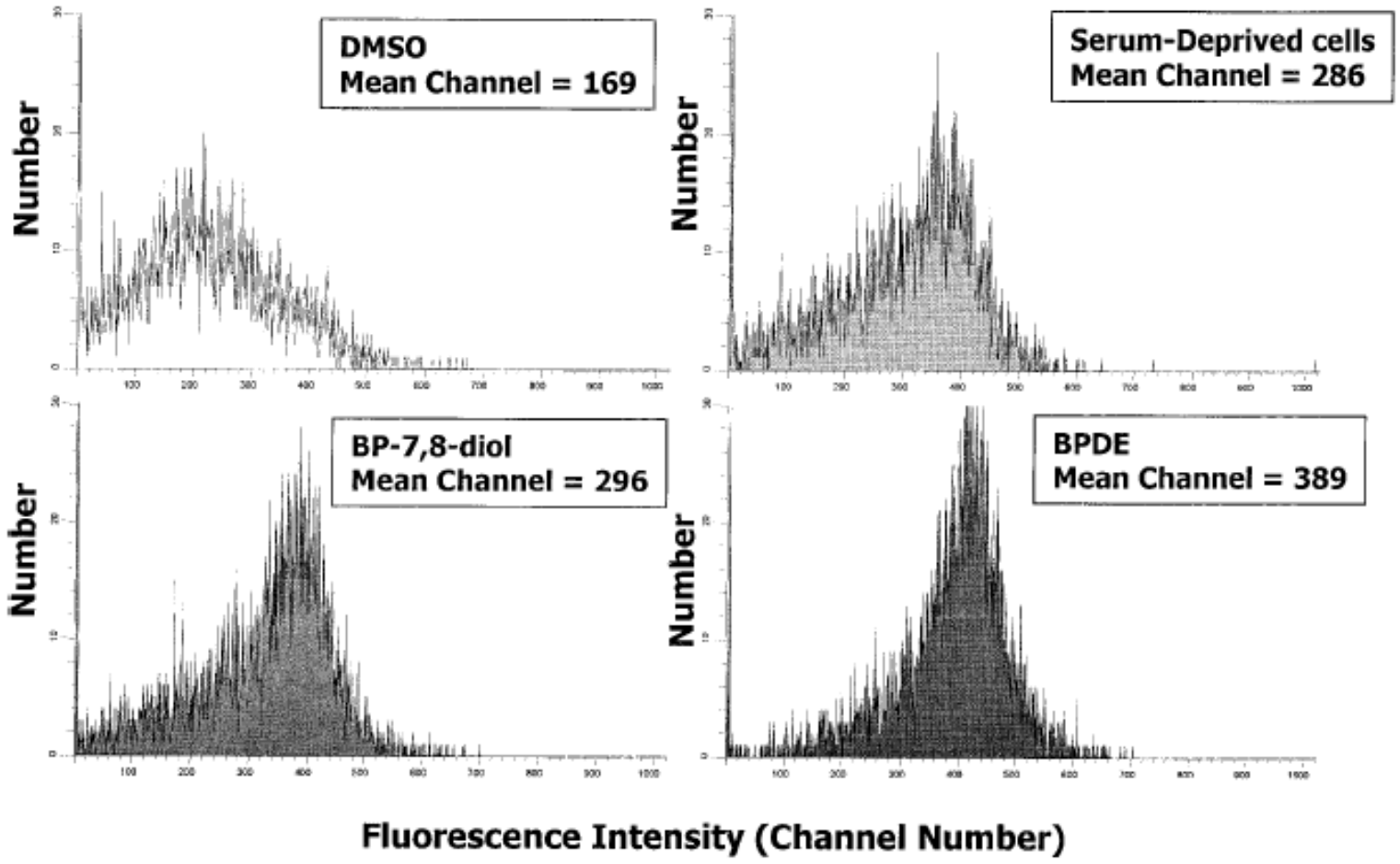


FIG. 3. Flow cytometric assay for detecting DNA strand breaks in human lymphoid cells. Representative TUNEL assay histograms obtained for Daudi human B cells demonstrating the change in FITC fluorescence induced by 3 μM BaP-7,8-dihydrodiol (lower left) and BPDE (lower right) compared with vehicle control (upper left) and a serum-deprived positive control (upper right), as described in the text. FITC fluorescence intensity data are expressed as channel number using a linear scale.

6. p53

53 kDa的核内磷蛋白。细胞周期中，在DNA损伤无法修复时启动细胞的“自杀”过程。P53突变或缺失是多种肿瘤发生的重要原因。

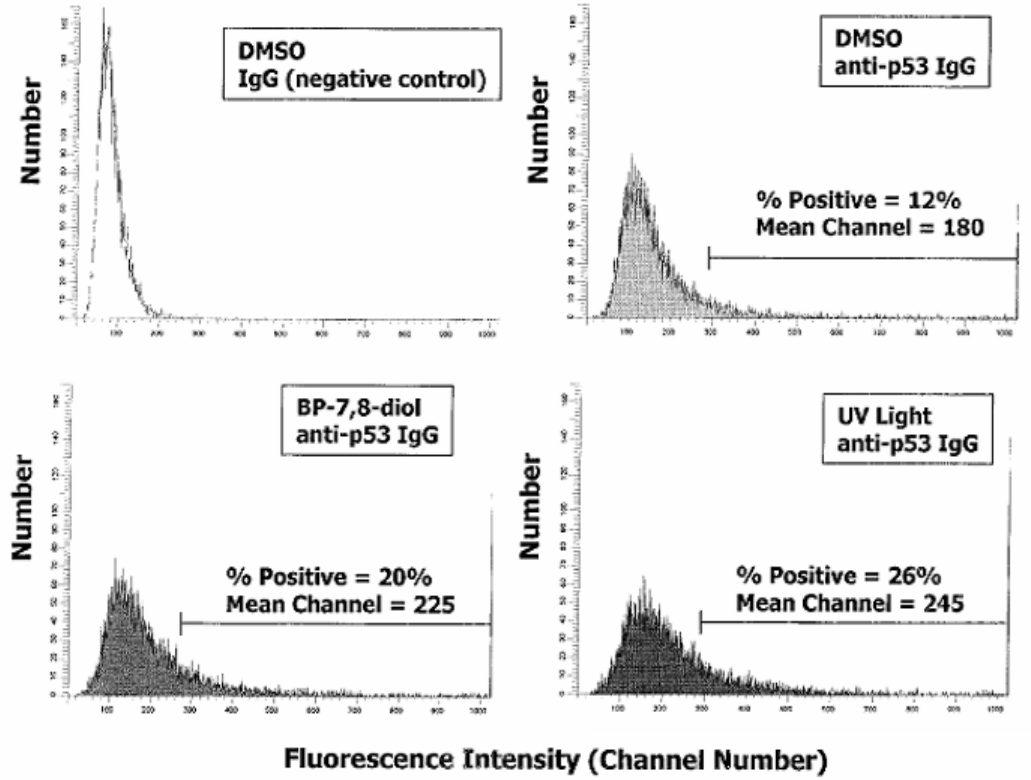
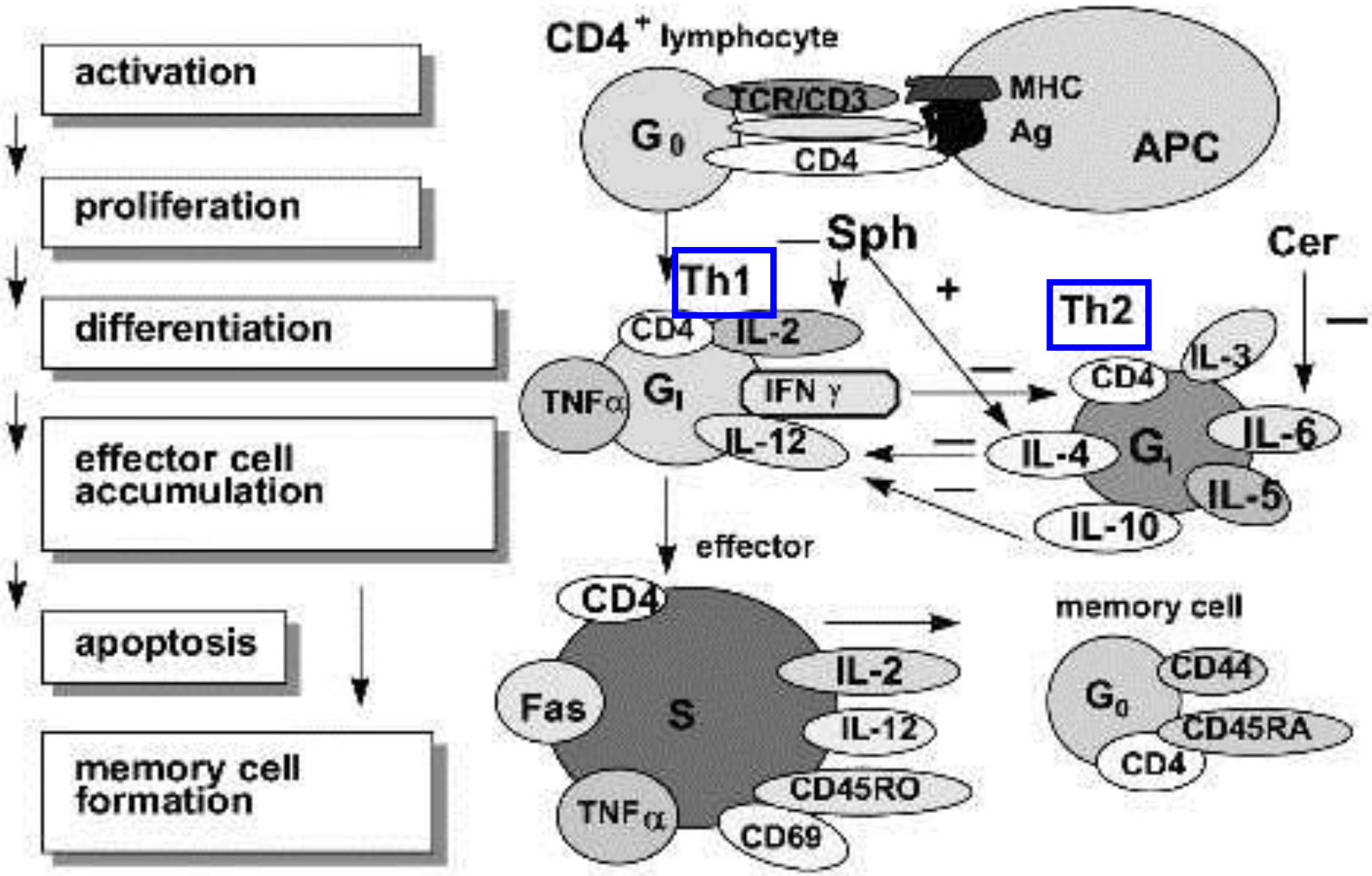
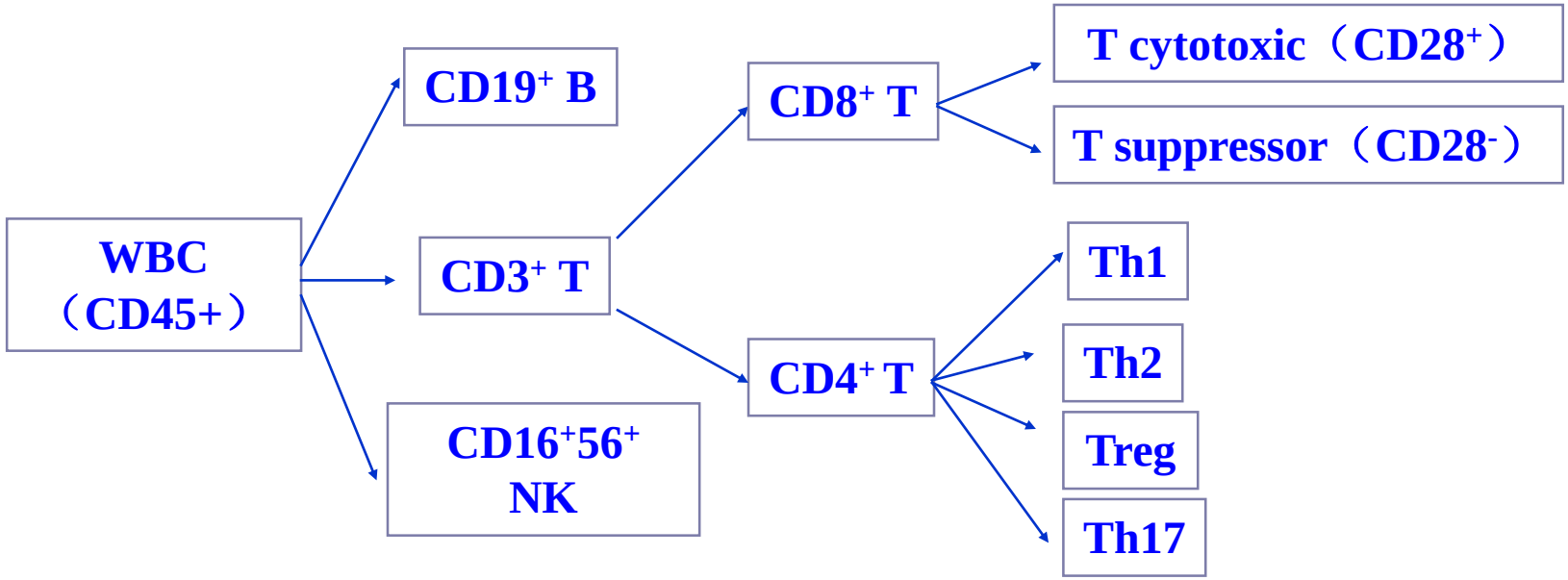


FIG. 2. Flow cytometric detection of p53 in human lymphoid cells. Representative histograms demonstrating the shift in FITC fluorescence compared with vehicle control (upper right) for anti-p53 antibodies in Daudi human B cells compared with IgG control following treatment with ultraviolet (UV) light (positive control) compared with an 18-h exposure to BaP-7,8-dihydrodiol. FI data are expressed as channel number using a linear scale, and the percentage of positive cells and the mean channel for these cells are shown in the gated regions.

（四）在免疫学领域的应用


activation
proliferation
differentiation
effector cell accumulation
apoptosis
memory cell formation
CD4+ lymphocyte
TCR/CD3
MHC
Ag
APC
CD4
G0
Sph
Cer
Th1
Th2
+
—
CD4
IL-2
TNFα
G1
IFN γ
IL-12
IL-3
IL-4
IL-6
IL-5
IL-10
effector
memory cell
Fas
S
CD44
CD45RA
CD45RO
CD69

1、淋巴细胞亚群分析



- NK细胞中有$CD8^+$细胞;
- 少量的单核细胞也能表达CD4;
- 结合淋巴细胞绝对数来看待某一亚群百分比的改变。

评价机体的免疫状态：自身免疫、免疫缺陷等

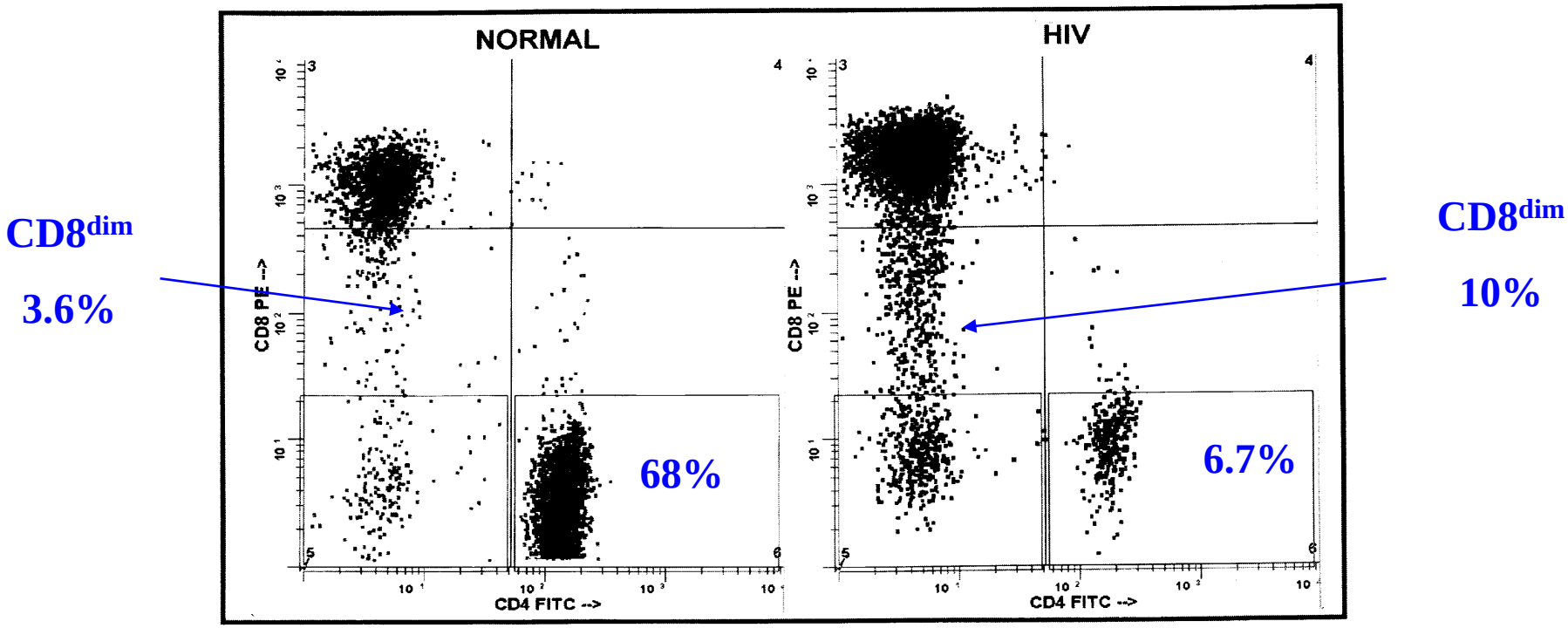


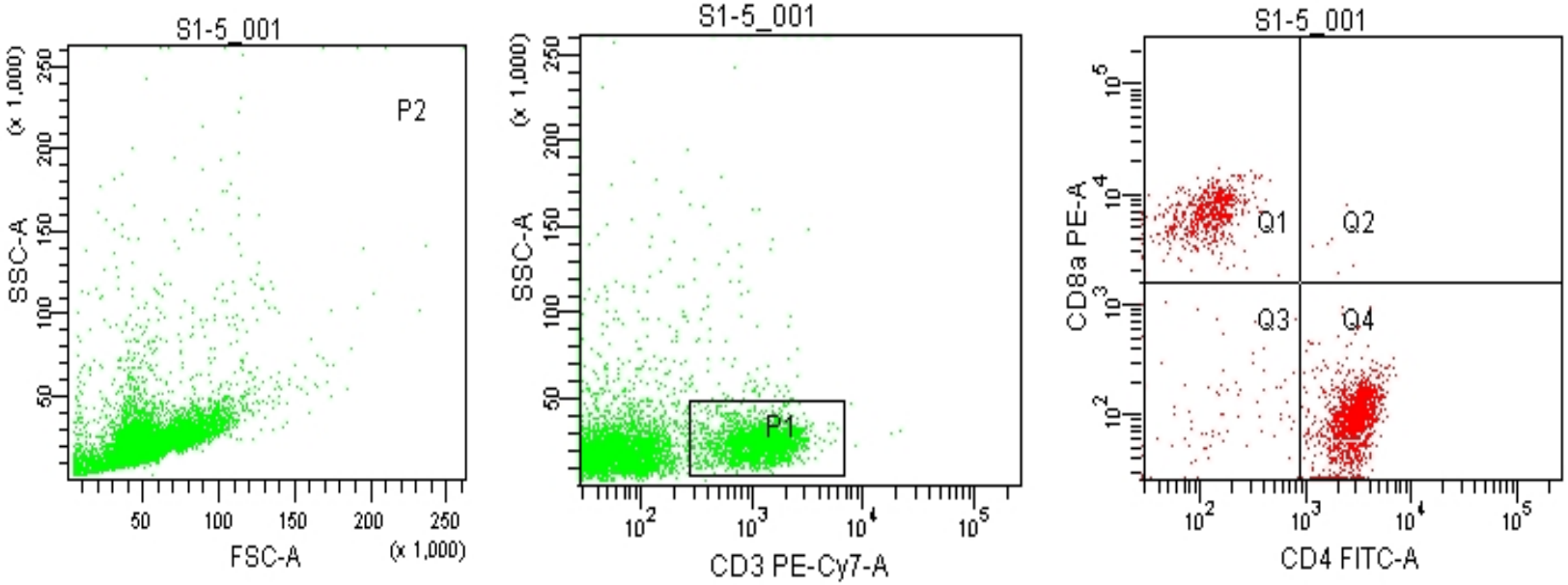
Figure 4
Bivariate scatter plots of peripheral blood leukocytes from healthy control (left panel) and HIV-1–infected (right panel) subjects. Cells were stained with antibodies to CD4 (FITC conjugated), CD8 (PE conjugated), and CD3 (PerCP conjugated). Events were gated on $CD3^+$ and lymphocyte scatter (not shown). This gating strategy confines the analysis to T lymphocytes. For analysis, the scatter plot was divided into six nonoverlapping regions corresponding to two categories for CD4 (negative and positive) and three categories for CD8 (negative, dim, and bright). T cells from the control subject were predominantly $CD4^+$ (68%). $CD8^{dim}$ cells and double negative cells comprised minor populations (3.6% and 3.4%, respectively). In the HIV-1–infected subject, $CD4^+$ cells accounted for only 6.7% of the total T cells. $CD8^{dim}$ cells and double negative cells were more prominent (10%, and 8.9%, respectively).

Naïve / Memory T lymphocyte

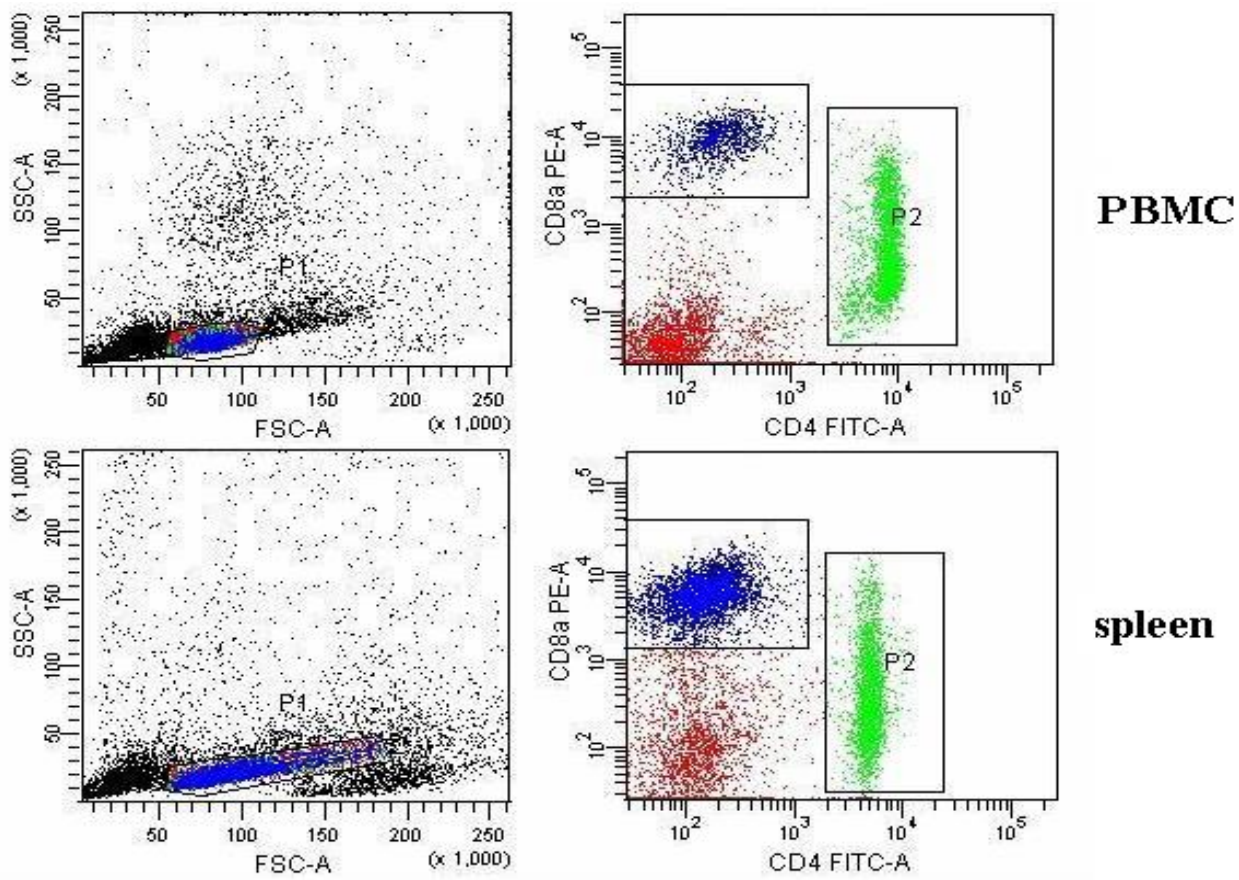
- 刚从胸腺移出到外周循环的Naive细胞，首次遇到抗原时，增殖反应强，产生细胞因子能力弱。
- 当Memory细胞遇到同类抗原时，产生大量的细胞因子，而不是增殖反应。
- Naive T细胞高表达CD45RA^{hi}、CD62L^{hi}。
- Memory T细胞表达CD45RO，一般不表达CD45RA
- 从Naive向Memory细胞转化过程中，CD11a表达增加、CD62L（L-selectin）表达丢失。

Th1/Th2 lymphocyte

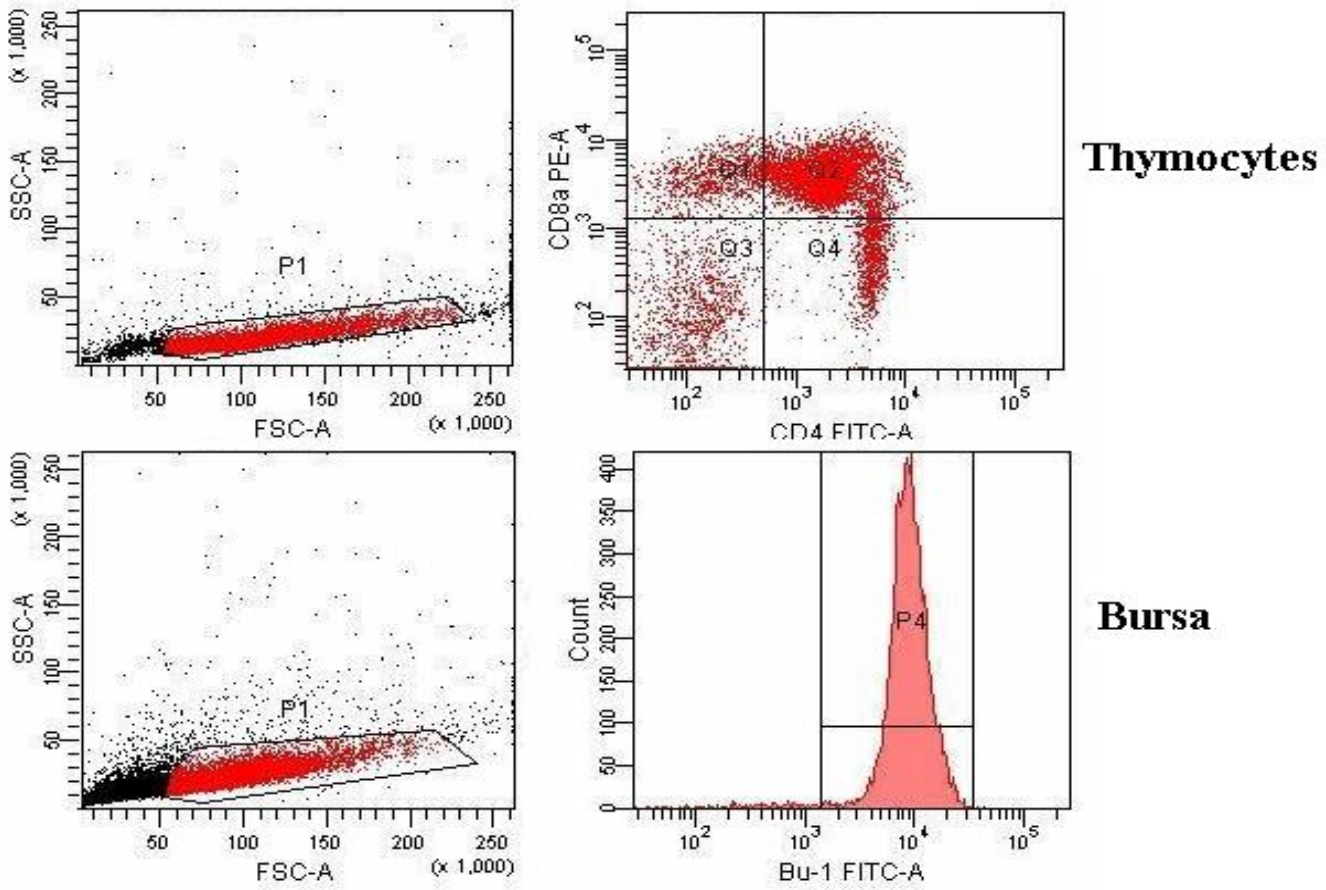
- T细胞根据其分泌的细胞因子分为两型。
- Th1类细胞分泌IL-2 ，IFN-γ和TNF-α，参与细胞免疫，对抗胞内病原体，如病毒；
- Th2类细胞分泌IL-4、IL-5、IL-10与IL-13，参与体液免疫，对抗胞外感染，如寄生虫。


S1-5_001
P2
SSC-A
(x 1,000)
FSC-A
(x 1,000)
S1-5_001
P1
SSC-A
(x 1,000)
CD3 PE-Cy7-A
S1-5_001
Q1
Q2
Q3
Q4
CD8a PE-A
CD4 FITC-A

Mouse

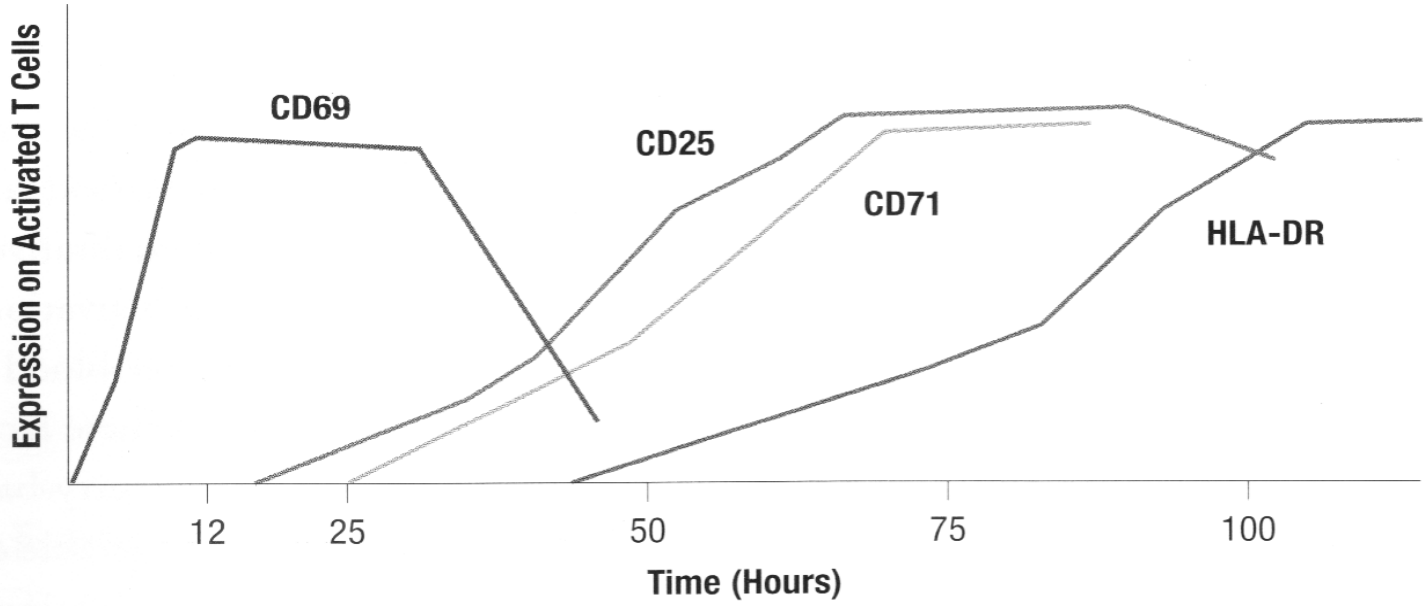


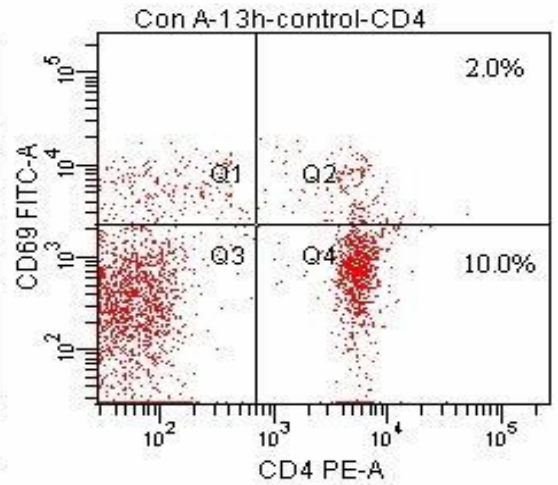
chicken （42d ）

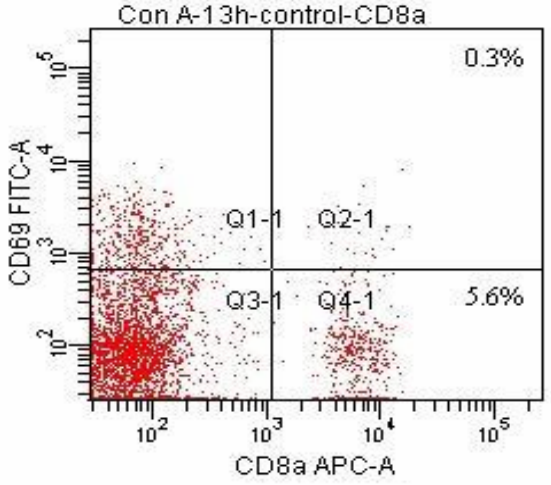

SSC-A
(x 1,000)
P1
FSC-A
(x 1,000)
CD8a PE-A
Q1
Q2
Q3
Q4
CD4 FITC-A
Thymocytes
SSC-A
(x 1,000)
P1
FSC-A
(x 1,000)
Count
P4
Bu-1 FITC-A
Bursa

chicken （42d ）

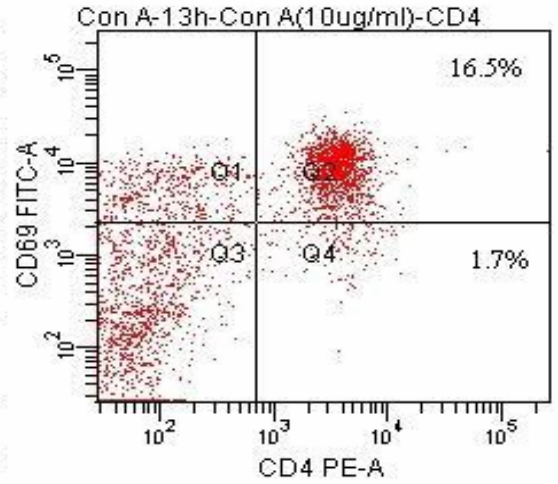
2、细胞表面活化抗原的表达

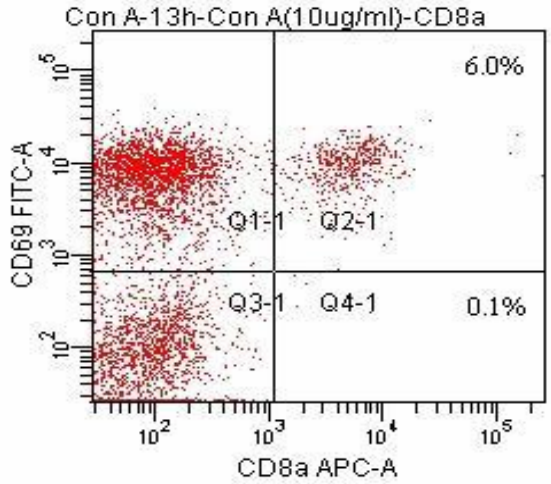






control

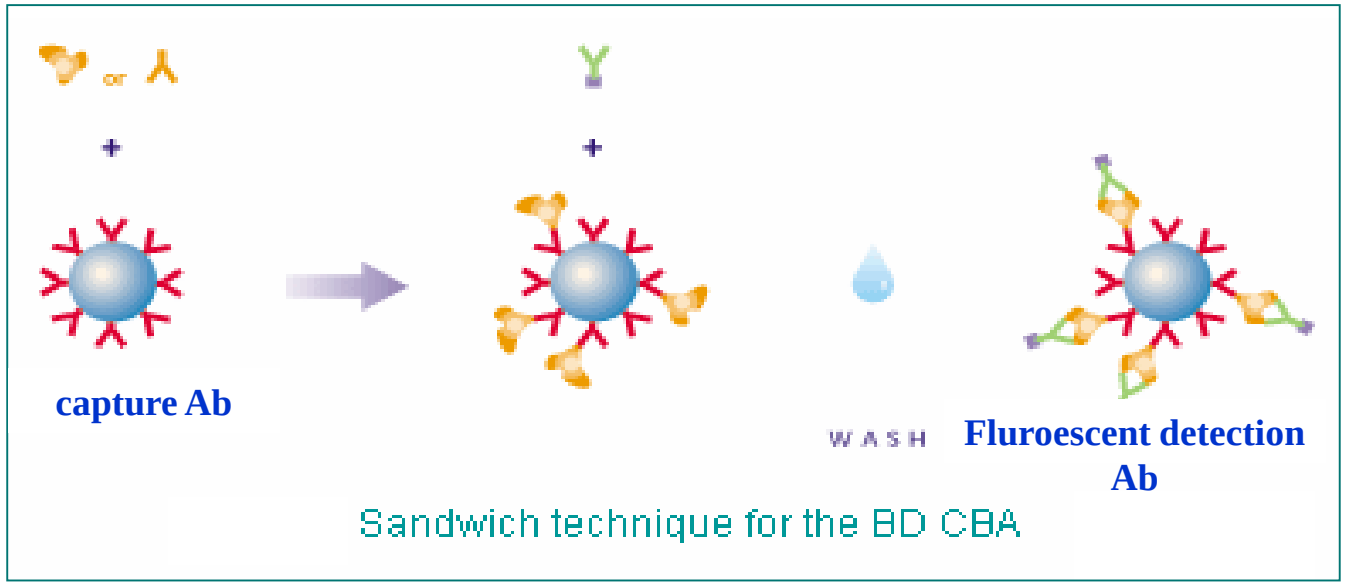




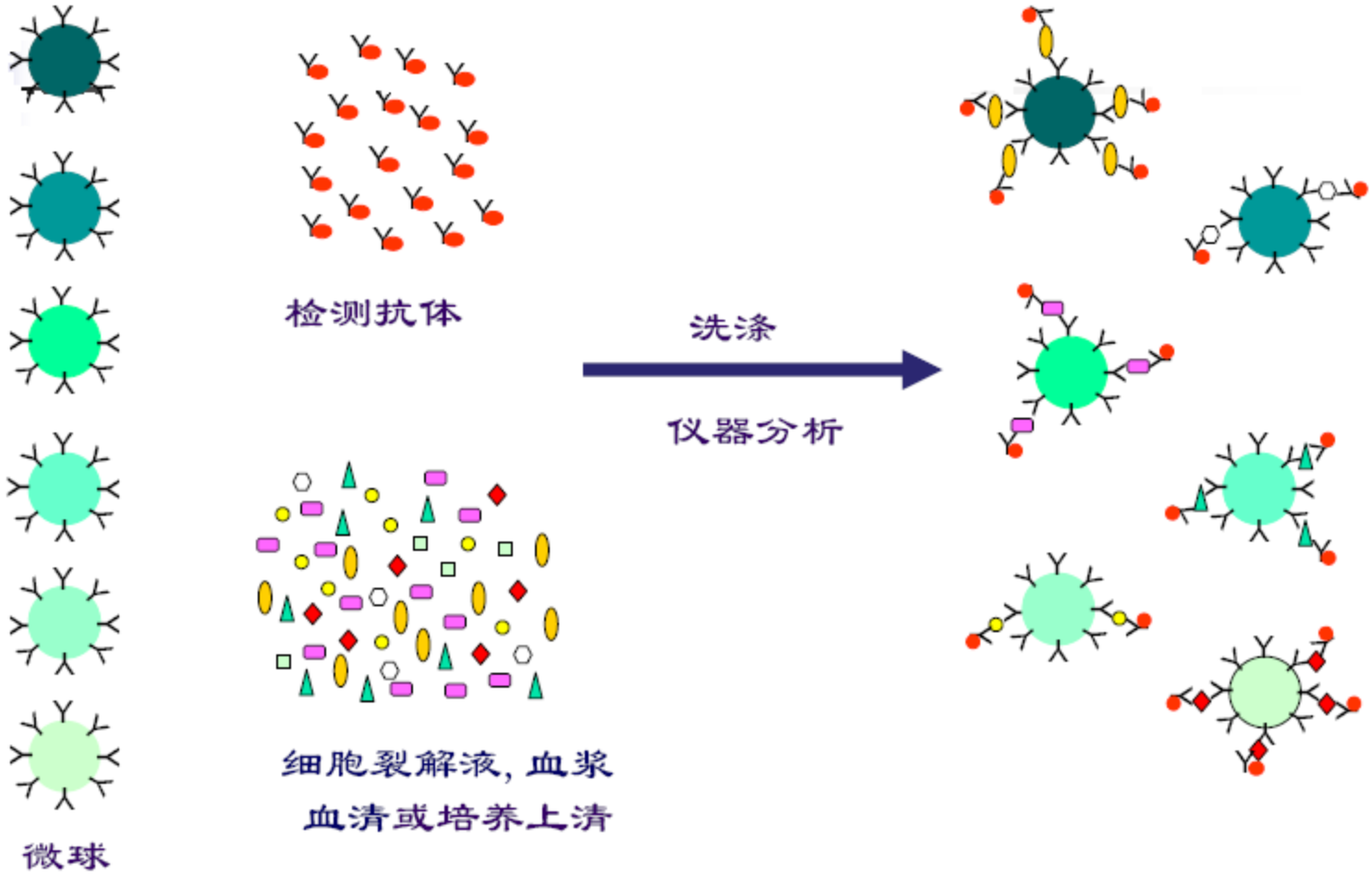
Con A

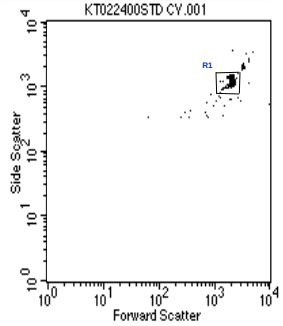
3、细胞因子分析

CBA （Cytometric Beads Assay）



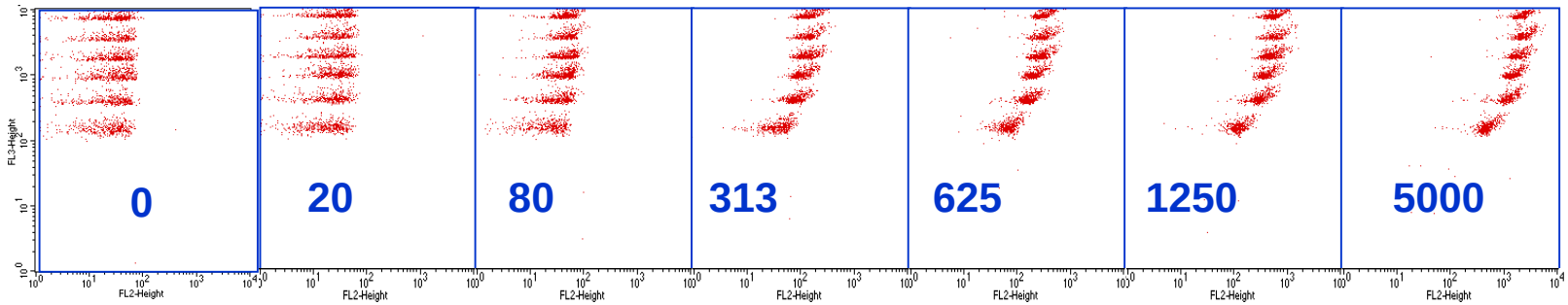
- 用一系列荧光强度不同的微球同时分析样本中多种可溶性成分。
- 每种微球大小一致，分别包被有适合于特定分析的特异性抗体。
- 所有分析只需一组标准曲线，检测的线性范围宽（**0--5000 pg/ml**）


检测抗体
洗涤
仪器分析
细胞裂解液，血浆
血清或培养上清
微球

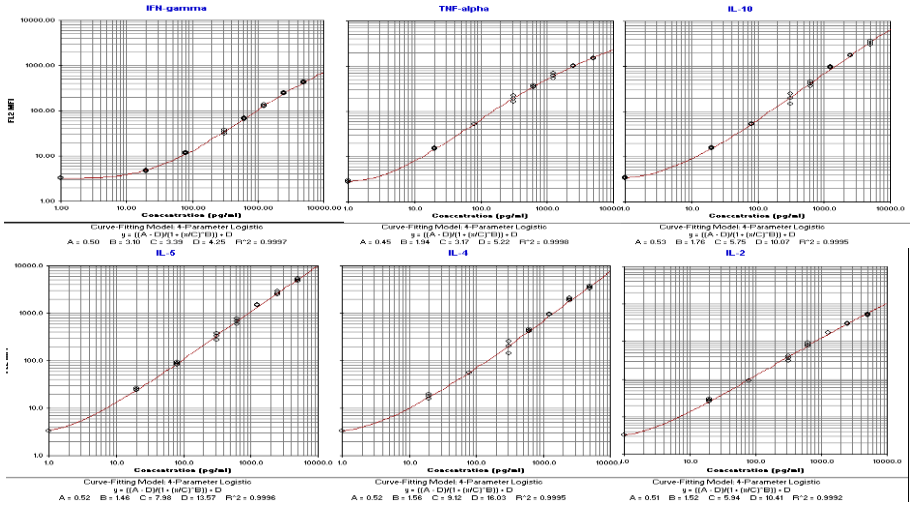


Scatter gate on singlets

Index Beads in FL3 - Read signals in FL2

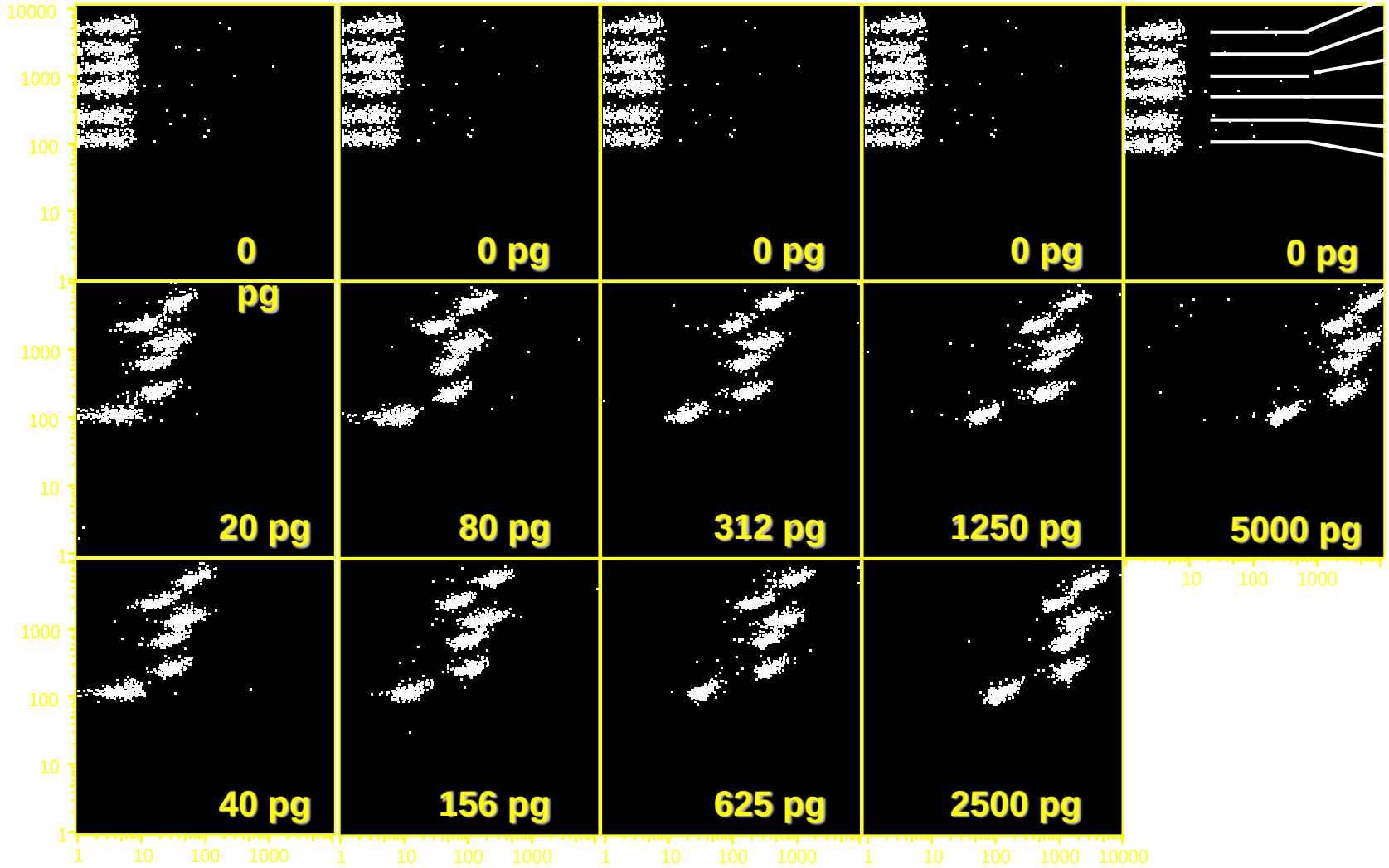


IL2
IL4
IL5
IL10
TNFα
IFNγ



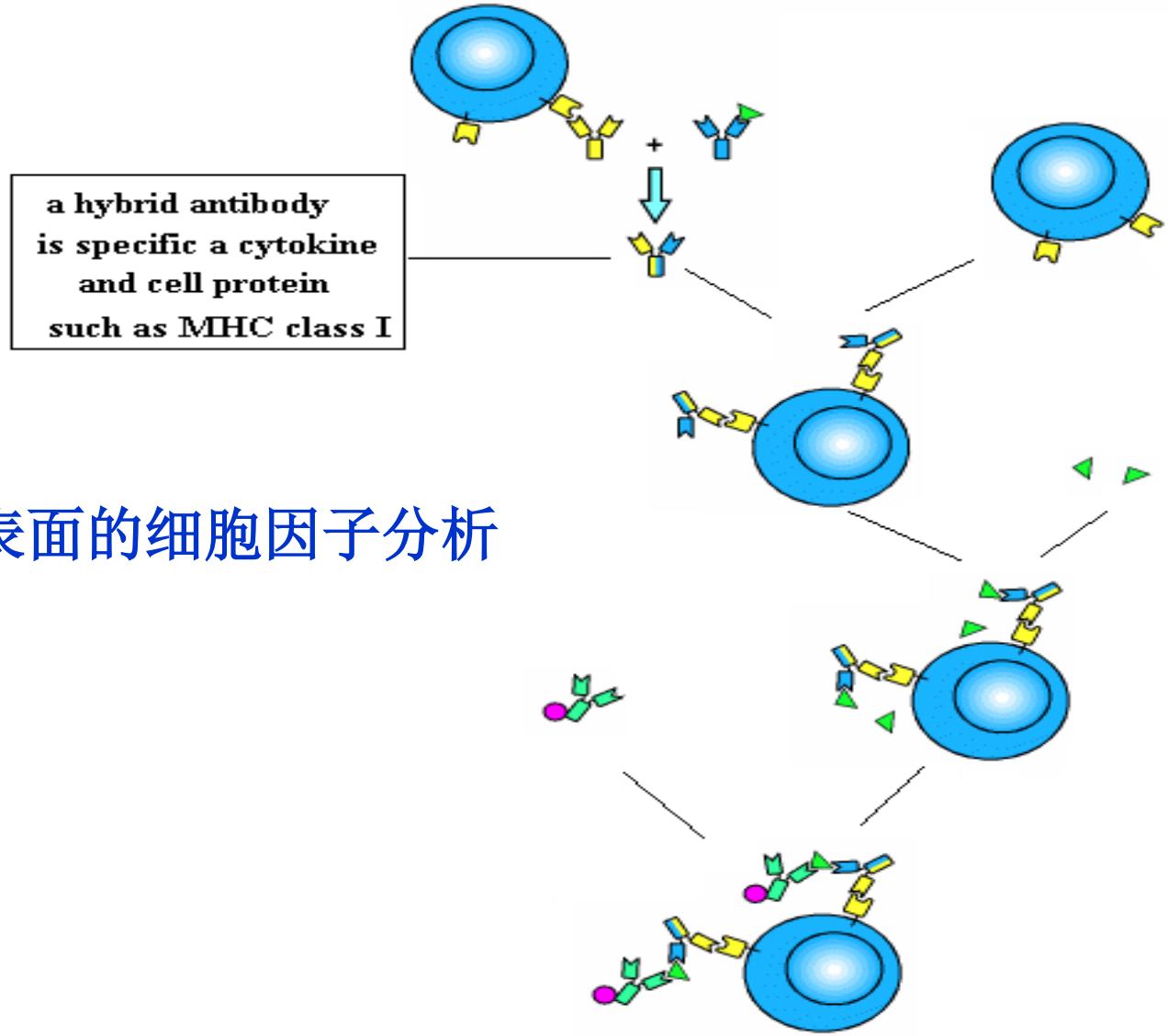
standard curves generated by CBA Software

Human Th1/Th2 Cytokine CBA

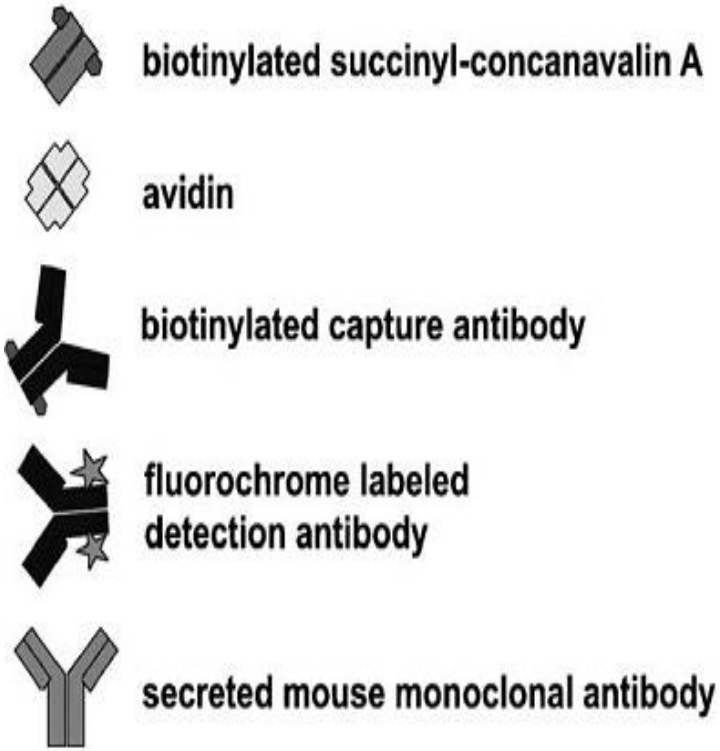


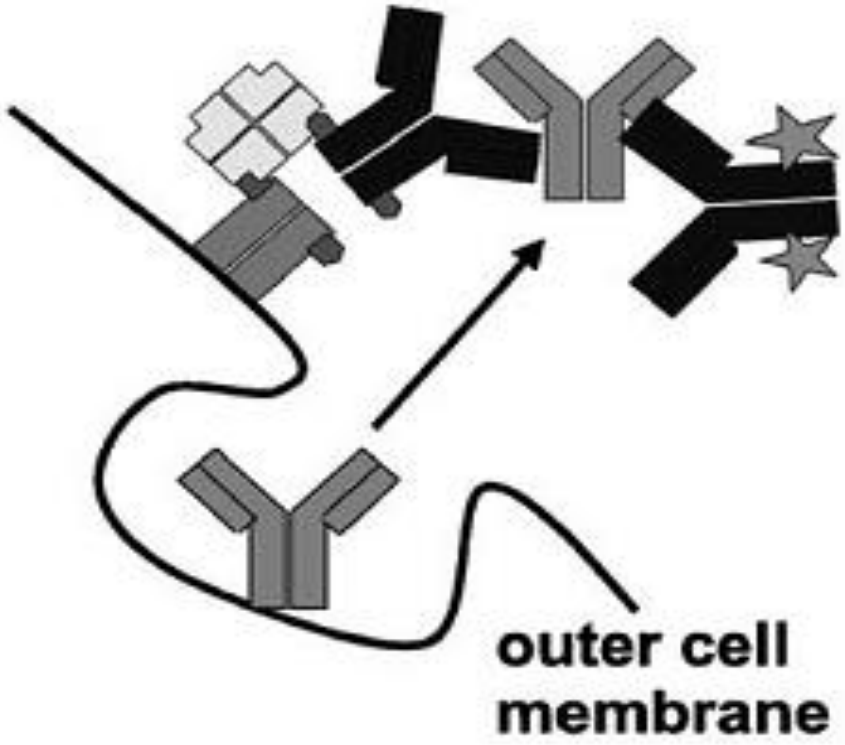
IL-2
IL-4
IL-5
IL-10
TNF-α
IFN-γ

Elisa	CBA
单一	多元和同步
样本量体积大	Elisa样本体积的1/6
灵敏度低，稳定性差	灵敏度高，稳定性好
每个蛋白分别检测，样本浓度高，时间长，效率低	芯片式集成测定，所需样本浓度低，且缩短时间，提高效率
酶联放大效应，结果易失真	流式荧光定量检测，避免假阳性

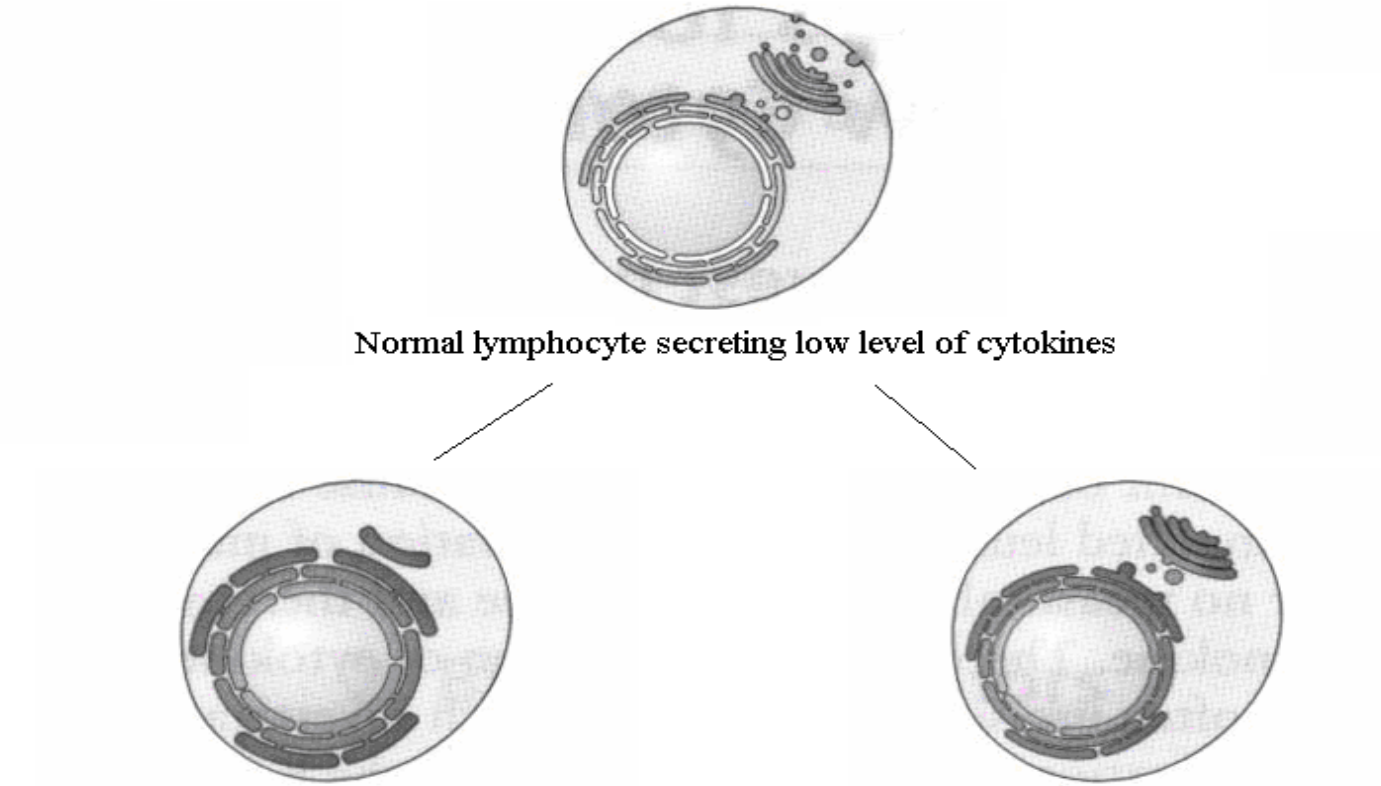


细胞膜表面的细胞因子分析


biotinylated succinyl-concanavalin A
avidin
biotinylated capture antibody
fluorochrome labeled
detection antibody
secreted mouse monoclonal antibody

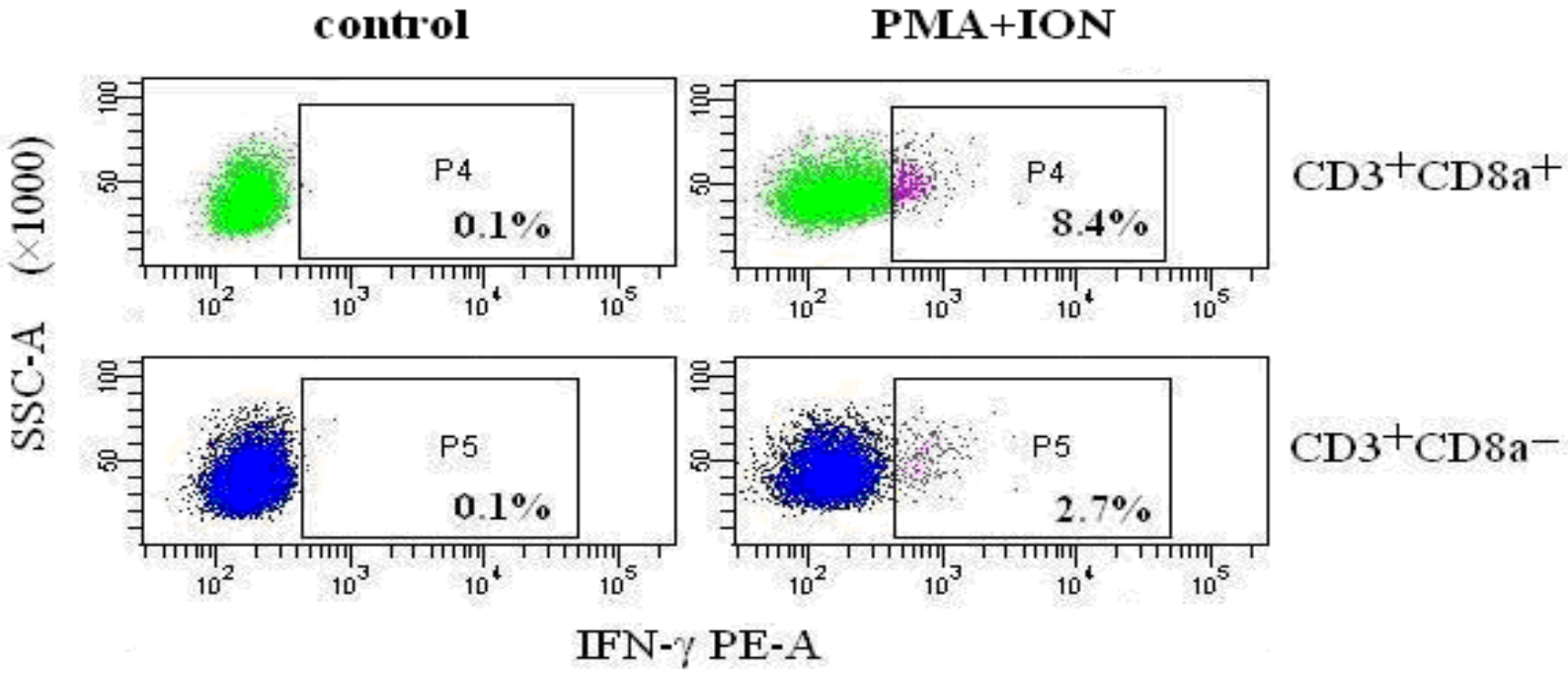

outer cell
membrane

将细胞表达的细胞因子阻断在细胞内



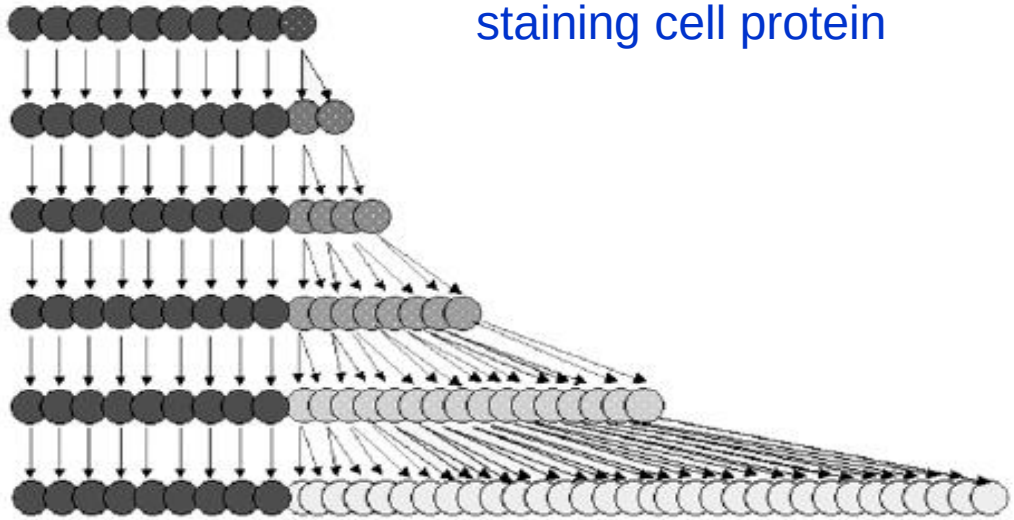
TNF-a

IL-10


control
PMA+ION
SSC-A (×1000)
P4
0.1%
P4
8.4%
CD3+CD8a+
P5
0.1%
P5
2.7%
CD3+CD8a−
IFN-γ PE-A

4、细胞分裂

generation (# of divisions)	proliferating cell(s) as % of total	brightness of proliferating cells relative to brightness of parent (≡ 100)
Parental	10%	[100]
1	18%	50
2	31%	25
3	47%	12.5
4	64%	6.25
5	78%	3.125



CFSE

staining cell protein

Fig. 2. A cartoon illustrating that dye intensity indicates the number of divisions that have occurred as a "specific" cell expands within a mainly resting population.

PKH

lipophilic(亲脂性)dye that inserts into the lipid bilayer of the outer membrane.

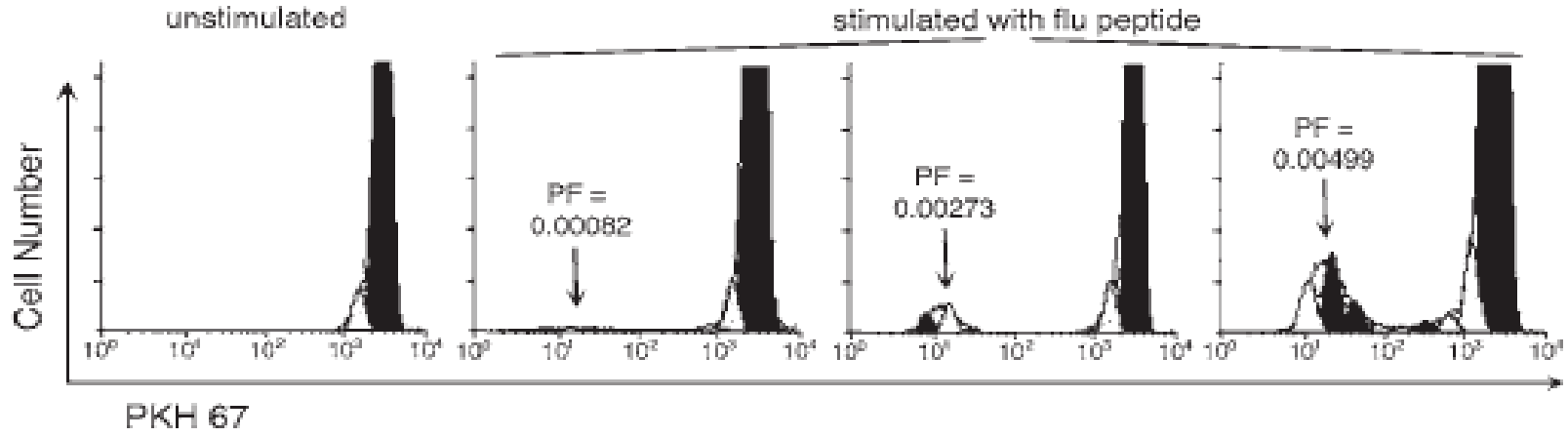
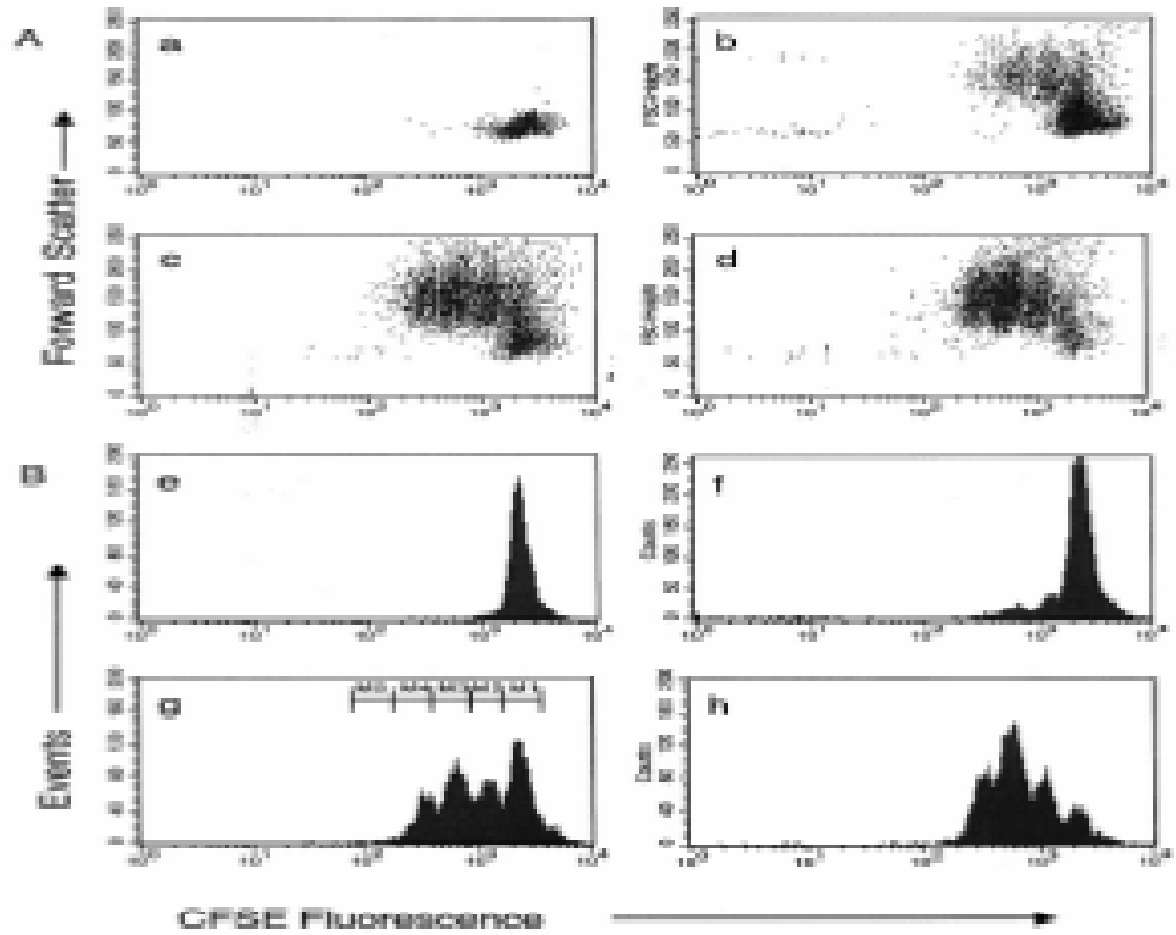


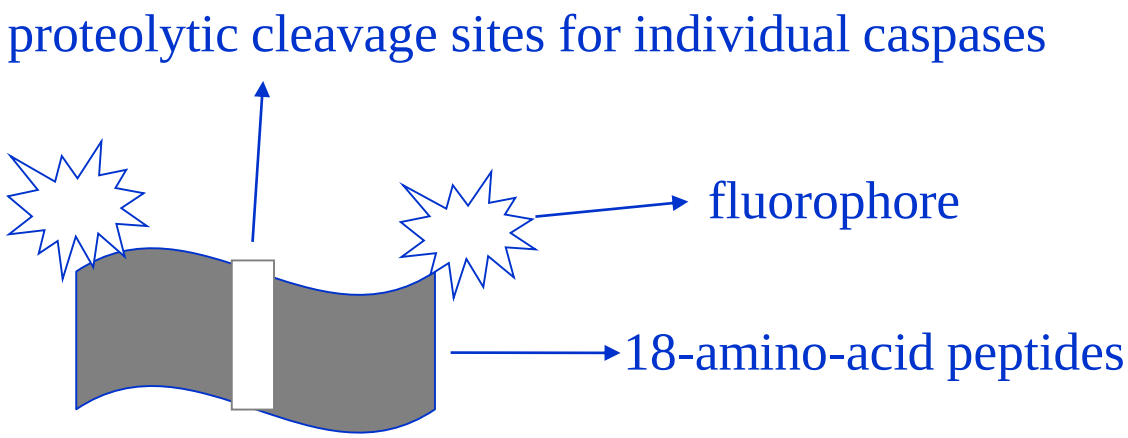
Fig. 3. PKH 67 histograms indicating a range of PBMC responses to flu peptide after 6 d in culture. Gaussian curves modeling each generation are drawn by ModFit software. Calculated precursor frequencies (PF) for proliferation are shown. Unstimulated cells are in the histogram on the **left**.



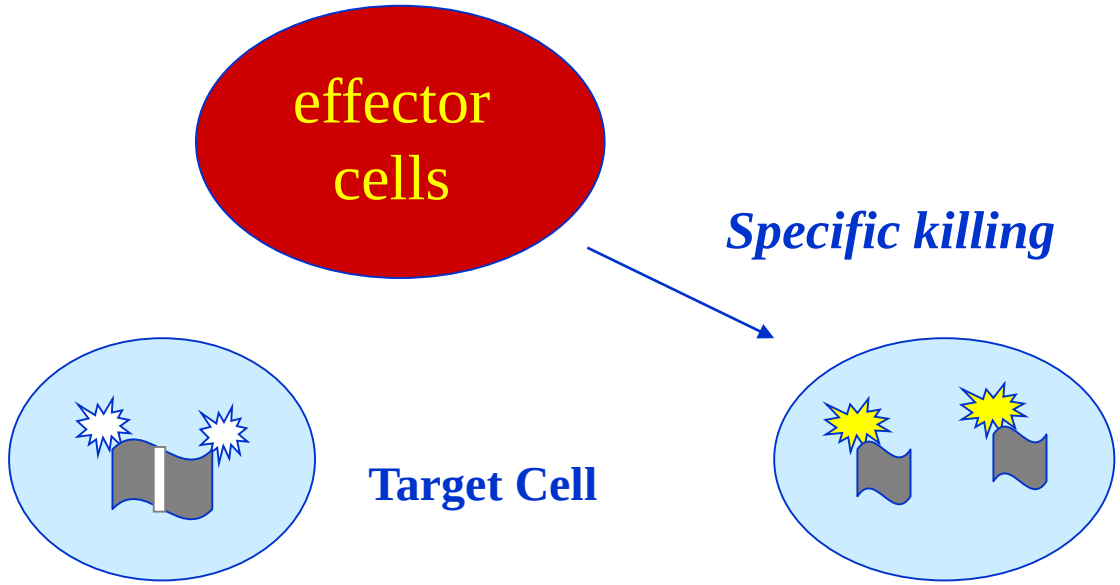
通过分析，可以计算分化前体频率（**precursor frequency**）

5、CTL功能分析

- 评价细胞杀伤过程的生物学变化：如效应细胞的凋亡，CTL介导的裂解功能的抑制。



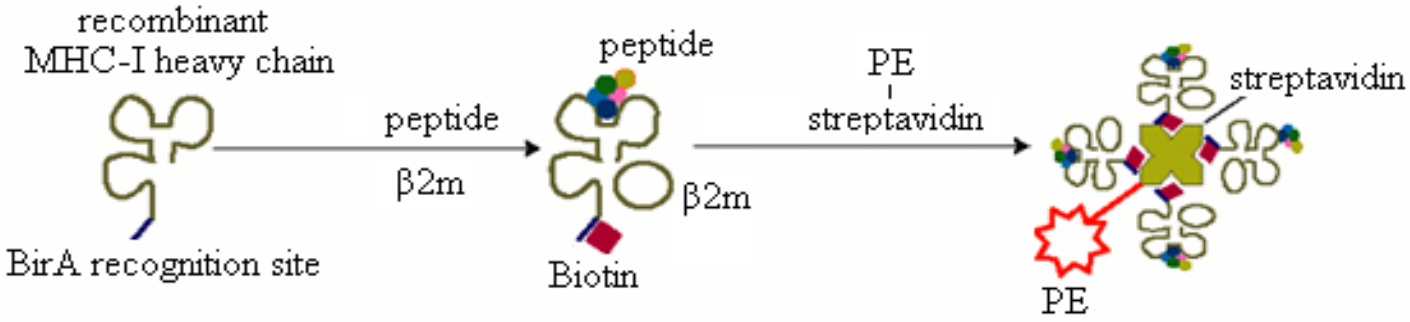
Fluorogenic Caspase Substrates 检测CTL介导的靶细胞凋亡

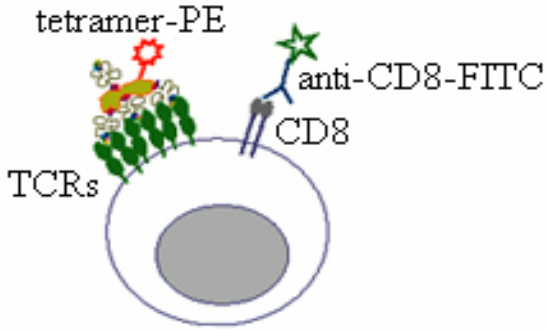


In the uncleaved substrates, fluorescence is quenched owing to the formation of intramolecular excitonic dimers.

On cleavage of the peptides by specific caspases, the fluorophore–fluorophore interaction is abolished, leading to an increase in fluoresence.

6、抗原特异性T淋巴细胞的分析





只要合成各种特异性抗原，即可定量体内抗原特异性CTL。

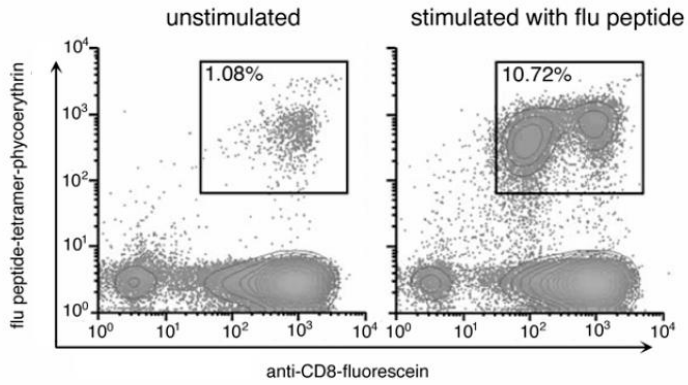
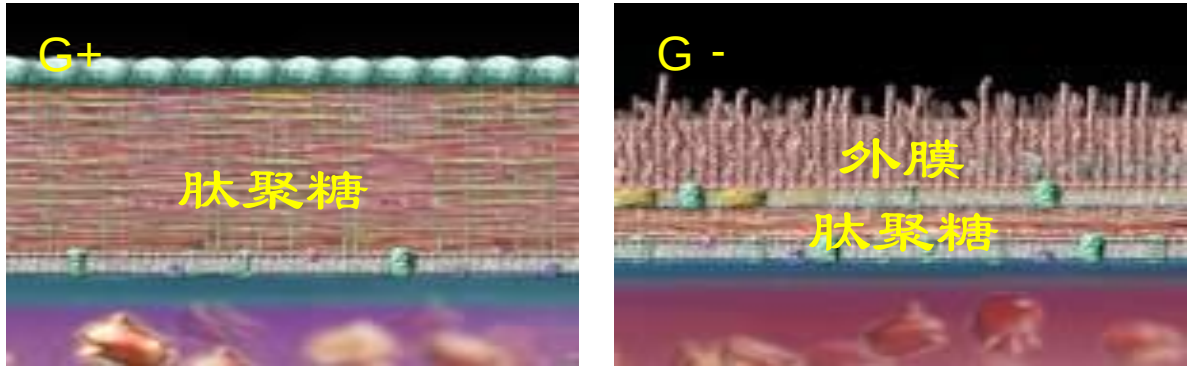


Fig. 1. Expansion of flu-peptide-tetramer-positive/CD8-positive T cells after 6 d of culture with flu peptide stimulation. The percentages indicate the tetramer-positive/CD8+ cells among live lymphocytes.

其他领域的应用

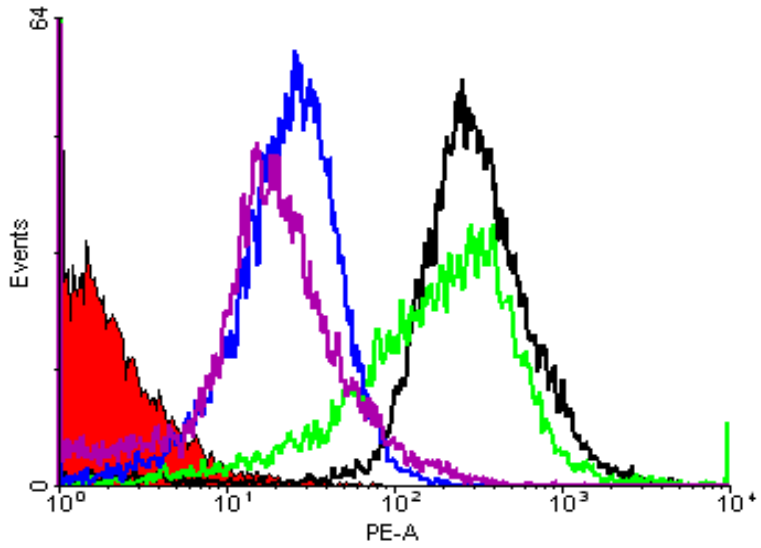
- 具有细胞壁的细胞及其细胞壁的主要成分：植物细胞（纤维素）、细菌（肽聚糖）、真菌（几丁质）
- 细菌的大小、质量、核苷酸和蛋白含量等大约是哺乳动物细胞的1/1000 。信号强度低，限制了检测的精确度。
- 细菌吸收和排除染料、药物等受细胞壁结构和孔、泵的影响。



细菌的细胞壁结构

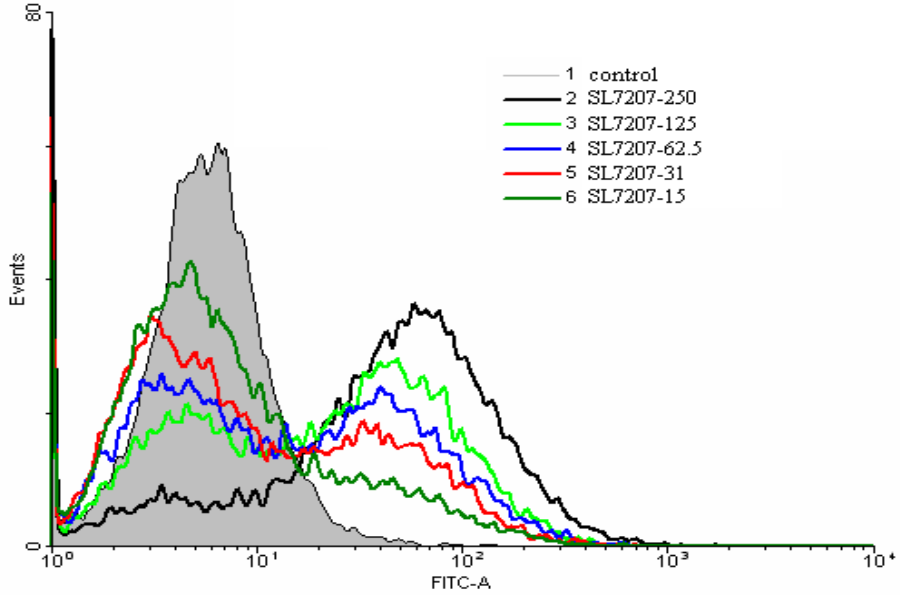
- G+：一般不需要额外的处理，便可以吸收大量的试剂。也有例外，如：Walberg等发现，吸入不同的核苷酸染料，也有不同的变化。
- G-：细胞膜排斥多数亲脂性或疏水分子，如cyanine染料。EDTA等破膜后，可使亲脂性化合物穿透细胞膜，但是，破膜后的特性与自然状态不同。

DsRed在重组菌中的表达

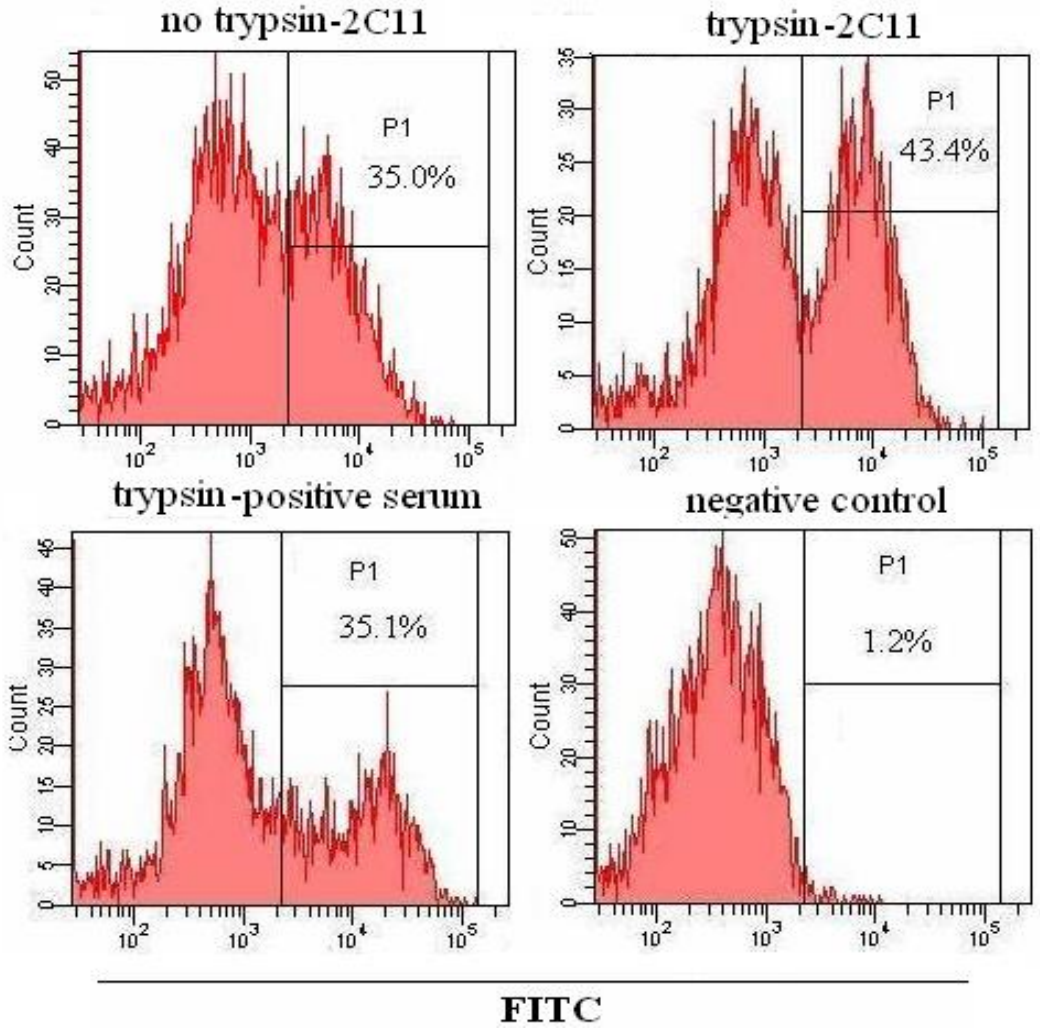


X4550，
X4550(pVAX1-trc-DsRed)，
X4550(p3334-DsRed),
X6212(p3334-DsRed)，
Xa(pVAX1-trc--DsRed)

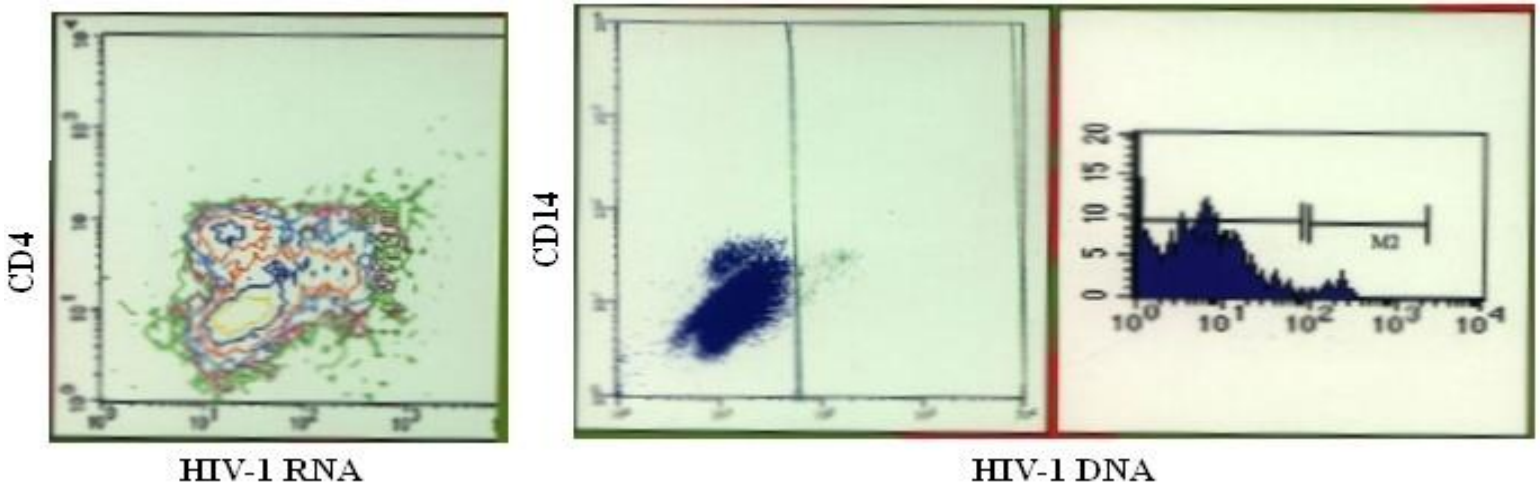
体外细菌感染效率



病毒感染细胞的检测


no trypsin-2C11
P1
35.0%
trypsin-2C11
P1
43.4%
trypsin-positive serum
P1
35.1%
negative control
P1
1.2%
Count
FITC

流式分子表型（Molecular phenotyping）



FCM-PCR-FISH：细胞中HIV特异性DNA或RNA

如：测定端粒长度

0514 – 87979383 –213