

# 流式細胞儀工作原理

劉 益 年

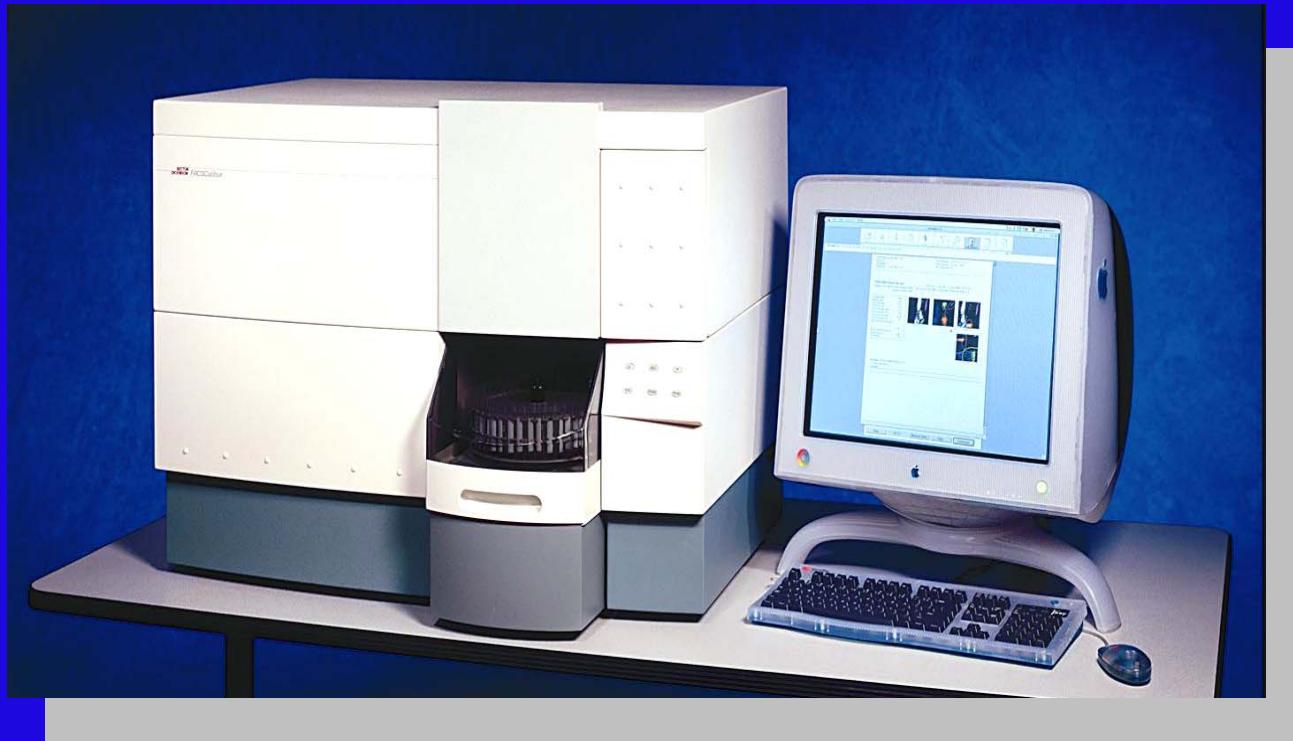
產品經理

美商必帝股份有限公司

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# FACSCalibur



# 大綱

- 實驗設計原理
- 流式細胞儀運作原理



# Introduction of the experiment design for Flow Cytometry

- 藉由螢光抗體標識細胞之抗原特性
  - Lymphocyte immunophenotyping
  - Stem cell enumeration
  - Cell signaling immunophenotyping
- 藉由螢光化合物標識細胞特性
- 藉由螢光蛋白標識細胞特性
- Cytometry Beads Array

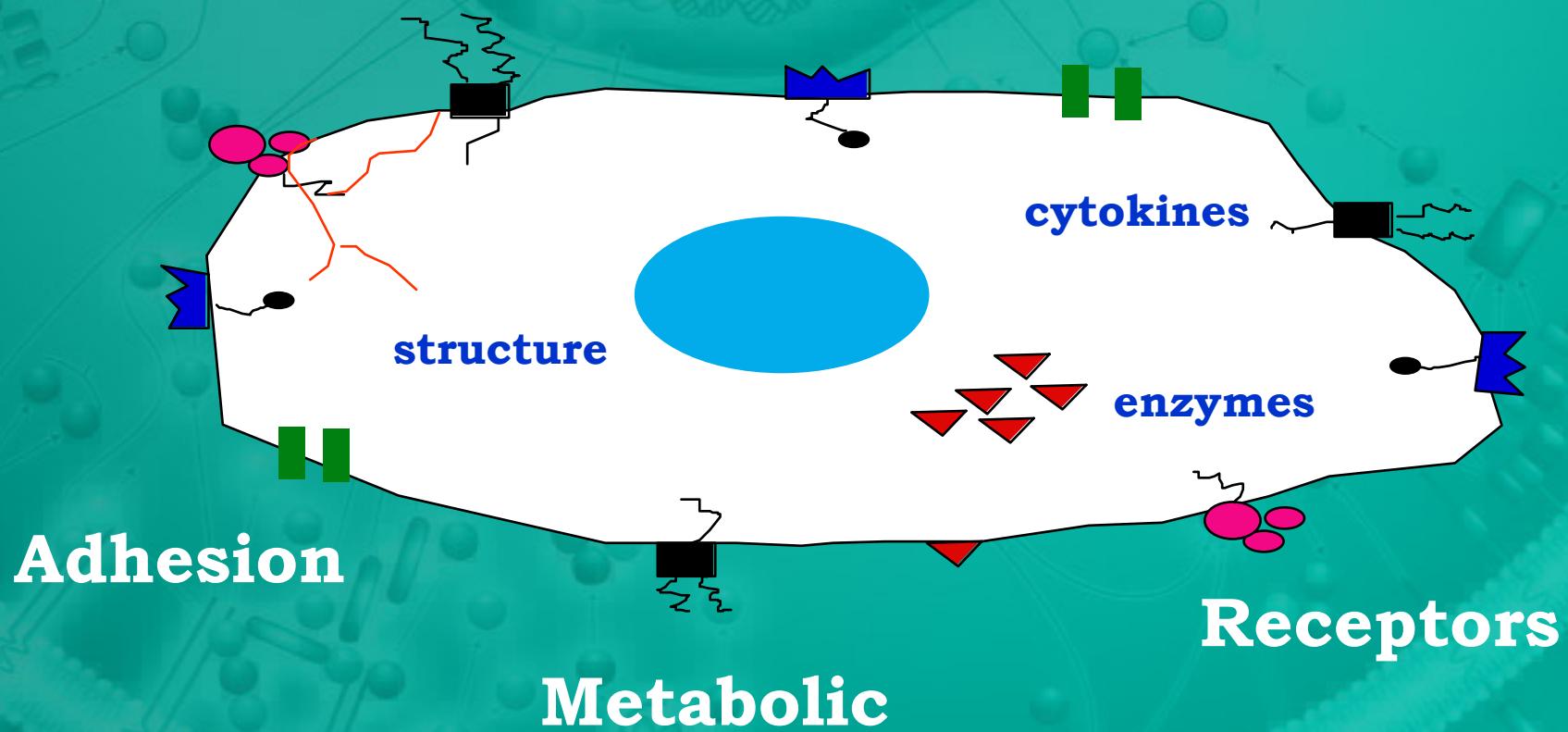


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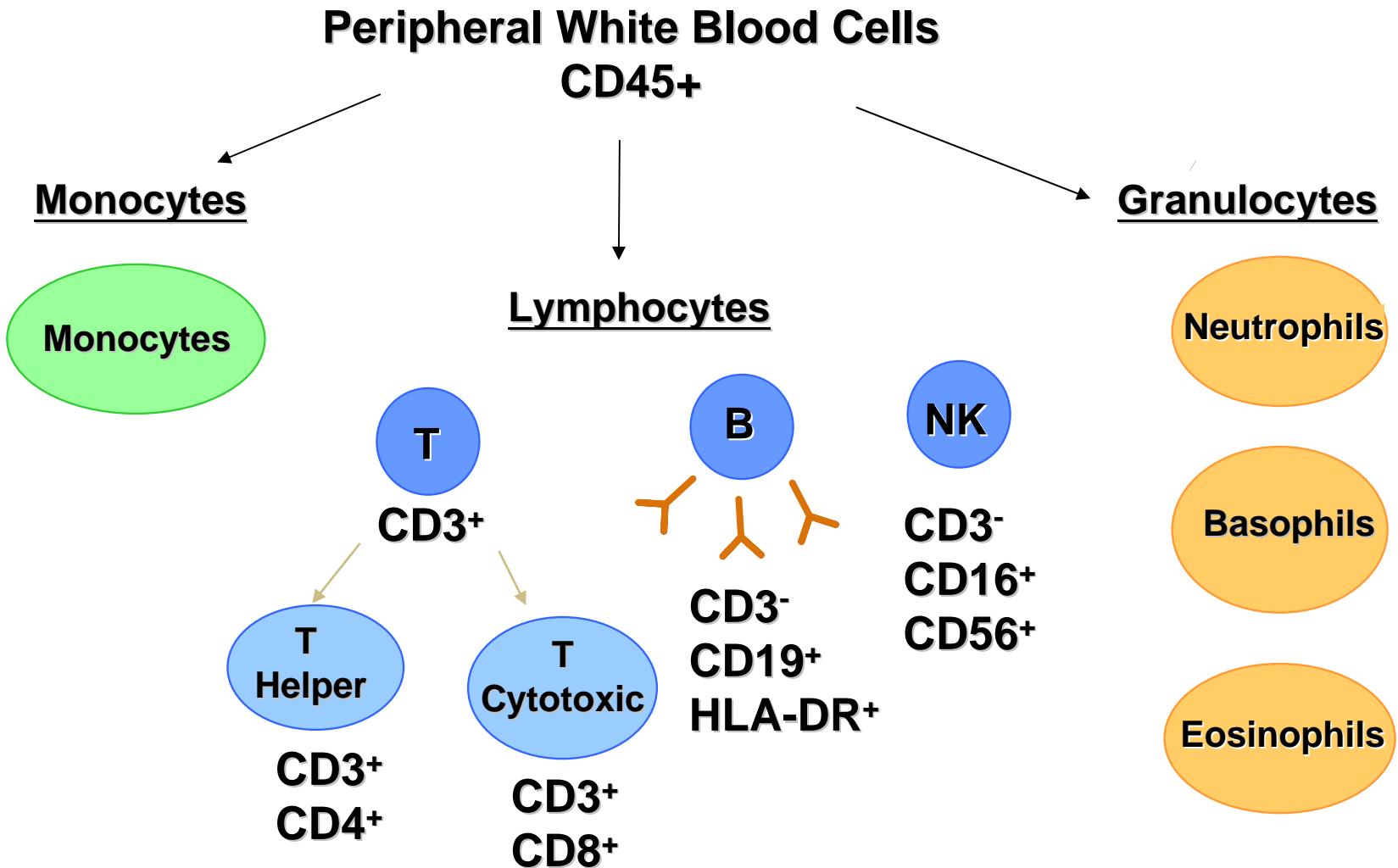
# 單一細胞特性之偵測



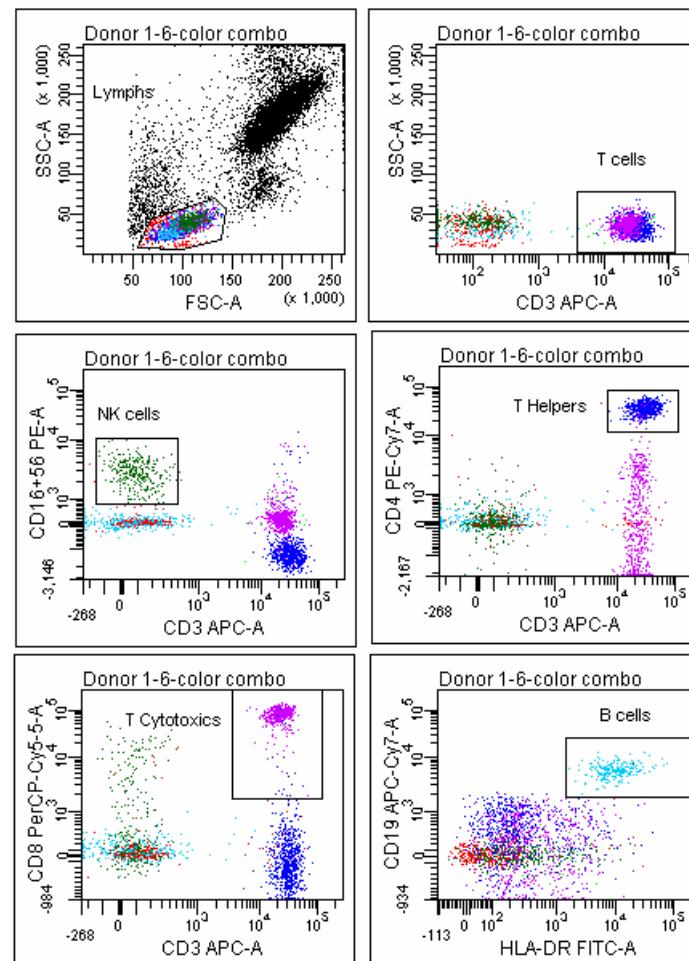
# 單一細胞特性之偵測



# Lysed Whole Blood Components



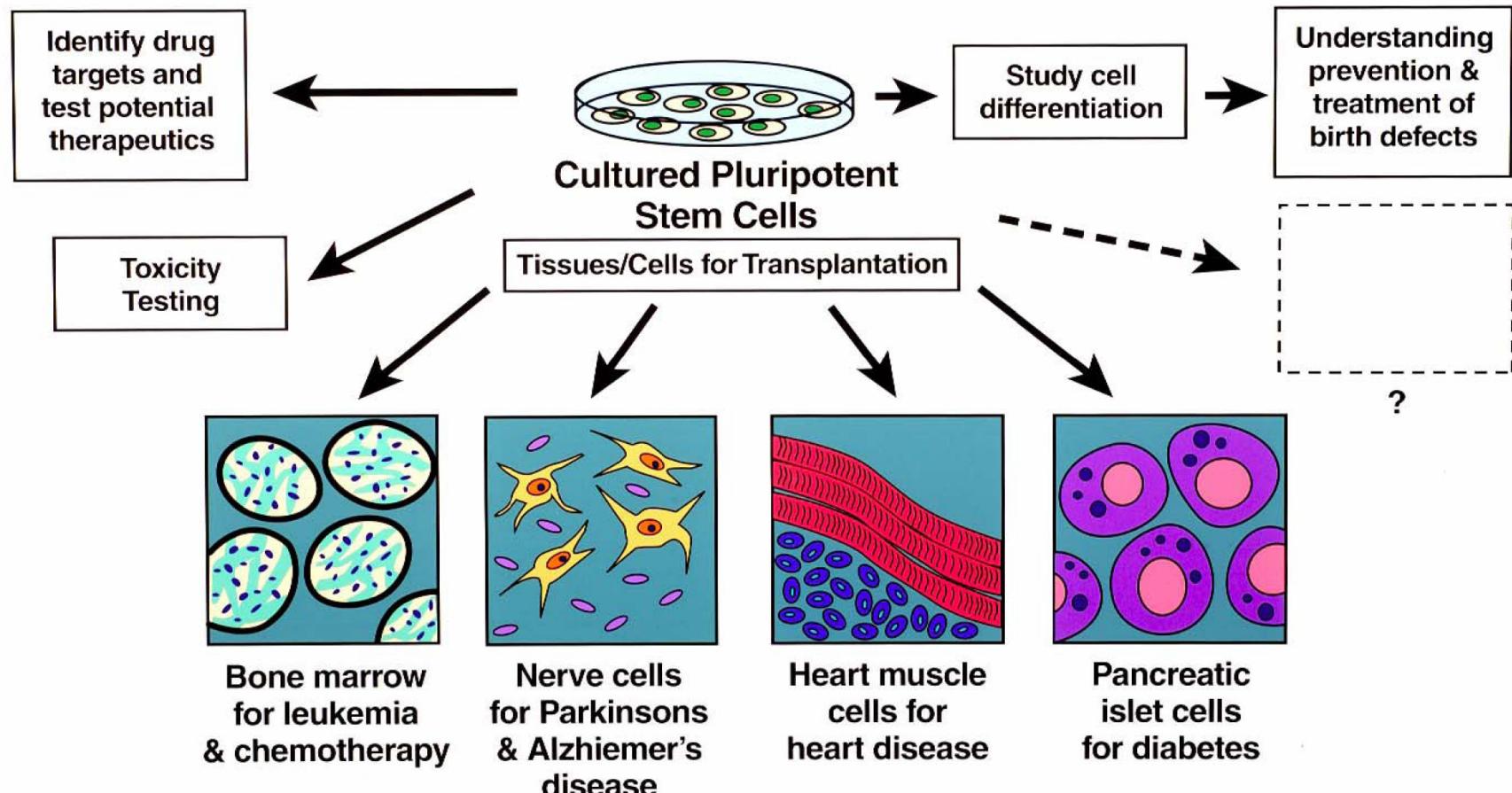
# Example



Tube: 6-color combo

Population	#Events	%Parent	%Total
All Events	10,000		100.0
Lymphs	2,136	21.4	21.4
T cells	1,275	59.7	12.8
T Helpers	716	56.2	7.2
T Cytotoxics	550	43.1	5.5
B cells	254	11.9	2.5
NK cells	288	13.5	2.9

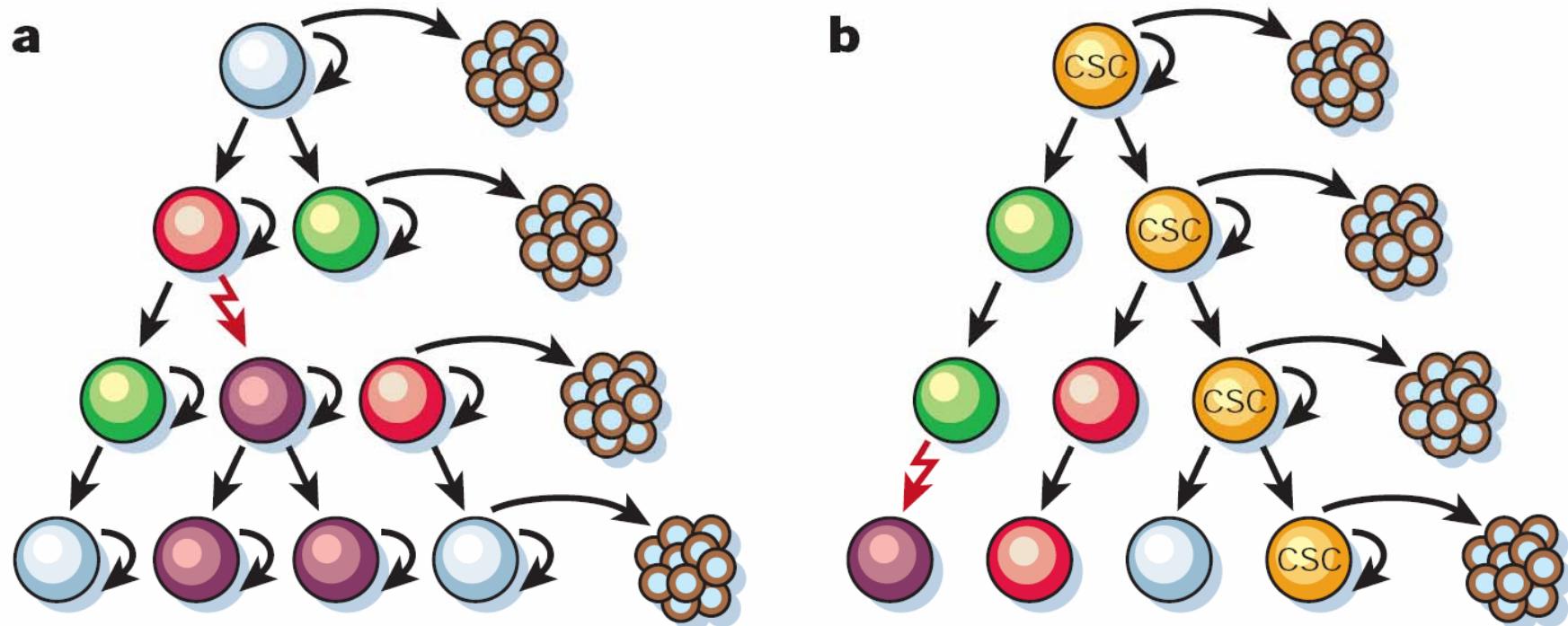
# Stem Cell Background



Schematic adapted from <http://stemcells.nih.gov/index.asp>



# Theory of Cancer Stem Cell

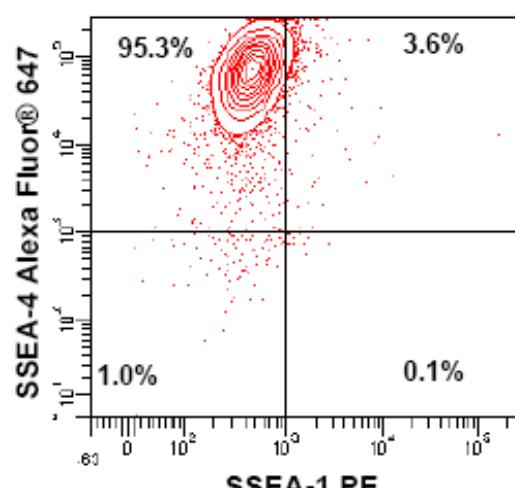
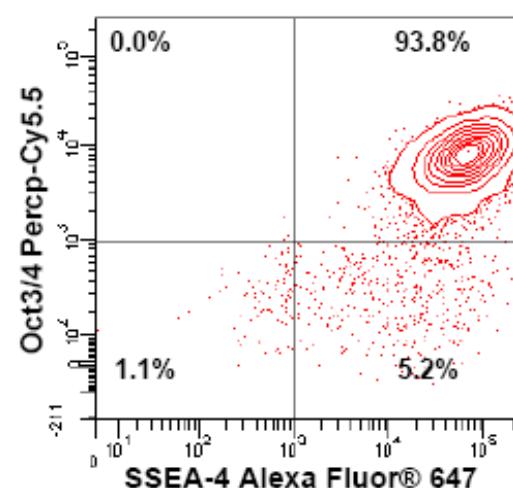
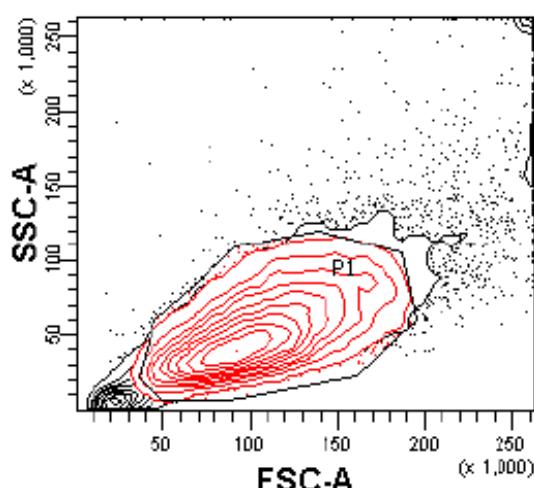
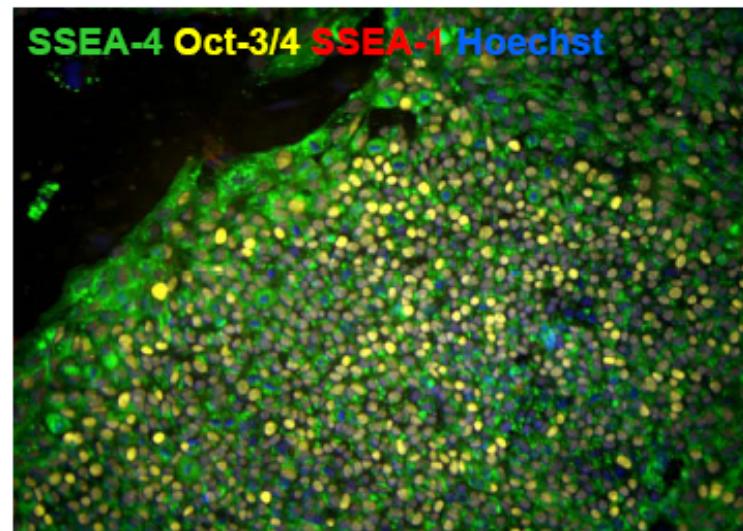
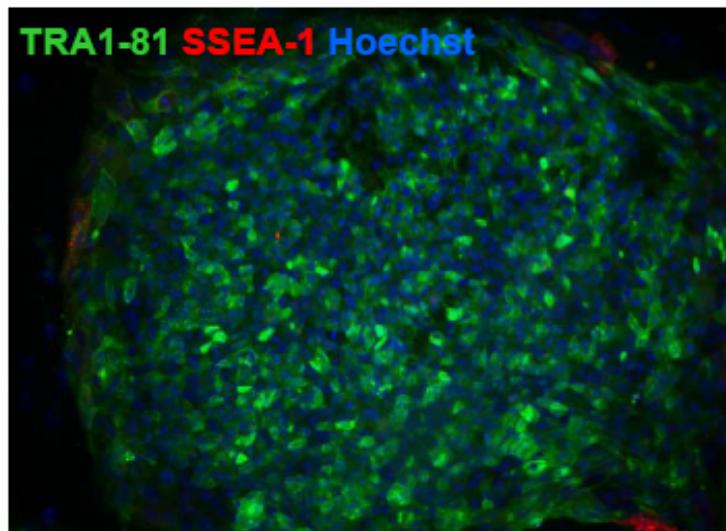


Tumour cells are heterogeneous, but most cells can proliferate extensively and form new tumours

Tumour cells are heterogeneous and only the cancer stem cell subset (CSC; yellow) has the ability to proliferate extensively and form new tumours

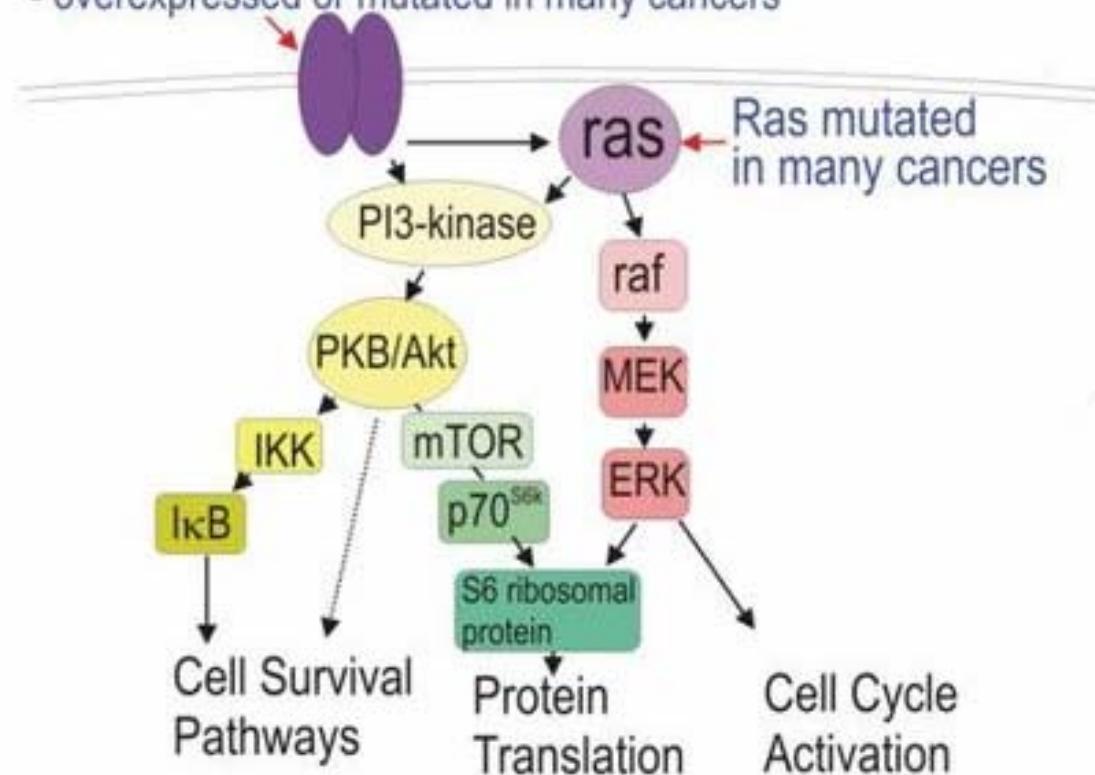
# Sorted H9 hESCs Express Pluripotency Markers

H9 P42 P6 post-sort

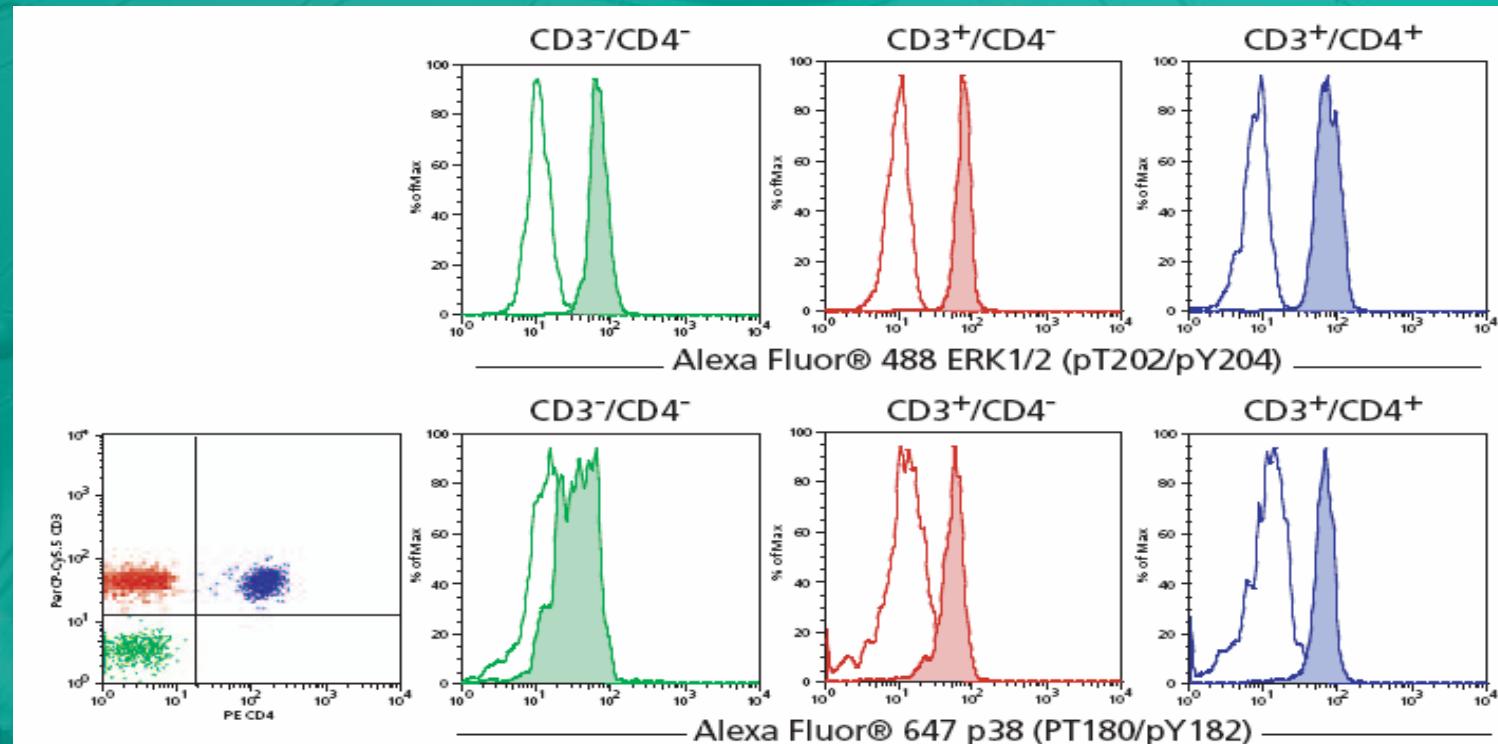


# 訊息傳導

Receptor tyrosine kinases -  
e.g. EGFR, Her2  
- overexpressed or mutated in many cancers



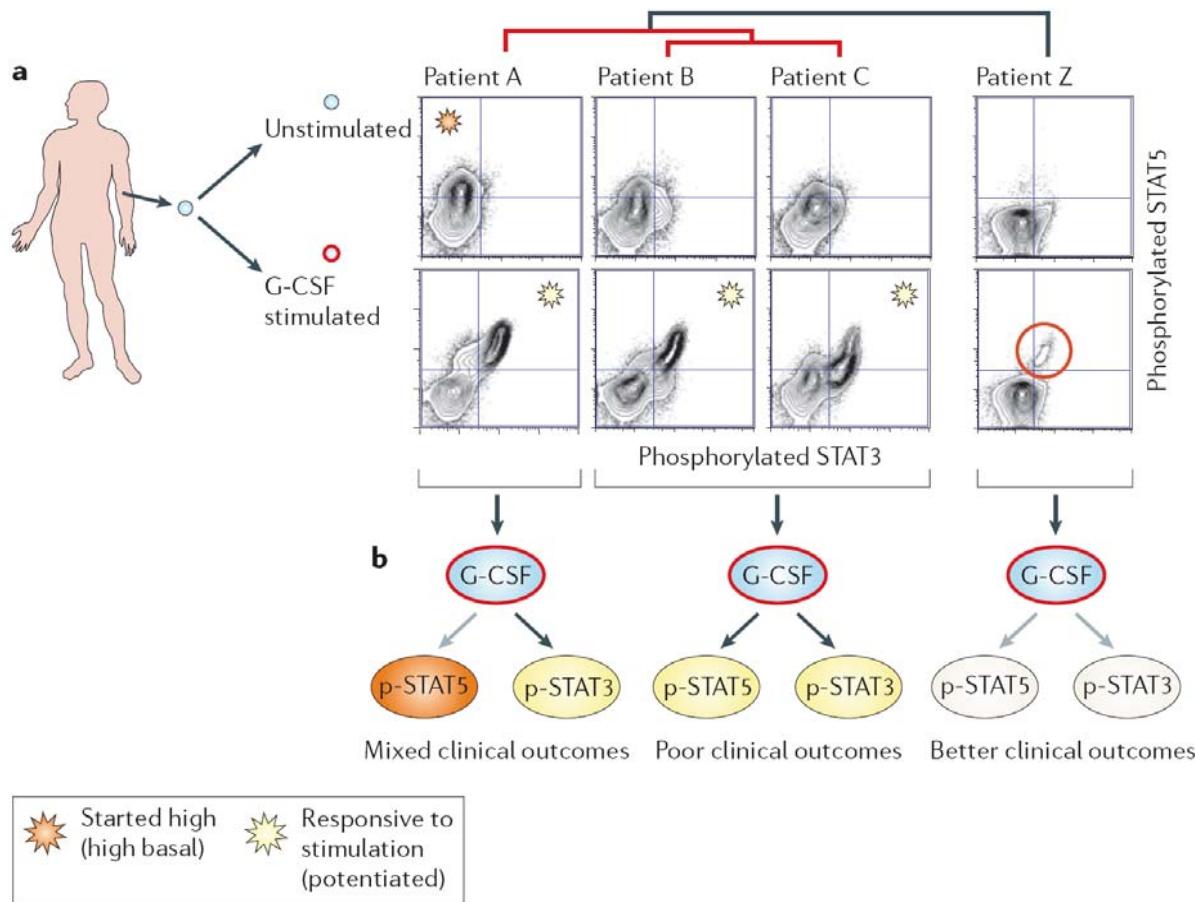
# BD™ PhosFlow for Whole Blood or PBMC Samples



<http://bdphosflow.com>



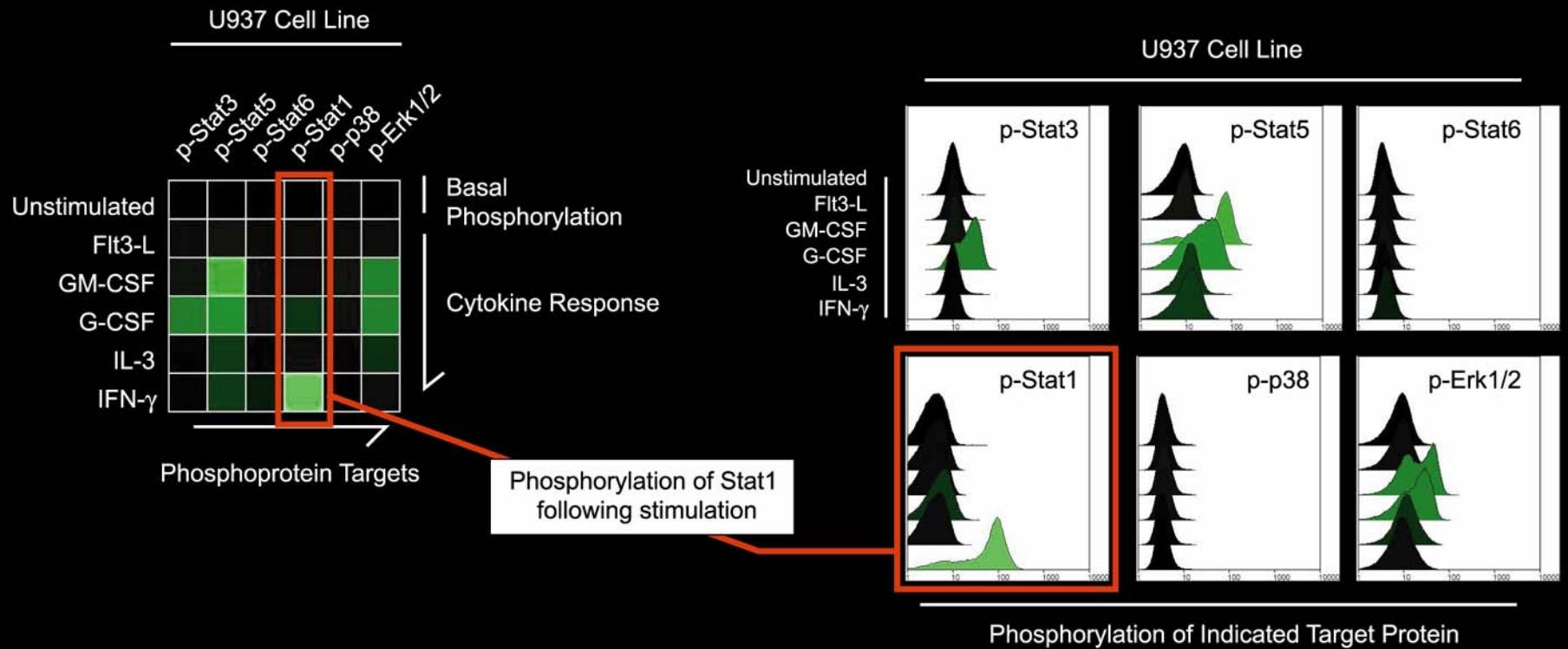
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**Mapping normal and cancer cell signalling networks:  
towards single-cell proteomics.**  
**Nat Rev Cancer. 2006 Feb;6(2):146-55**



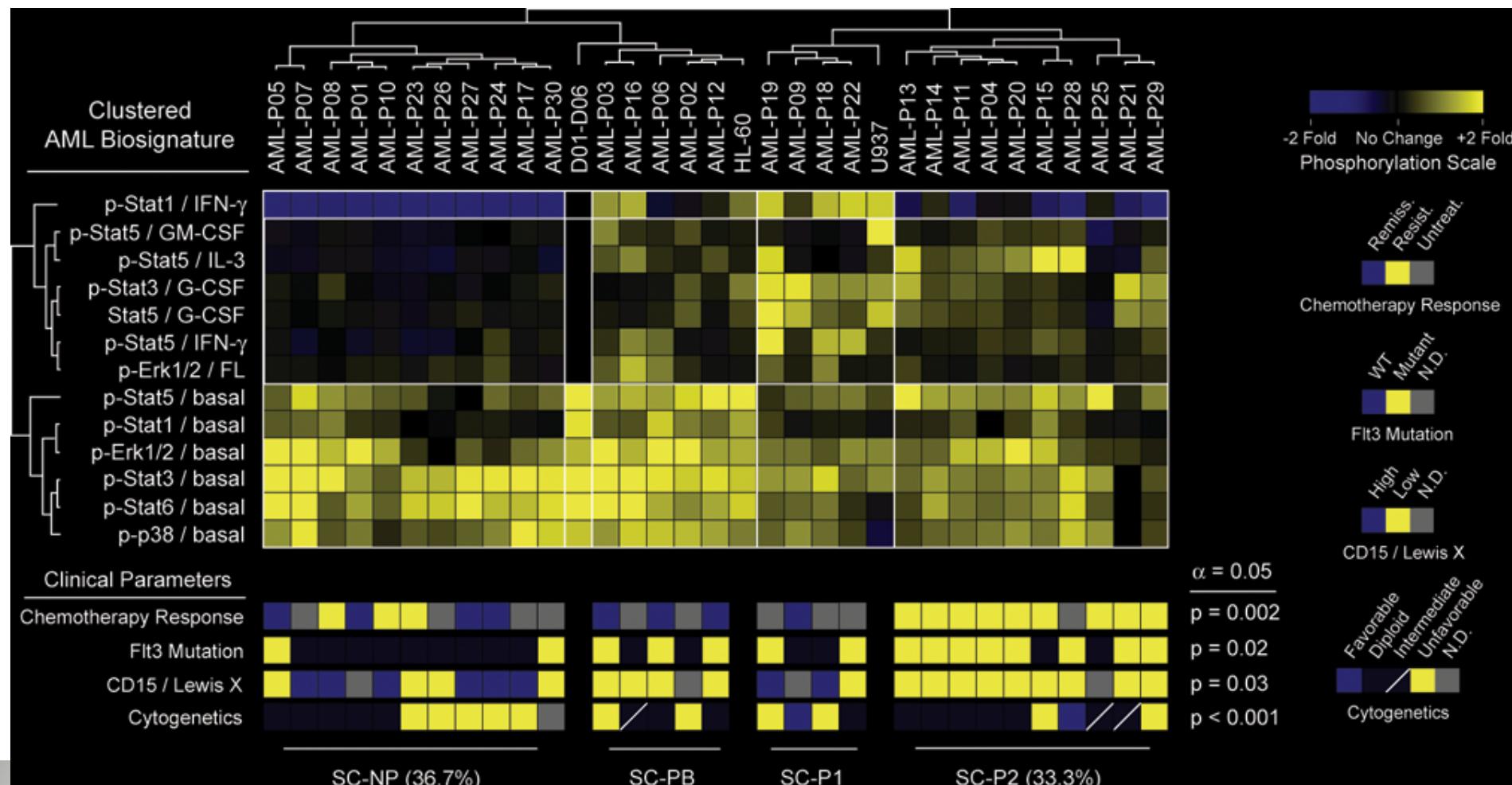
# Development Of Visualization Tools



Nolan et al.



# Clustering of Biosignature, Clinical Significance



Irish JM, Hovland R, Krutzik PO, Perez OD, Bruserud O, Gjertsen BT, Nolan GP.  
 Single cell profiling of potentiated phospho-protein networks in cancer cells. *Cell*.  
 2004 Jul 23;118(2):217-28.



# 實驗設計原理

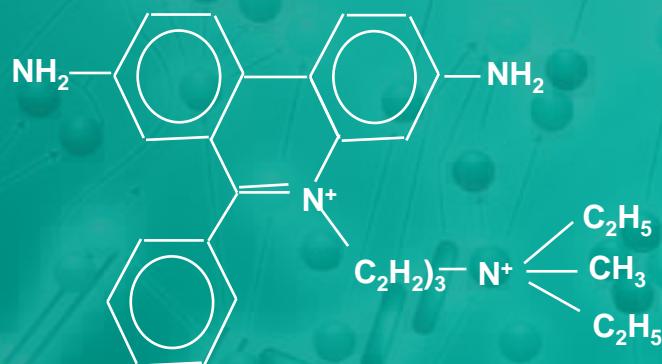
- 藉由螢光抗體標識細胞之抗原特性
- 藉由螢光化合物標識細胞特性
  - DNA content determinant
  - Cell proliferation determinant
  - Apoptosis detection
- 藉由螢光蛋白標識細胞特性
- Cytometry Beads Array



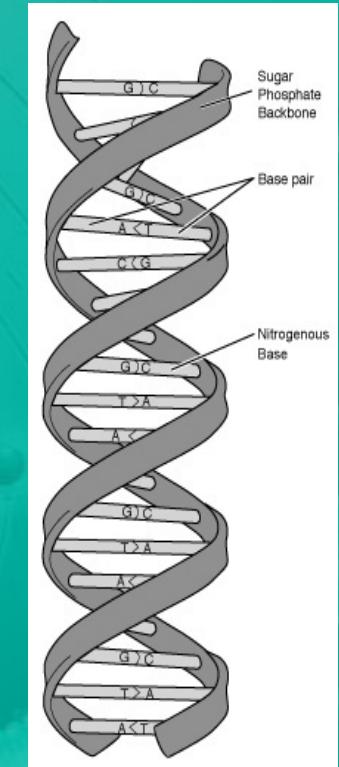
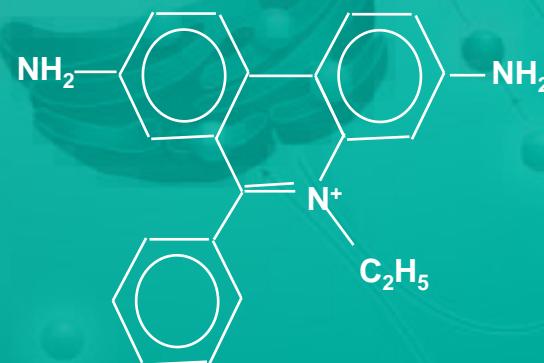
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# DNA 特異性染劑

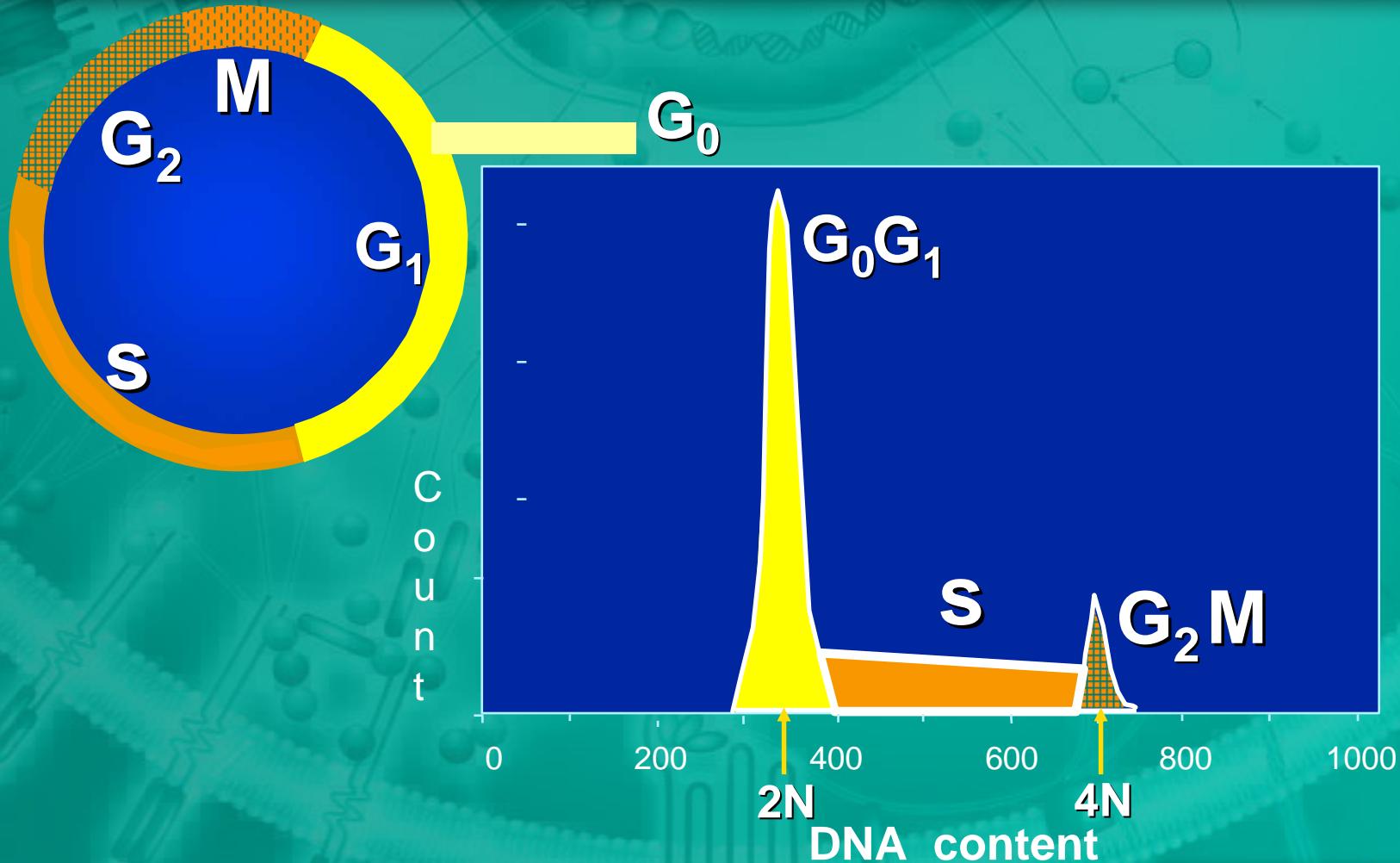
Propidium



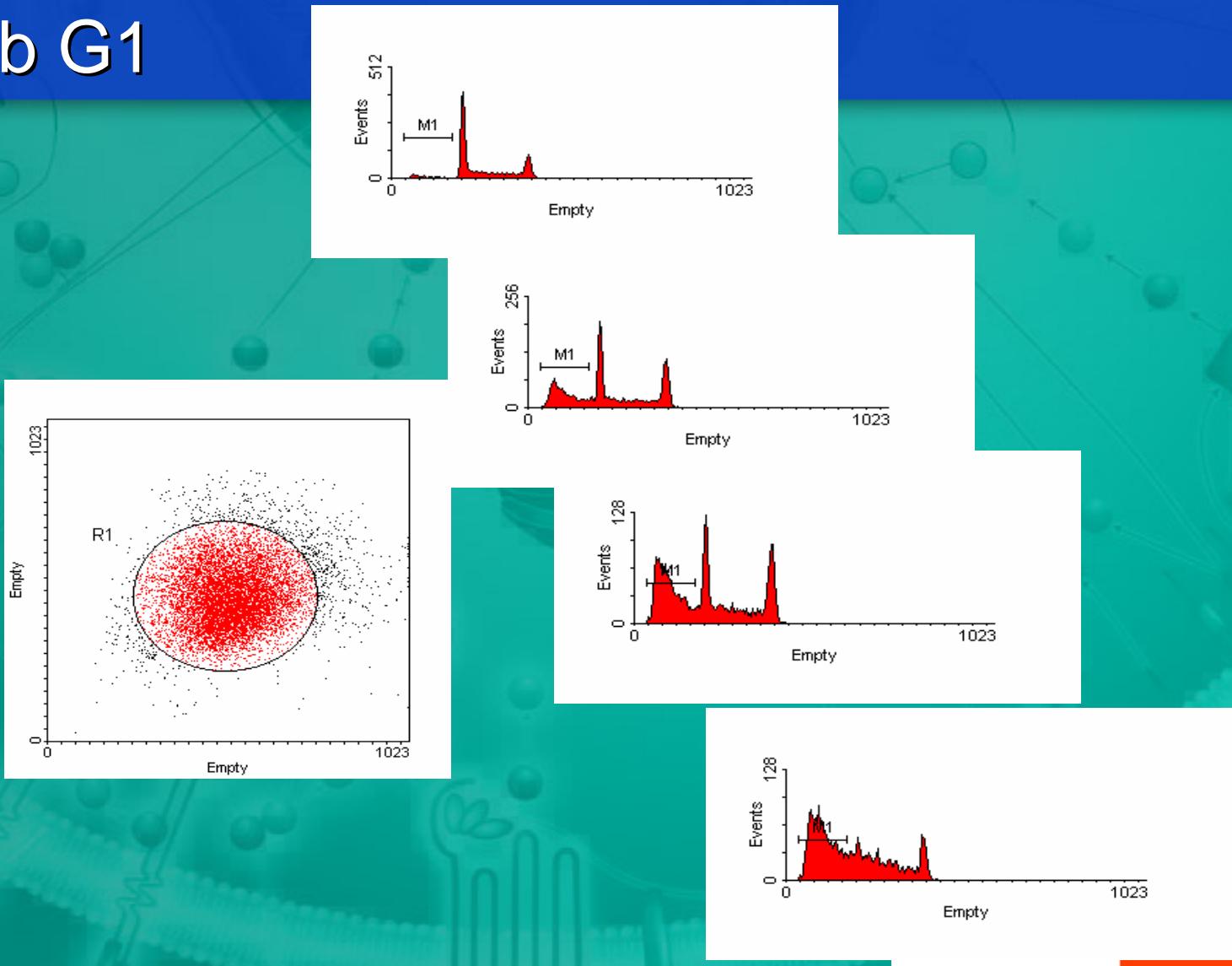
Ethidium



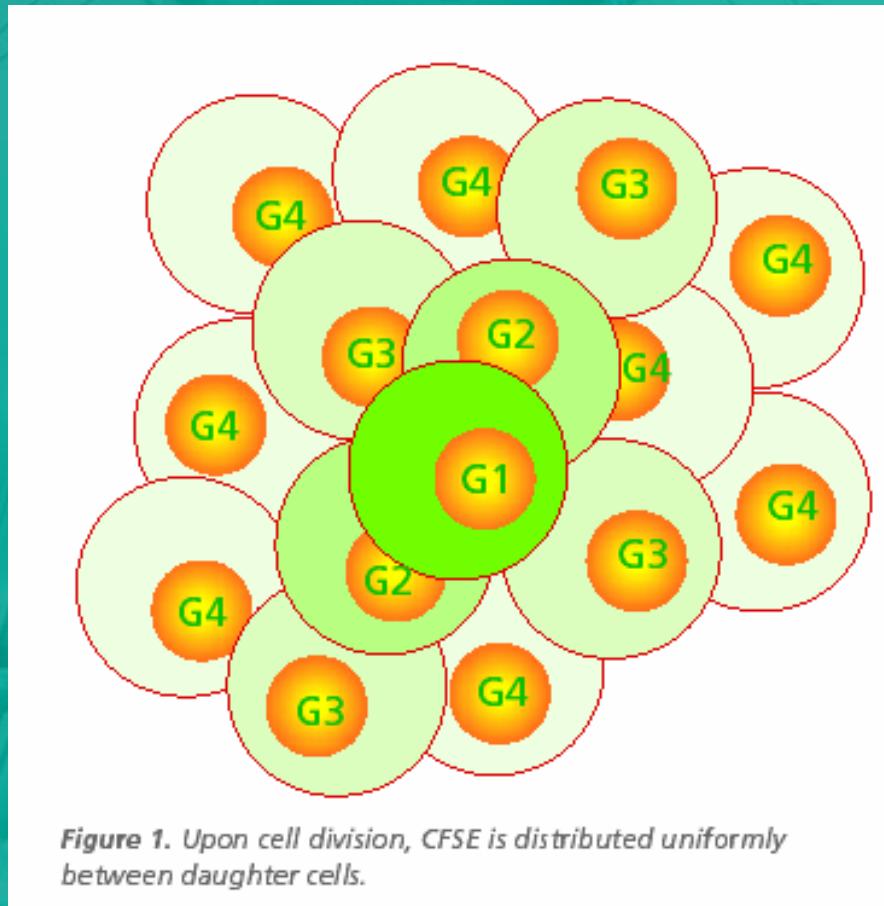
# 細胞周期位相的決定



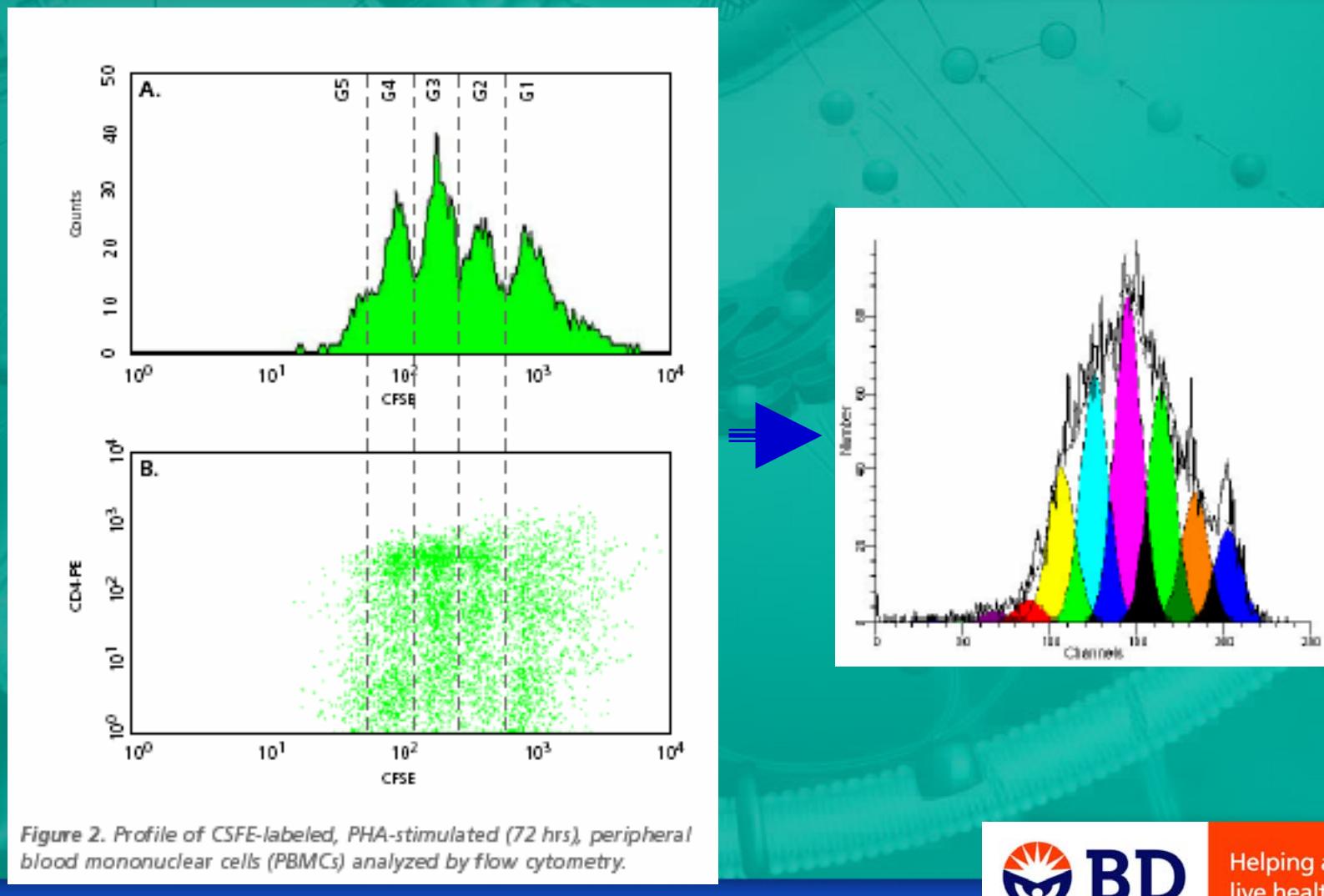
# Sub G1



# 細胞增殖反應

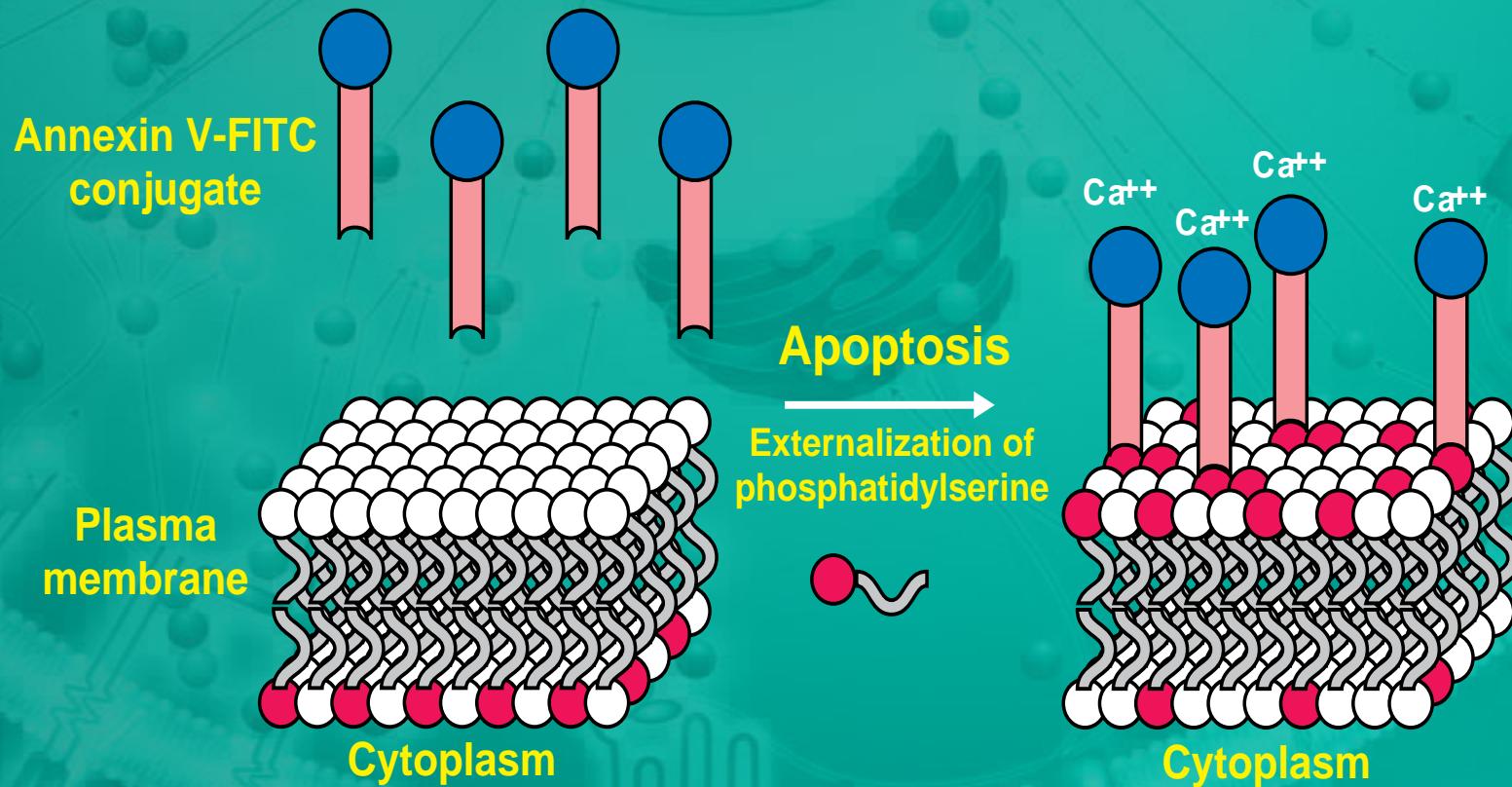


# 細胞增殖反應



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# Annexin V Assay



# Flow Cytometric Analysis of Apoptosis in Jurkat T Cells

Relative Cell Number

Incubation with Camptothecin (hr)

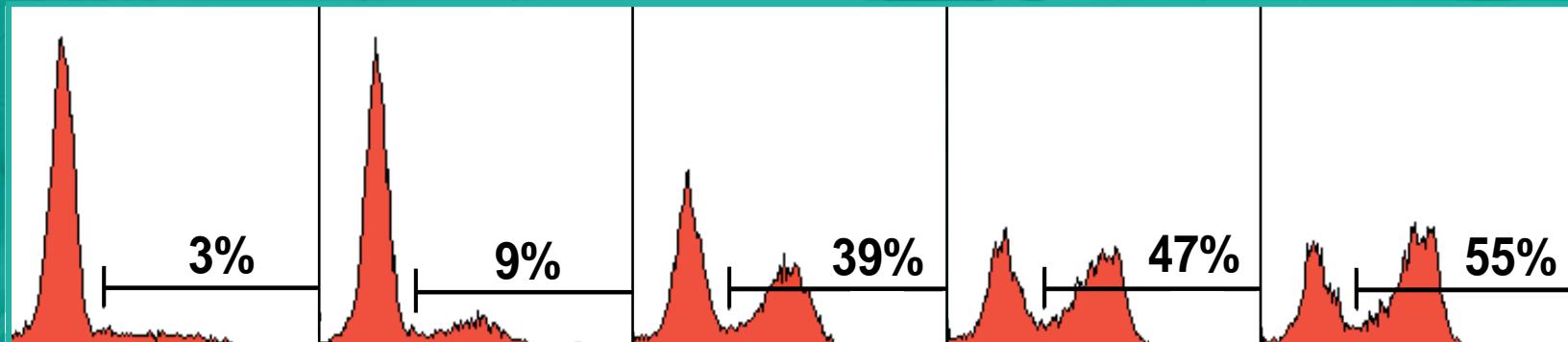
0

0.5

1

2

4



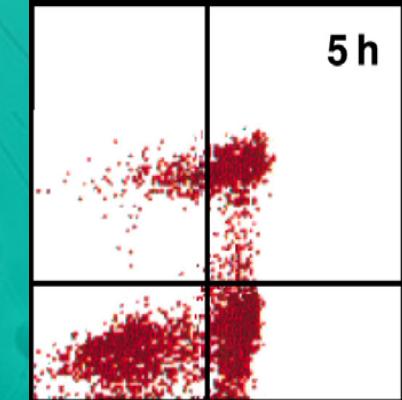
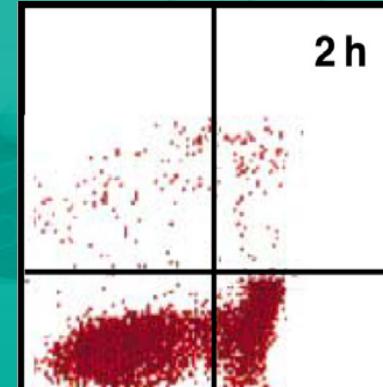
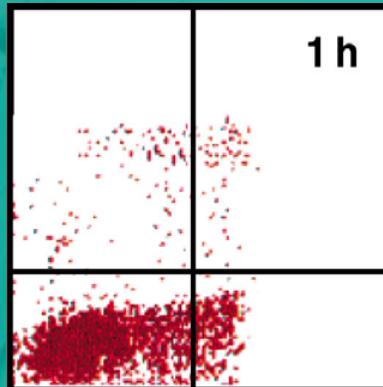
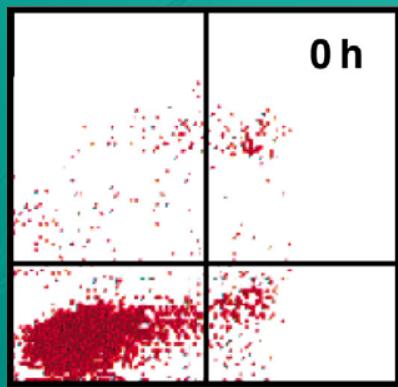
Annexin V-PE



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# Fas-induced Apoptosis in Jurkat Cells

PI



Annexin V-FITC



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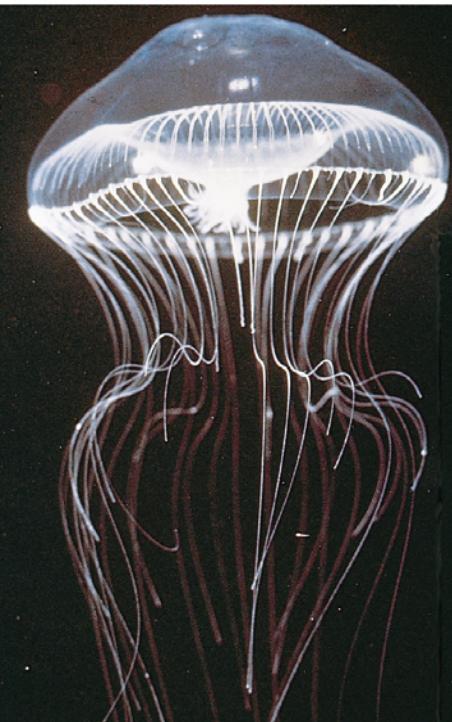
# 實驗設計原理

- 藉由螢光抗體標識細胞之抗原特性
- 藉由螢光化合物標識細胞特性
- 藉由螢光蛋白標識細胞特性
  - Reporter gene
  - Screening platform
- Cytometry Beads Array



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# GFP as a Reporter



**Figure 1.** Photograph of the jellyfish *Aequorea victoria* which produces the green fluorescent protein (GFP). (Photograph courtesy of Claudia Mills at the Friday Harbor Research Laboratories, WA, USA.)

**Table 1. Fluorescence properties of enhanced green fluorescent protein (GFP) variants**

Variant	Excitation max. (nm)	Emission max. (nm)	$E_m$	QY
Wild-type	395 (470)	509	21,000 (7150)	0.77
EGFP	488	597	55,000	0.70
EBFP	380	440	31,000	0.20
EYFP	513 (498)	527	36,500	0.63
ECFP	433 (453)	475 (501)	25,000	0.24



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# GFP as a Reporter

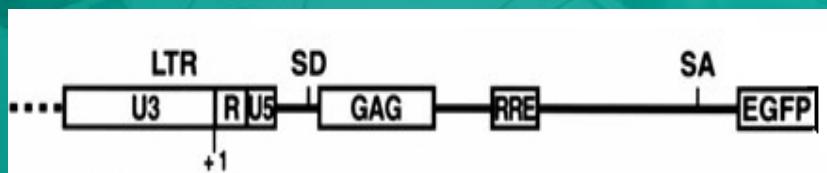
- The Usage of GFP

Biosensor construct	GFP type	Fluorescence measurement	Event detected
<b>Transcription reporter biosensors</b>			
NF-κB-dEGFP	d2EGFP	Intensity change	TNF or IL-1 signalling
AP-1-dEGFP	d2EGFP	Intensity change	GF stimulation or AP-1 activation
SRE-dEGFP	d2EGFP	Intensity change	Serum stimulation or SRF activation
GRE-dEGFP	d2EGFP	Intensity change	Glucocorticoid stimulation
CRE-dEGFP	d2EGFP	Intensity change	cAMP stimulation or PKA activation
NFAT-dEGFP	d2EGFP	Intensity change	Ca <sup>2+</sup> stimulation or NFAT activation
HRE-dEGFP	d2EGFP	Intensity change	Stress or heat stimulation
p53-dEGFP	d2EGFP	Intensity change	DNA damage or tumour suppressor activation
Myc-dEGFP	d2EGFP	Intensity change	Cell growth
HIV-1 LTR-GFP	EGFP	Intensity change	HIV infection

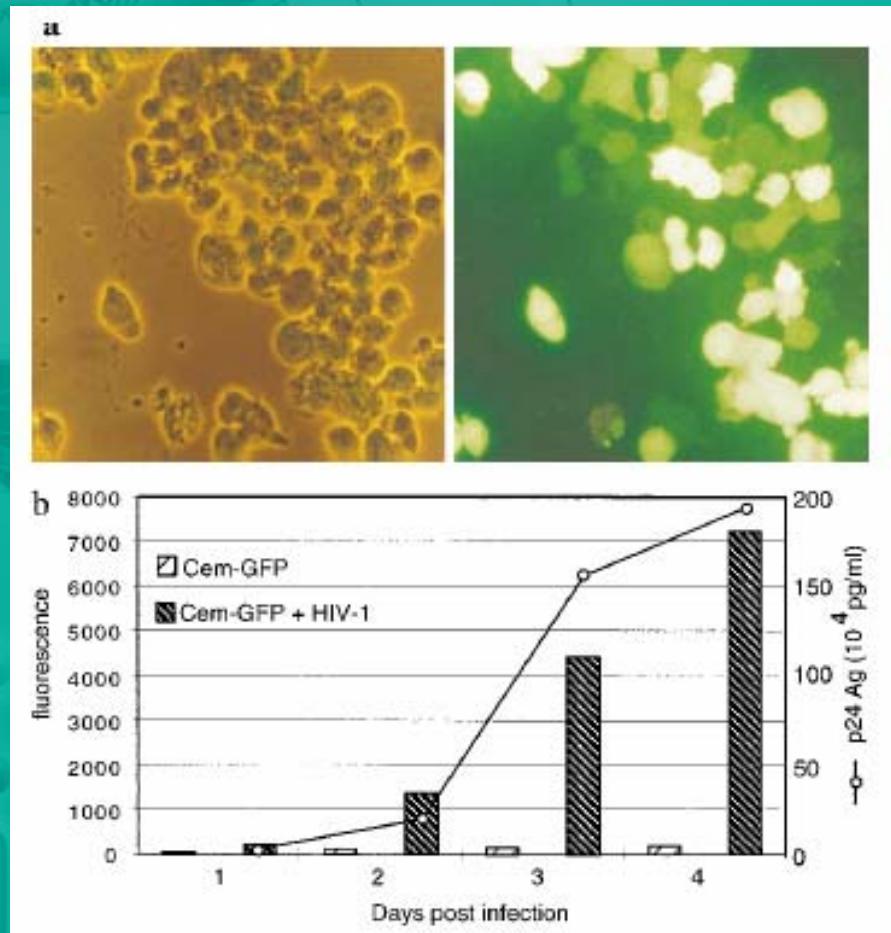


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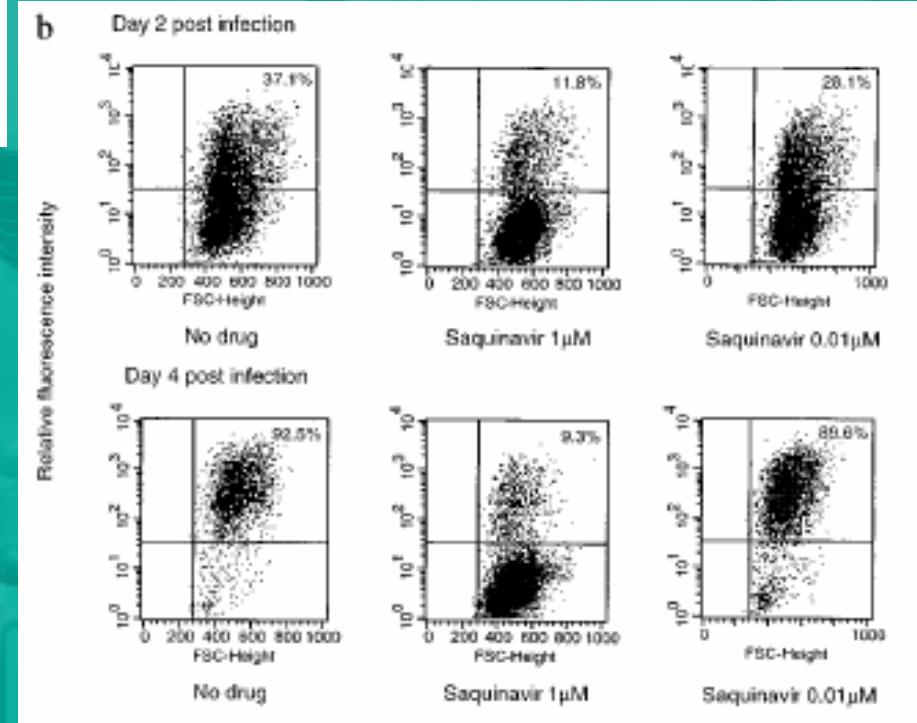
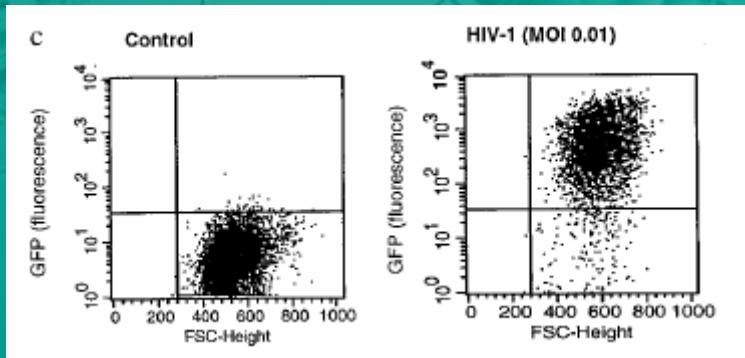
# GFP as a Reporter



**Gervaux, A. et. al. 1997.** A new reporter cell line to monitor HIV infection and drug susceptibility in vitro. PNAS vol. 94.



# GFP as a Reporter



# 實驗設計原理

- 藉由螢光抗體標識細胞之抗原特性
- 藉由螢光化合物標識細胞特性
- 藉由螢光染劑標識細胞特性
- Cytometry Beads Array
  - Cytokine detection
  - Active kinase profile
  - Fusion protein detection



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# Cytometric Beads Array (CBA)



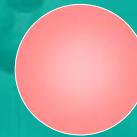
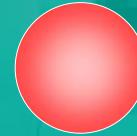
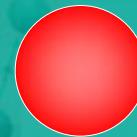
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# Beads Provide a Flexible Platform

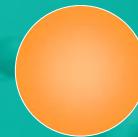
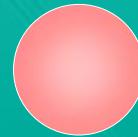
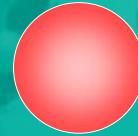
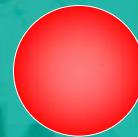
Beads provide an expandable assay platform for use with a flow cytometer



Multiple sizes



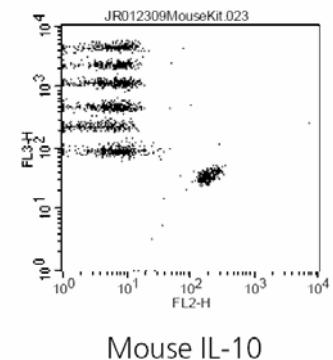
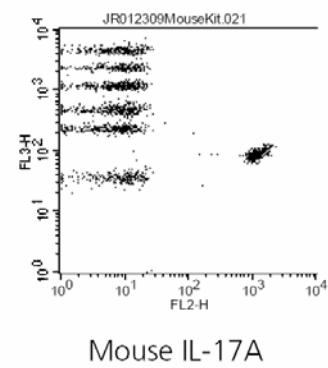
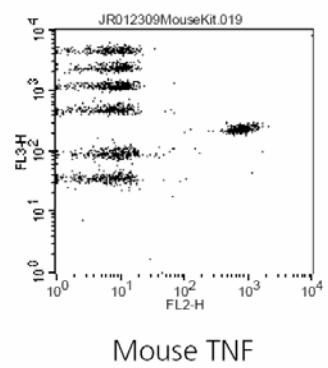
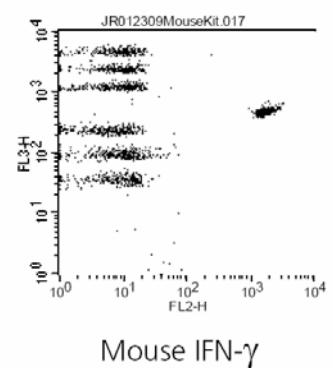
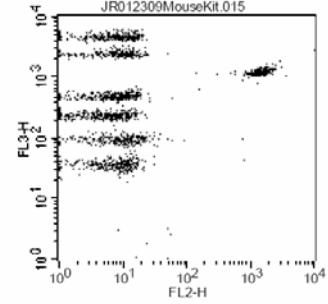
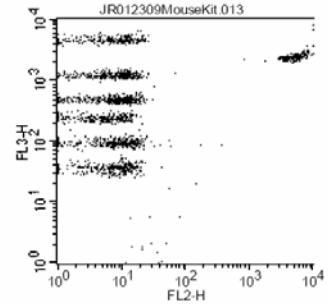
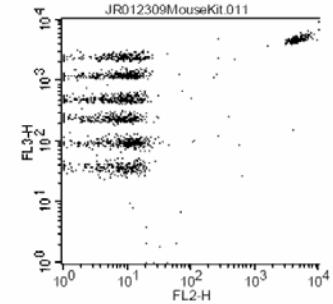
Different intensities\*



Different colors with different intensities



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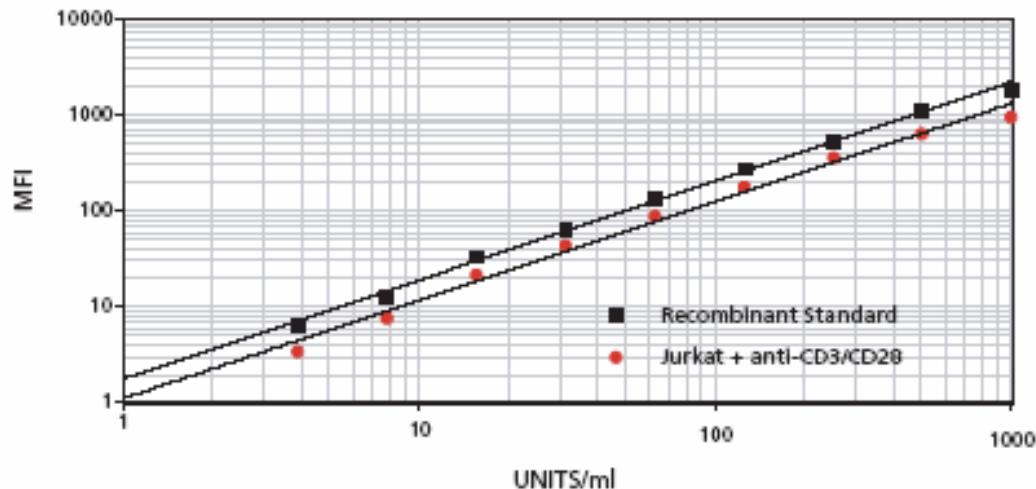
## Proteins Measured

- A. Interleukin (IL)-2
- B. IL-4
- C. IL-6
- D. Tumor Necrosis Factor
- E. Interferon- $\gamma$
- F. IL-17a
- G. IL-10

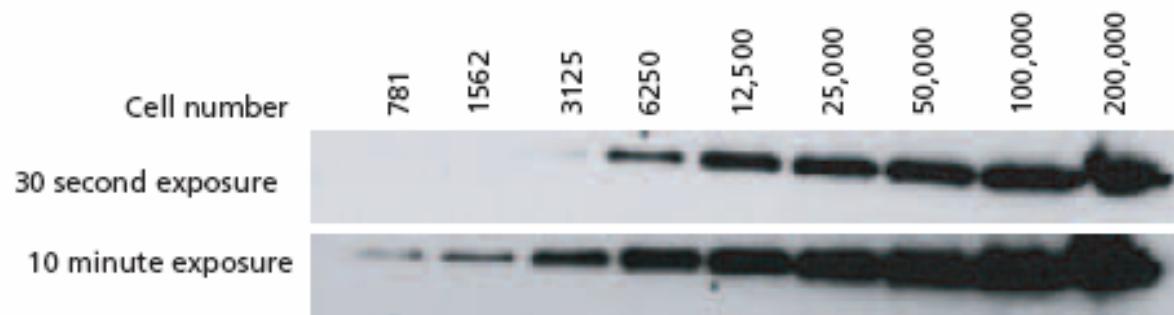


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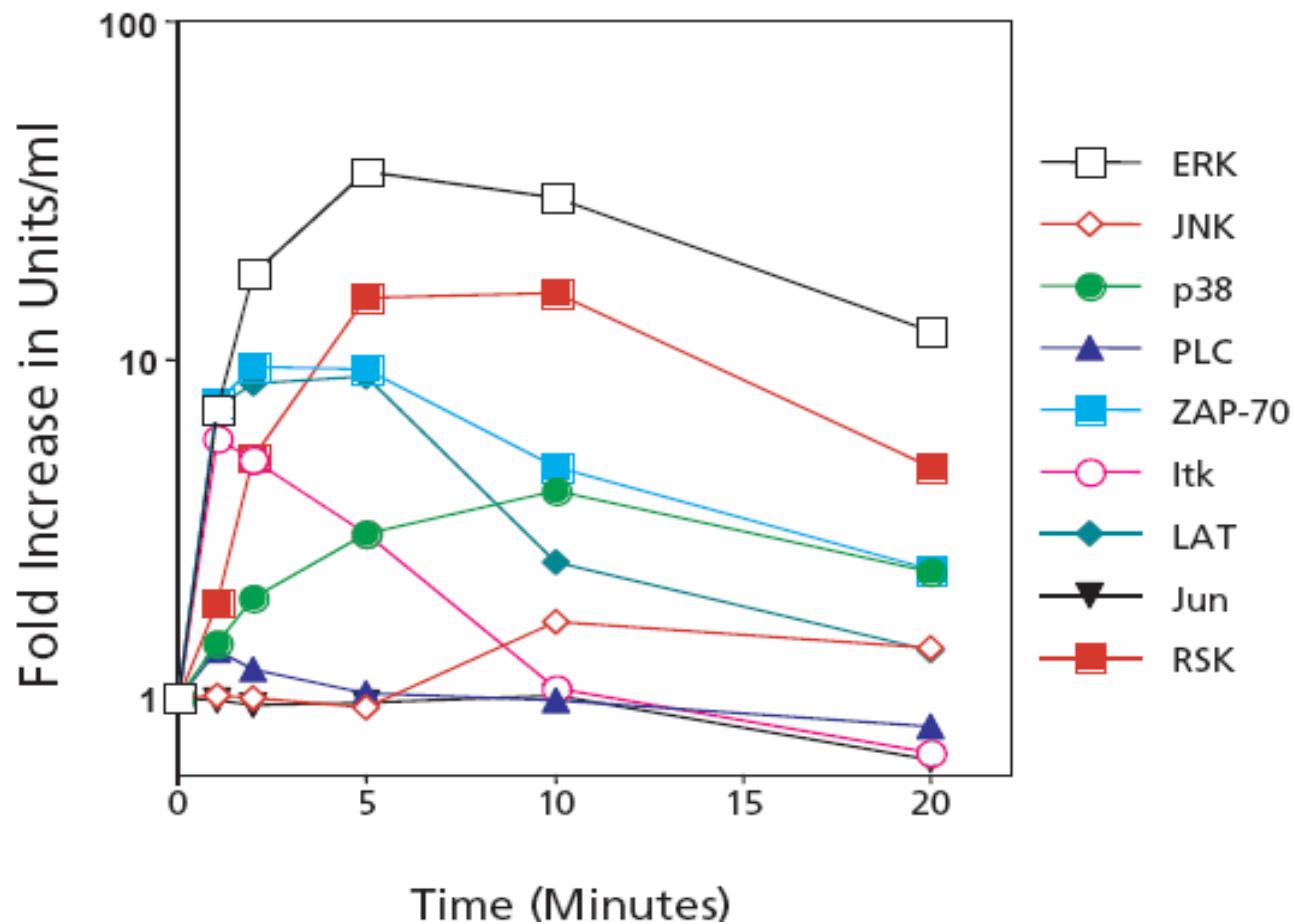
CBA



## Western Blot

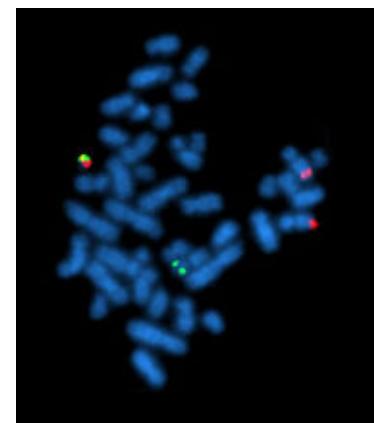
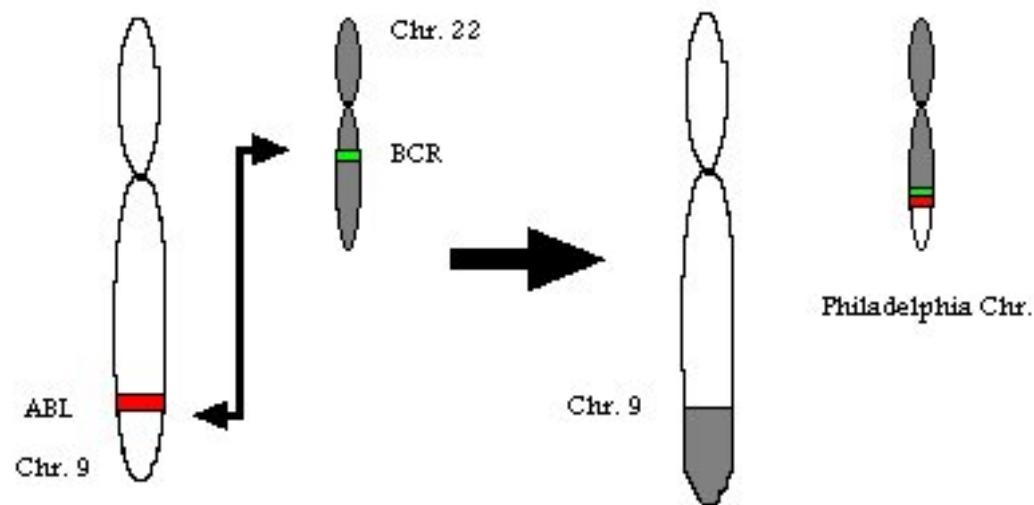


# Kinetic analysis of T cell activation by anti-CD3/CD28



# Philadelphia chromosome

- The presence of this translocation is a highly sensitive test for CML, since 95% of people with CML have this abnormality



# Philadelphia chromosome

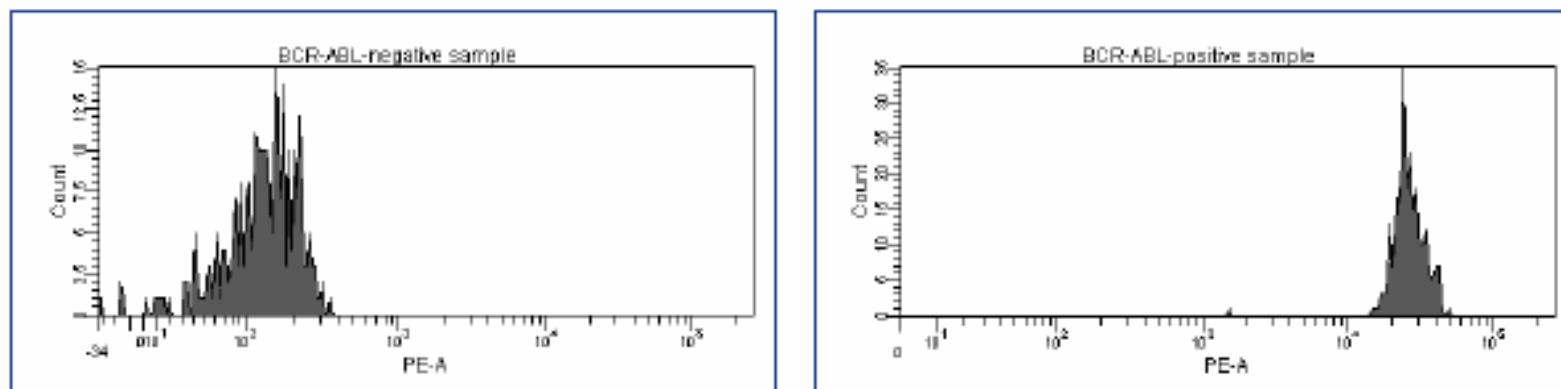
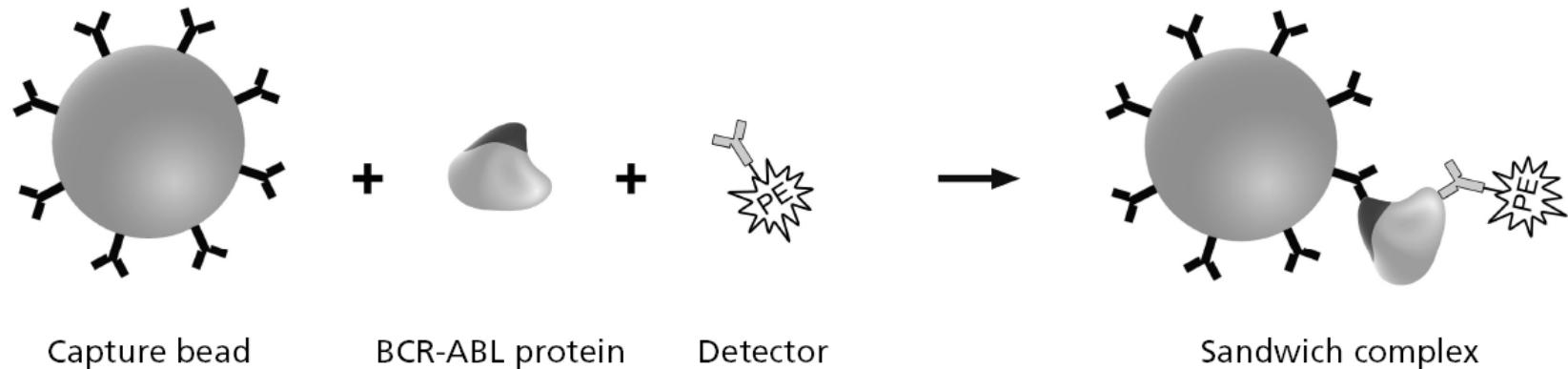
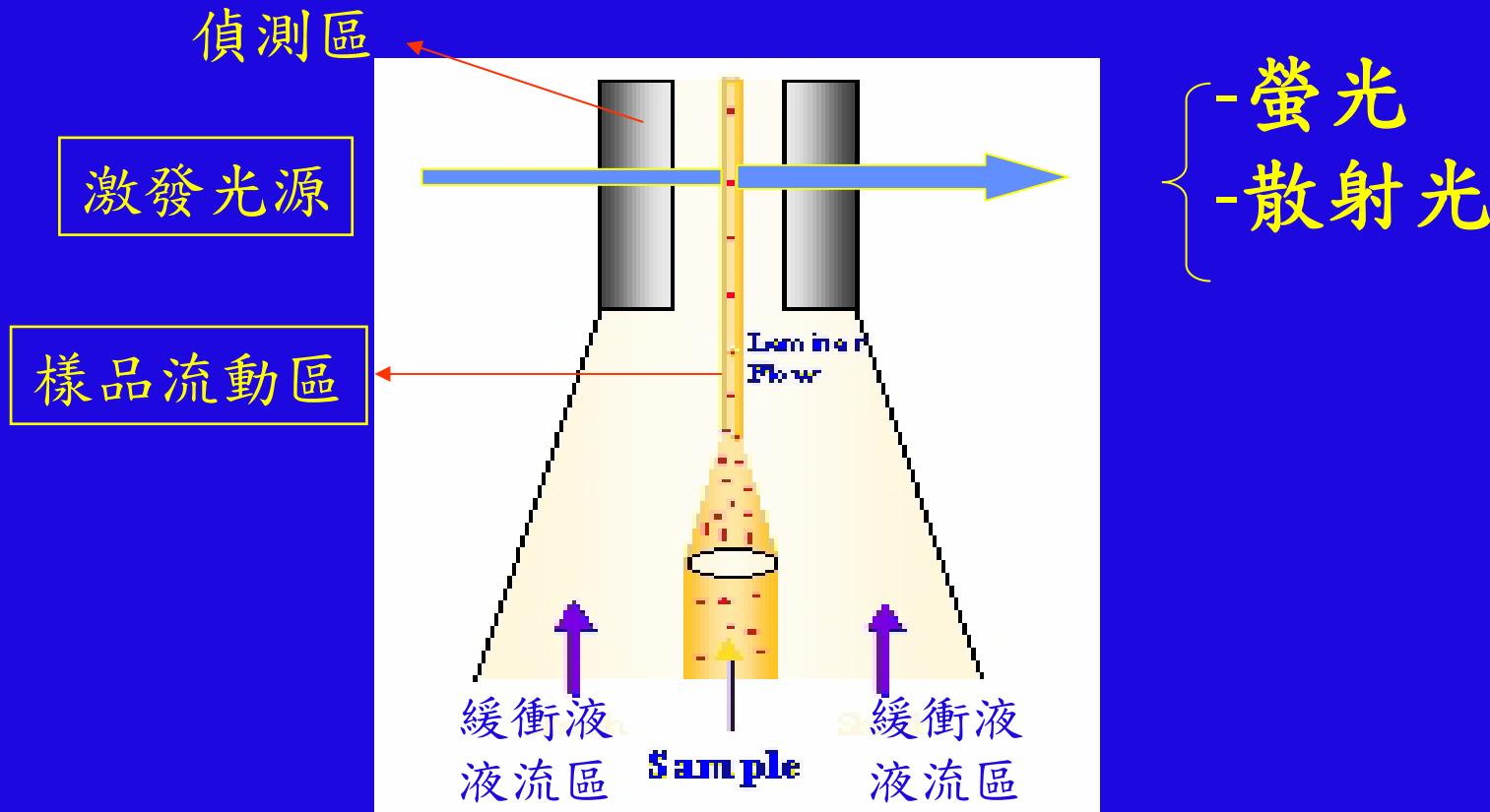


Figure 1. Once serum is prepared using the BD BCR-ABL Protein Kit and samples are run in the flow cytometer, histograms show the presence or absence of BCR-ABL fusion proteins.



# 流式細胞儀的工作原理 -Hydro-dynamic Focusing



# 流式細胞儀能測量：

- 散射光
  - 細胞大小 (前方散射光)
  - 細胞折射率 (側方散射光)
- 各色螢光

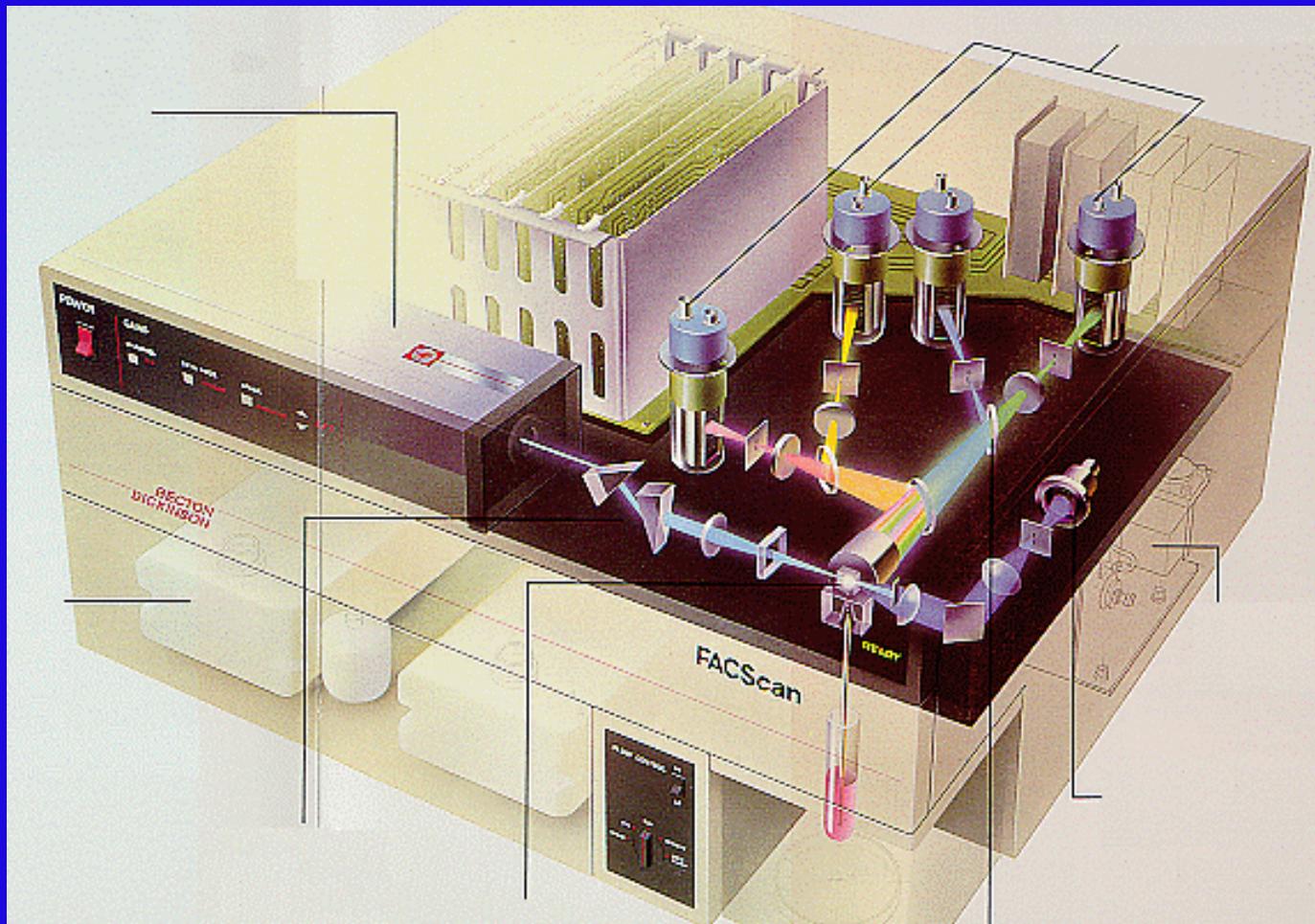


# 綜合三個系統的功能：

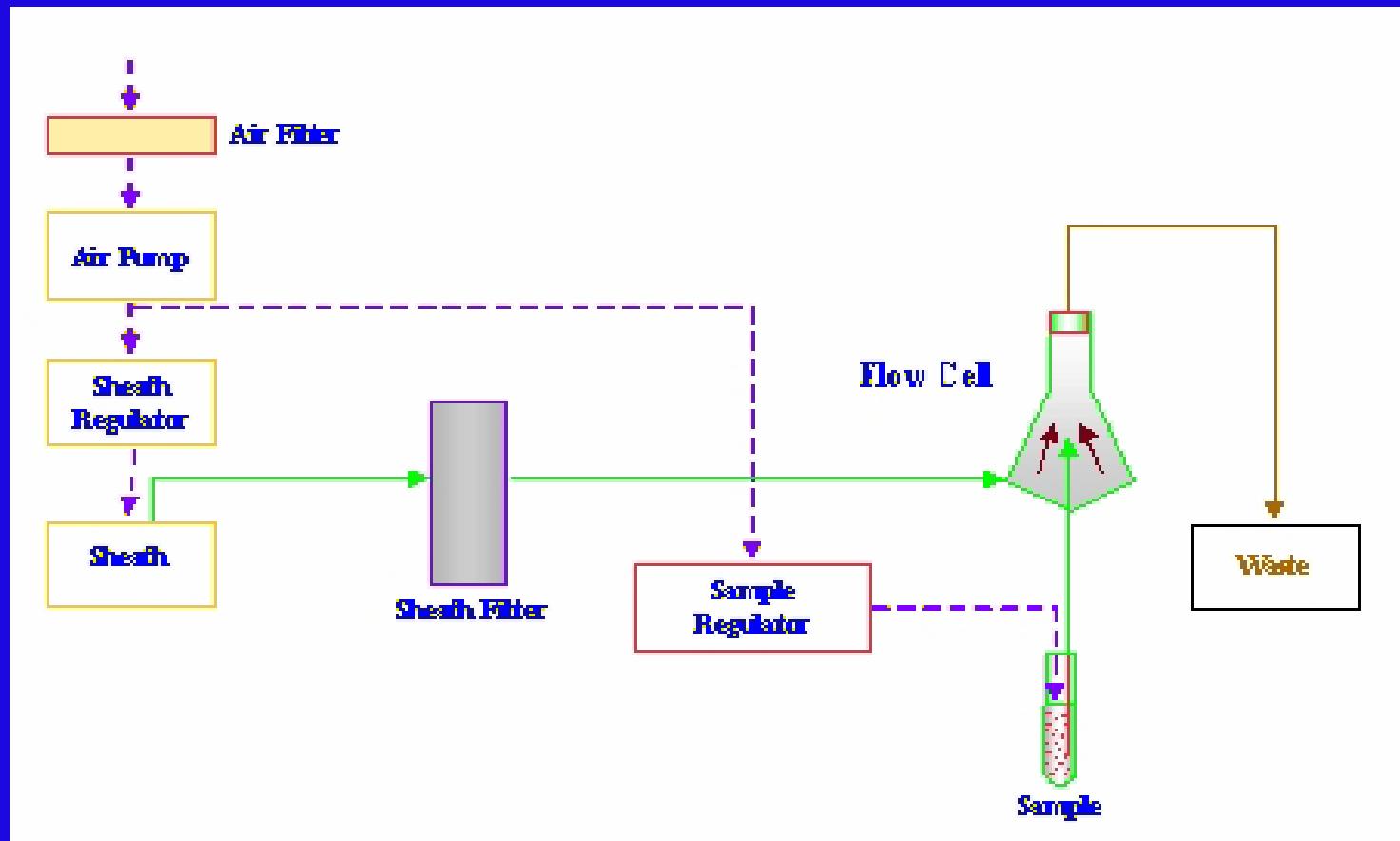
- 液流系統：將細胞依序送到測量區受檢。
- 光學系統：產生並收集螢光、光散射等信號。
- 電子系統：
  - 將光學訊號轉換成電子訊號。
  - 分析所輸出的電流訊號，以脈衝高度、寬度、積分面積顯示。
  - 量化訊號並傳至電腦。



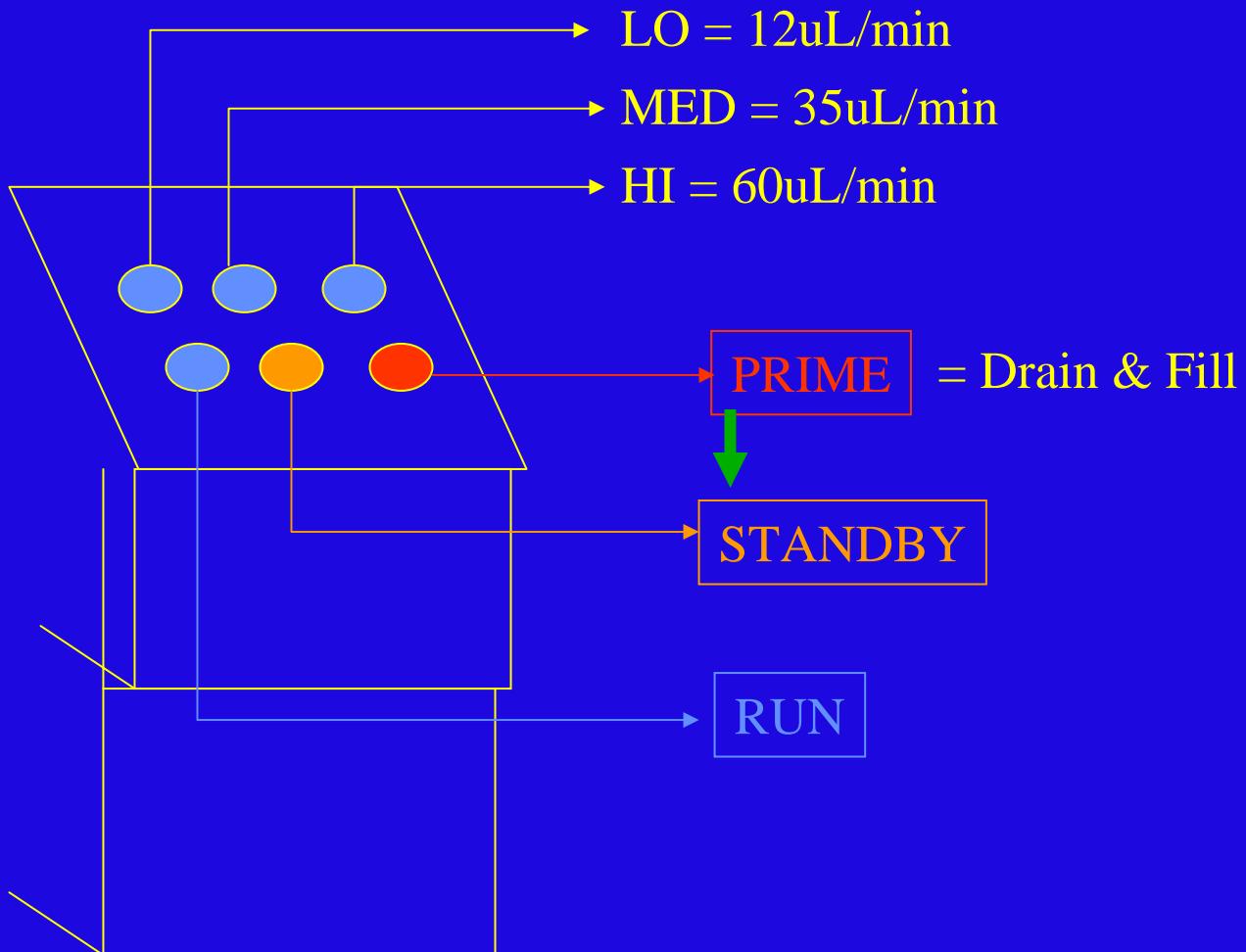
# 綜合三個系統的功能-液流系統



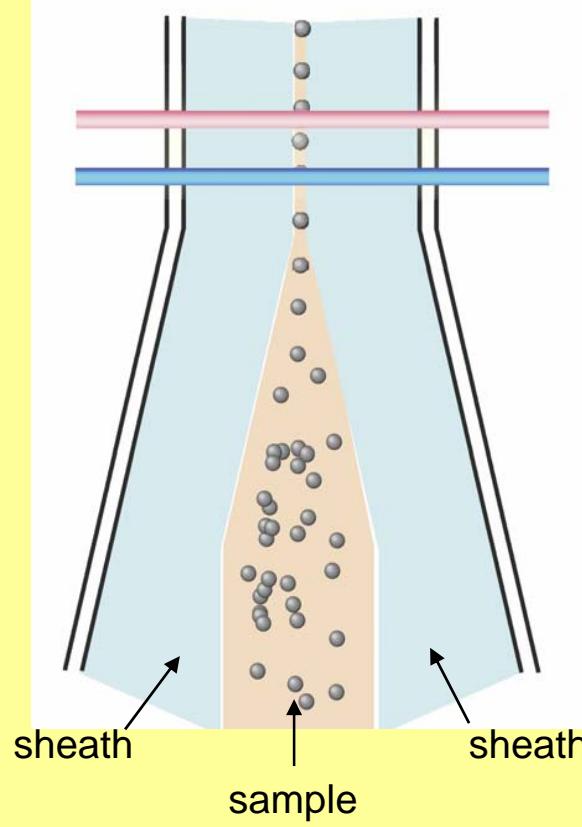
# FACS Calibur 的液流系統



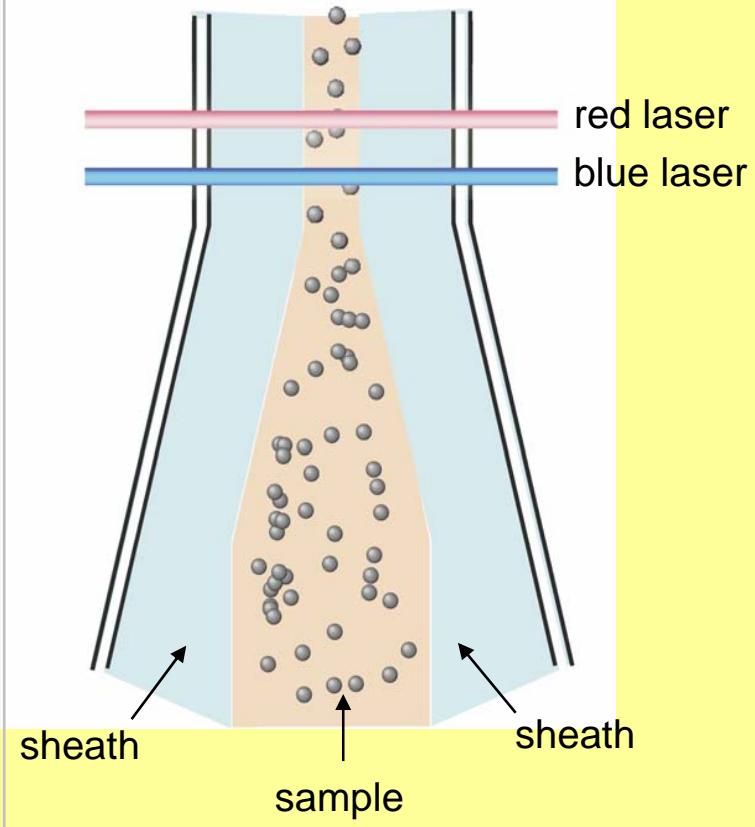
# FACS Calibur 儀表板



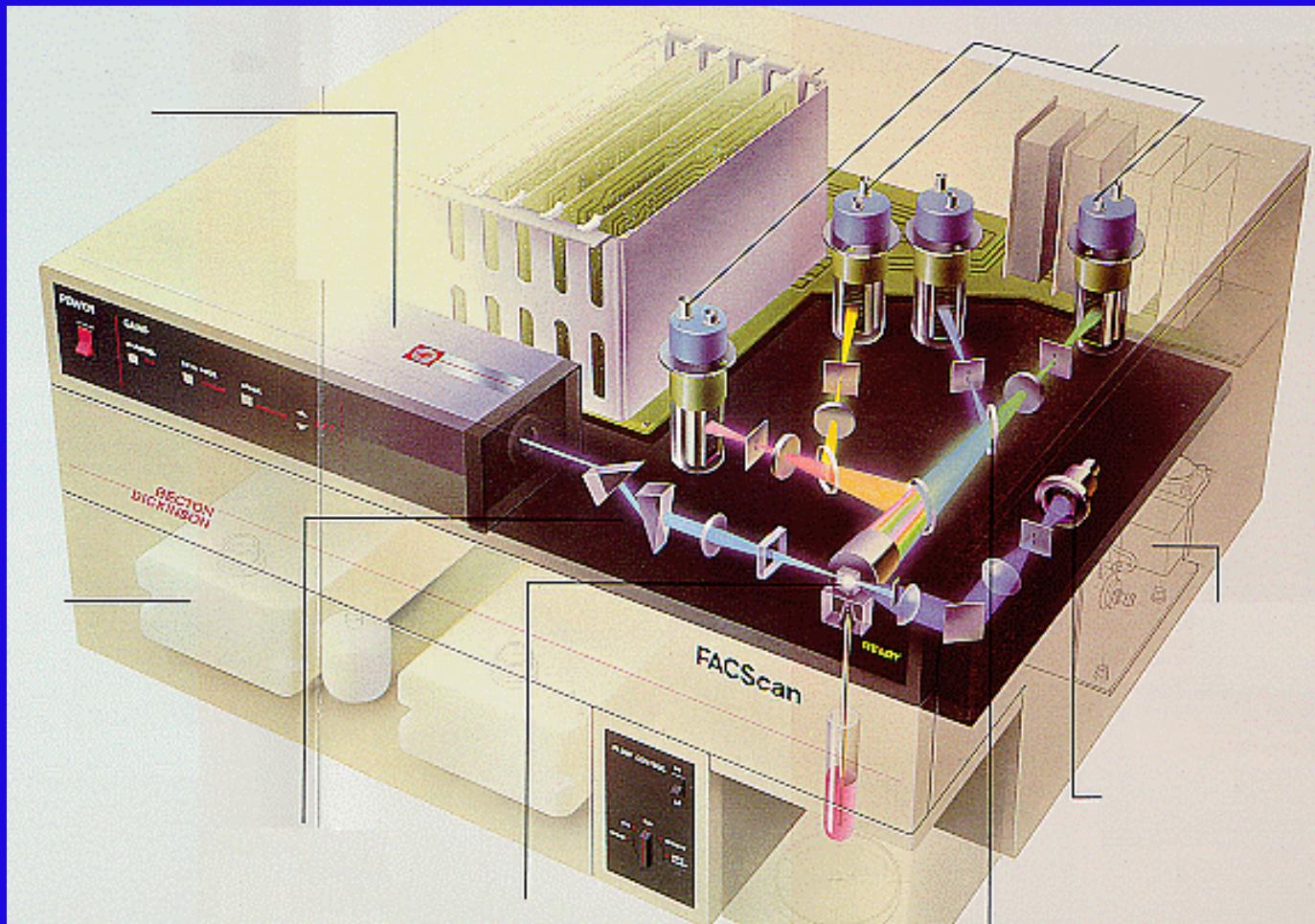
### Low Sample Pressure



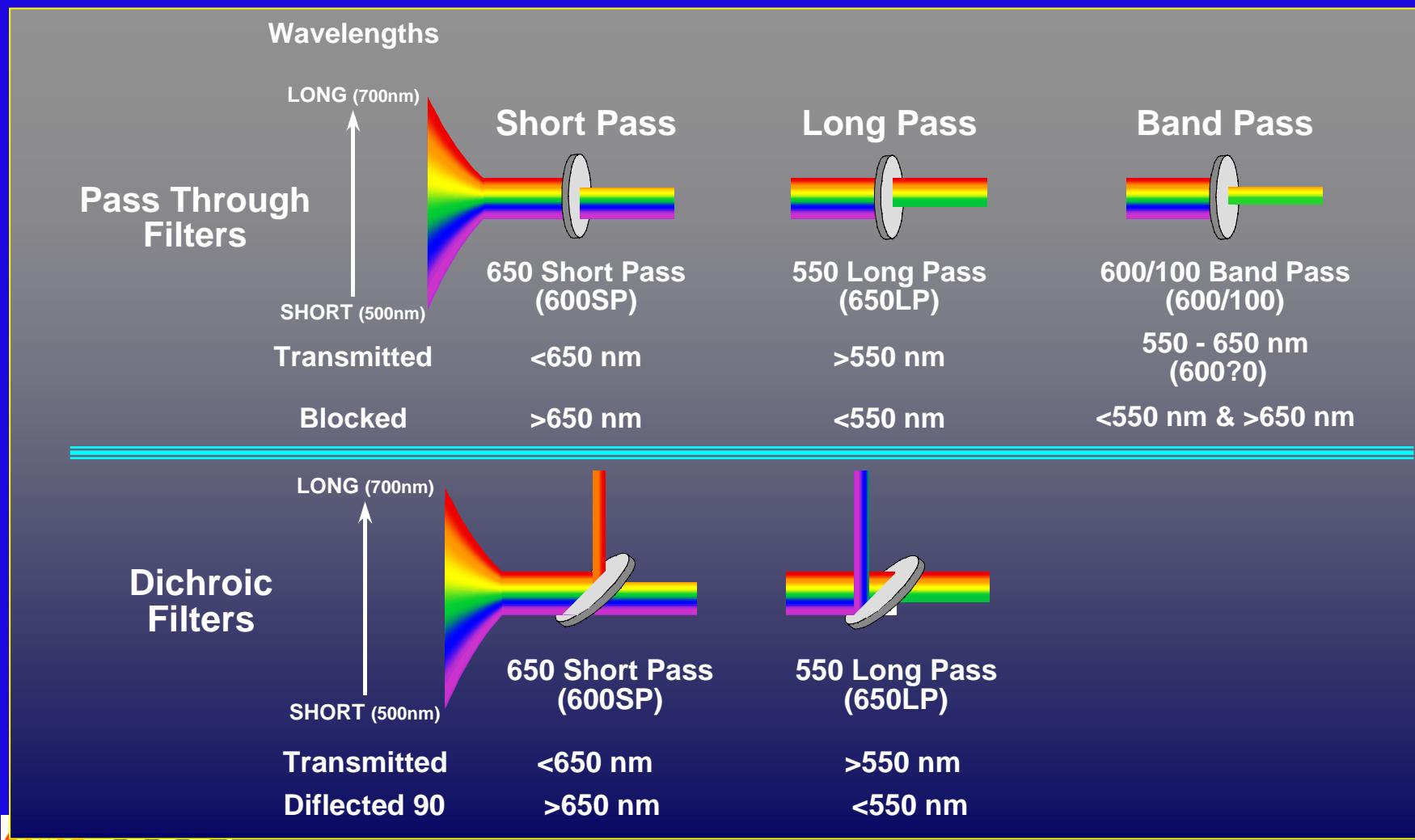
### High Sample Pressure



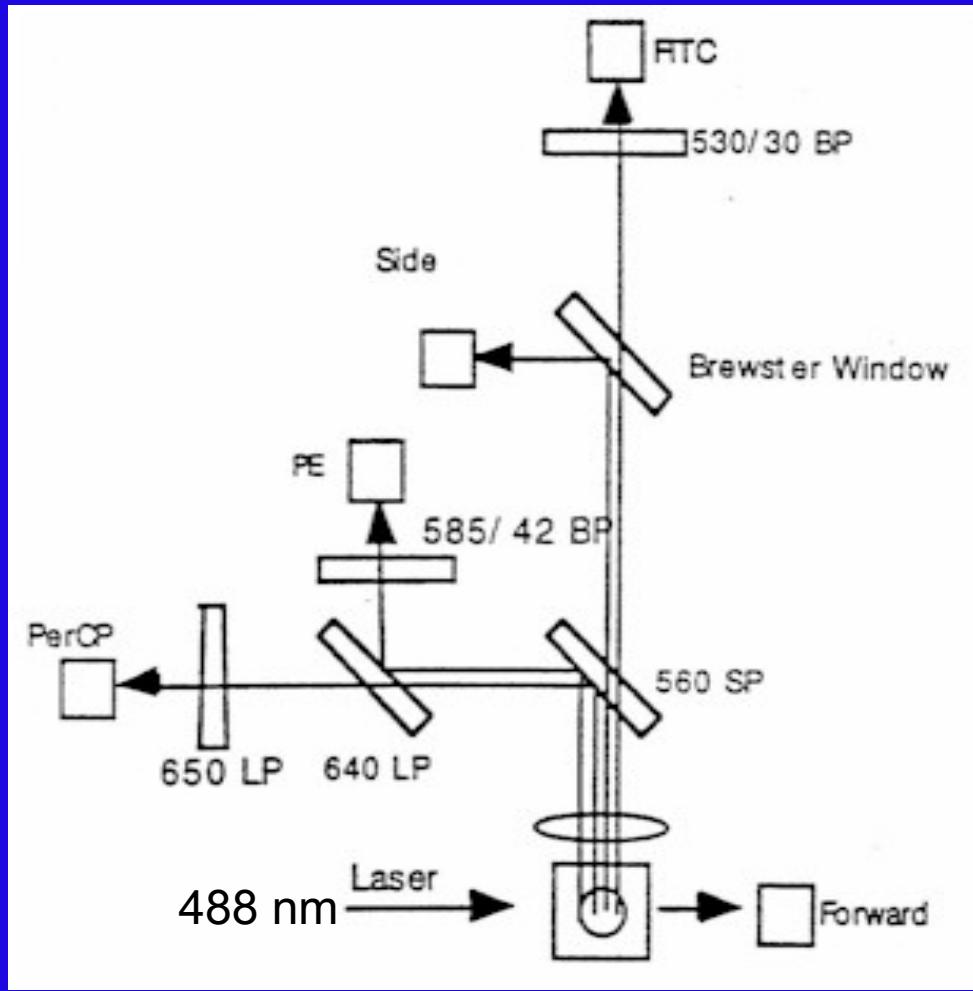
# 綜合三個系統的功能-光學系統



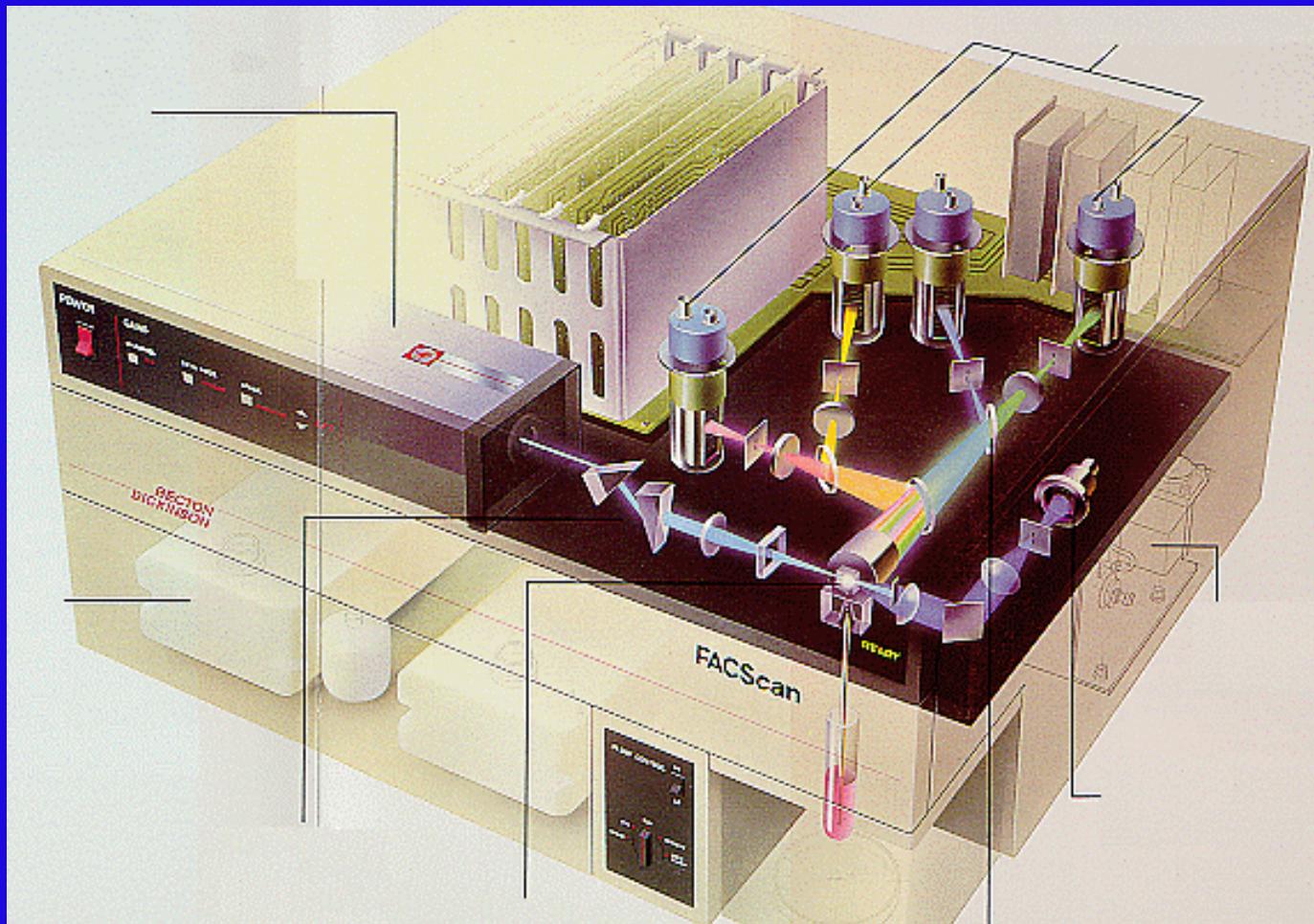
# 螢光濾片



# FACS Calibur 的光學系統



# 綜合三個系統的功能-電子系統



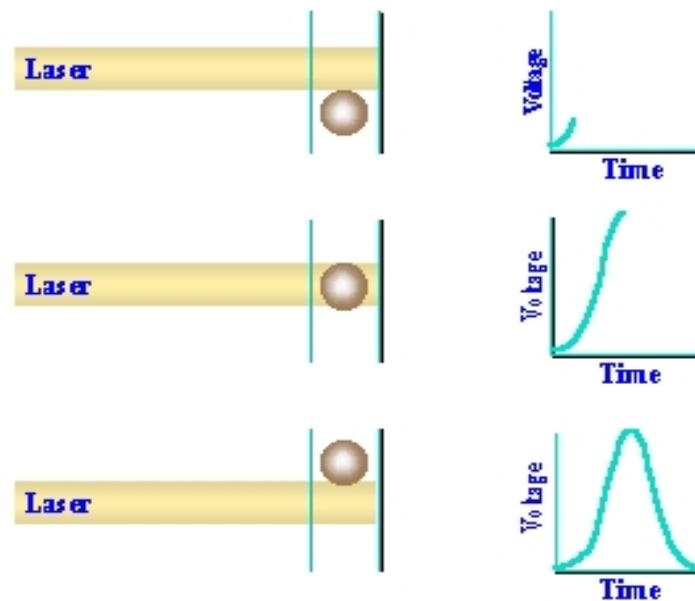
# 電子系統

- 將光學訊號轉換成正比例的電子訊號。
- 分析所輸出的電子訊號，以脈衝高度、寬度、積分面積顯示。
- 將量化訊號傳至電腦。



# 電子系統

## Creation of a Voltage Pulse

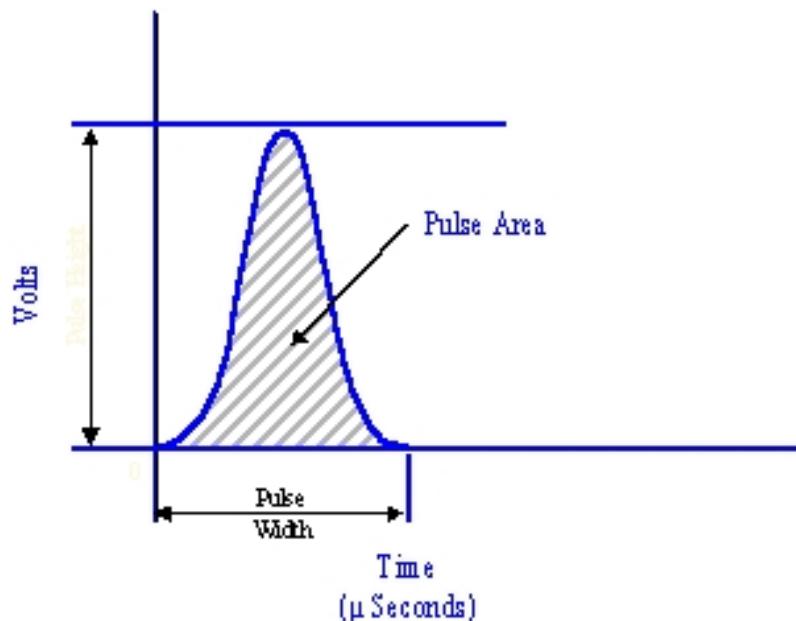


FACS  
Academy

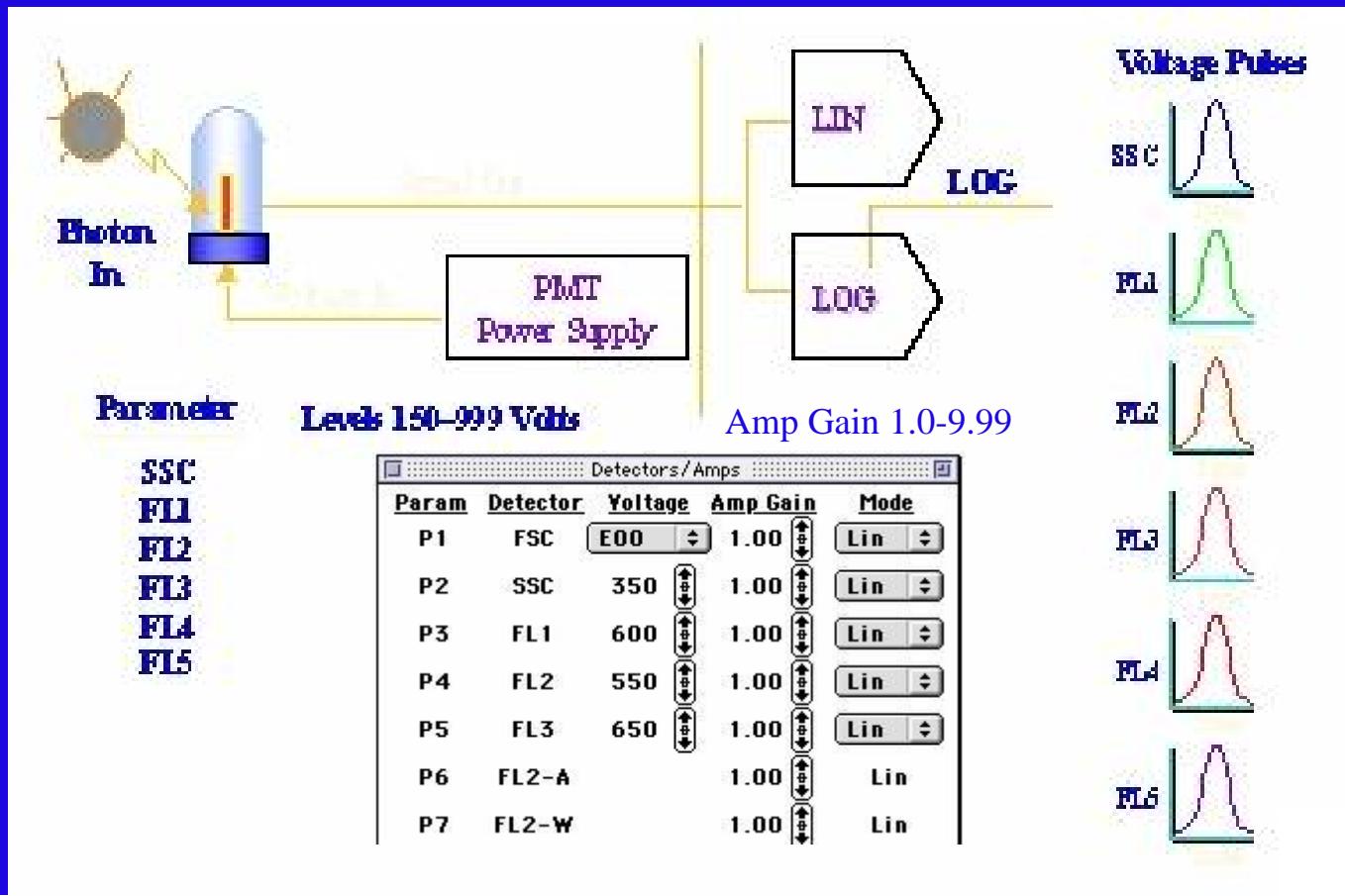
12

# 電位脈衝之定量

## Quantification of a Voltage Pulse

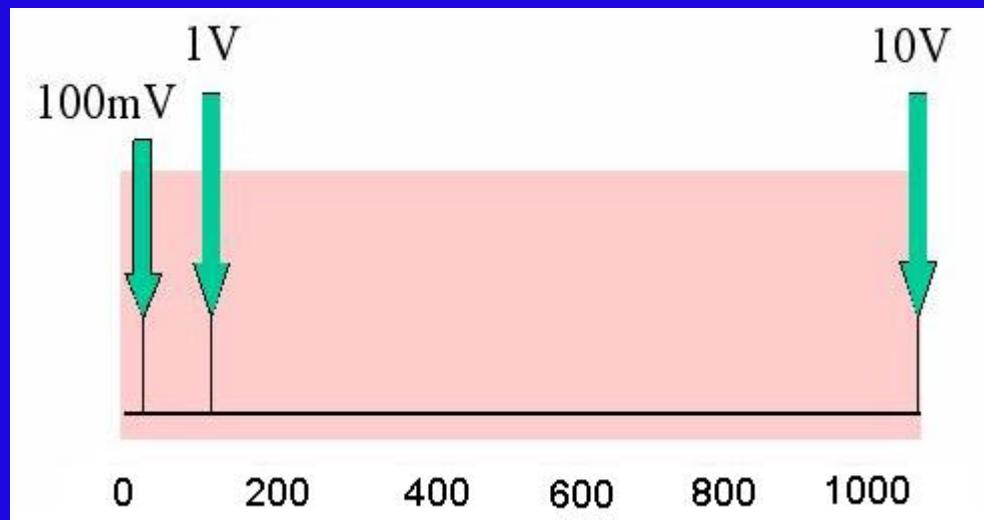


# 訊號之產生與處理

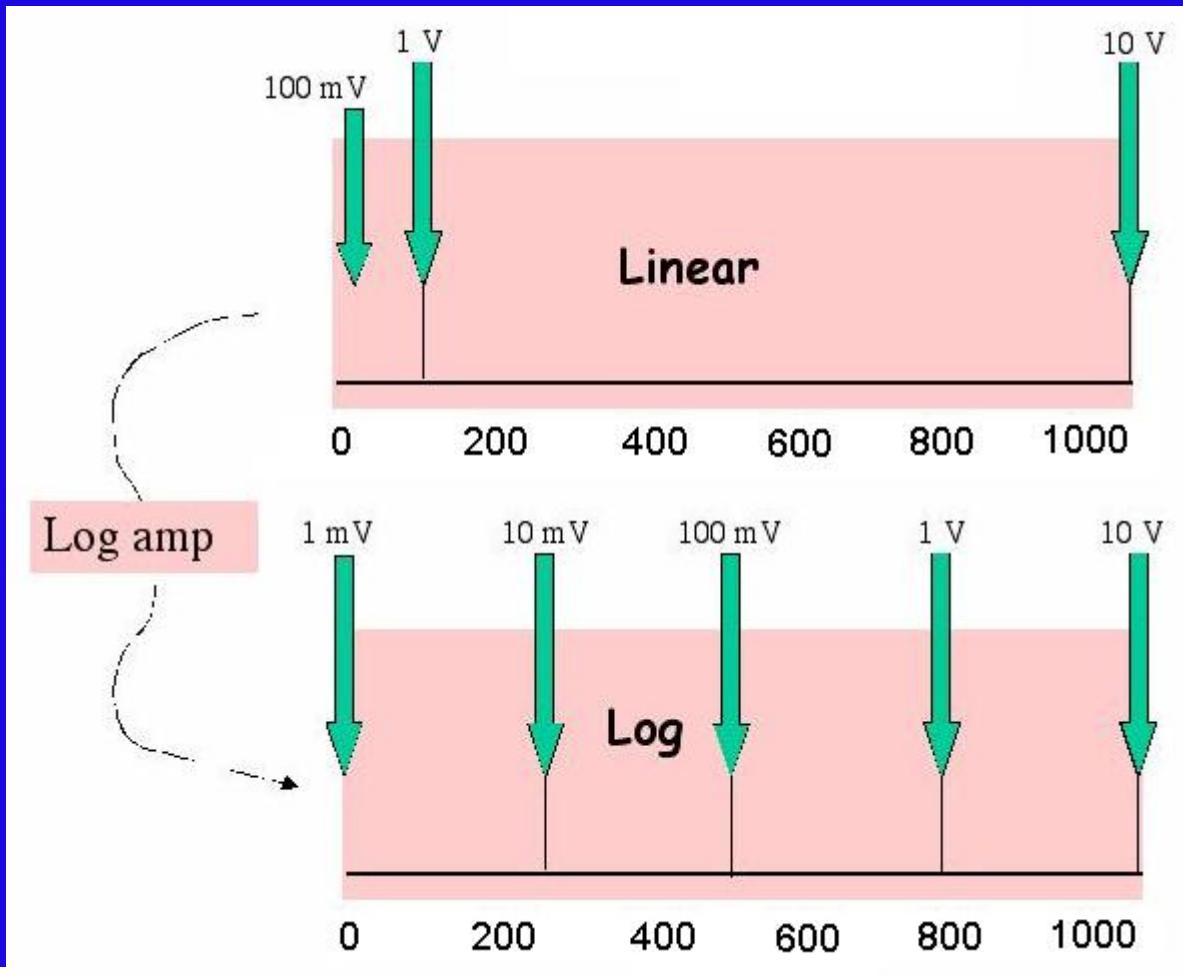


# The Use of Linear Amplification

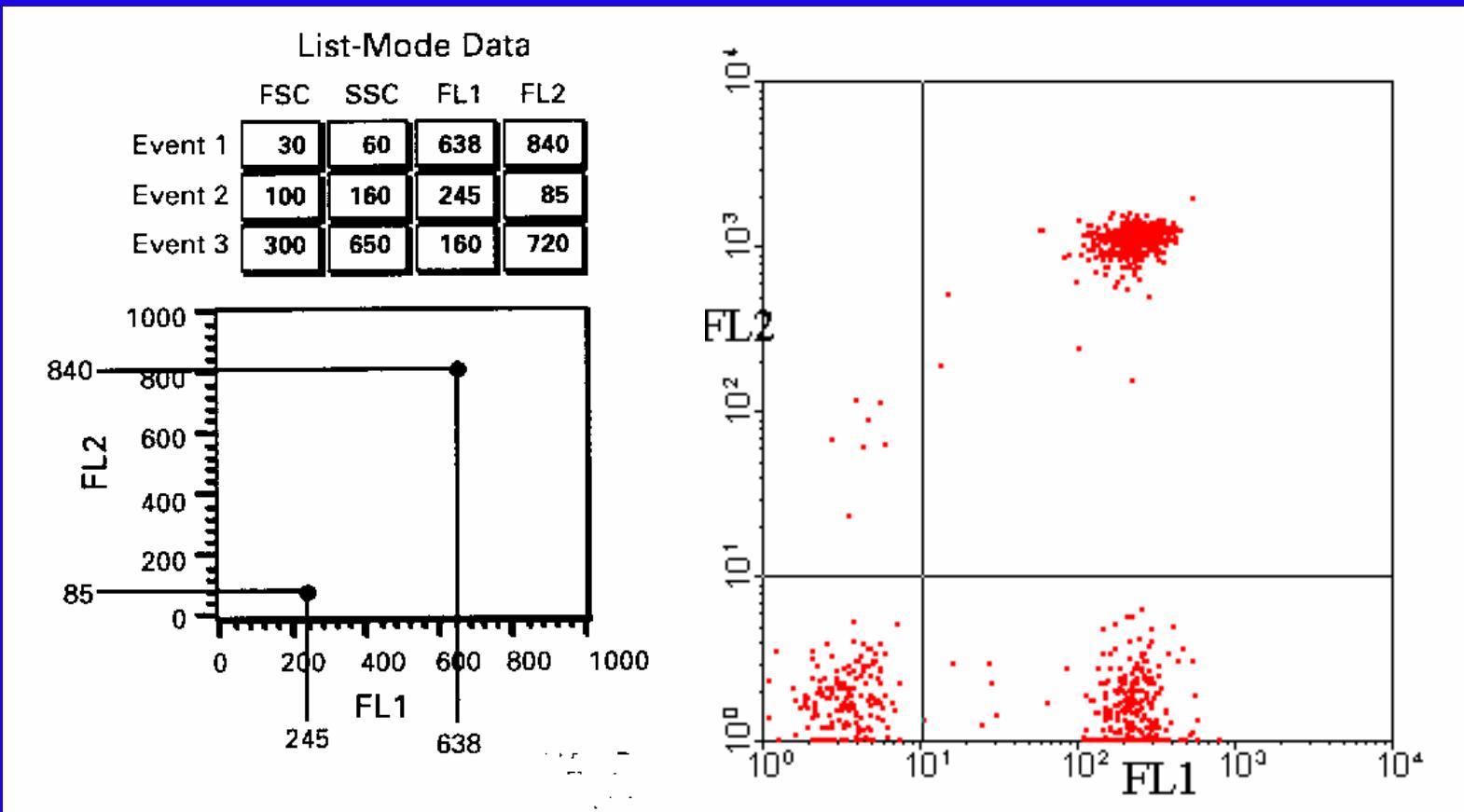
Assume we convert linear analog signals using a 10 bit ADC--we have 1024 channels of range corresponding to 0-10 V.  
Channel difference is  $10V/1024 = 0.01V$



# Ideal Lin To Log Conversion

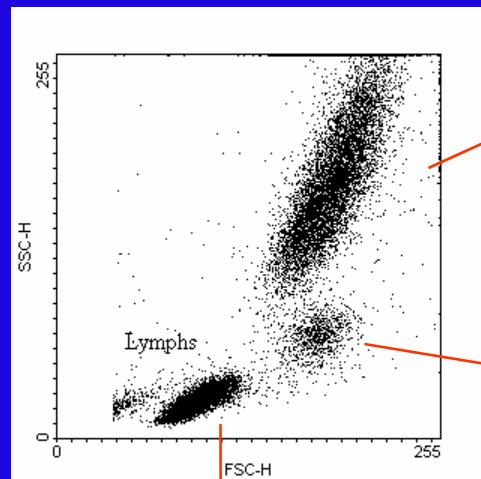


# 實驗數據的呈現



# 常見的數據呈現

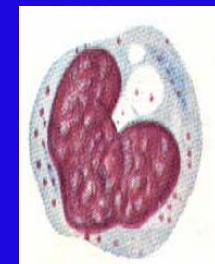
散點圖反映細胞形態



8 to 10  $\mu\text{m}$   
Lymphocyte

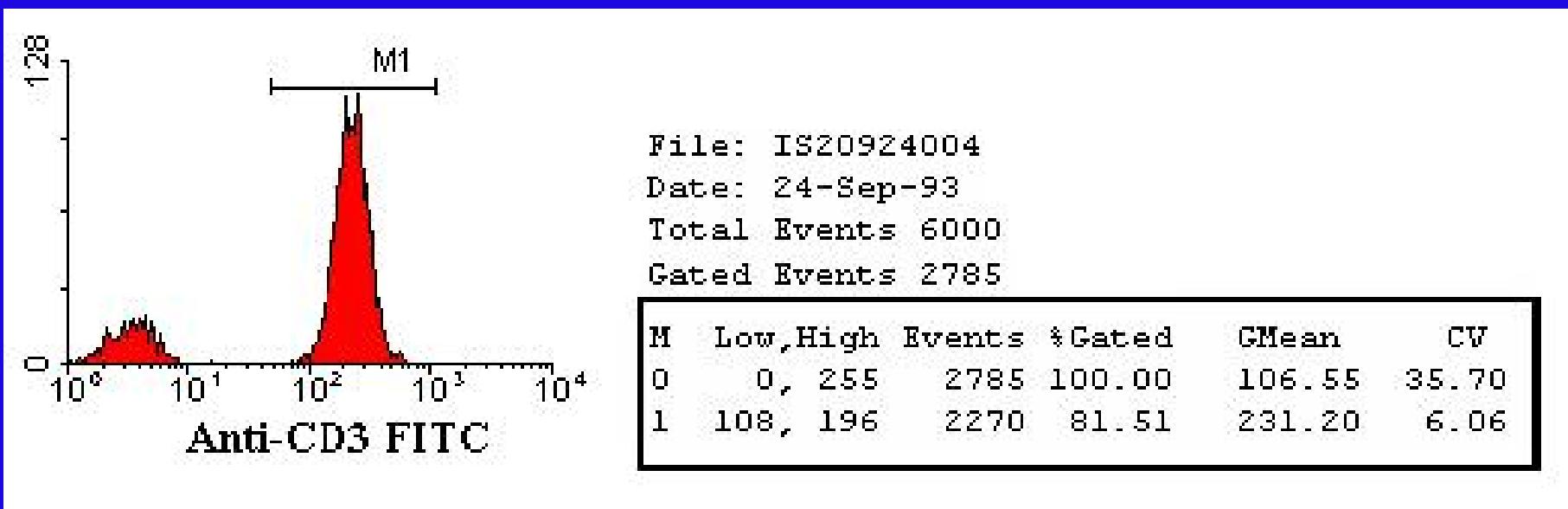


10 to 14  $\mu\text{m}$   
Neutrophil



15 to 20  $\mu\text{m}$   
Monocyte

# 直方圖分析報告



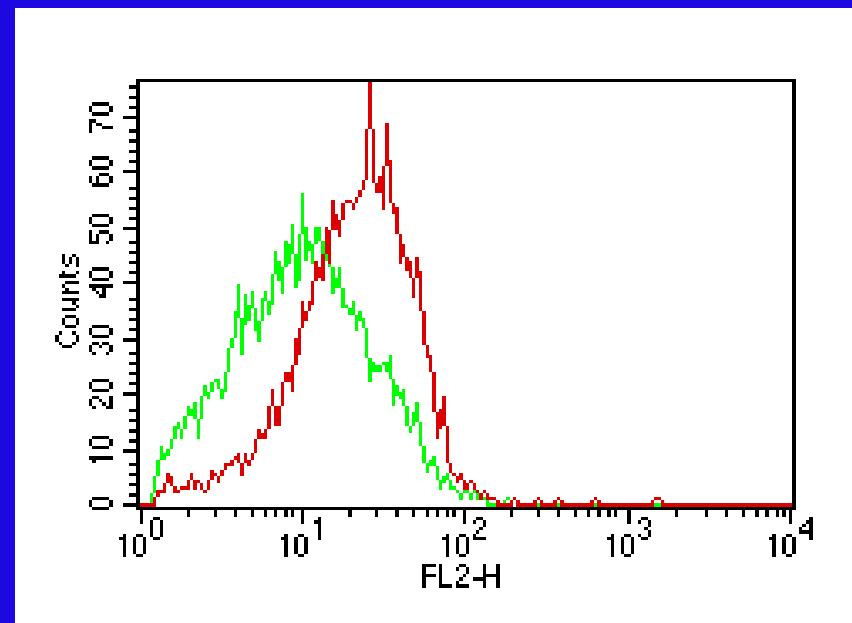
CV=S.D./Mean



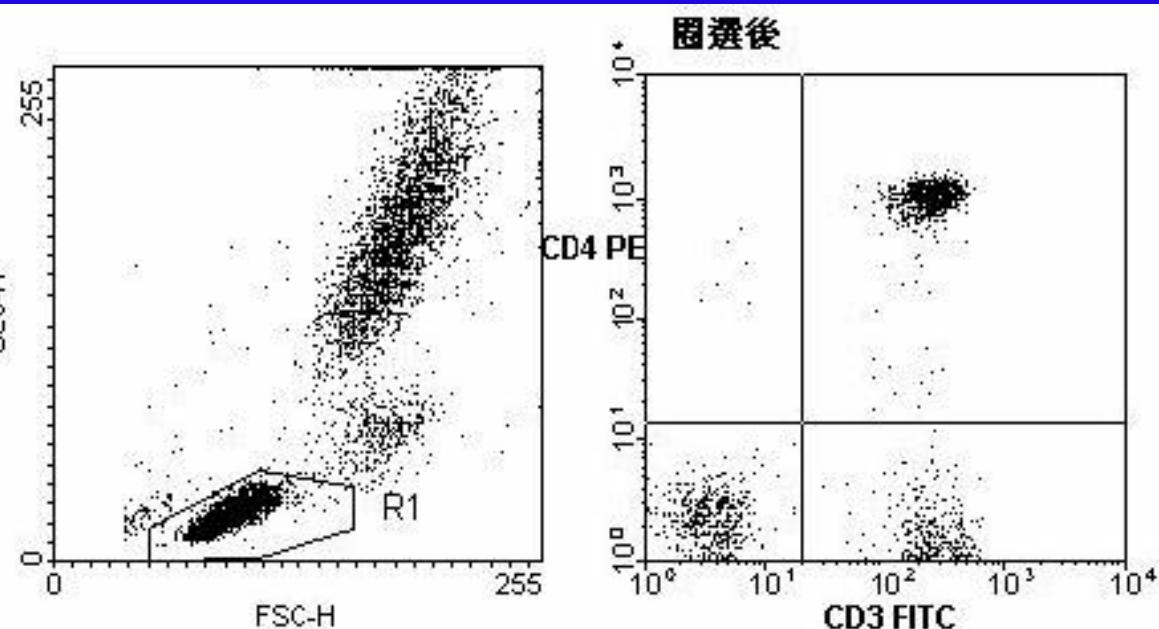
# 直方圖分析報告例二

## 細胞螢光強度

- Mean
  - Mean
  - Geo Mean
- Median (50th)
- Mode



# 散點圖分析報告



## 四象限統計 Quadrant Statistics

Gates:	R1			
Gated Events:	2354			
Quad Stats				
FL1-H(Log) vs FL2-H(Log)				
Quadrant x,y:	83,72			
Quad	X-Mean	Y-Mean	Events	*Gated
UL	5.2	262.6	6	0.25
UR	252.9	977.9	1139	48.39
LL	3.4	2.0	508	21.58
LR	288.1	1.3	701	29.78

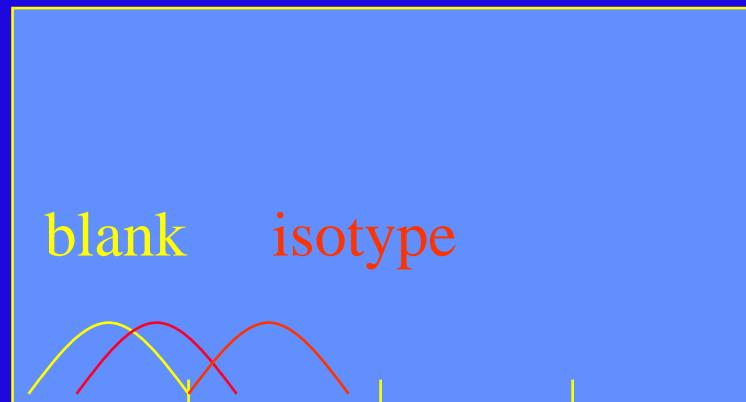
# 對照組

- 空白對照組: blank, no antibody used.
- 陰性對照組: isotypic antibody control.
- 間接法對照組: isotype + 2nd antibody.
- 陽性對照組: use experimental antibody + cell line with known positive reaction.

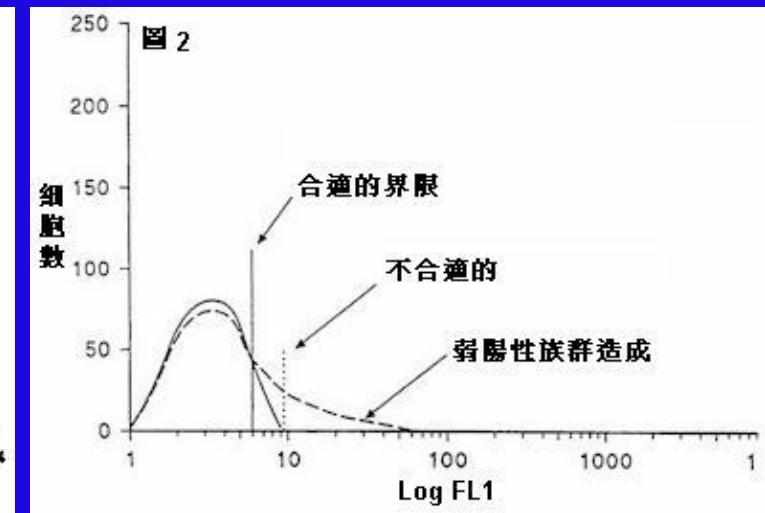
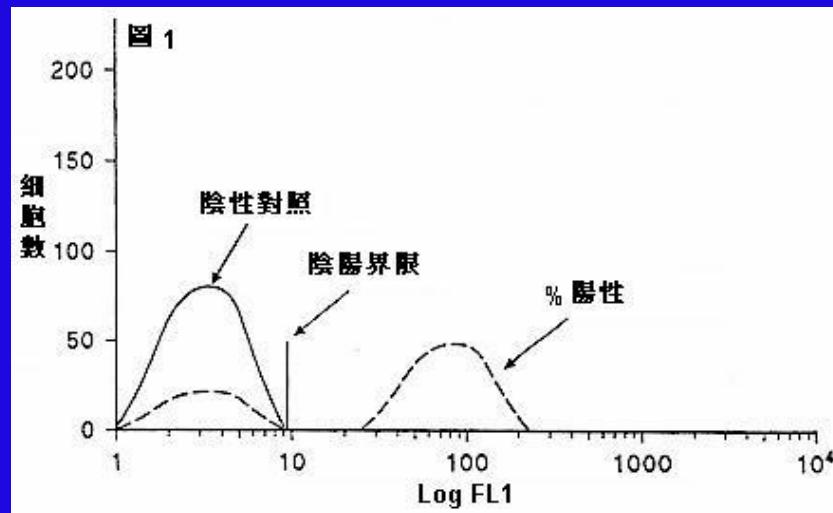


# 對照組的重要性

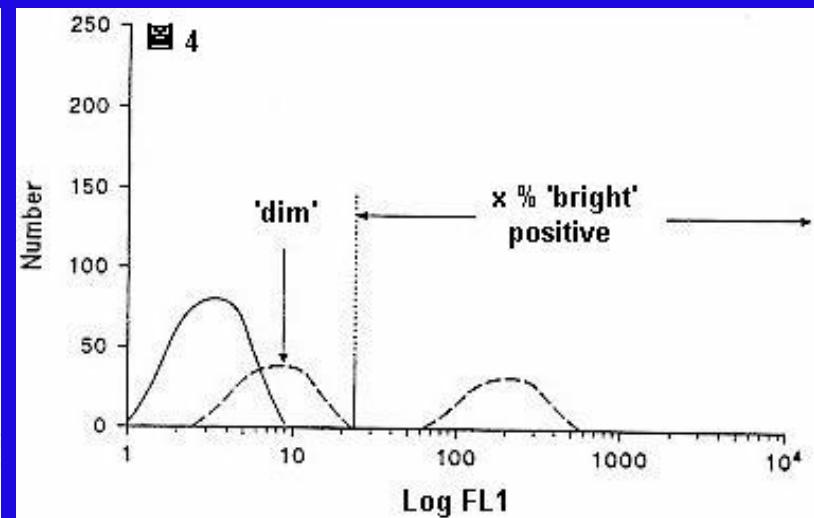
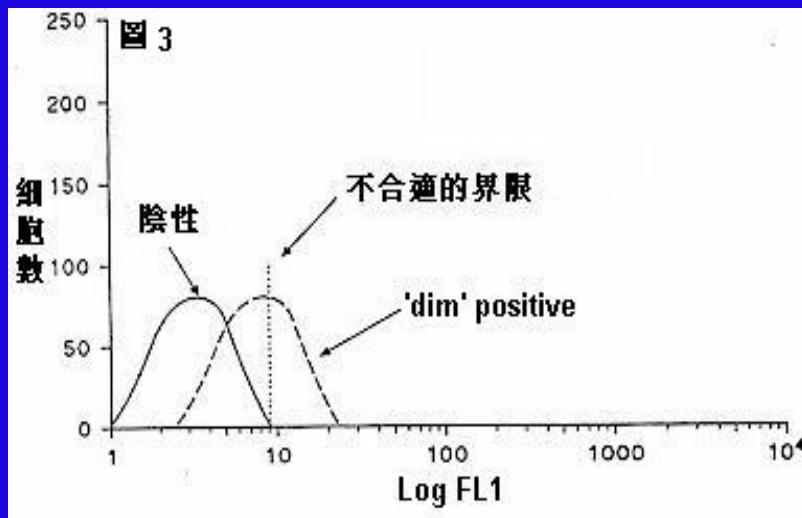
- 空白對照組: autofluorescence, instr setup.
- 陰性對照組: the extent of non-specific staining
- 間接法對照組: for indirect staining
- 陽性對照組: antibody functionality



# 陽性界限設定原則



# 陽性界限設定原則



# 公共儀器的維護



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# 流式細胞儀應用之細胞凋亡研究

# Apoptotic Phenotypes

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## Morphological disintegration

- Plasma membrane loses asymmetry (1-2 h)
- DNA fragmentation (6-8 h by TUNEL, 12-24 h by Hypoploid Analysis)
- Cell shrinkage (12-24 h)
- Chromatin condensation (18-36 h by gel analysis)
- Cells are phagocytosed before loss of membrane integrity -No inflammatory response



# **Apoptotic Phenotypes**

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## **Biochemical Phenotypic Changes**

- Calcium influx, pH changes (0.5 - 1 h)
- Energy pump shuts off (0.5 - 1 h)
- Mitochondria depolarization (1-3 h)
- Caspase-3 activation (2-3 h)

# Flow Cytometry Assays

## Live (unfixed cells)

- Annexin V-PI 分析法
- MitoScreen (JC-1)

## Fixed cells

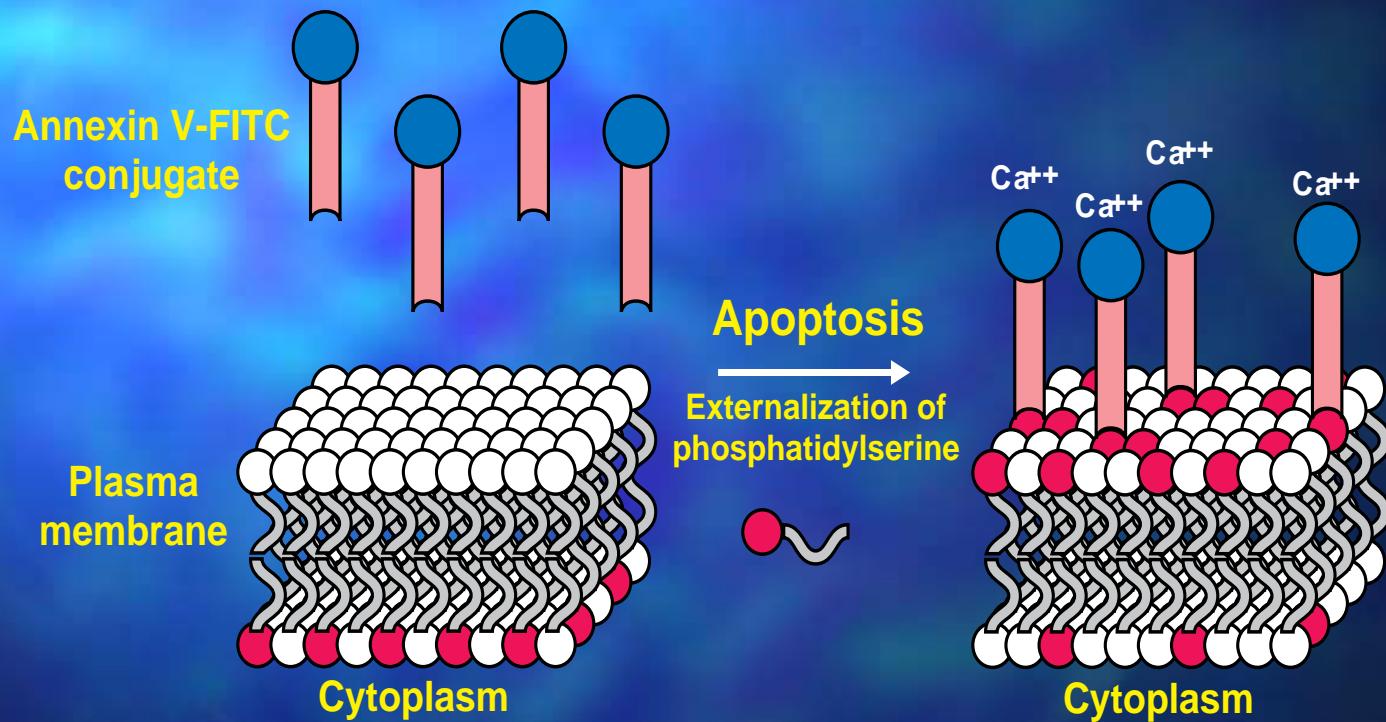
- Active Caspase-3
- APO-BRDU™ (DNA 鏈斷分析法)
- Hypoploidy Analysis (Sub-G1分析法)

## Cell Lysate

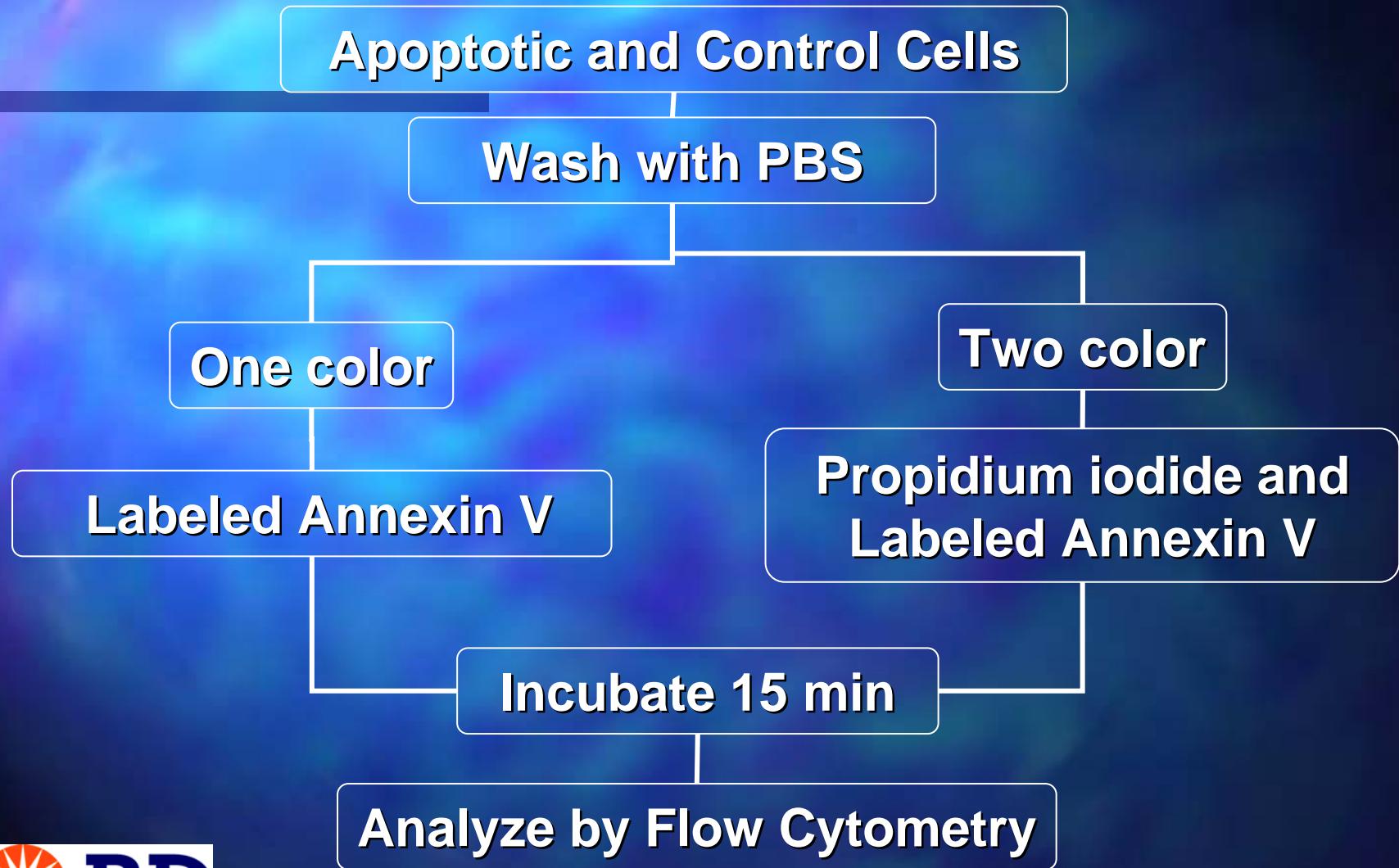


- CBA Apoptosis

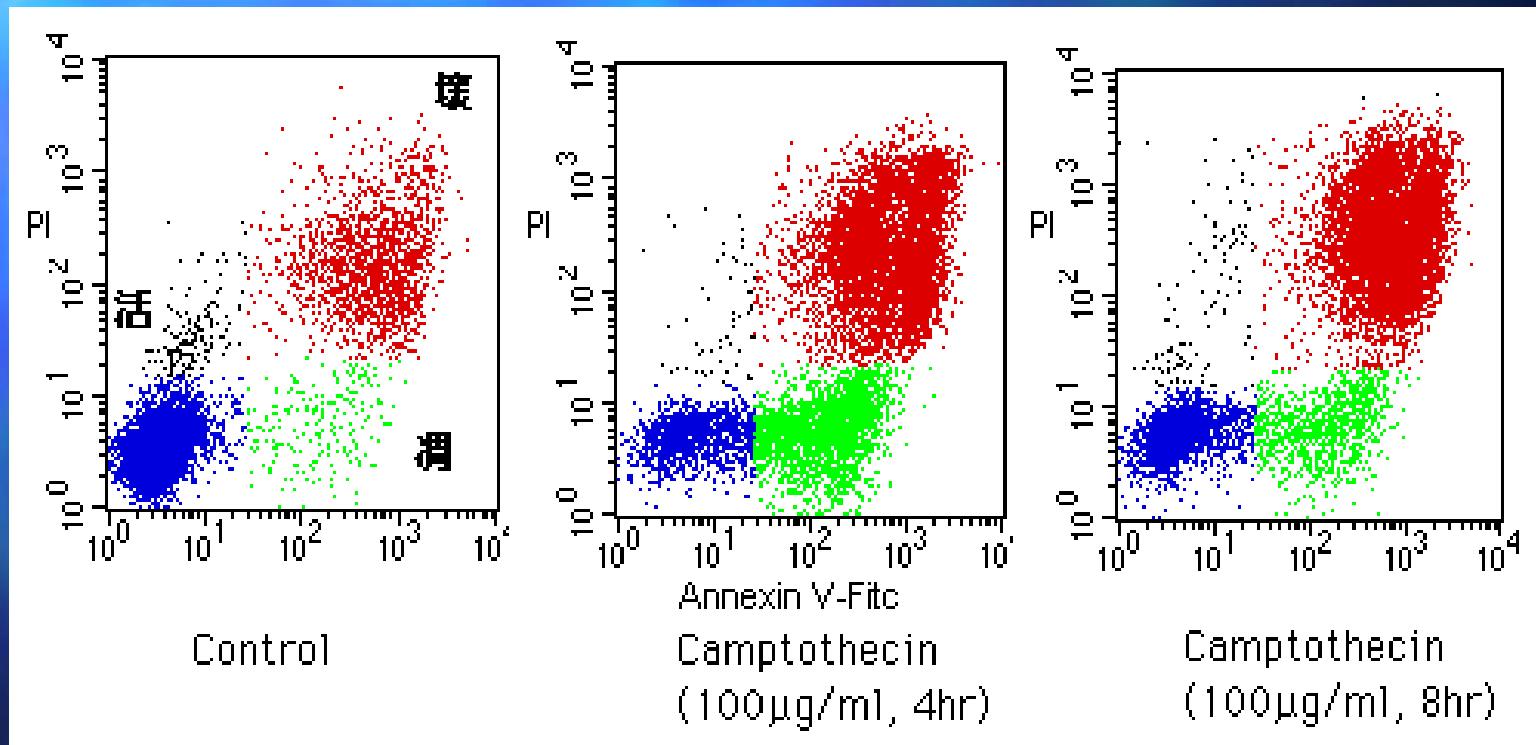
# Annexin V Assay



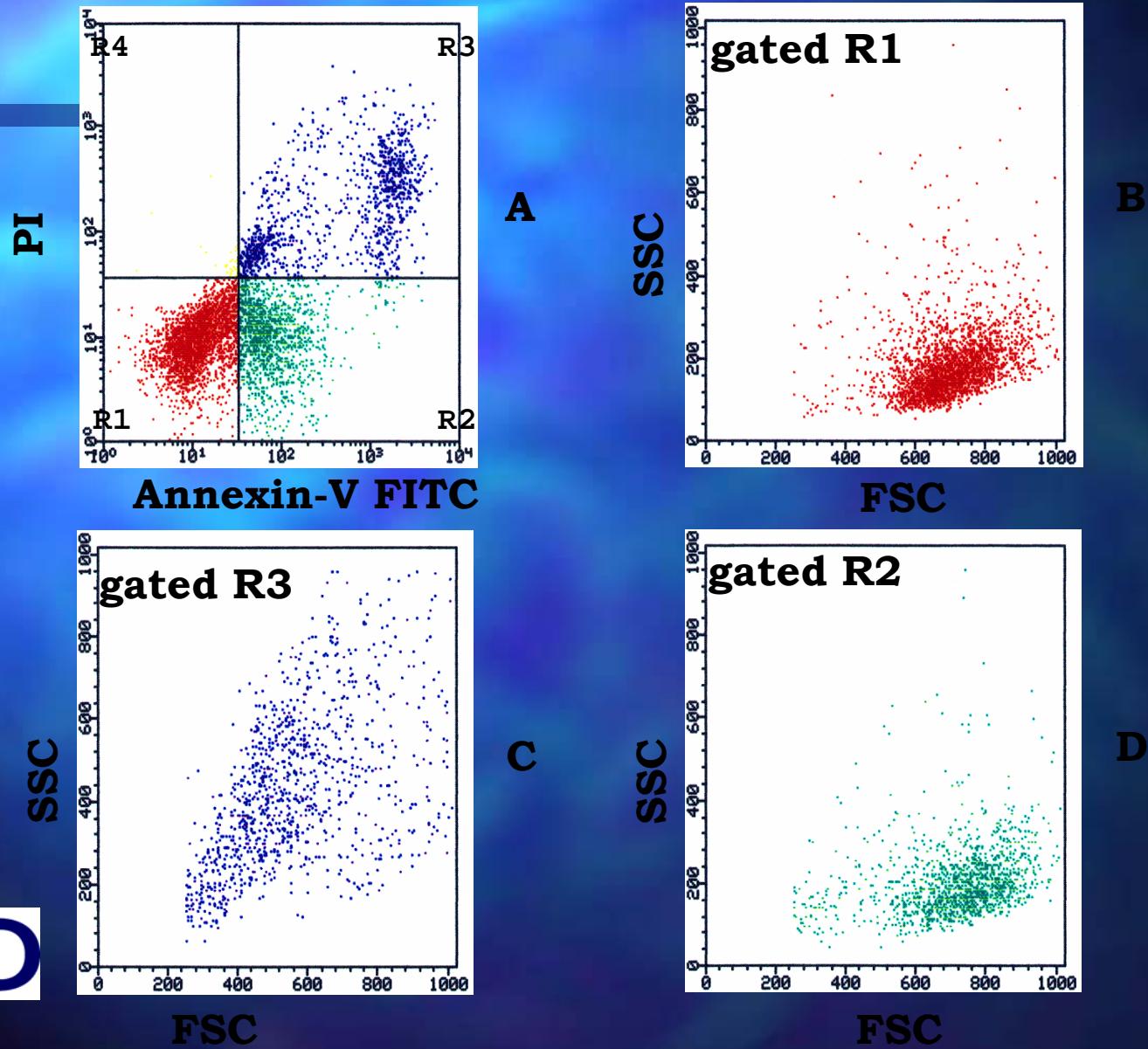
# Annexin V is a Quick Assay



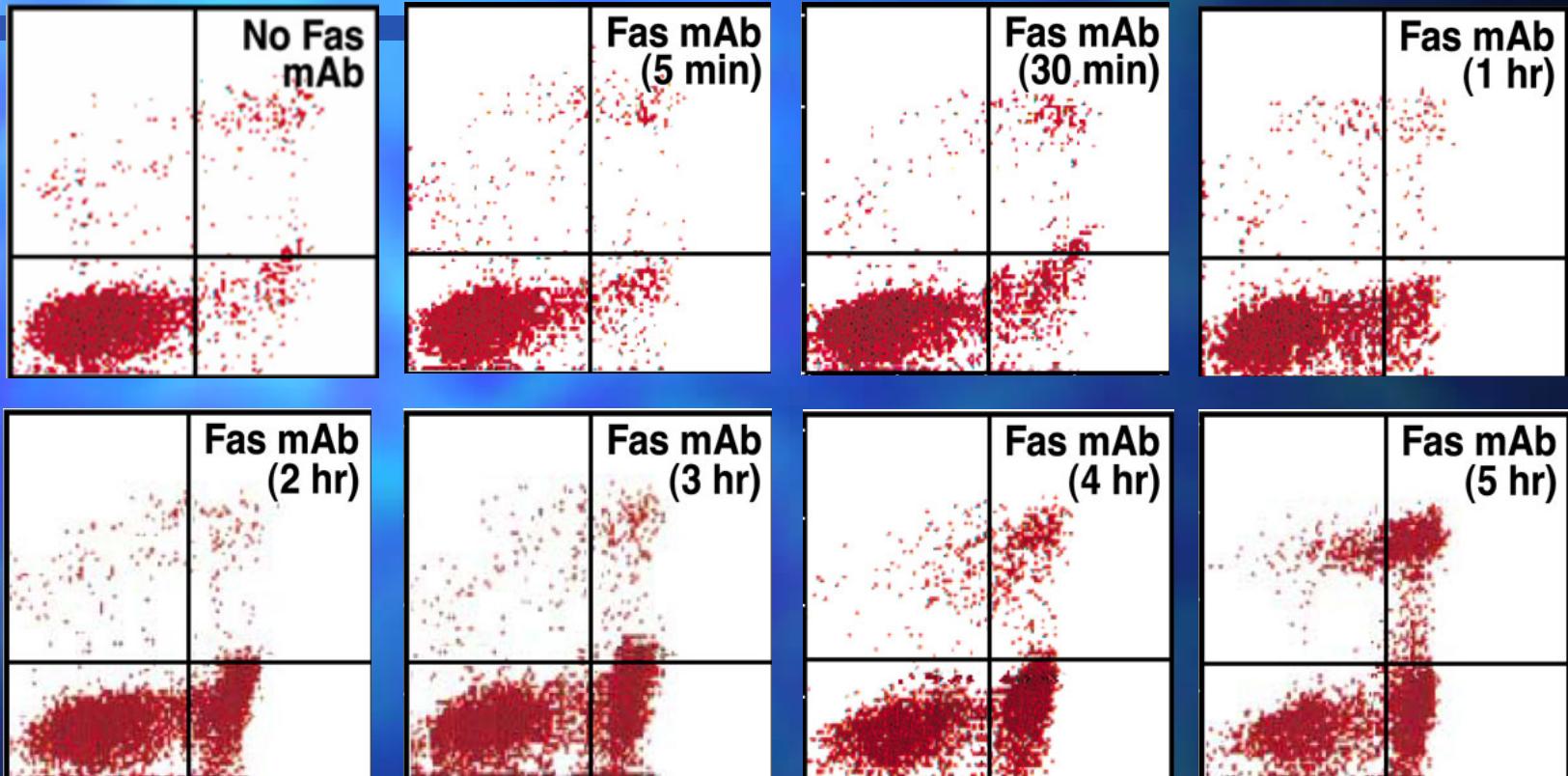
# Annexin V-PI Analysis



# ANALYSIS OF VIABLE APOPTOTIC CELLS



# Annexin V Assay: A Time Course



Annexin V-FITC

# Flow Cytometry Assays

## Live (unfixed cells)

- Annexin V-PI 分析法
- MitoScreen (JC-1)

## Fixed cells

- Active Caspase-3
- APO-BRDU™ (DNA 鏈斷分析法)
- Hypoploidy Analysis (Sub-G1分析法)

## Cell Lysate



- CBA Apoptosis

# Mitoscreen (JC-1): Monitor Mitochondrial Membrane Potential ( $\Delta\psi$ )

- Energy stored as a negative electrochemical gradient  
 $\Delta\psi$  polarized
- Apoptosis is often associated with a decrease in  $\Delta\psi$   
 $\Delta\psi$  depolarized

JC-1:

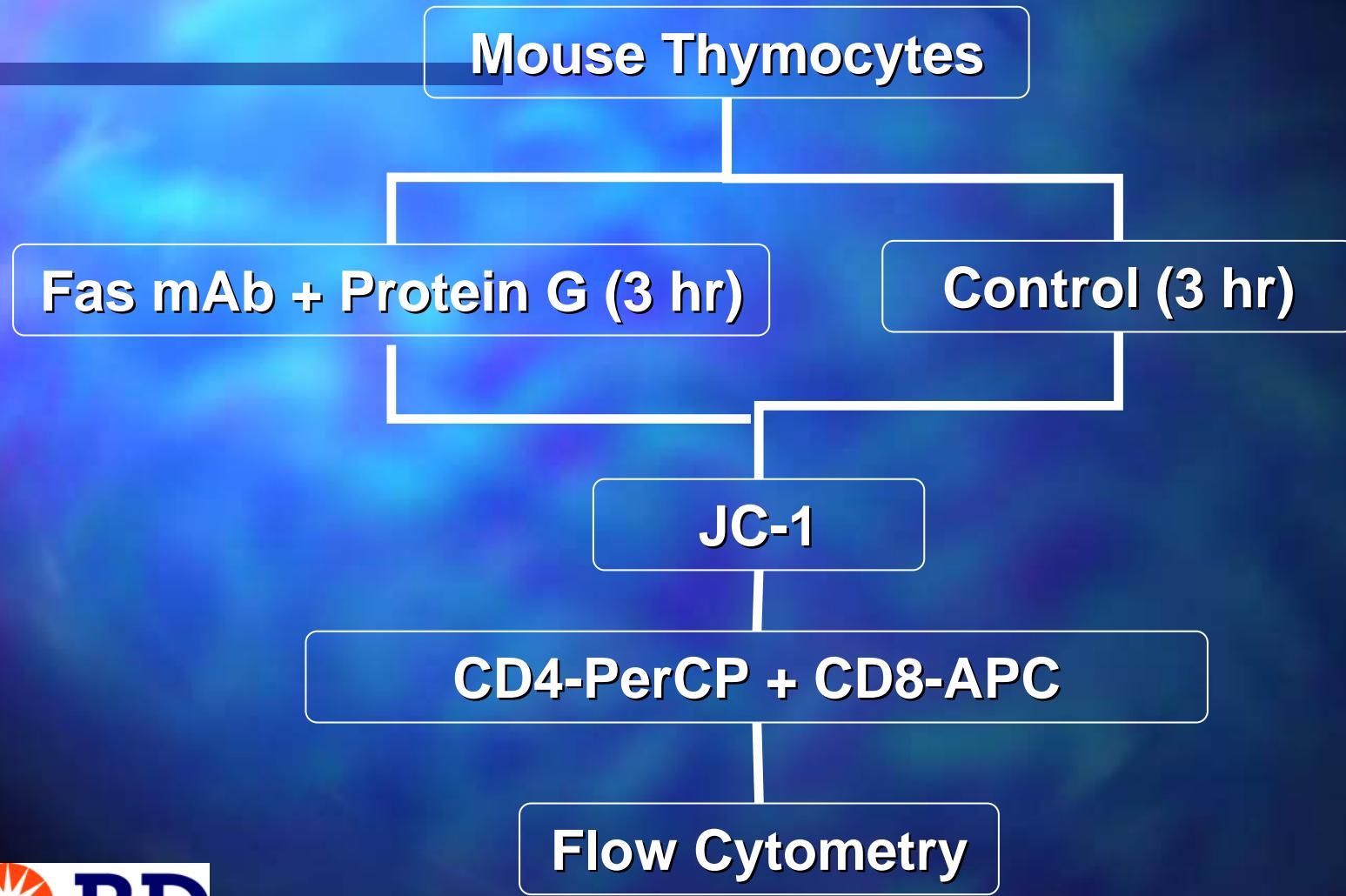
- 1st J-aggregate-forming Cationic dye found to be  $\Delta\psi$  sensitive
- Fluorescence color dependent on  $\Delta\psi$

Analysis by flow cytometry:

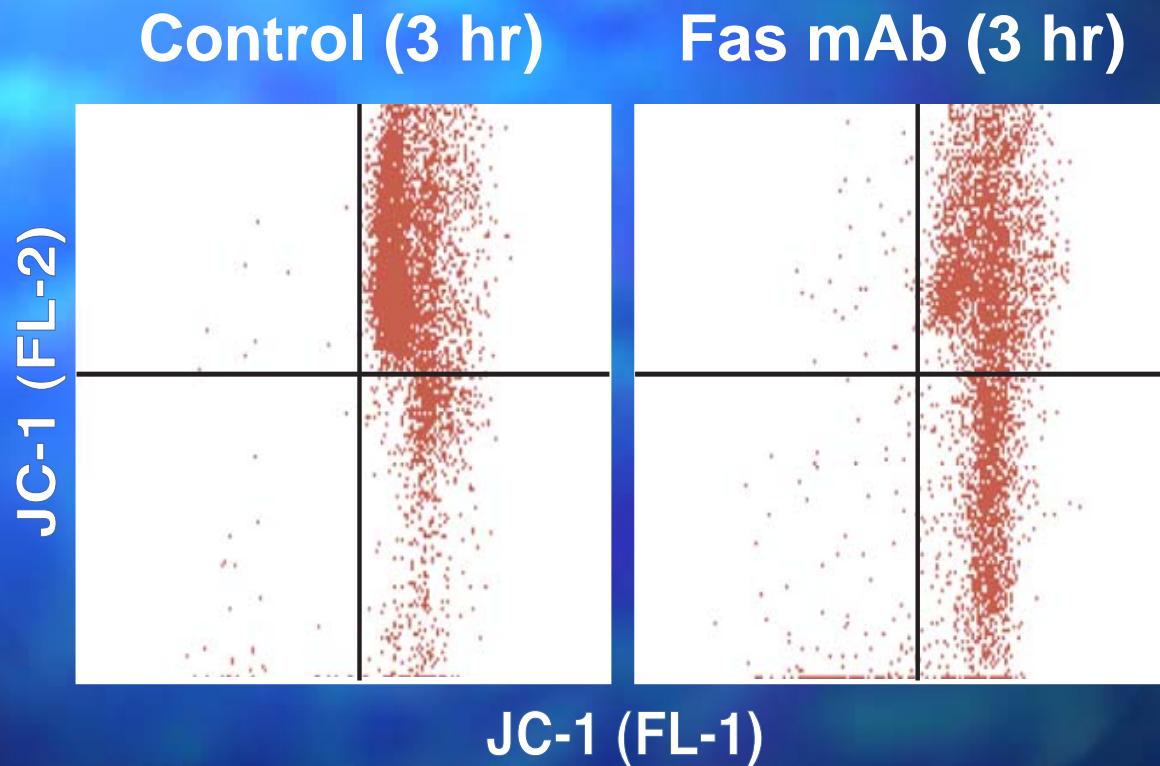
- $\Delta\psi$  polarized (J-aggregates): FITC-PE channel
- $\Delta\psi$  depolarized (monomers): FITC channel



# Model System: Mouse Apoptosis



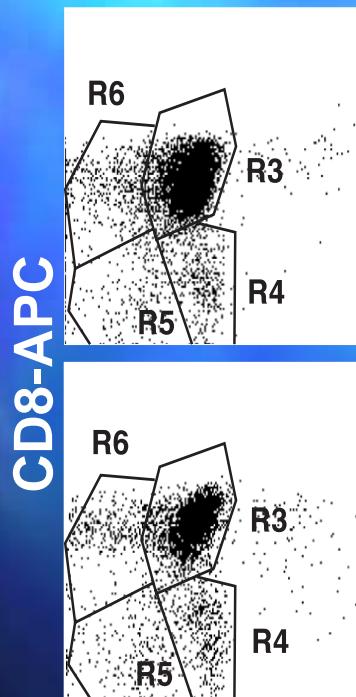
# JC-1 Staining in Mouse Thymocytes



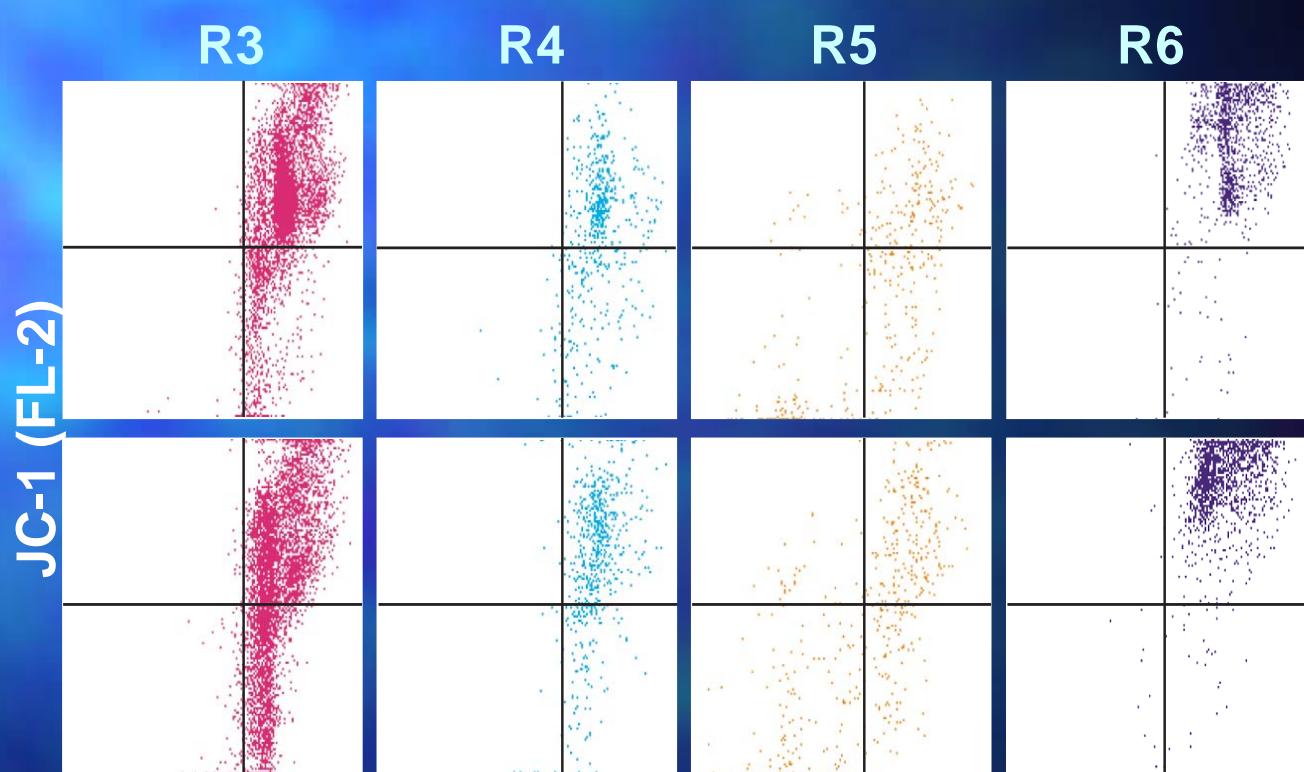
# JC-1 Staining in Mouse Thymocyte Subpopulations

Gated on CD4/CD8

Fas mAb Control



CD8-APC



CD4-PerCP



JC-1 (FL-1)

# Flow Cytometry Assays

## Live (unfixed cells)

- Annexin V-PI 分析法
- MitoScreen (JC-1)

## Fixed cells

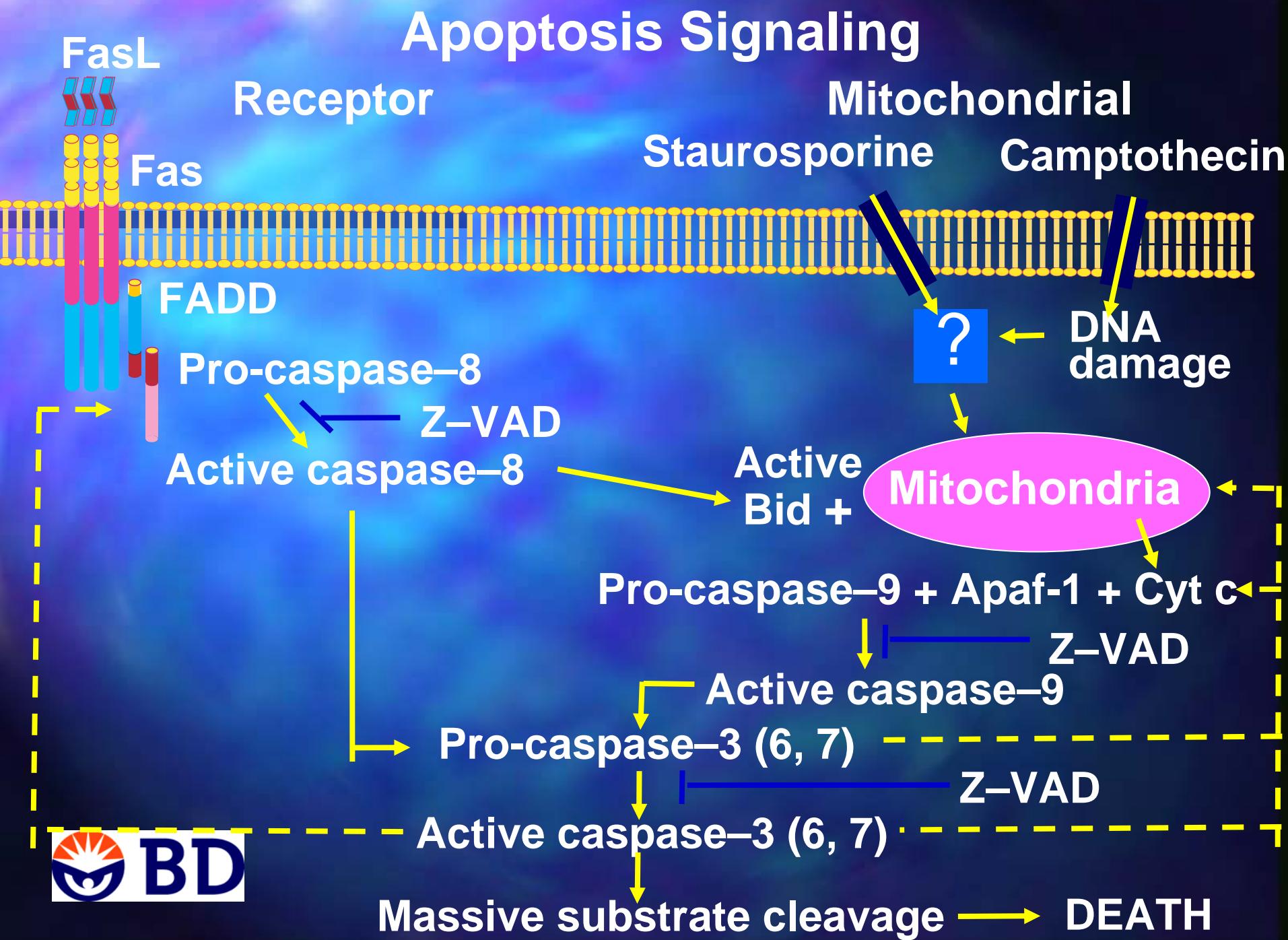
- Active Caspase-3
- APO-BRDU™ (DNA 鏈斷分析法)
- Hypoploidy Analysis (Sub-G1分析法)

## Cell Lysate



- CBA Apoptosis

# Apoptosis Signaling



# Annexin V and Caspase-3 Staining Protocol

Jurkat T Cells

Incubation in camptothecin  
0, 0.5, 1, 2 or 4 hr

Apoptosis Validation

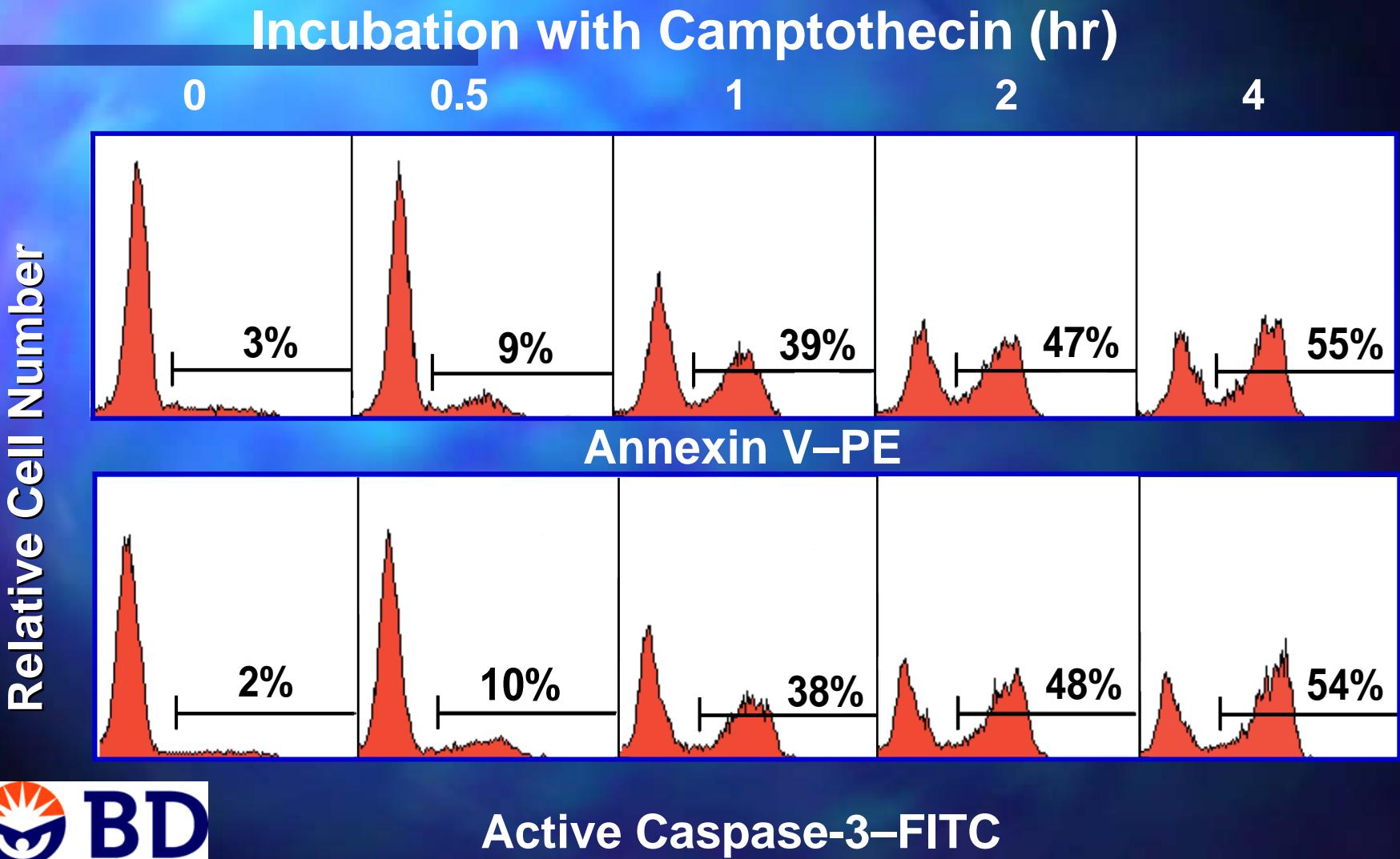
Annexin V-PE → Flow Cytometry

Cytofix / Cytoperm™

Active Caspase-3-FITC → Flow Cytometry



# Flow Cytometric Analysis of Apoptosis in Jurkat T Cells



# Flow Cytometry Assays

## Live (unfixed cells)

- Annexin V-PI 分析法
- MitoScreen (JC-1)

## Fixed cells

- Active Caspase-3
- APO-BRDU™ (DNA 鏈斷分析法)
- Hypoploidy Analysis (Sub-G1分析法)

## Cell Lysate

- CBA Apoptosis



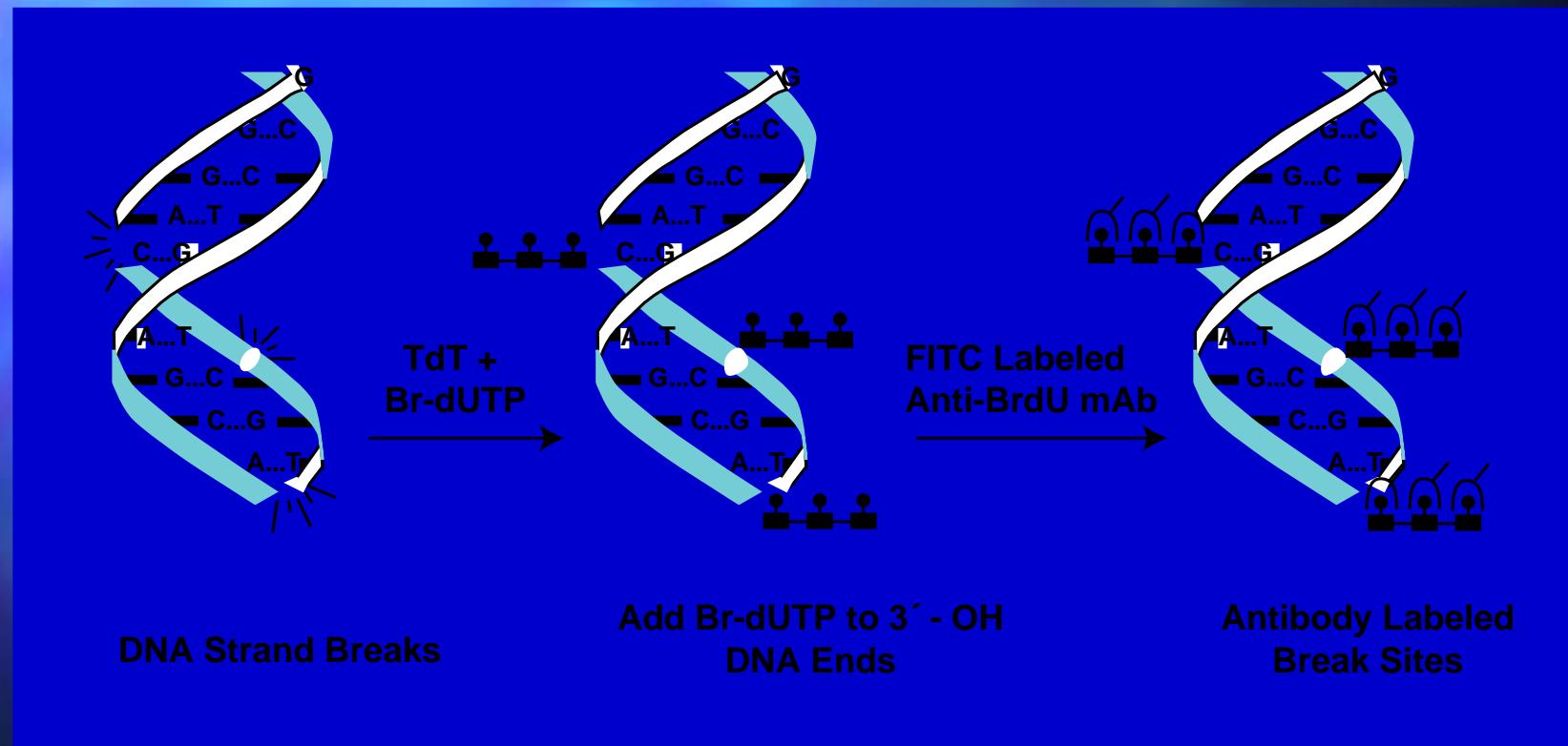
# APO-BRDU™ Exploits the Large Number of DNA Fragments in Apoptotic Cells

---

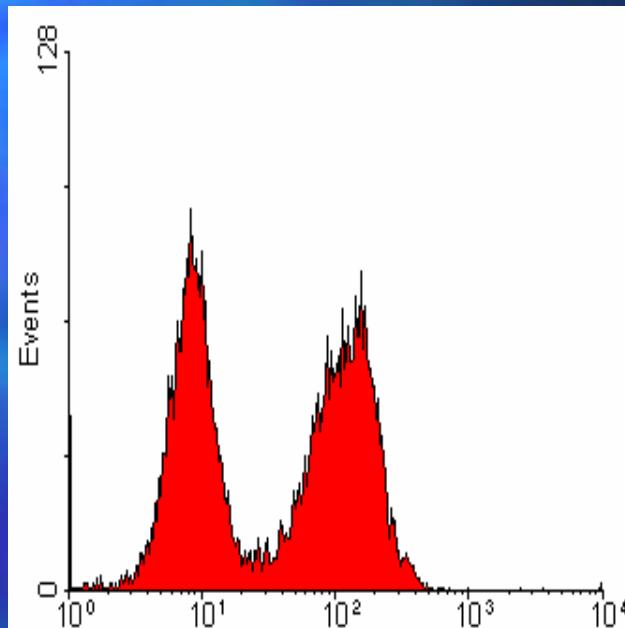
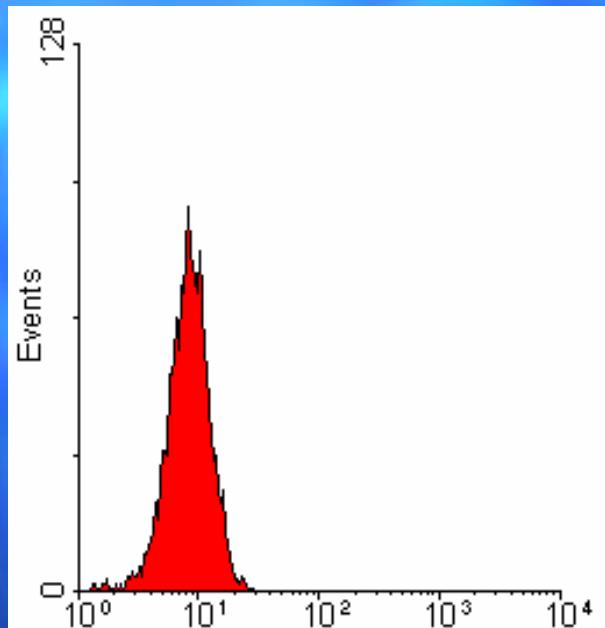
- DNA 3'-hydroxyl terminal are labelled with bromolated deoxyuridine triphosphate nucleotides (Br-dUTP)
  - Reaction catalyzed by terminal deoxynucleotidyl transferase (TdT)
- Non-apoptotic cells lack exposed 3'-hydroxyl ends and therefore incorporate little Br-dUTP
- Br-dUTP sites are detected with a BrdU-FITC mAb



# APO-BRDU™ Assay

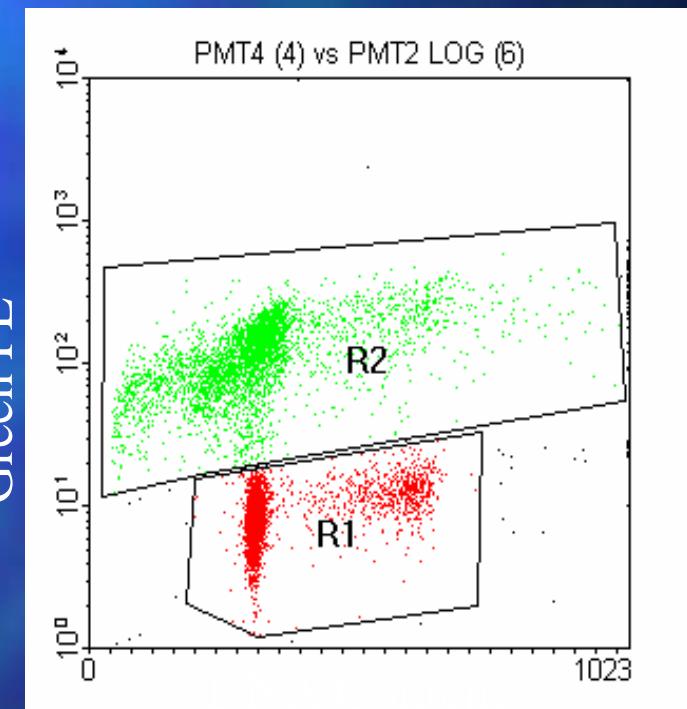
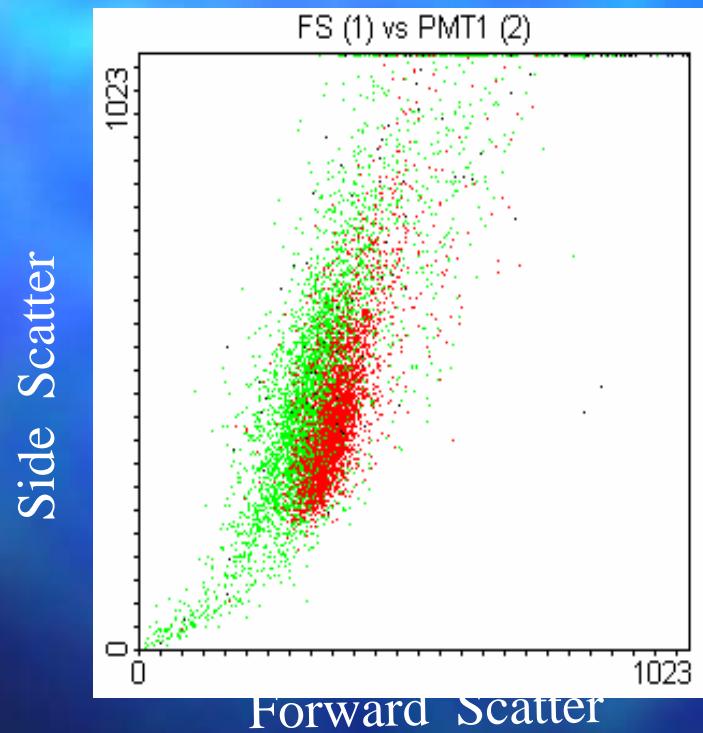


# APO-BRDU™ Assay



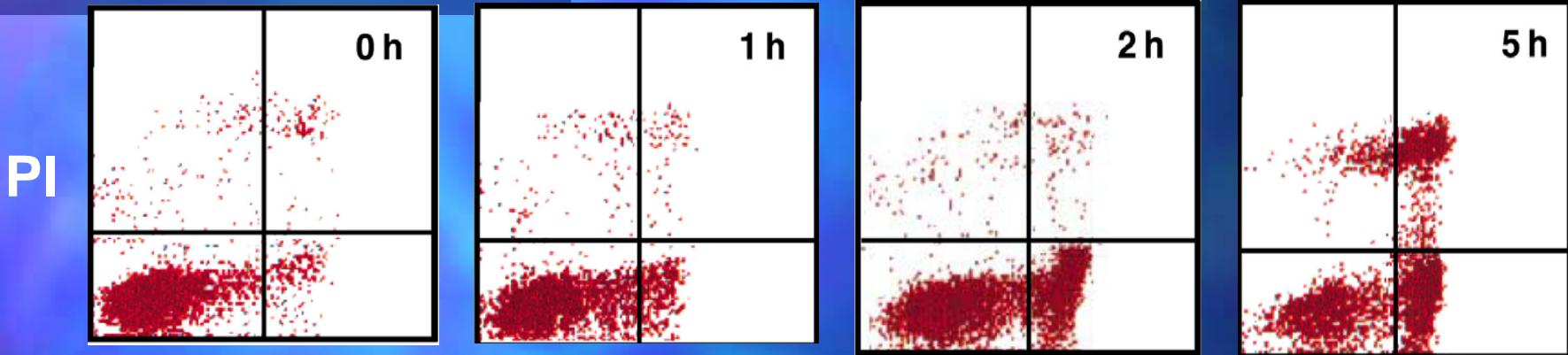
Green Fluorescence

# APO-BRDU™ Assay

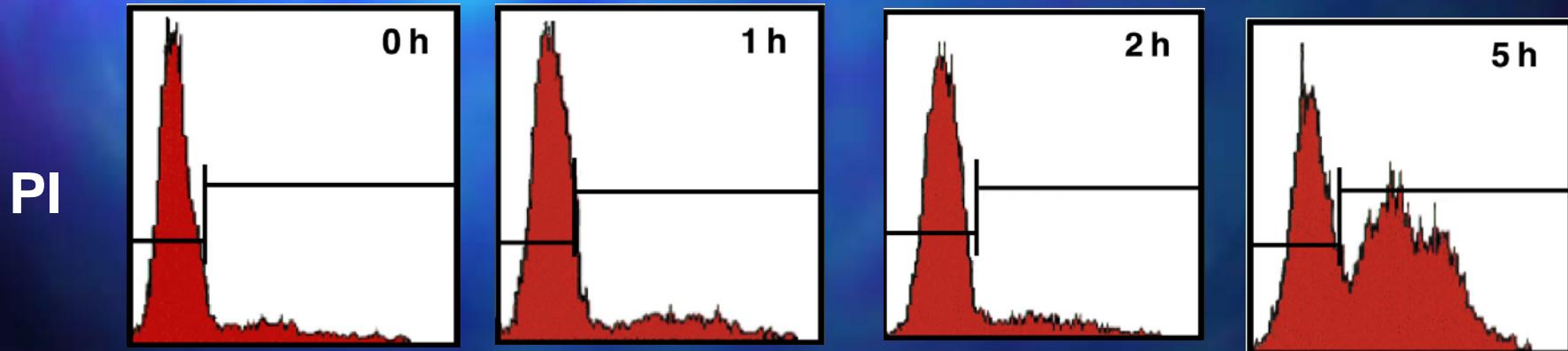


R1: Normal Cells  
R2: Apoptotic Cells

# Fas-induced Apoptosis in Jurkat Cells



Annexin V-FITC



BrdU-FITC

# Flow Cytometry Assays

## Live (unfixed cells)

- Annexin V-PI 分析法
- MitoScreen (JC-1)

## Fixed cells

- Active Caspase-3
- APO-BRDU™ (DNA 鏈斷分析法)
- Hypoploidy Analysis (Sub-G1分析法)

## Cell Lysate



- CBA Apoptosis

# Sub-G1 分析法

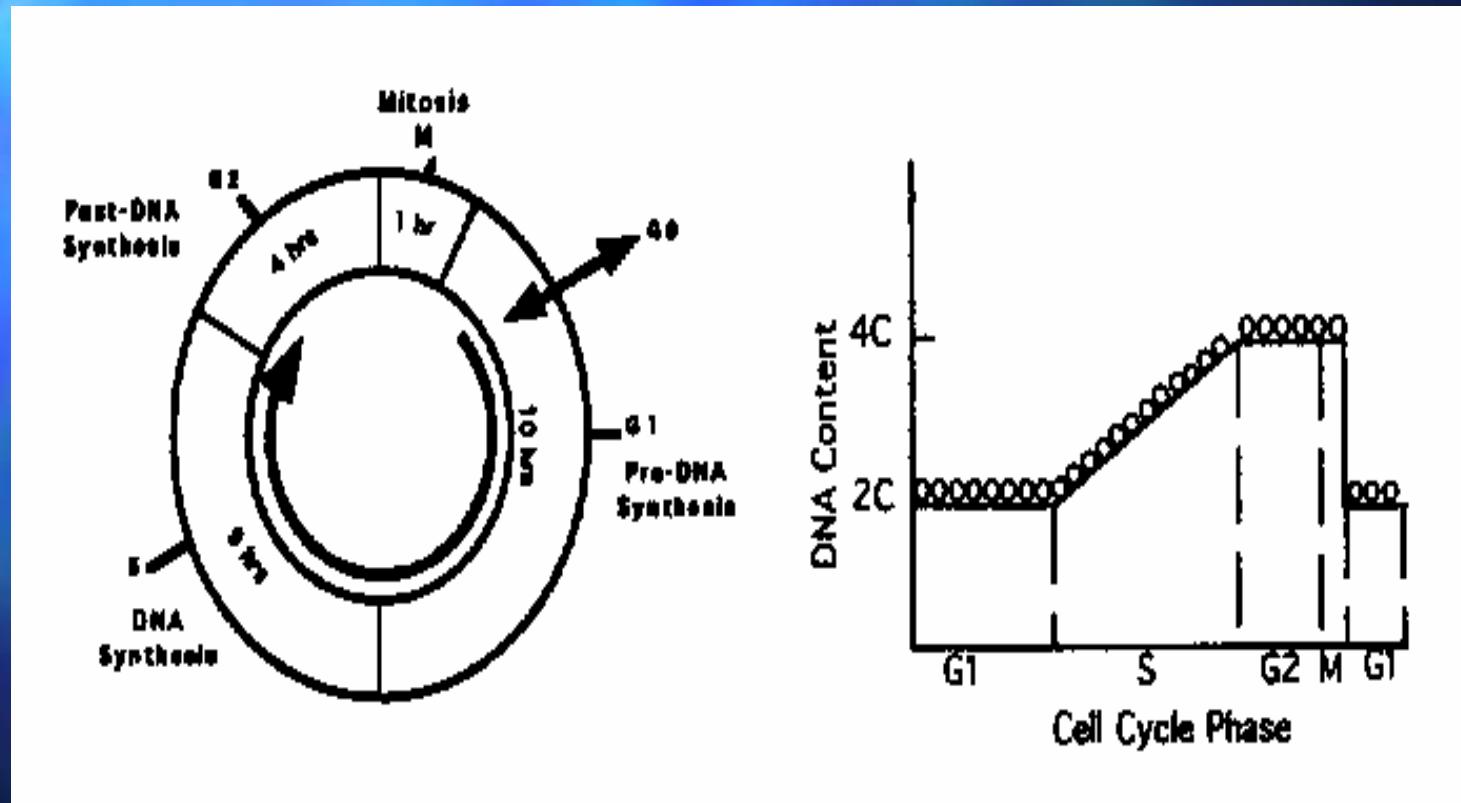
---

## 染色原則同細胞周期分析

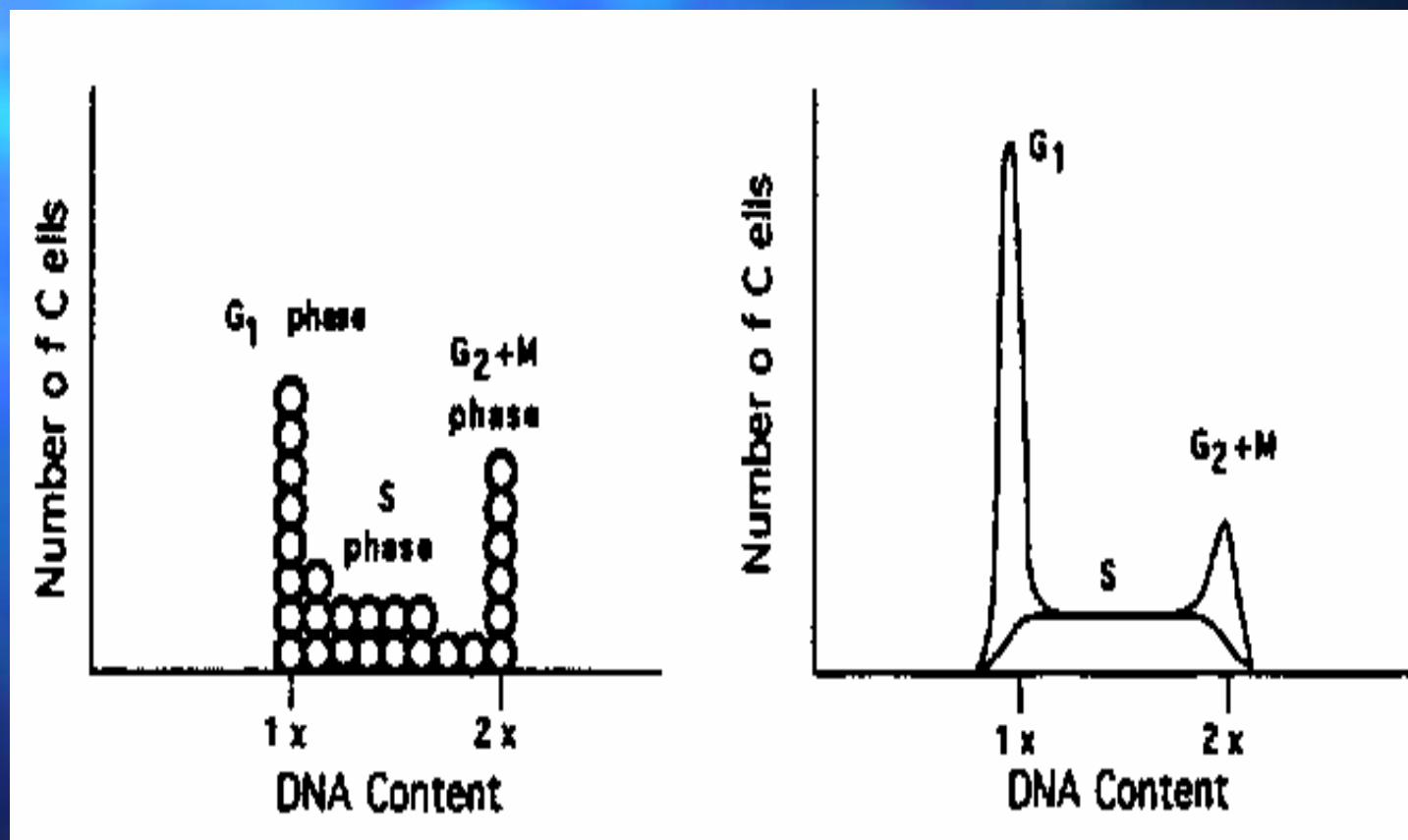
- Overnight incubation to extract small DNA fragments, then PI stain.
  - Pre-treat with Triton X.
  - Use of phosphate buffers with high osmolarity.



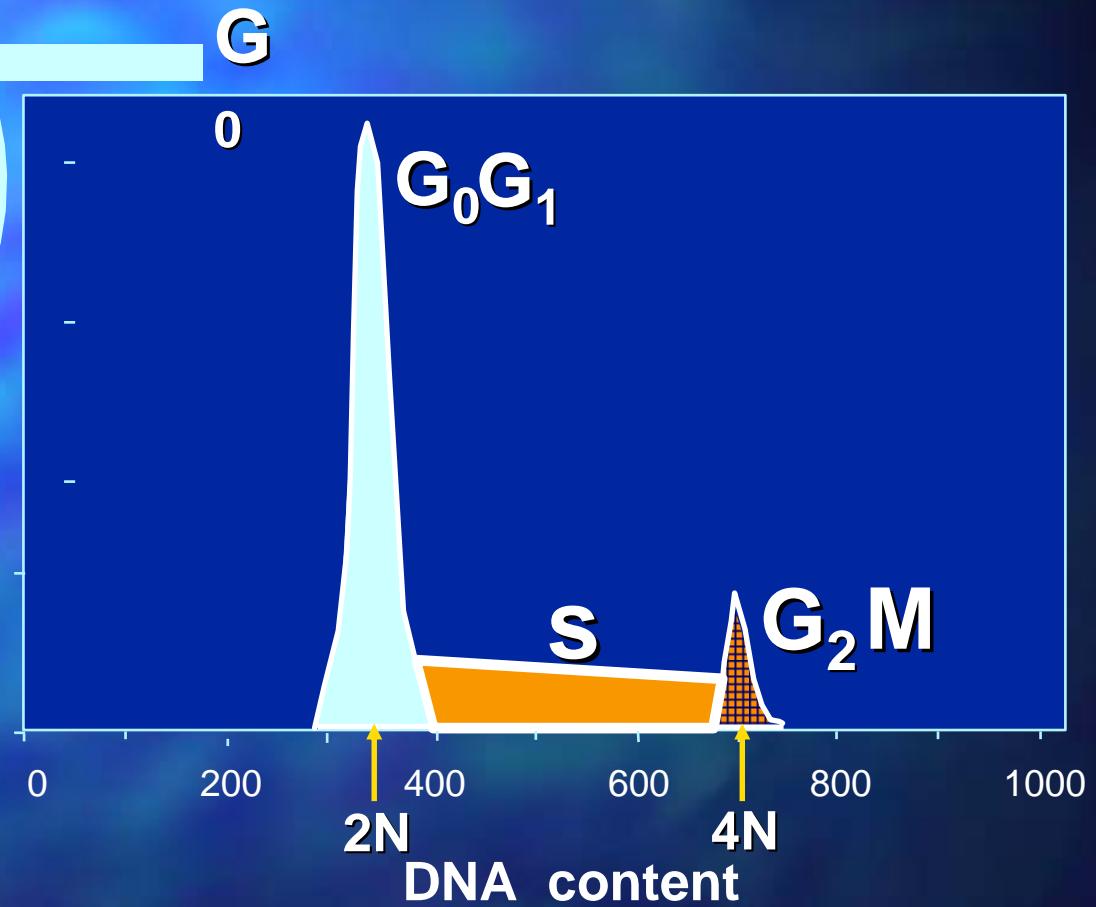
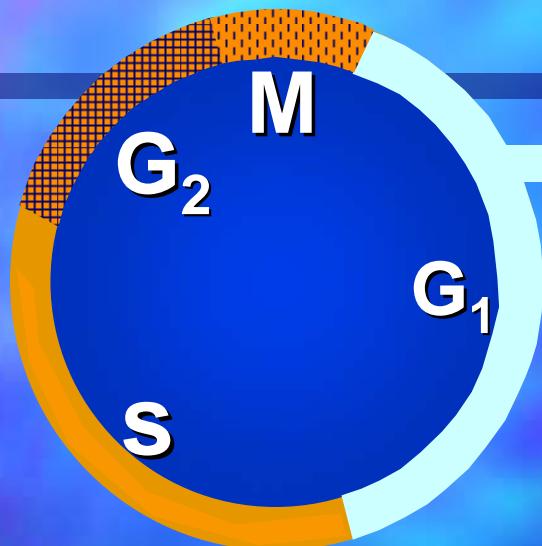
# 細胞周期與 DNA 含量



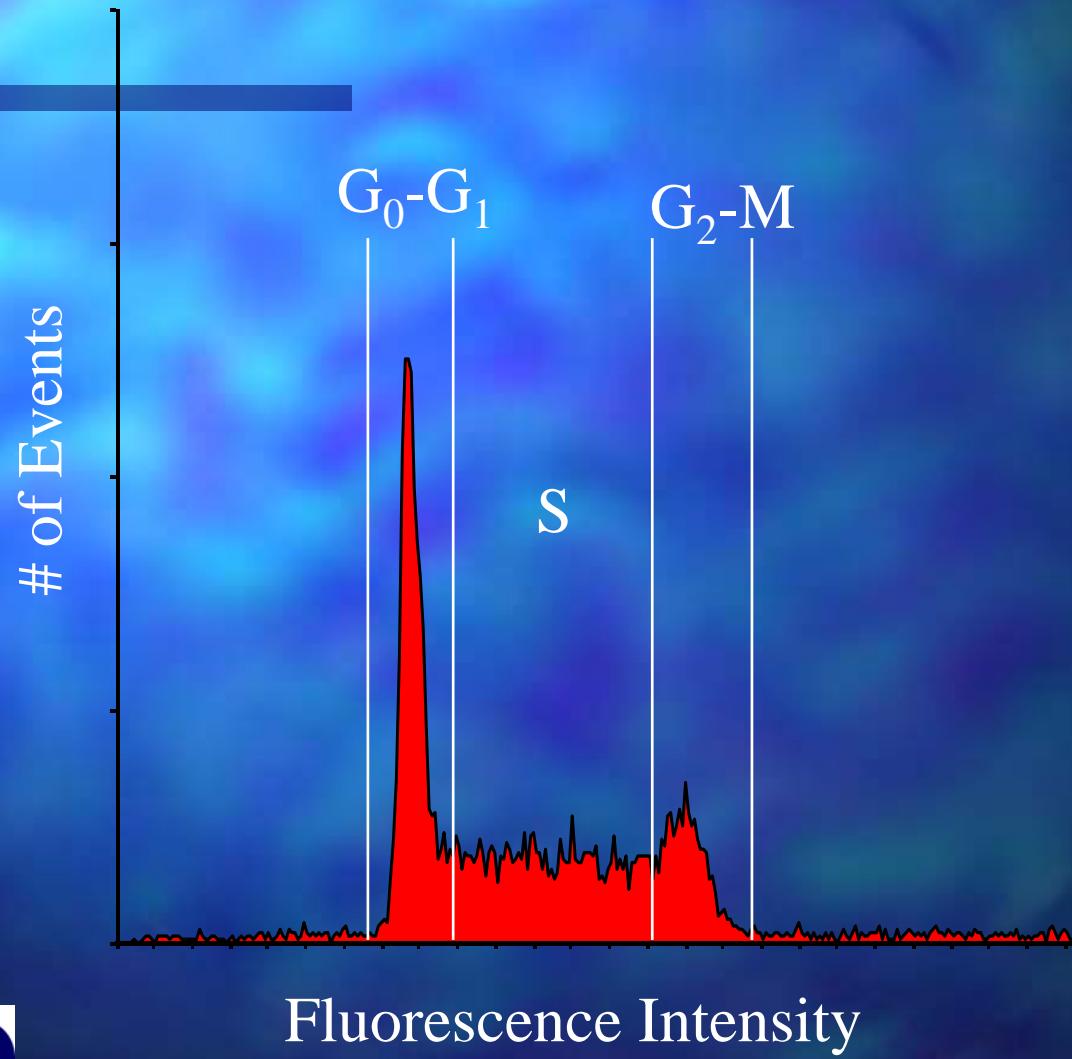
# DNA 直方圖-理論與實際



# 細胞周期位相的決定



# 典型的 DNA 直方圖



# Sub-G1分析法

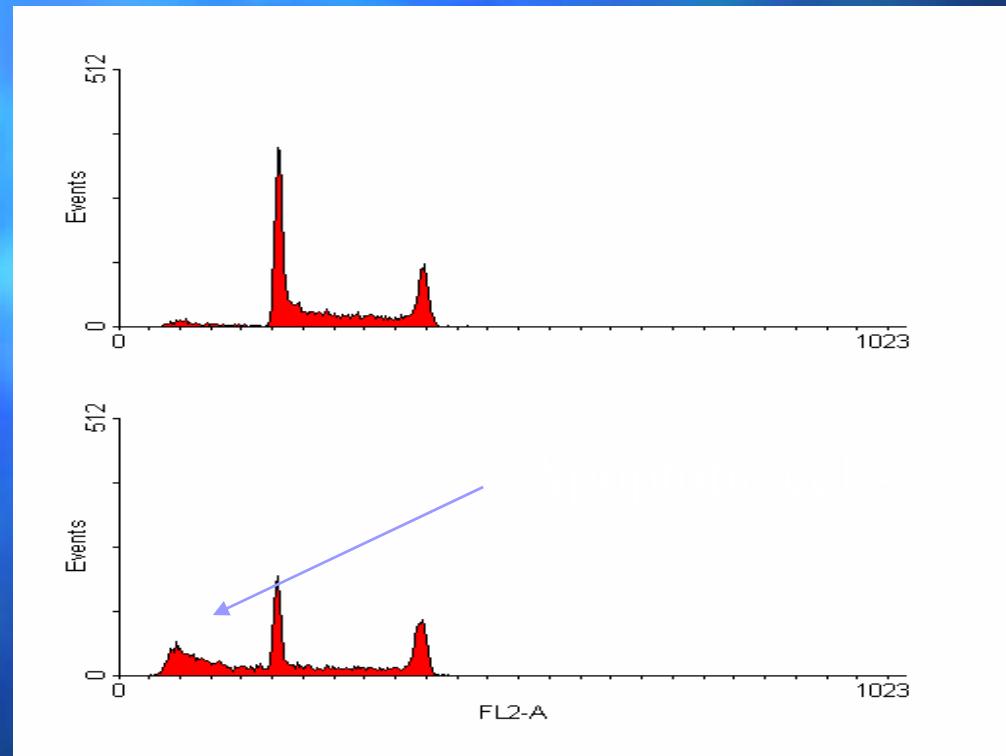
---

## ■ 檢體前處理的注意事項

- Healthy, viable cells before ethanol fixation.
- For adherent cells, avoid over-trypsinization. Use serum containing medium to stop trypsinization.
- Add ethanol while vortexing.
- Avoid the reuse of RNase.

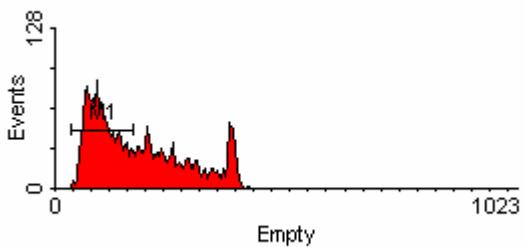
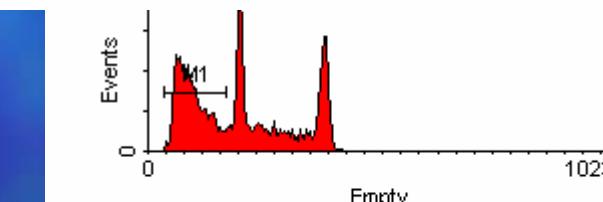
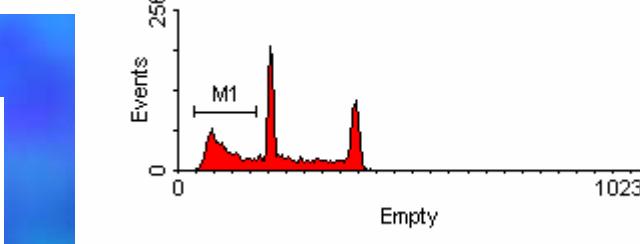
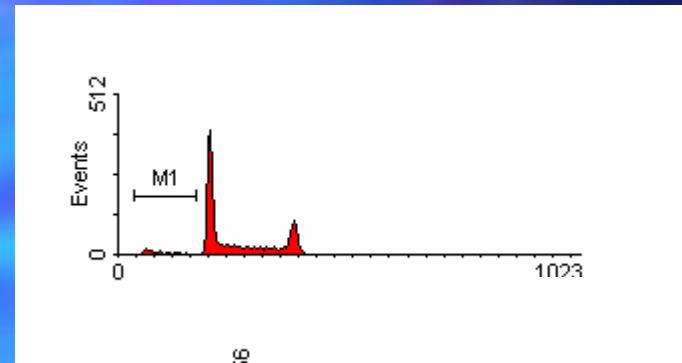
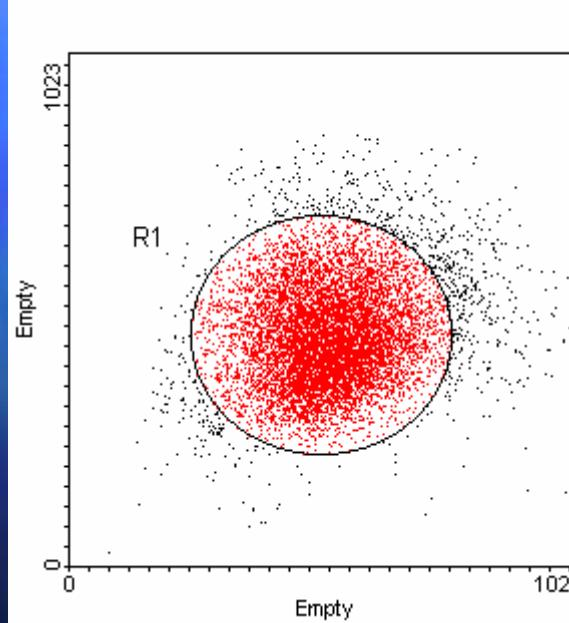
# Sub-G1 分析法

# of Events



PI Fluorescence Intensity

# Sub-G1分析法



# Flow Cytometry Assays

## Live (unfixed cells)

- Annexin V-PI 分析法
- MitoScreen (JC-1)

## Fixed cells

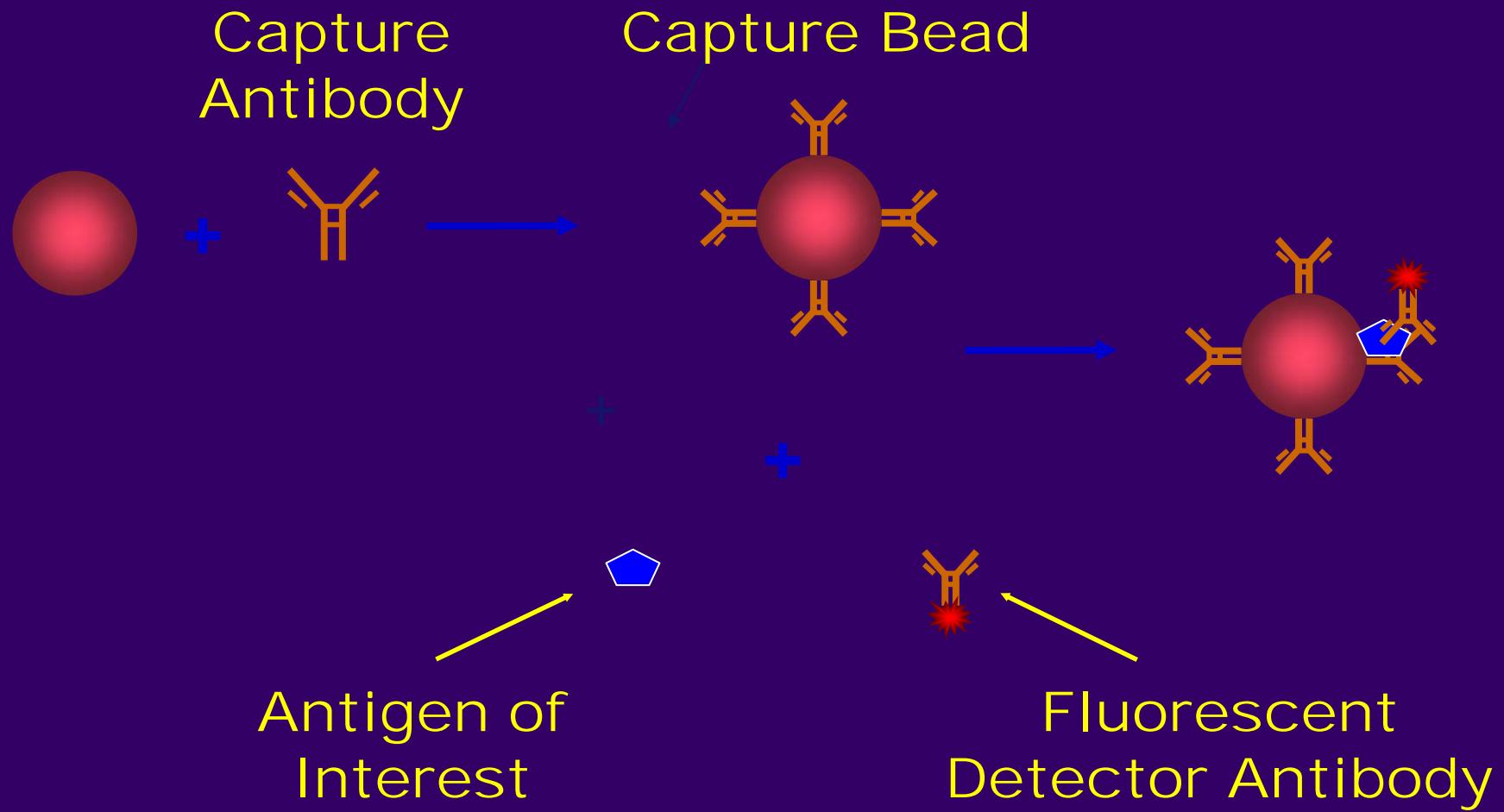
- Active Caspase-3
- APO-BRDU™ (DNA 鏈斷分析法)
- Hypoploid Analysis (Sub-G1分析法)

## Cell Lysate

- CBA Apoptosis



# Overview of the CBA Assay Configuration



# Advantages of Bead-Based Immunoassays

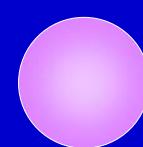
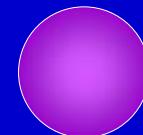
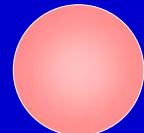
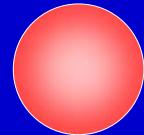
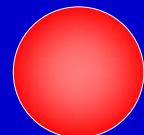
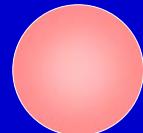
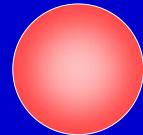
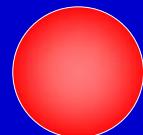
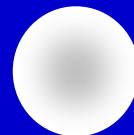
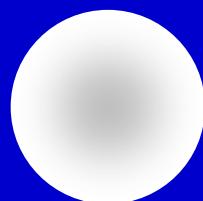
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- Analyze multiple analytes simultaneously
- Reduced sample volume requirements
- Reduced hands-on time by parallel analysis of samples
- Wide dynamic range of fluorescence detection (requires fewer sample dilutions)



# Beads Provide a Flexible Platform

Beads provide an expandable assay platform for use with a flow cytometer



Multiple sizes

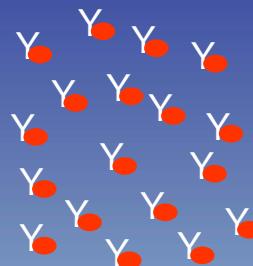
Different intensities\*

Different colors  
with different  
intensities

# 3 Bead Apoptosis CBA Assay



Active Caspase-3



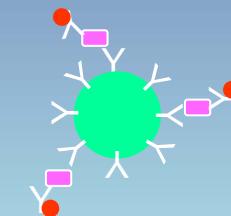
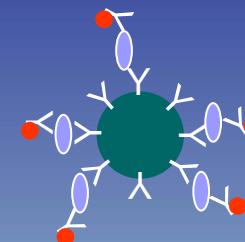
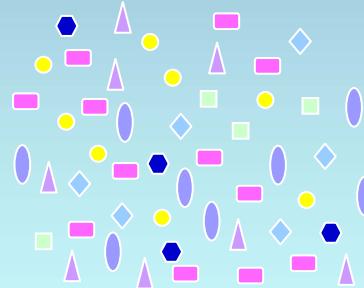
Cleaved PARP

BEADS

LYSATE OR  
SUPERNATANT

DETECTOR  
ANTIBODY

Wash  
Read on  
Flow Cytometer

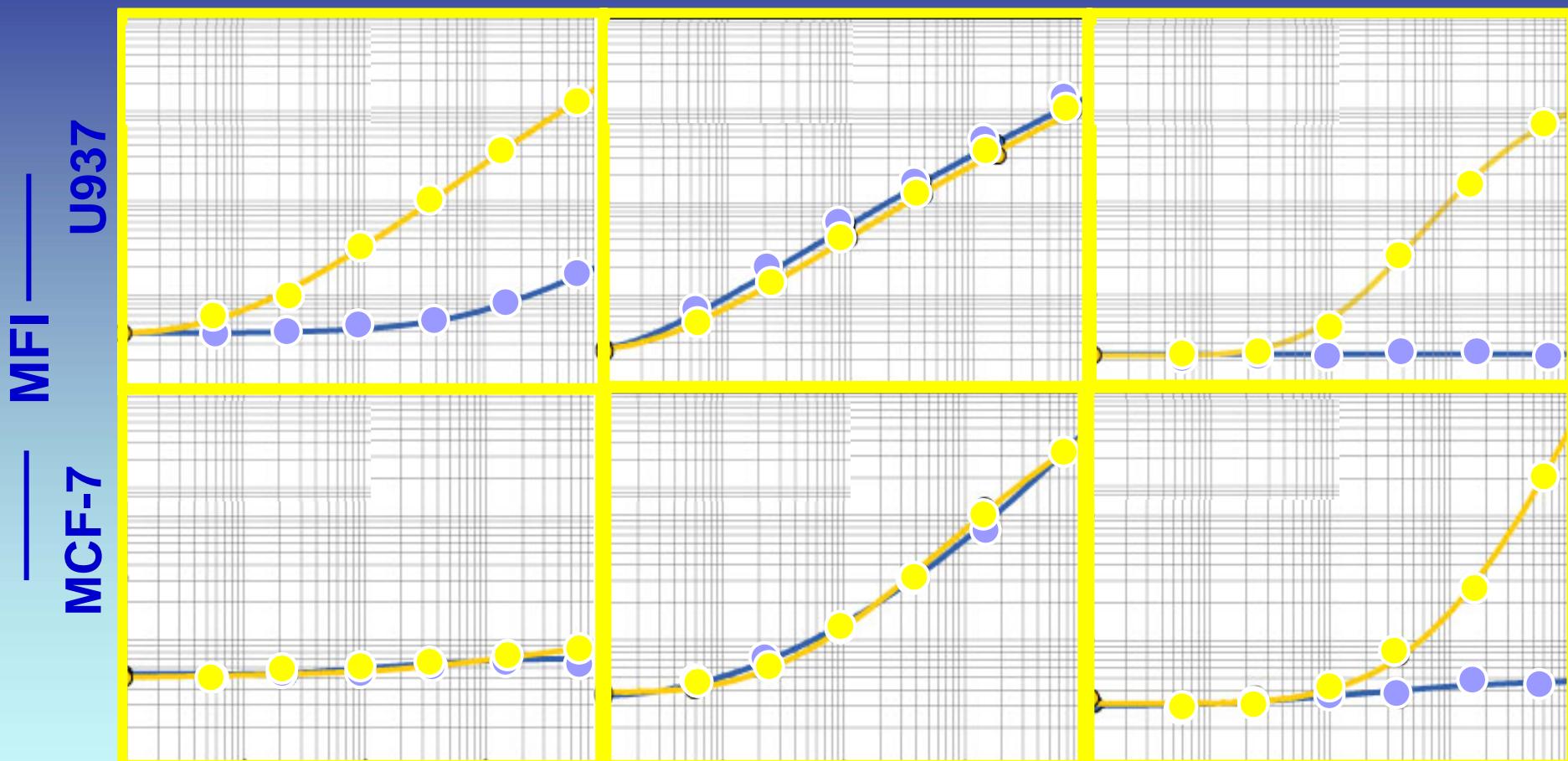


# Cytometric Bead Array Analysis of Cell Lysates

Active Caspase-3

Bcl-2

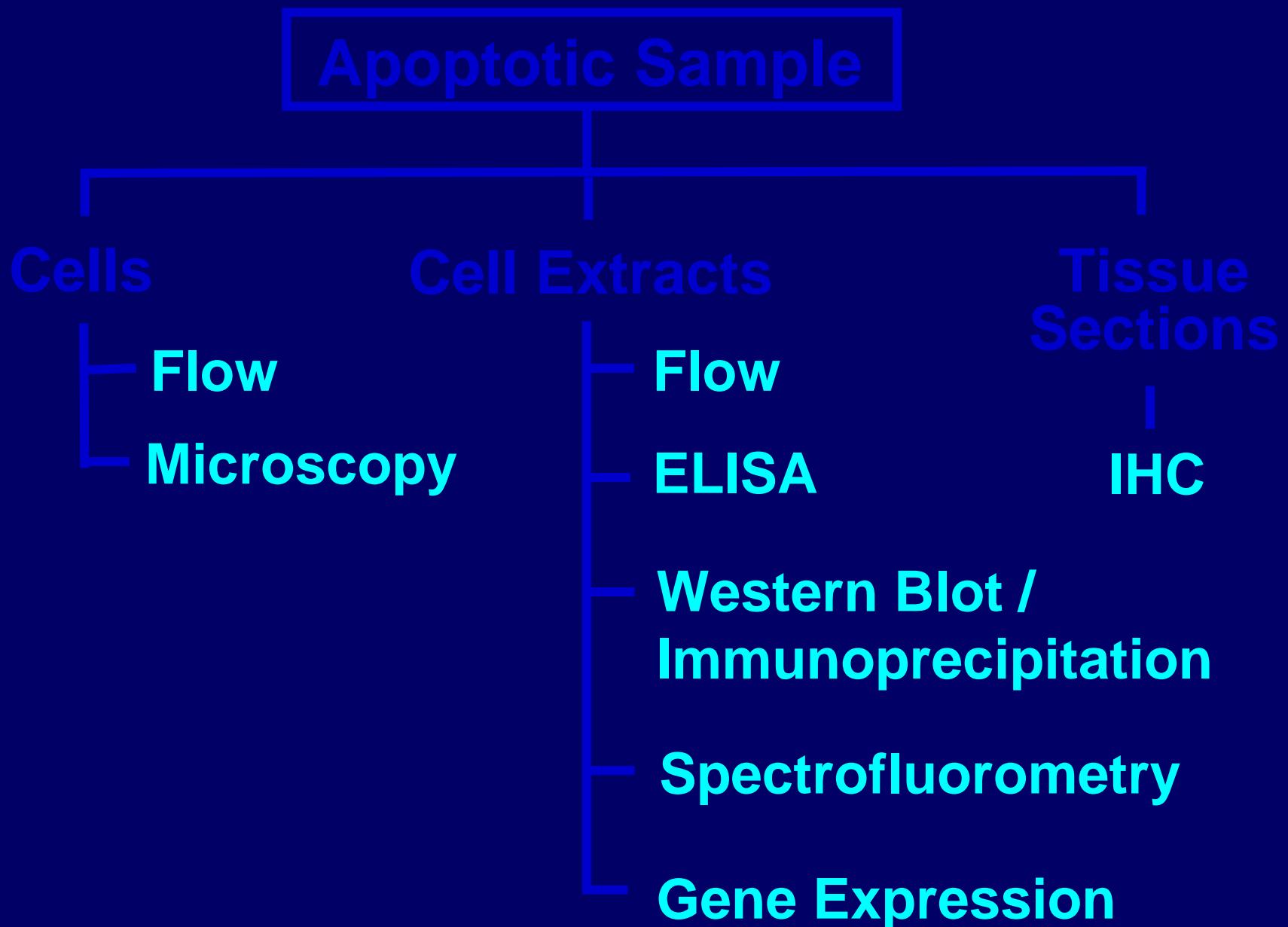
Cleaved PARP



○ Control  
● Camptothecin

Protein (units)

# Apoptosis Decision Tree



# Advantage of using flow cytometer as research tools

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- Identify unique cells from heterogeneous population by fluorescence labeled probe
- Multi-parameter detection in one assay\_ 3 to 18 colors
- Statistically meaningful result within limit time period\_ 10,000 to 70,000 cells per second
- Multiple proteins detection in a single assay by Cytometry Beads Assay



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**Thank you for your attention !!**

