Flow Cytometry
Basic Introduction and Principle

Eric Wei 魏昱齊
Product Specialist
BD Bioscience

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Topic

- **Basic Concept of Flow Cytometer**
- **Introduction to Instrument**
  - **Fluidics/Optics/Electronic System**
  - **Compensation Theory**
- **Application**

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What is Flow Cytometry?

Flow = Fluid

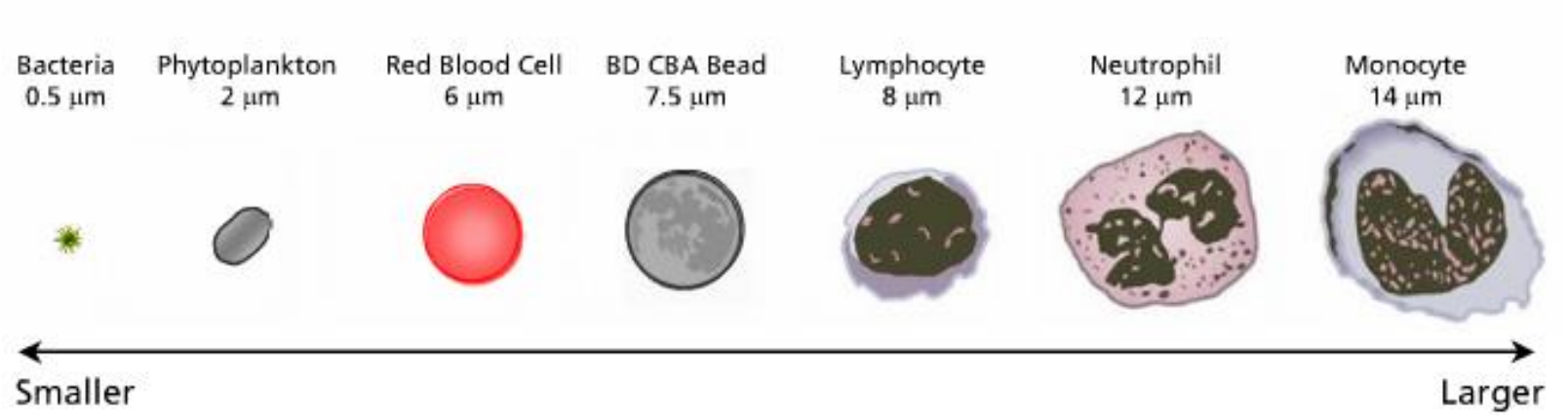
Cyto = Cell

Metry = Measurement

A variety of measurements are made on cells, cell organelles, and other objects suspended in a liquid and flowing at rates of several thousands per second through a flow chamber.

Particle Size

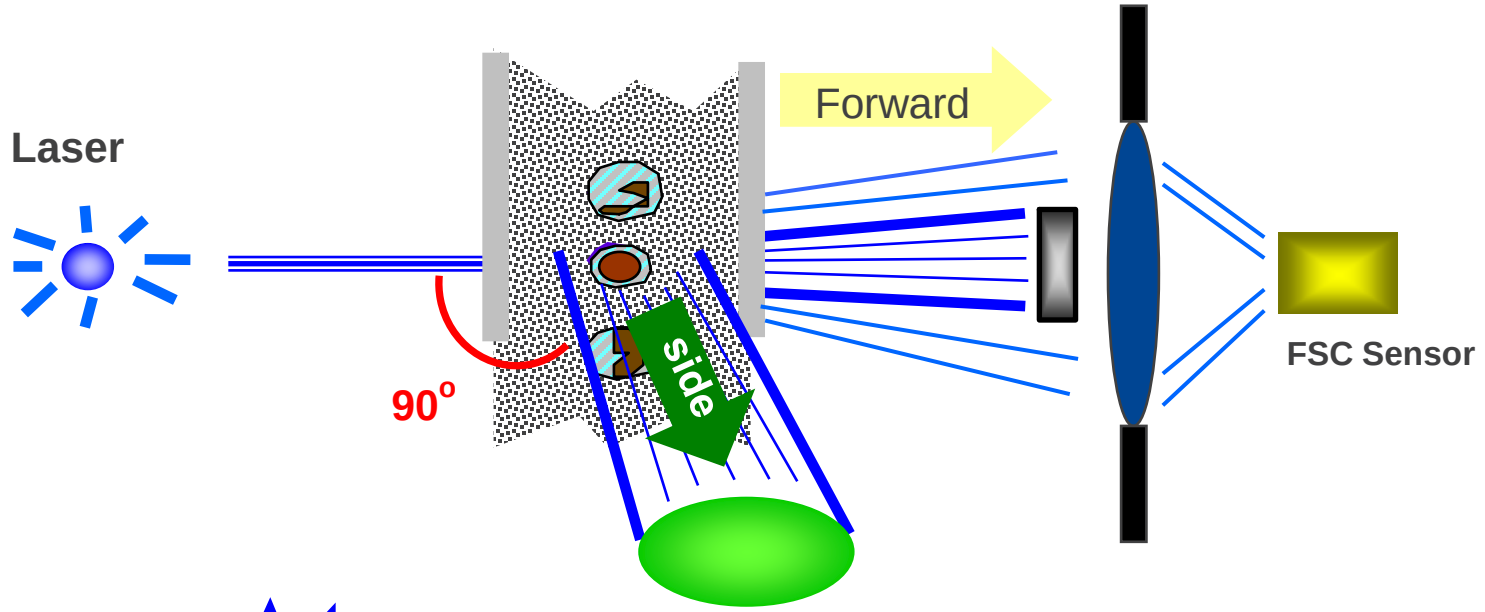
- Detection range: 0.5~50μm

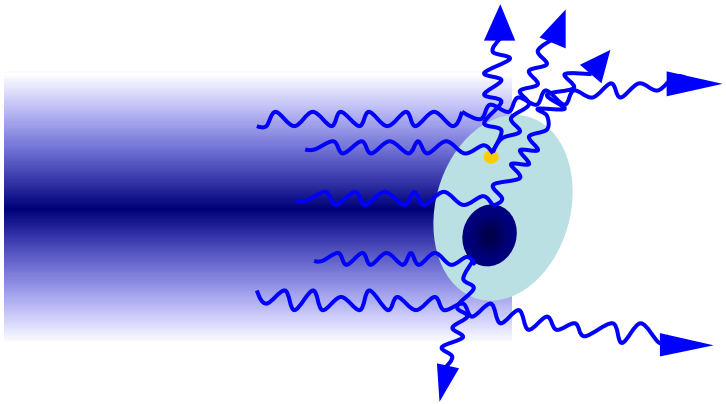


What Can a Flow Cytometer Tell Us About a Cell?

- Its relative size (Forward Scatter—**FSC**；前向散射光)
- Its relative granularity or internal complexity (Side Scatter—**SSC**；側向散射光)
- Its relative **fluorescence intensity**

Scatter Light





Forward Scatter—diffracted light
- **Related to cell surface area**
- **Detected along axis of incident light in the forward direction**

Side Scatter—reflected and refracted light
- **Related to cell granularity and complexity**
- **Detected at 90° to the laser beam**

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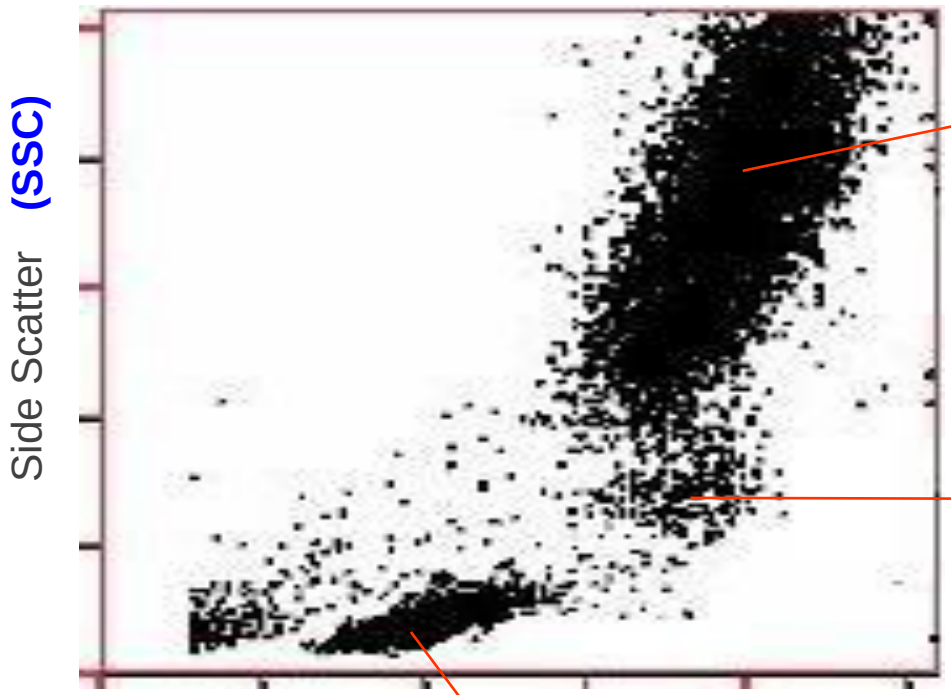
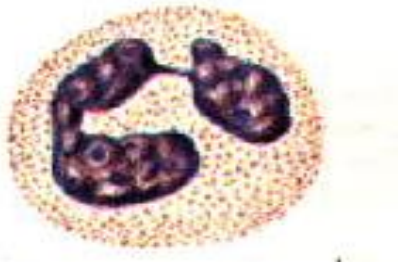
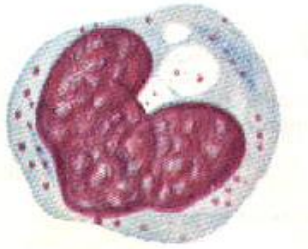
Ex. Lysed Human Whole Blood





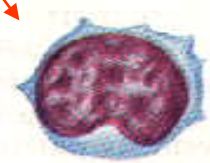
Neutrophils

10 – 14 µm



Monocytes

>10 µm



Lymphocytes

8 – 10 µm

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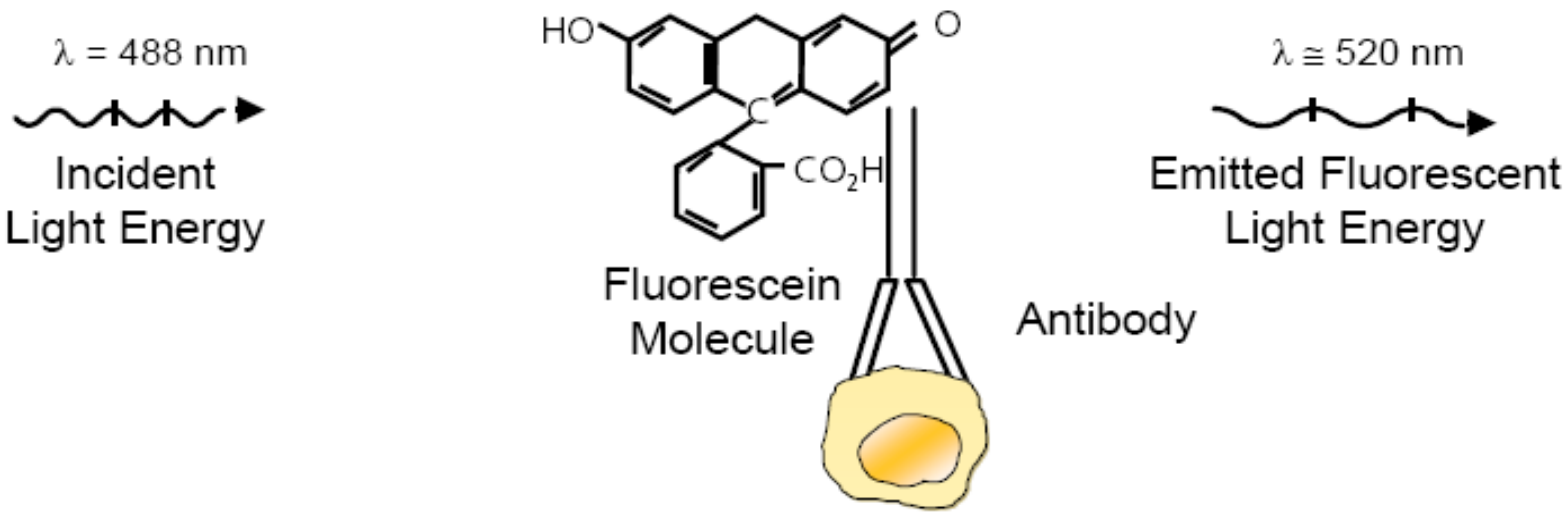
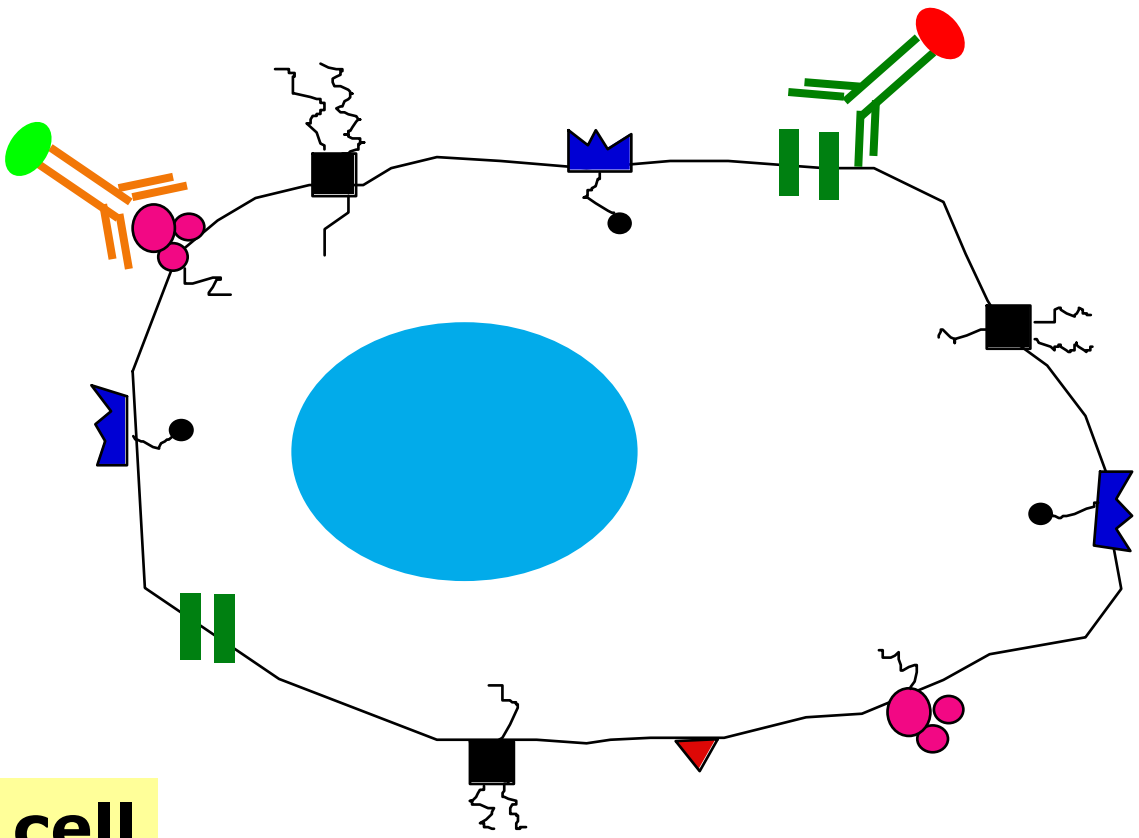
Fluorescence Light



- The fluorochrome absorbs energy from the laser.
- The fluorochrome releases the absorbed energy by:
  - vibration and heat dissipation.
  - emission of photons of a longer wavelength.

Flow Cytometry Detection Principle



T cell

:Antigen CD3

:Antigen CD4

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BD Flow Cytometers – Cell Analyzer

FACSCalibur

2 Lasers,
4 Colors

Main Component

Fluidics 液流系統

To introduce and focus the cells for interrogation.

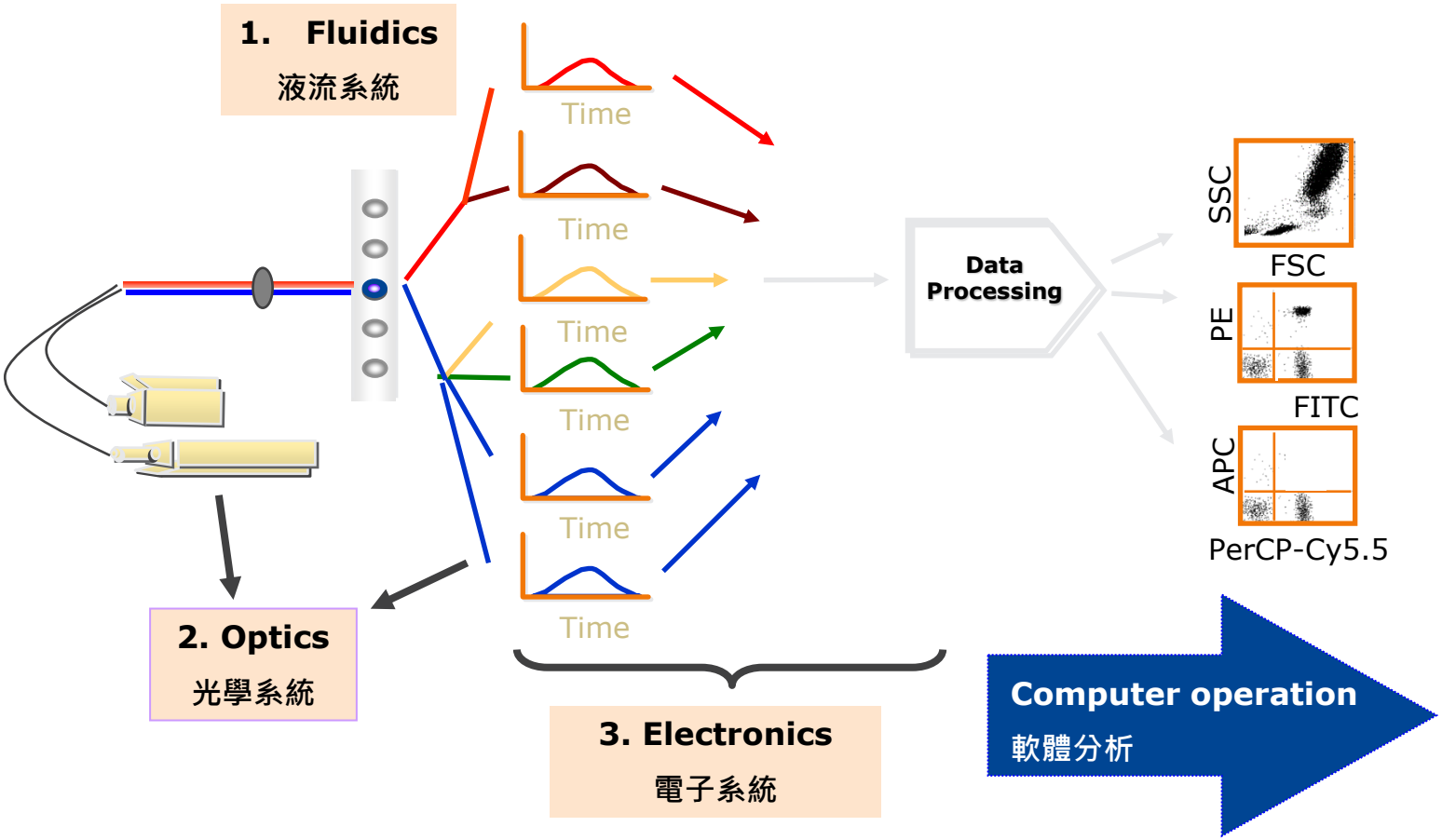
Optics 光學系統

To generate and collect the light signals.

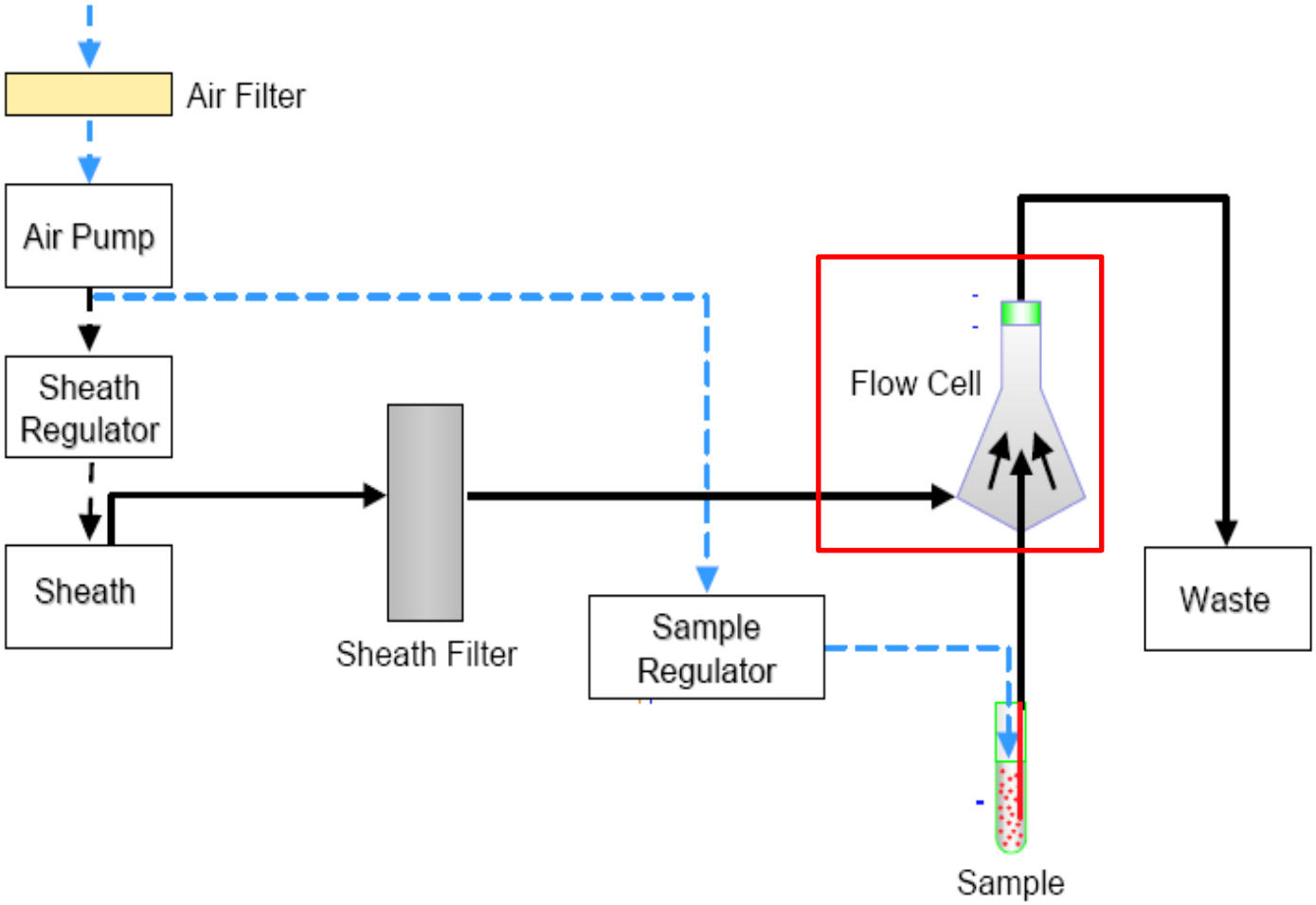
Electronics 電子系統

To convert the optical signals to proportional digital signals, process the signals, and communicate with the computer.

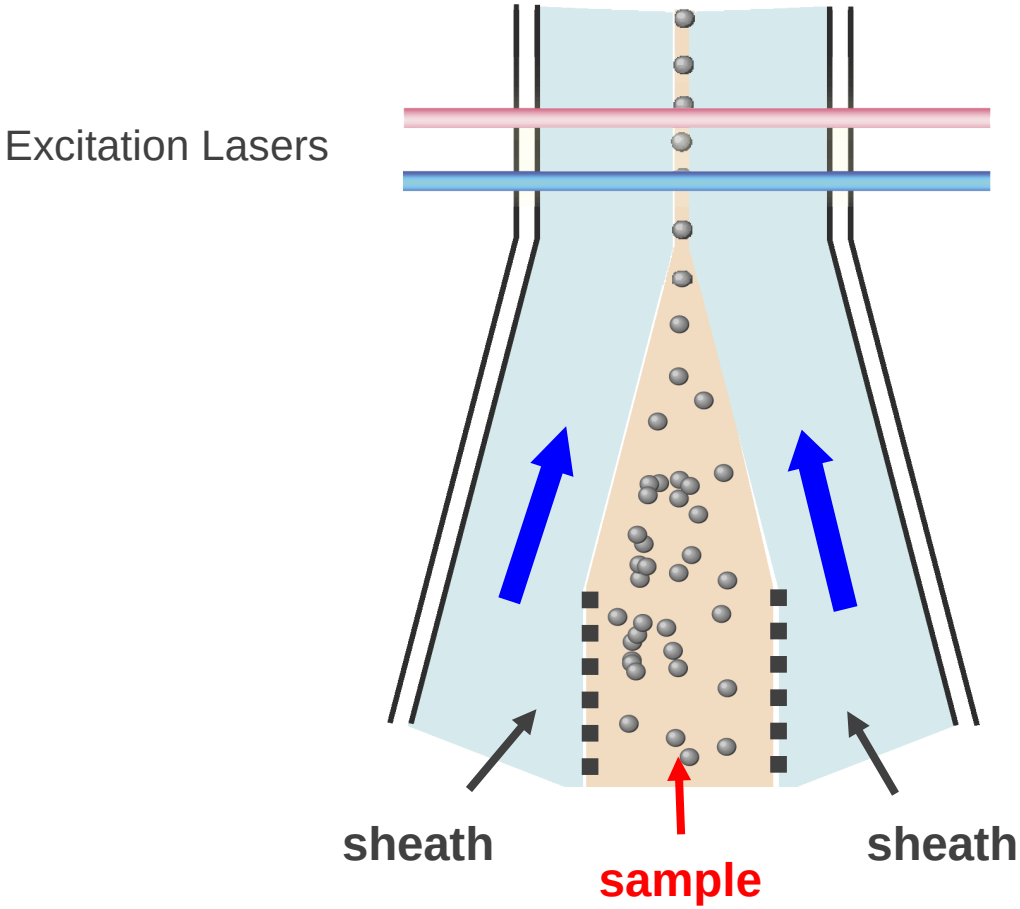
Flow Cytometry Detection Overview



Fluidics Pressurized System



Sample Flow



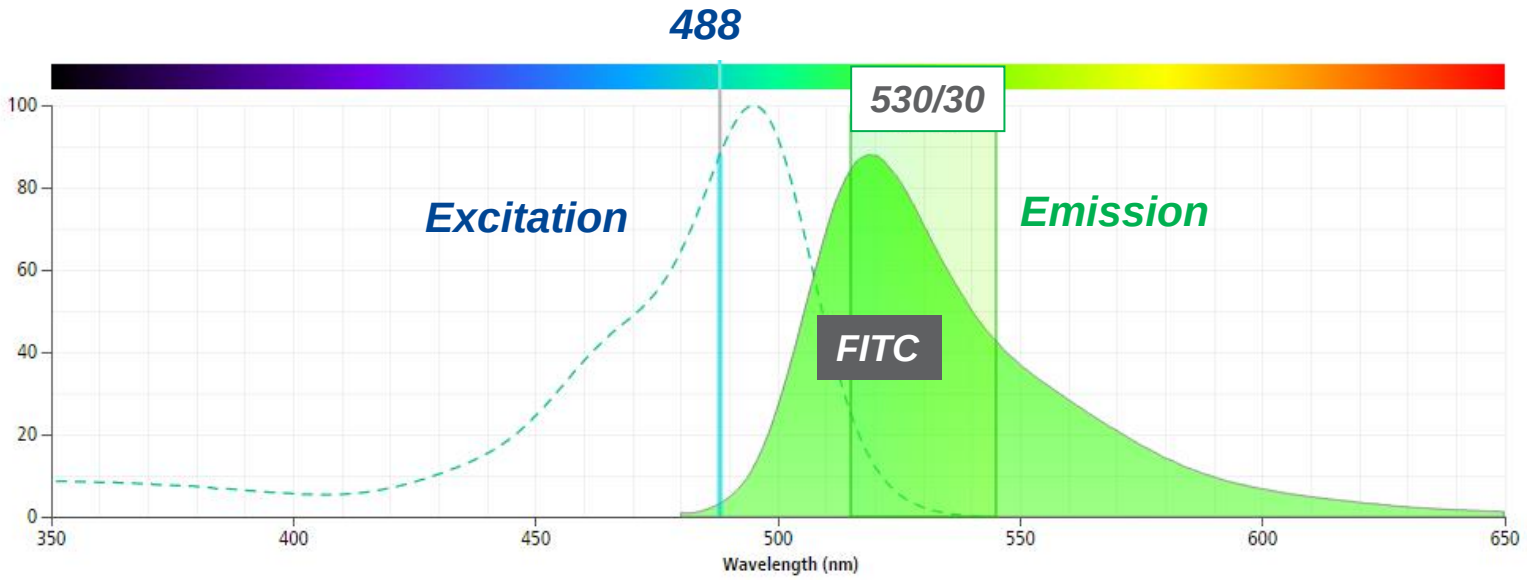
Hydrodynamic Focusing

Flow Cell

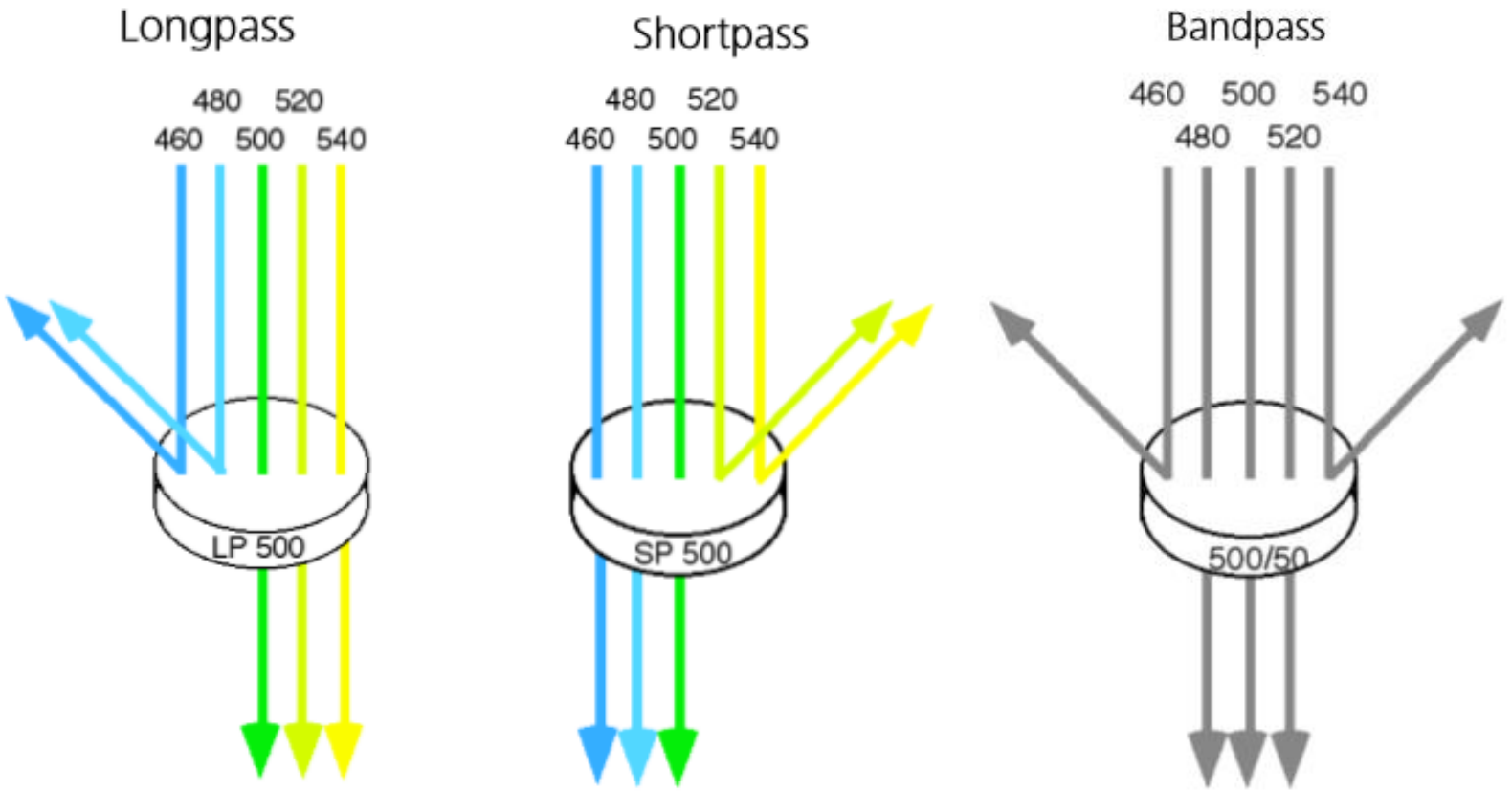
Excitation and Emission

- Use the maximum excitation wavelengths to **determine lasers** that can be used to excite the fluorochrome.

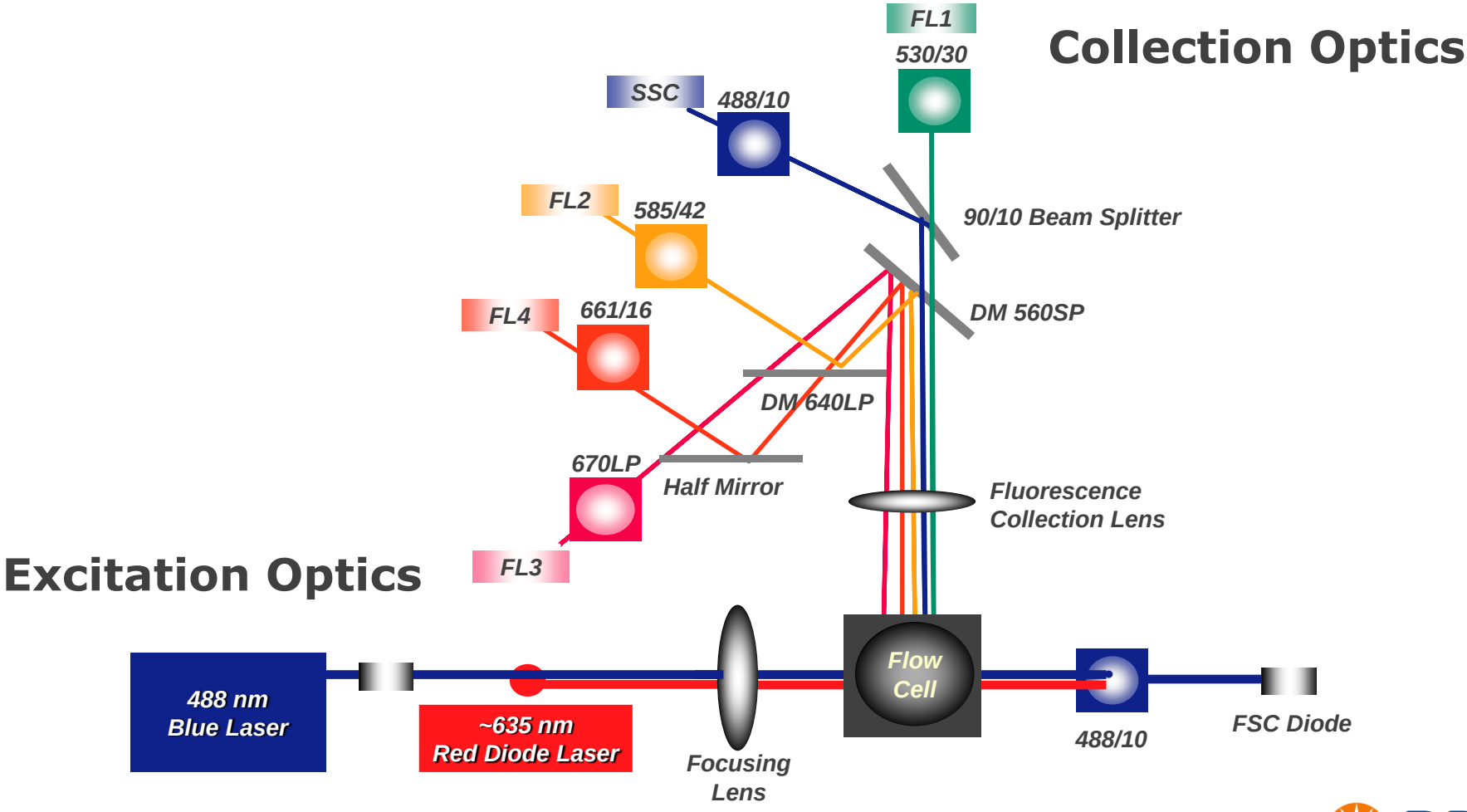
- Use the maximum emission wavelengths to **determine filters and PMTs** that can be used to measure the signal.



Types of optical filters



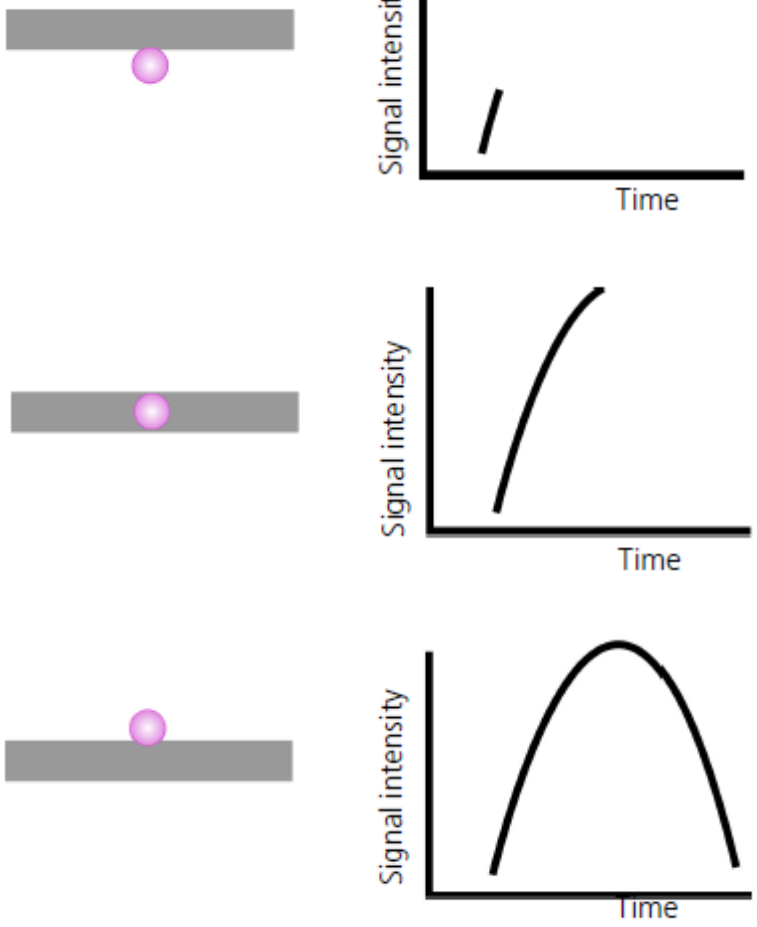
FACSCalibur Optics

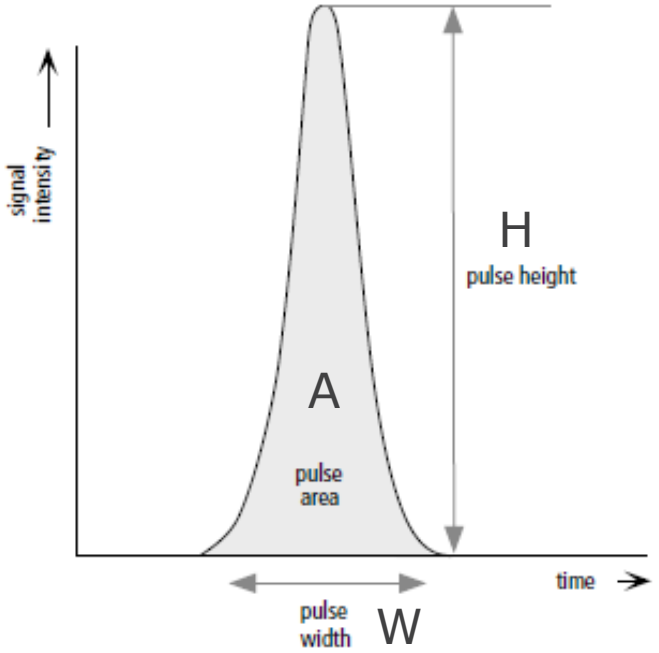


FACSCalibur Configuration

	Detector	Filter	Color	Fluorochrome
488nm	FL1	530/30 nm	Green	FITC, BB515, Alexa Fluor 488
	FL2	585/42 nm	Yellow/Orange	PE, PI
	FL3	670 nm LP	Dark Red	PerCP, PerCP-Cy5.5, BB700
635nm	FL4	661/16 nm	Red	APC, Alexa Fluor 647

Electronics





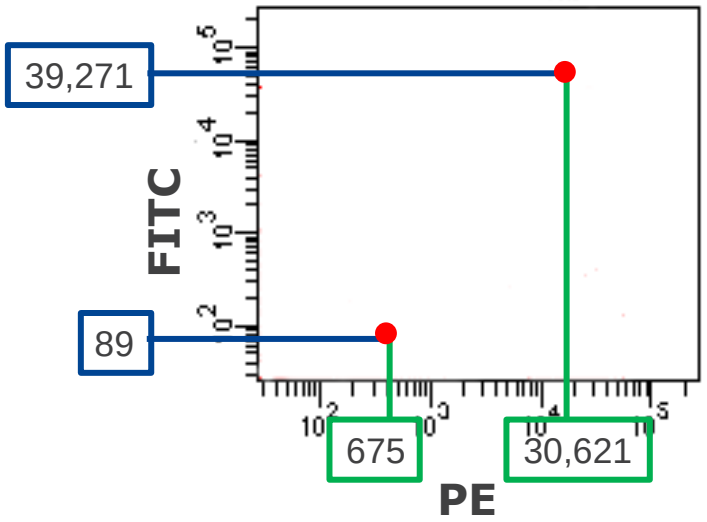
PMT偵測器將光學訊號轉換成電子訊號
H:電子訊號最高時數值
A:電子訊號總面積
W:電子訊號存在時間(細胞通過雷射時間)

Data Storage

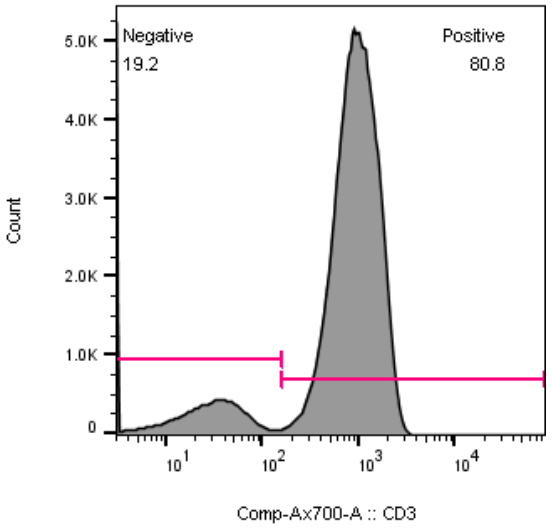
- FCS File: Flow Cytometry Standard

List-Mode Data

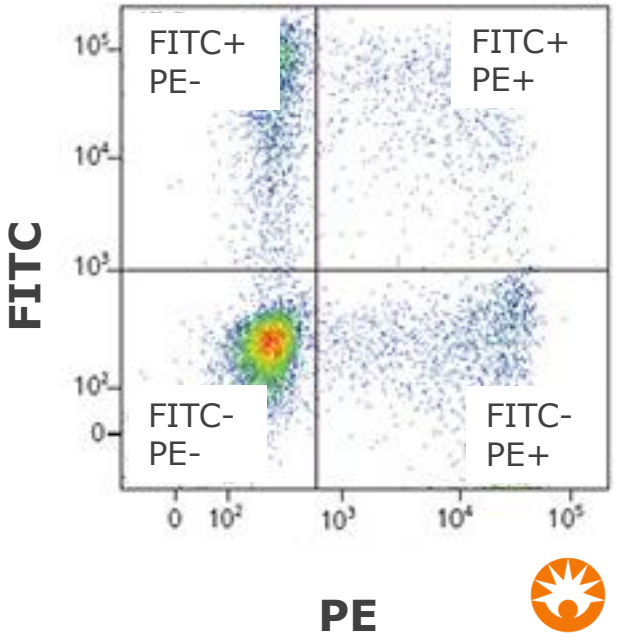
	Time	FSC	SSC	FITC	PE
Event 1	0	60	120	89	675
Event 2	10	160	65	39,271	30,621
Event 3	30	650	160	22,688	6,189



Histogram

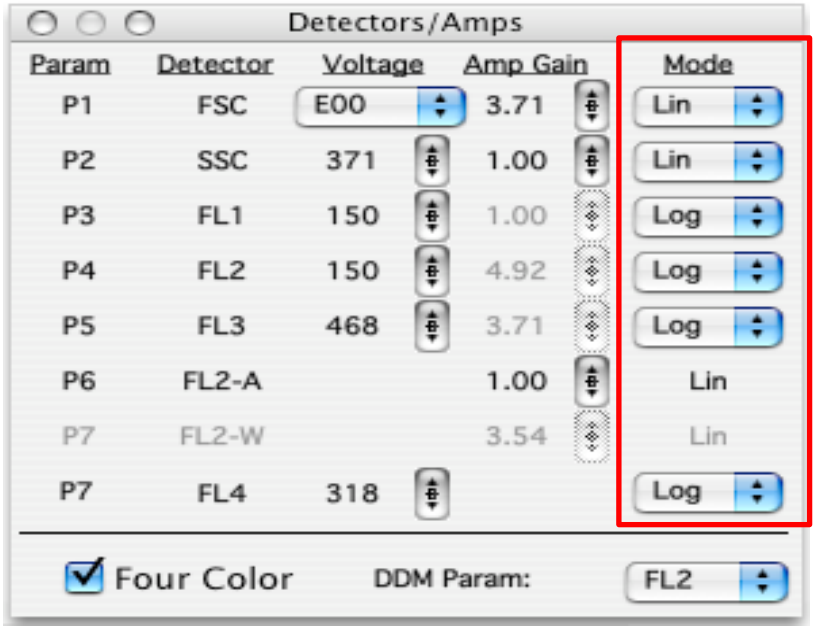


Dot Plot



Data Display

- Linear Scaling
- Log Scaling



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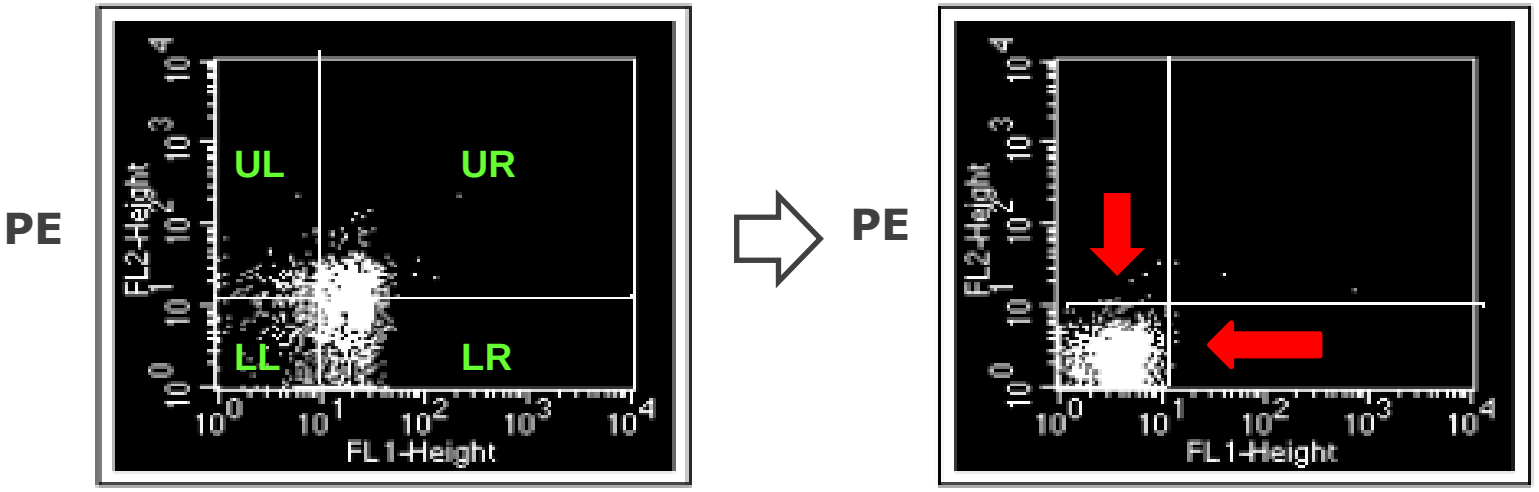
Auto-fluorescence

Non-stain sample

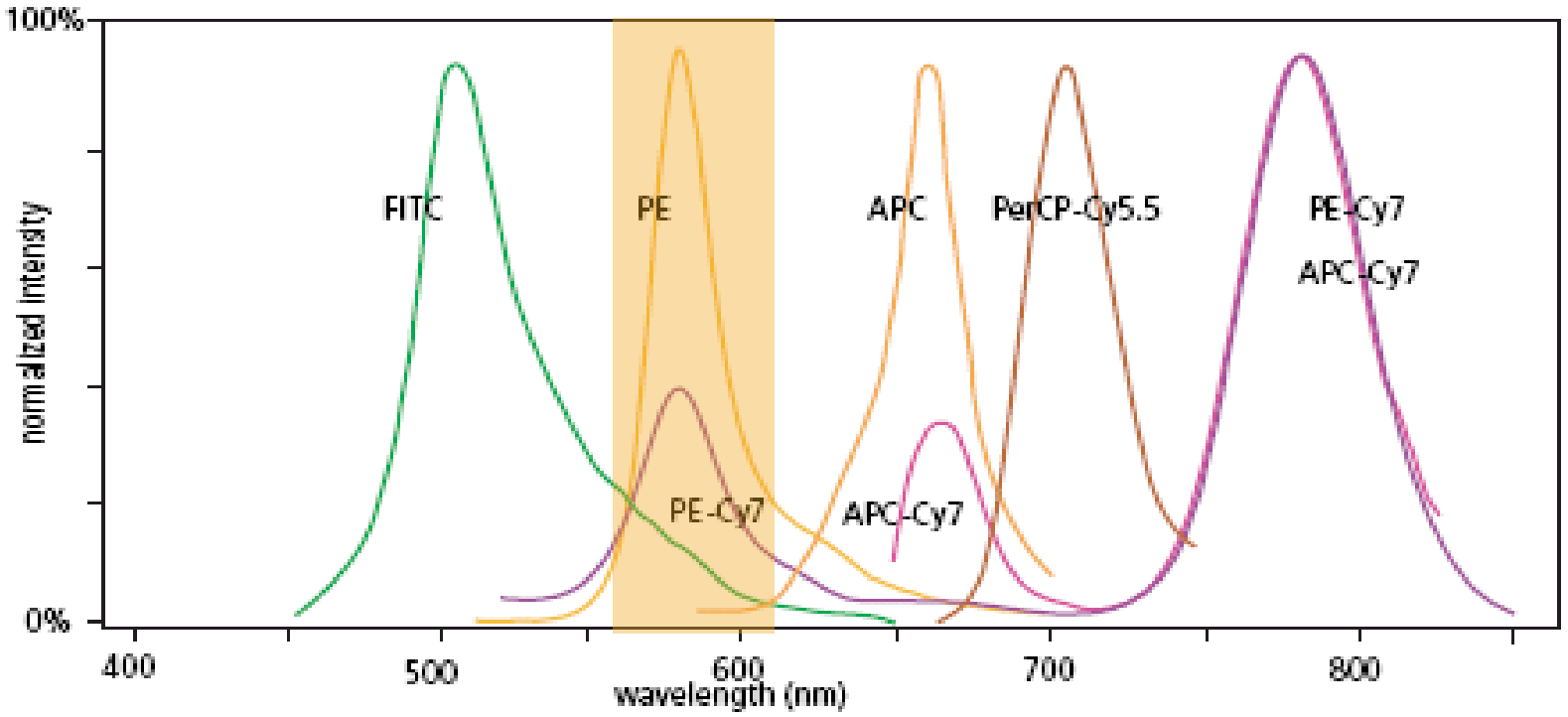


Auto-fluorescence

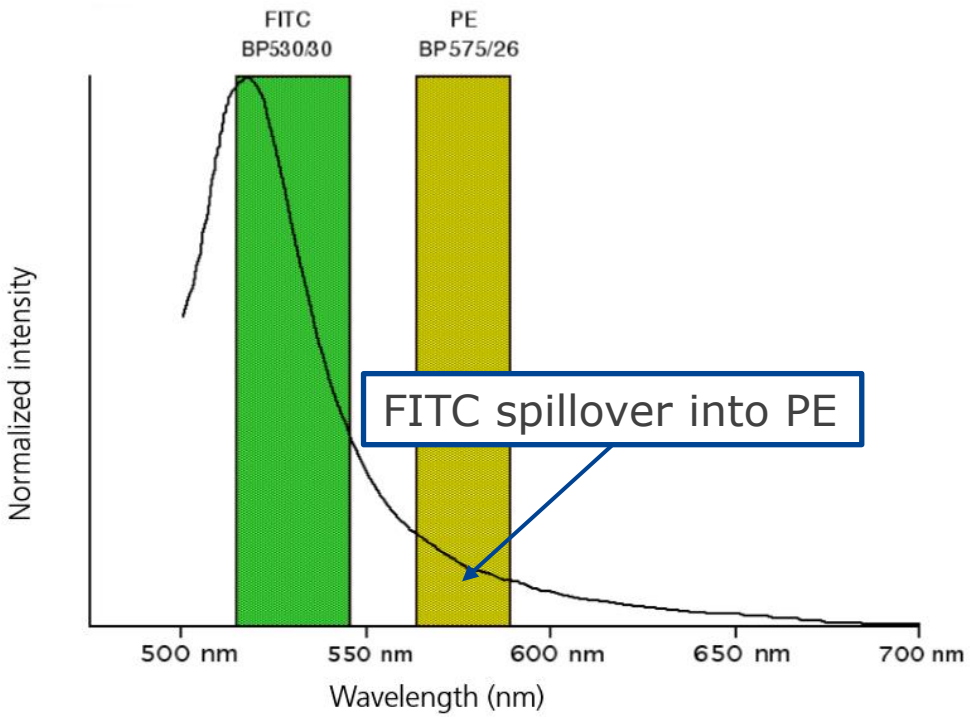
After voltage adjustment

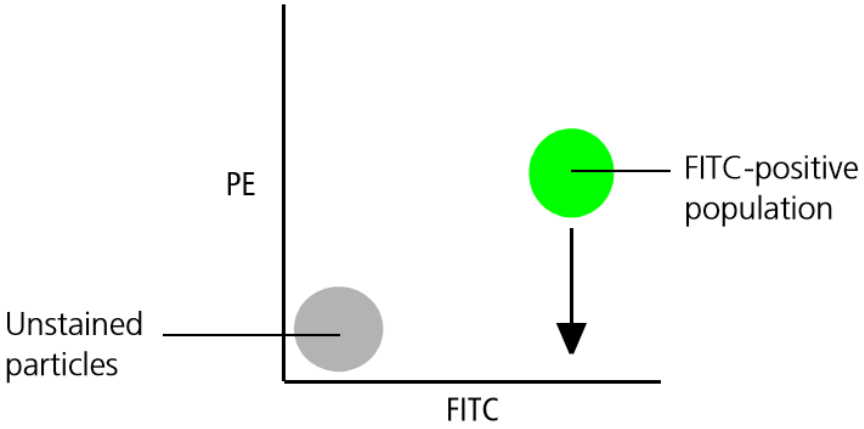
Compensation theory

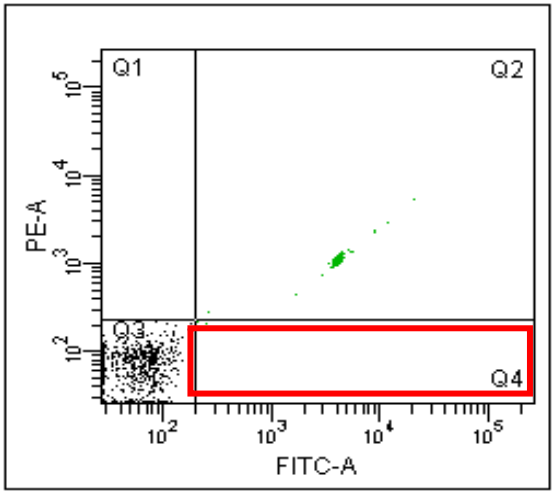
Emission Optics



FITC Spillover



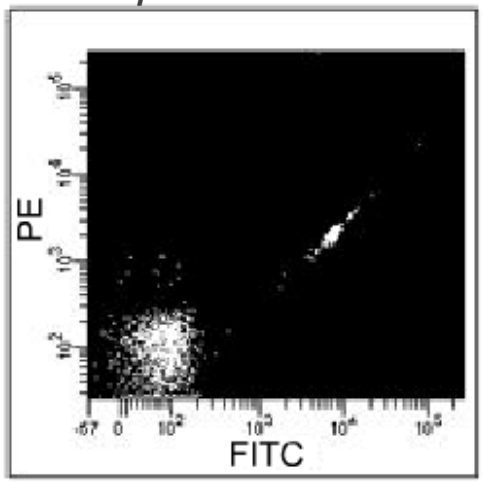




FITC Compensation

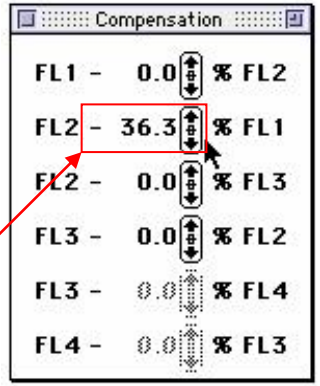
FITC single stained sample

Before Compensation

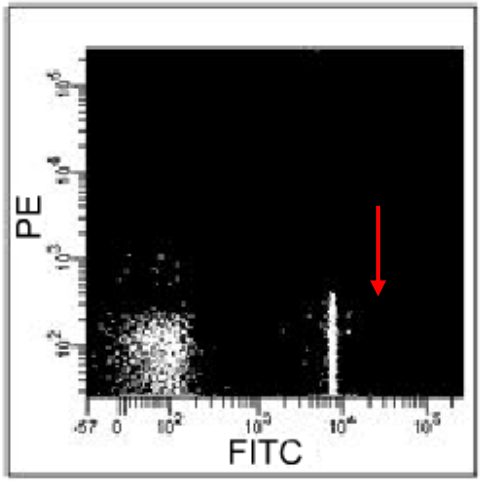


FL1 = FITC + x% PE

FL2 = Background + y% FITC



After Compensation



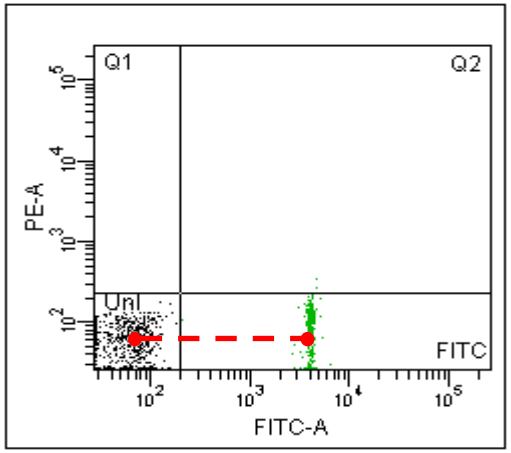
FL2 = Background

FL1 = FITC + x% PE

Compensation Examples

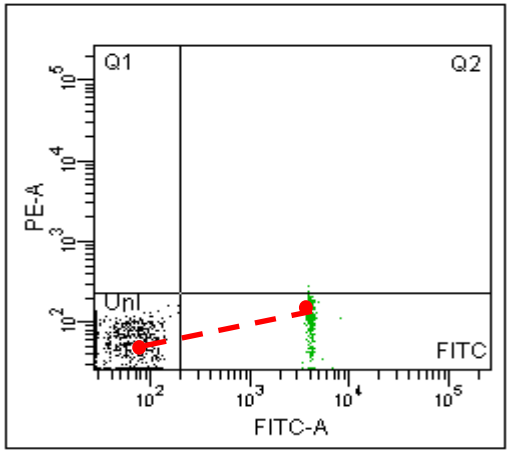
Incorrect Compensation

Correct Compensation



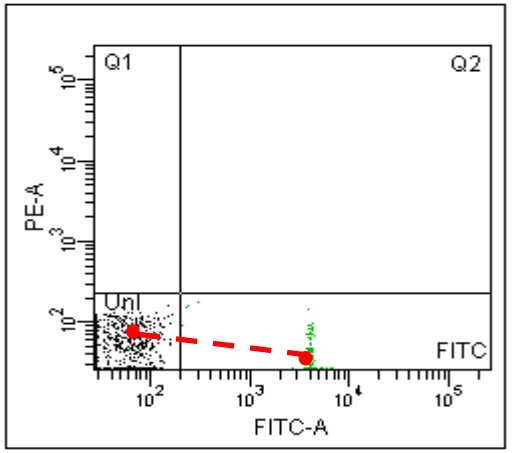
Population	PE-A Mean
Unl	64
FITC	69

Undercompensation



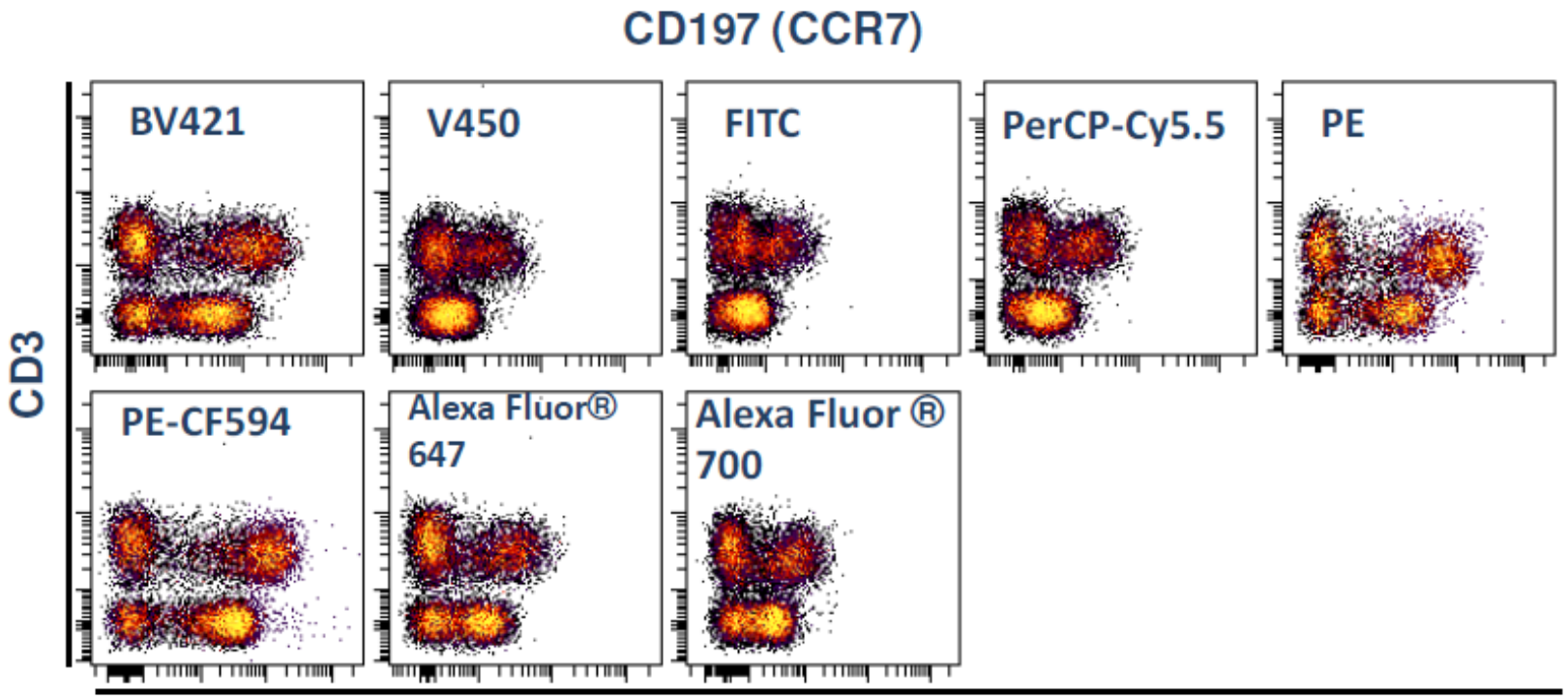
Population	PE-A Mean
Unl	61
FITC	96

Overcompensation



Population	PE-A Mean
Unl	62
FITC	-1

Fluorochrome Choice is the Key to Reveal Dim Marker



- The proper choice of fluorochrome helps us understand more about the biology of the experiment.
- Bright dyes are important when looking at dim antigens.

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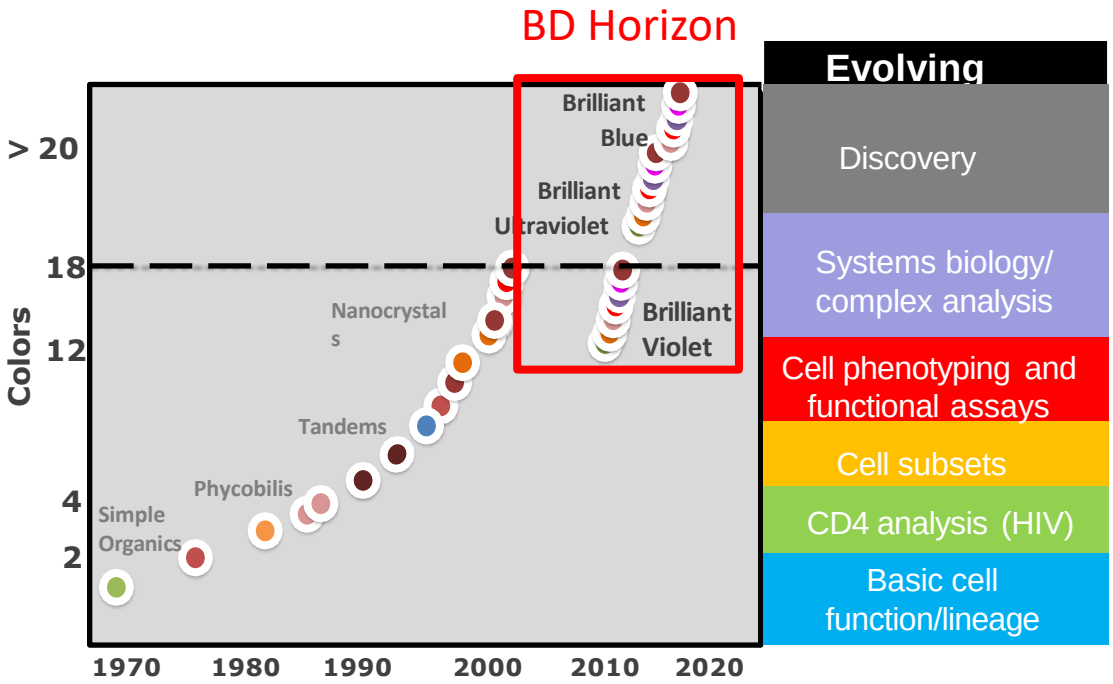
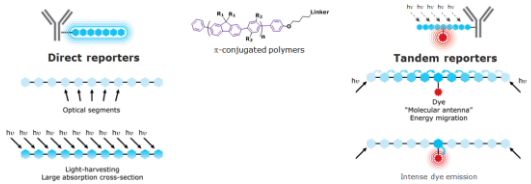
Colors continue to drive advances in Flow Cytometry



- More Colors and Greater Sensitivity

2012年BD收購Sirigen公司



- Bright fluorescent materials
- Large collective optical response
- Efficient energy donors
- Amplified dye emission
- Reproducible synthetic framework

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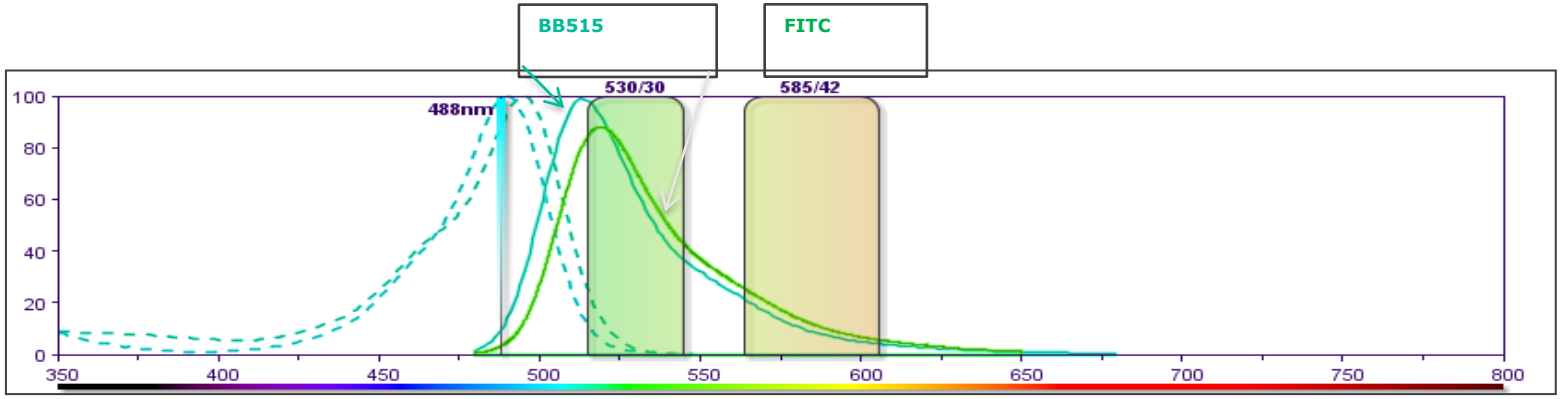
BD Horizon Brilliant Blue515 -for 488nm Blue laser

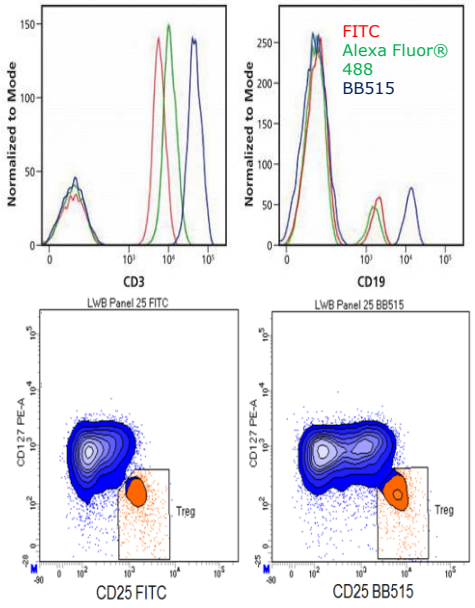


特點：替代FITC的最佳選擇

- 亮度: 是FITC 的 7倍
- 干擾: 對 PE 干擾更小 (幾無螢光補償)
- 抗體使用量更少
- 所有儀器標配偵測: FITC 檢測偵測器

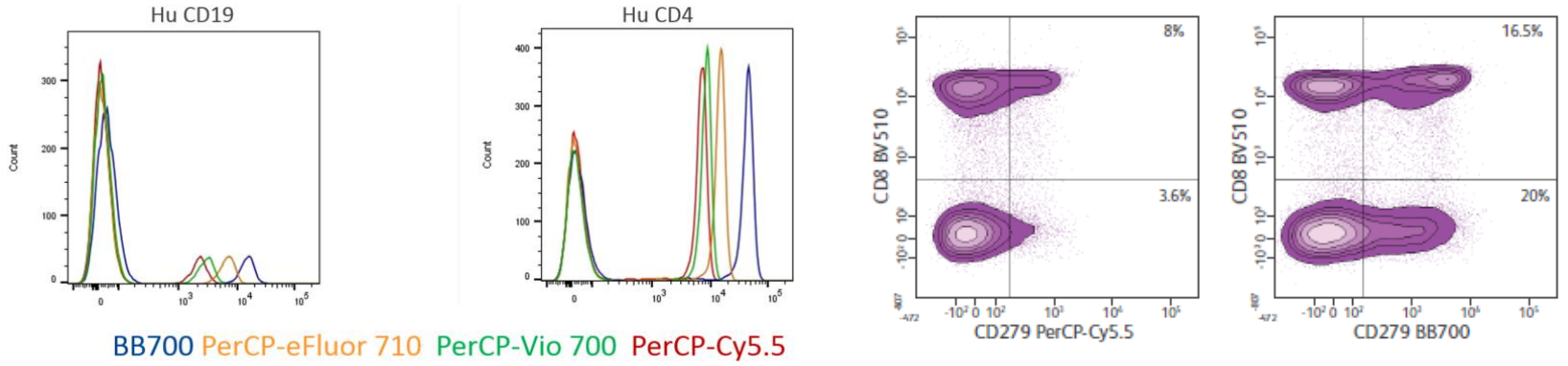
好處

- 高亮度低干擾有更好的細胞群分辨能力
- 入門等級的流式細胞儀也可使用
- 弱表達的族群更易辨識, 低干擾使實驗的設計更簡單



BD Horizon Brilliant Blue700 -for 488nm Blue Laser

特點：更加明亮、較不易被其它雷射激發

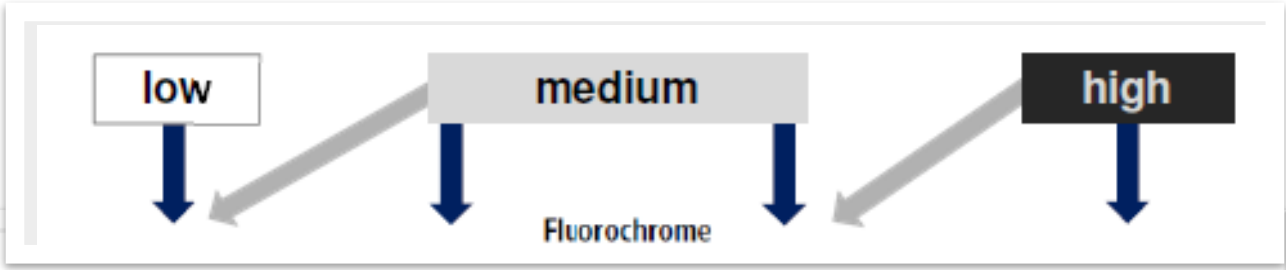


	BUV395	BUV496	BUV661	BUV737	BV421	BV510	BV605	BV650	BV711	BV786
CD4-BB700	0%	0%	6%	7%	0%	0%	2%	8%	50%	18%
CD4-PerCP-Cy5-5	0%	0%	25%	17%	0%	0%	0%	18%	87%	32%
CD4-PerCP-Vio700	0%	0%	12%	17%	0%	0%	0%	10%	92%	44%
CD4-PerCP-eFluor710	0%	0%	7%	20%	0%	0%	0%	6%	105%	47%

	FITC	PE (Y-G)	PE-CF594 (Y-G)	PE-Cy7 (Y-G)	APC	APC-H7	APC-R700
CD4-BB700	1%	0%	0%	2%	39%	16%	18%
CD4-PerCP-Cy5-5	0%	0%	0%	19%	46%	16%	20%
CD4-PerCP-Vio700	0%	0%	0%	22%	20%	19%	25%
CD4-PerCP-eFluor710	0%	0%	0%	23%	13%	21%	34%

Fluorochrome/Antigen Combination

Antigen Density



Laser	Very Bright	Bright	Moderate	Dim
Ultraviolet (355 nm)		BD Horizon™ BUV737	BD Horizon™ BUV395	
Violet (405 nm)	BD Horizon™ BV421 BD Horizon™ BV650 BD Horizon™ BV711	BD Horizon™ BV605 BD Horizon™ BV786	BD Horizon™ BV510	BD Horizon™ V450 BD Horizon™ V500
Blue (488 nm)	BD Horizon™ BB515 BD Horizon™ PE-CF594 PE-Cy™5	PE PE-Cy™7	FITC Alexa Fluor® 488 PerCP-Cy™5.5	PerCP
Yellow/Green (561 nm)	PE BD Horizon™ PE-CF594 PE-Cy™5 PE-Cy™7			
Red (640 nm)		APC Alexa Fluor® 647		Alexa Fluor® 700 APC-H7 APC-Cy7

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Applications

- **Phenotype Analysis**
  (Cell Surface Antigens/Markers)
- **Intracellular Analysis**
  -- Eg. Cytokines, Signal Transduction molecules…etc.
- **DNA Analysis**
  -- Eg. Viability, Cell cycle, Apoptosis…etc.
- **Cell Fuction Analysis**
  -- Eg. Free radicals, Ca^{2+}, Reporter genes…etc.
- **CBA (Cytometric Bead Array)**

  -- cytokine detection

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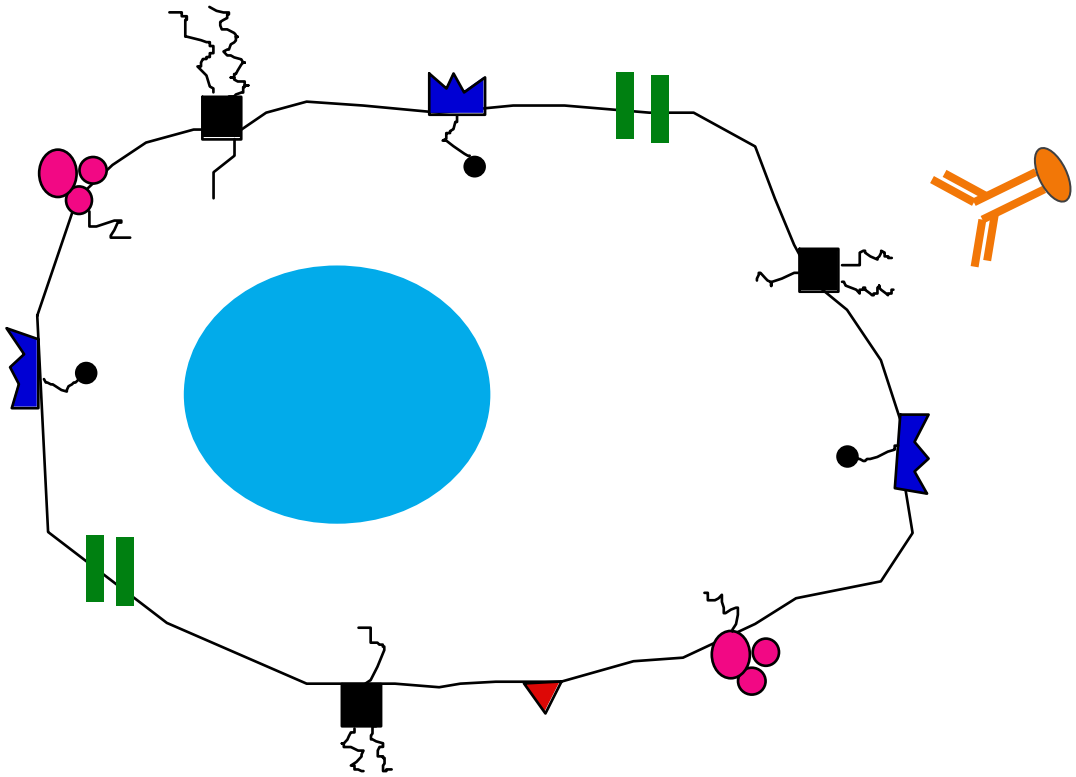
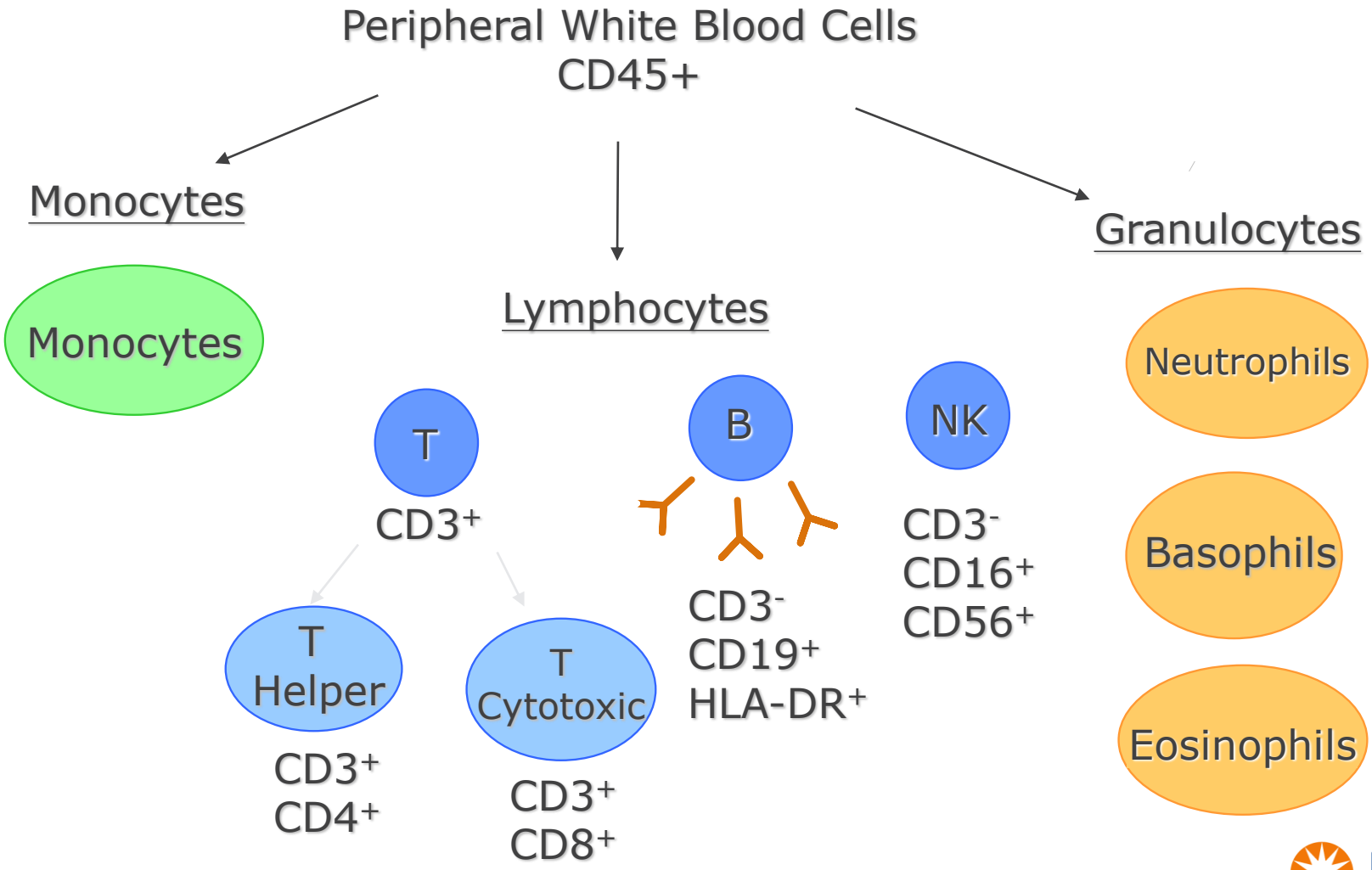
Phenotype Analysis

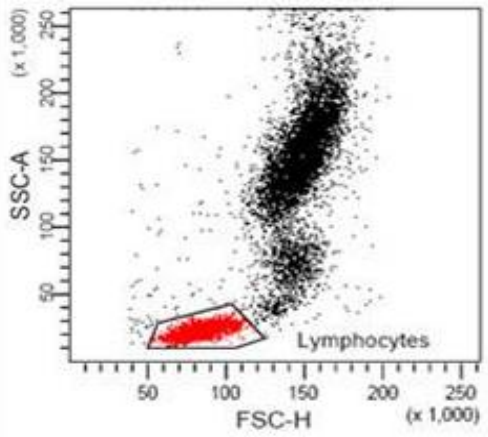


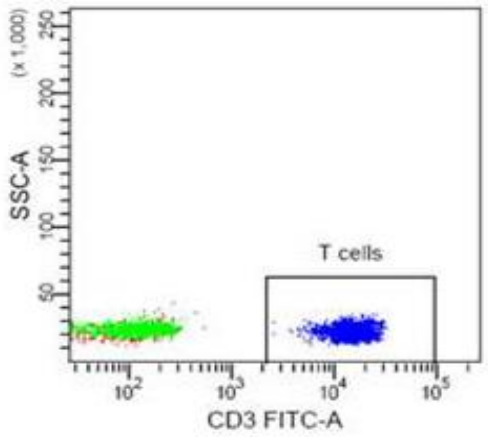
- Ligand
- Receptor
- Adhesion molecule
- …etc

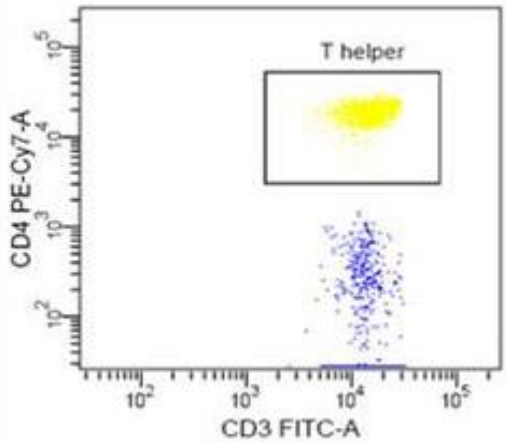
Lymphocyte Immunophenotyping

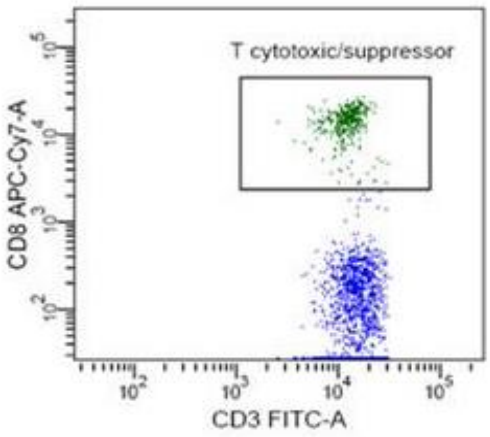


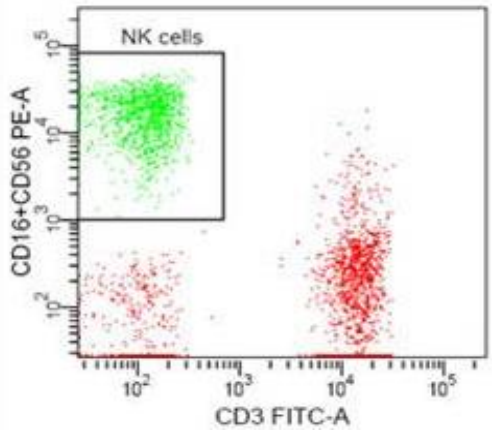
Lymphocyte Phenotype -TBNK Subset

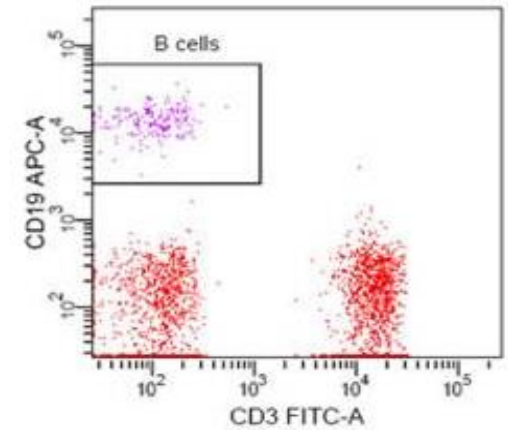












Major Known T-Cell Markers

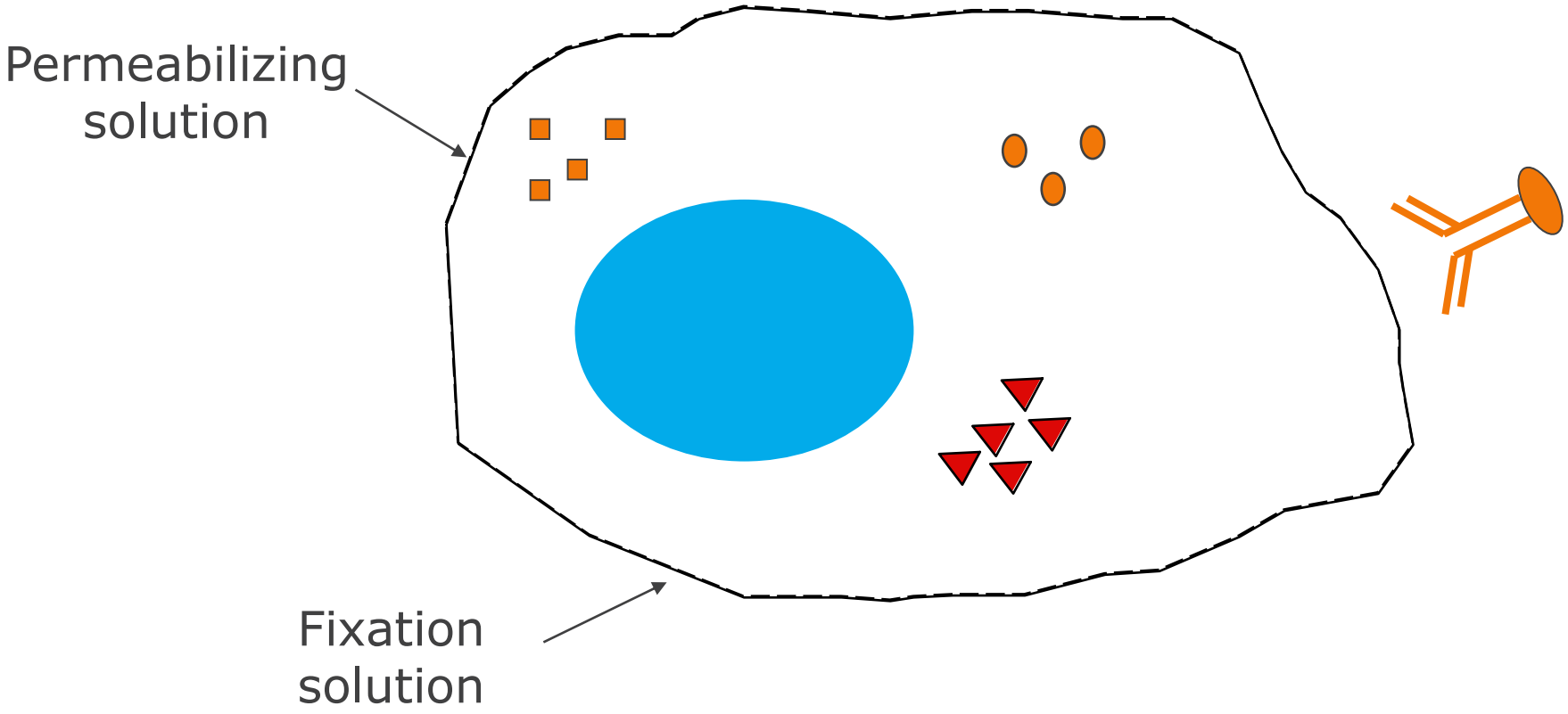
Type of Cell	Cytotoxic	Th1	Th2	Th9 [1]	Th17	Tfh [2]	Treg
Main Function	Kill virus-infected cells	Activate microbicidal function of infected macrophages, and help B cells to produce antibody	Help B cells and switch antibody isotype production	T cell proliferation and enhanced IgG and IgE production by B cells	Enhance neutrophil response	Regulate development of antigen specific B cell development and antibody production	Immune regulation
Pathogens Targeted	Viruses and some	Intracellular pathogens	Parasites	Parasites	Fungi and extracellular bacteria	-	-
Harmful Function	Transplant rejection	Autoimmune disease	Allergy, asthma	Allergy	Organ-specific autoimmune disease	Autoimmune disease	Autoimmune disease, cancer
Extracellular Markers	CD8	CD4, CXCR3	CD4, CCR4, Crth2 (human)	CD4	CD4, CCR6	CD4, CXCR5	CD4, CD25
Differentiation Cytokines	-	IFN-γ, IL-2, IL-12, IL-18, IL-27	IL-4, IL-2, IL-33	IL-4, TGF-β	TGF-β, IL-6, IL-1, IL-21, IL-23	IL-12, IL-6	TGF-β, IL-12
Effector Cytokines	IFN-γ, TNF, LT-α	**IFN-γ**, LT-α, TNF	**IL-4**, IL-5, IL-6, IL-13	IL-9, IL-10	**IL-17A**, IL-17F, IL-21, IL-22, IL-26, TNF, CCL20	IL-21	TGF-β, IL-10
Transcription Factors	-	T-bet, Stat1, Stat6	GATA3, Stat5, Stat6	GATA3, Smads, Stat6	RORγt, RORα, Stat3	Bcl-6, MAF	FoxP3, Smad3, Stat5

Phenotype Analysis

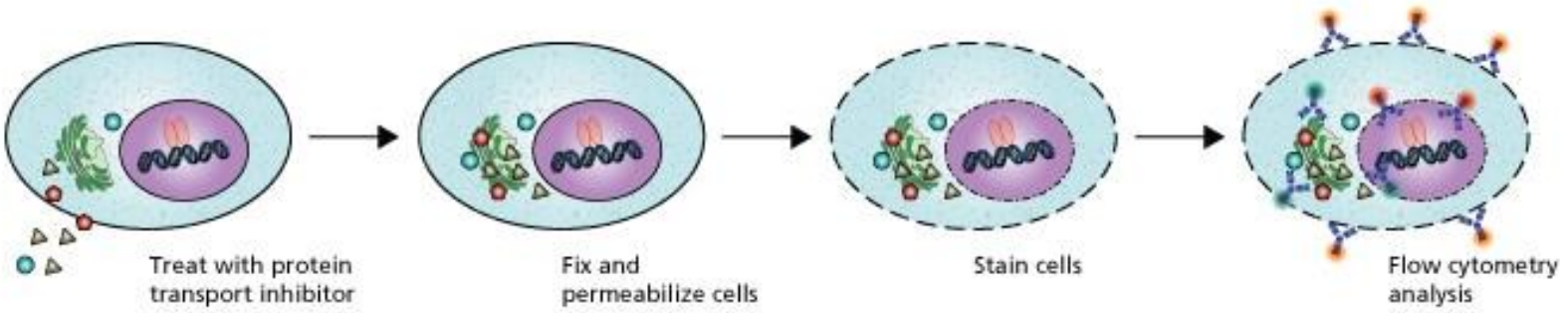
Intracellular Analysis

BD

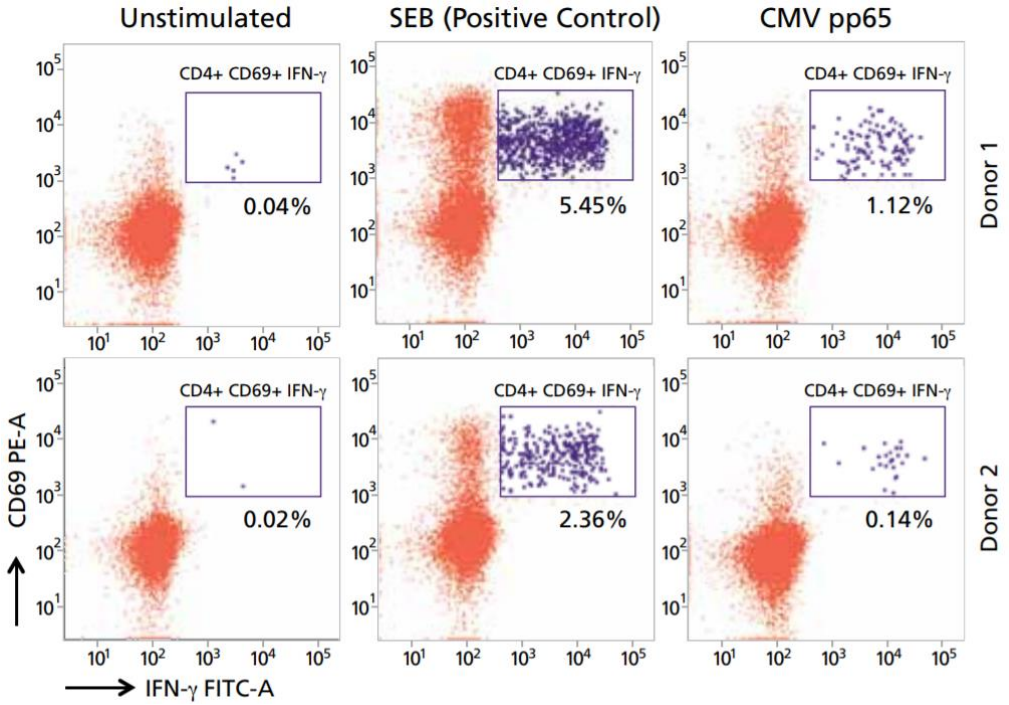
Intracellular Analysis



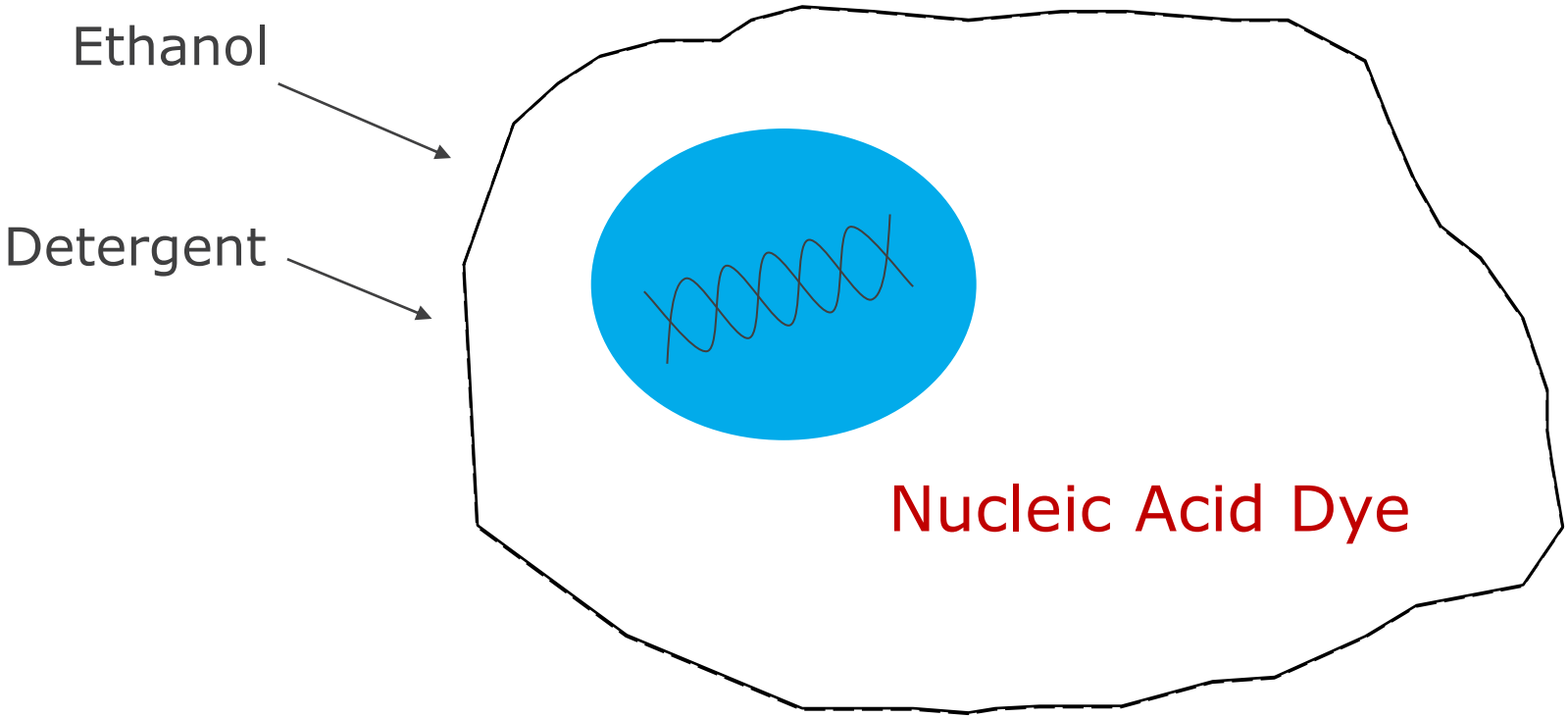
Cell Surface and Cytoplasmic Stain protocol



Antigen-specific IFN-γ response in CD4+ and CD69+ lymphocytes



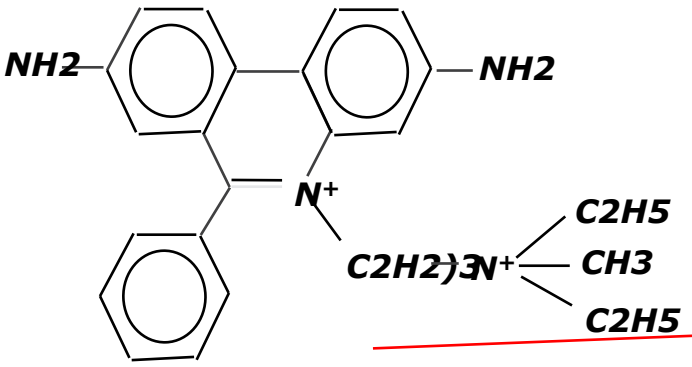
DNA Analysis

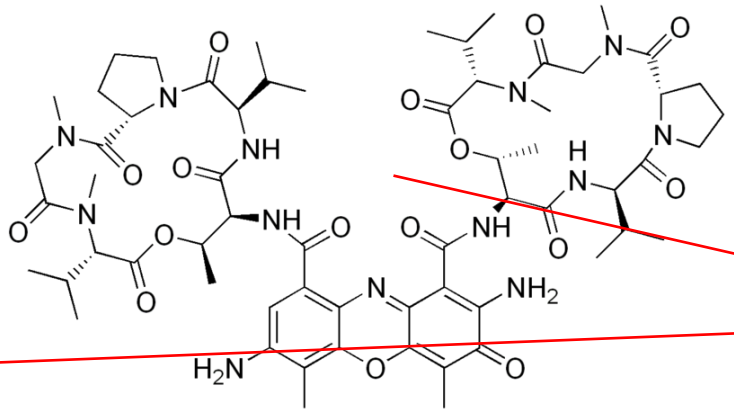


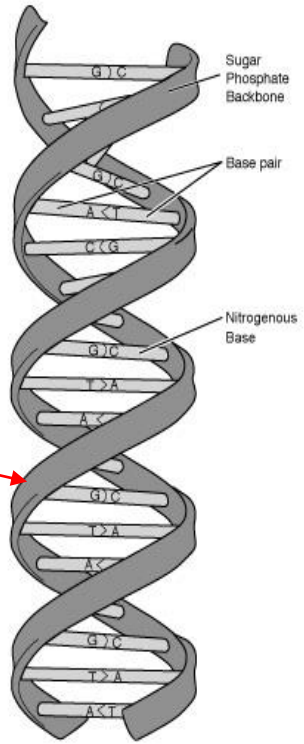
DNA Dye

Propidium

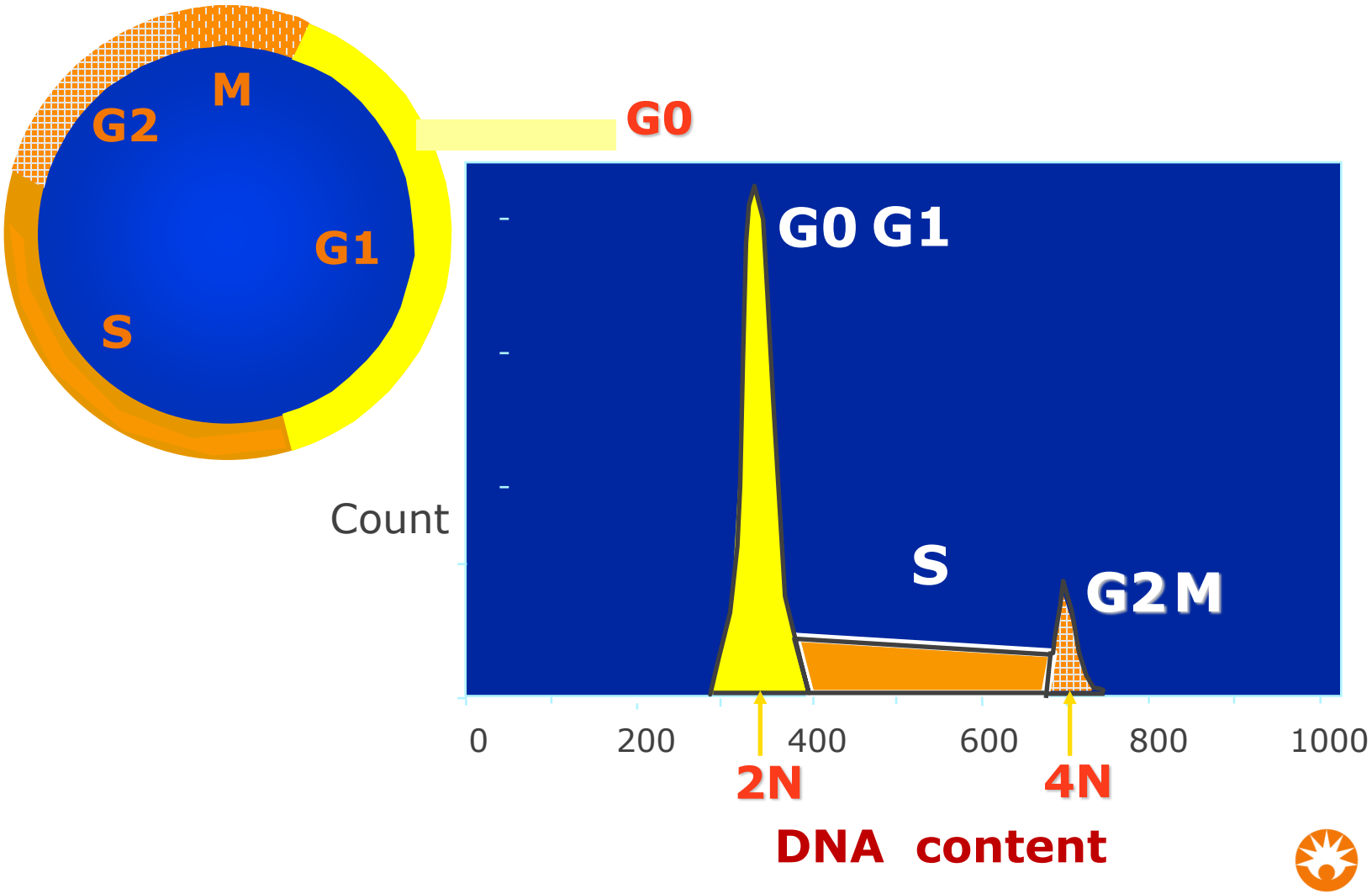
7-AAD



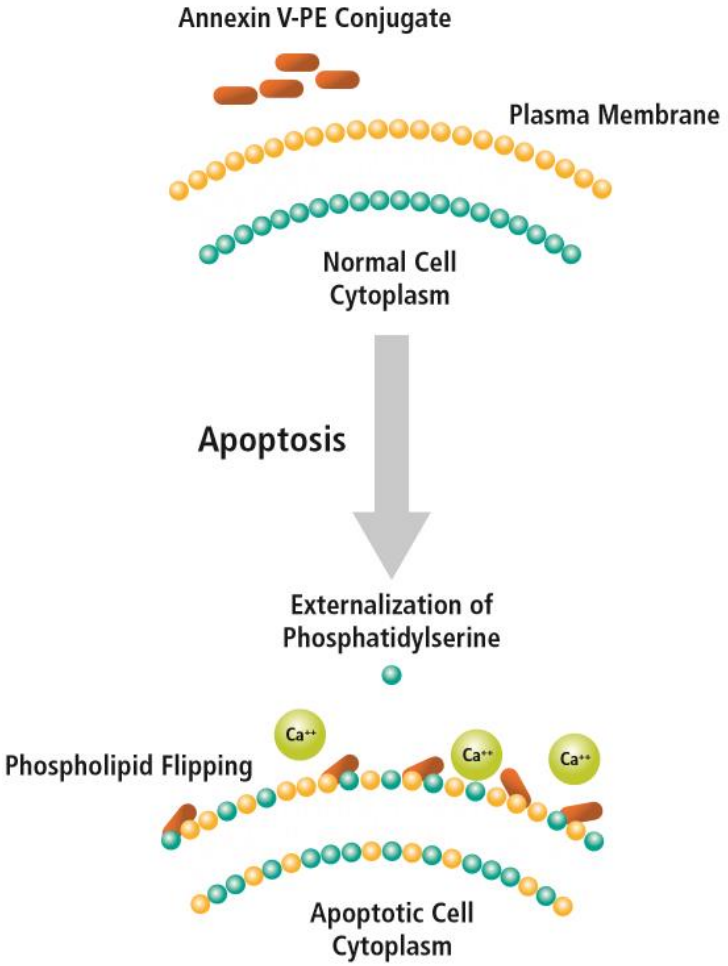




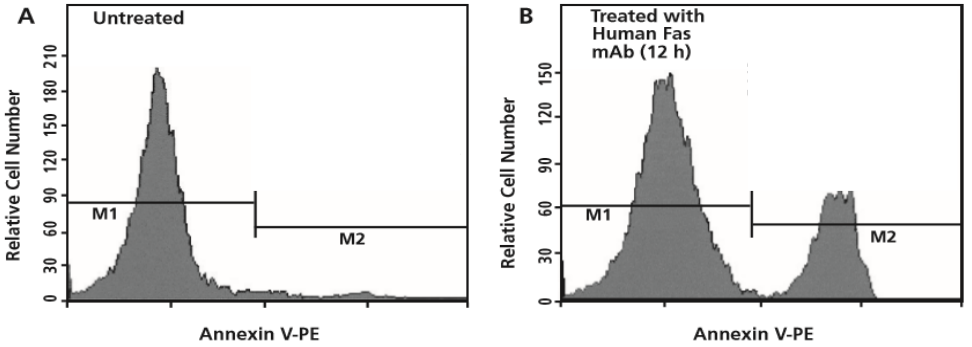
Cell Cycle Analysis



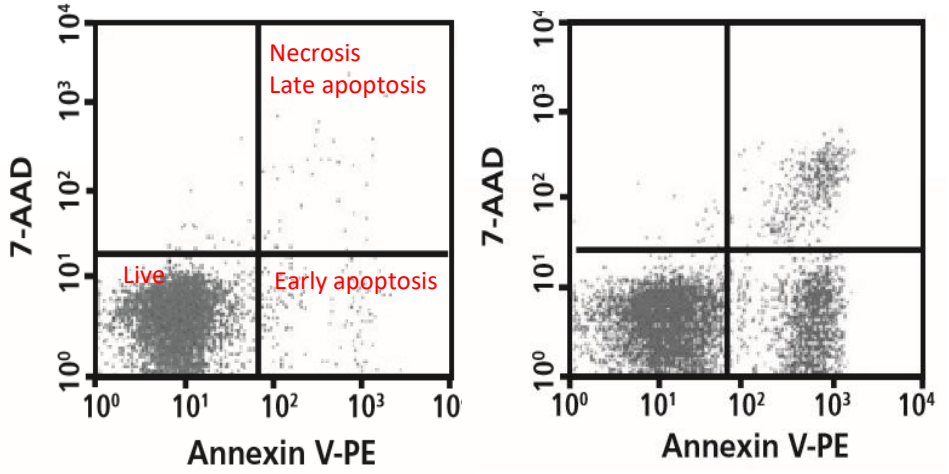
Cell Apoptosis
-Annexin V Apoptosis Assay



■ Annexin V-PE

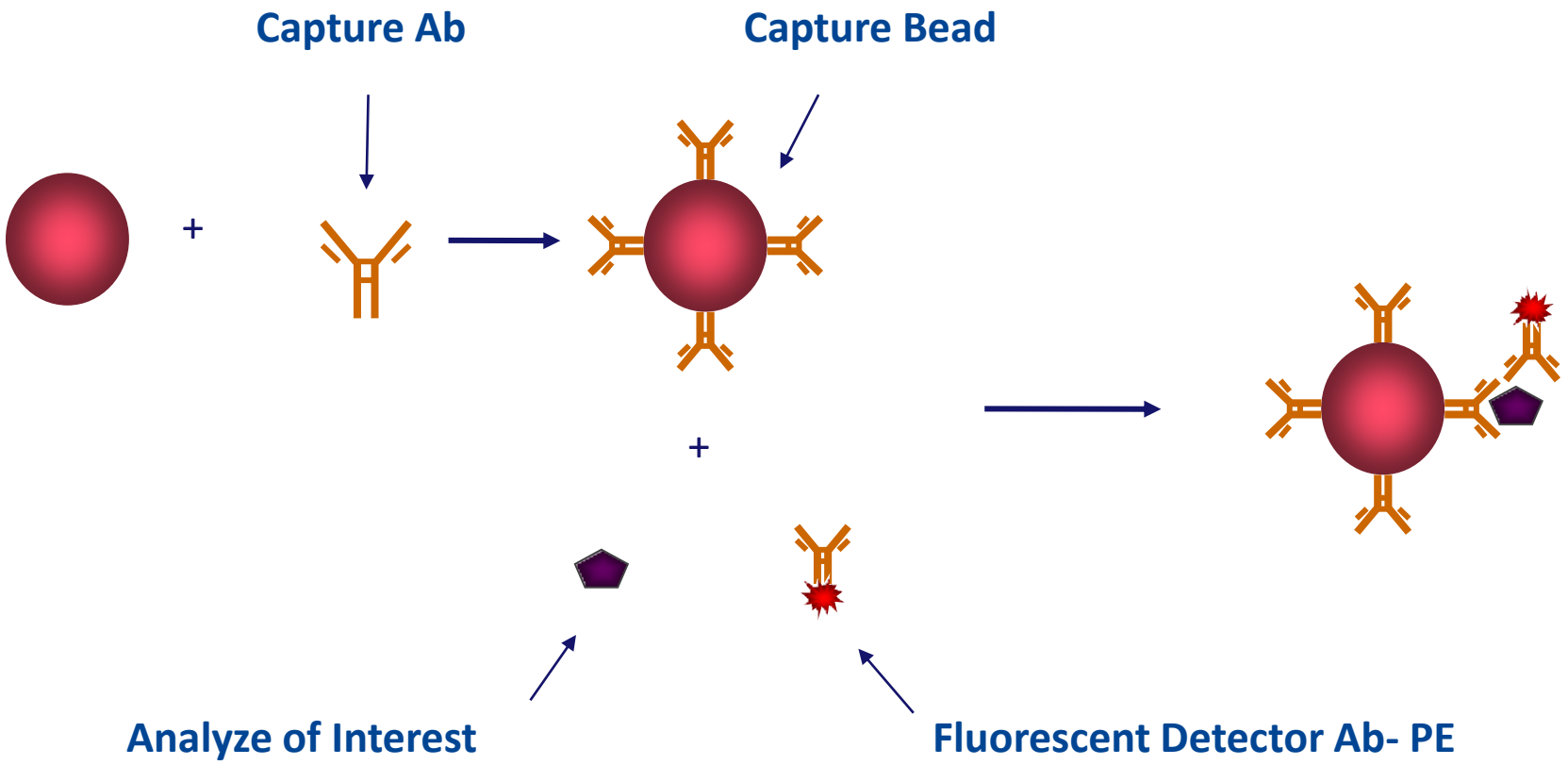


■ Annexin V-PE and 7-AAD staining

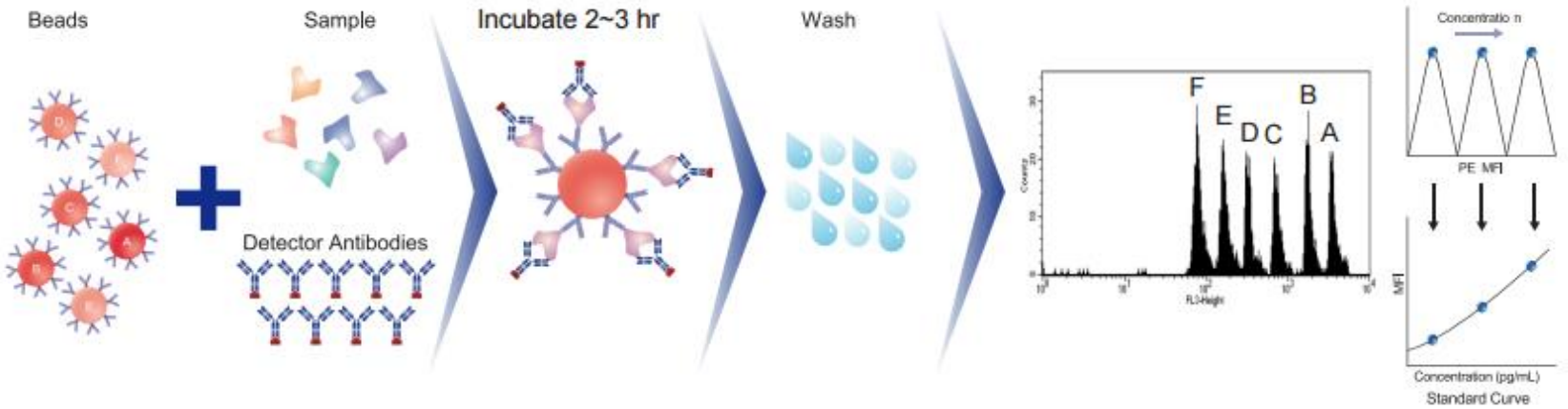


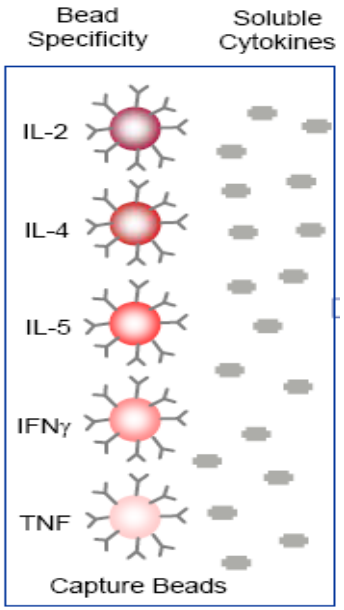
BD

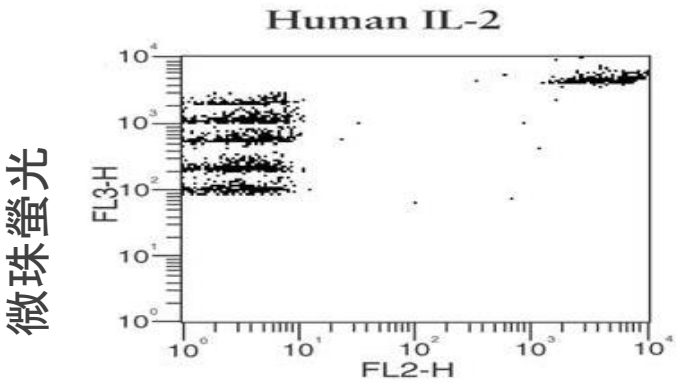
Soluble form protein detection -Cytometric Beads Array (CBA)



CBA Assay Procedure

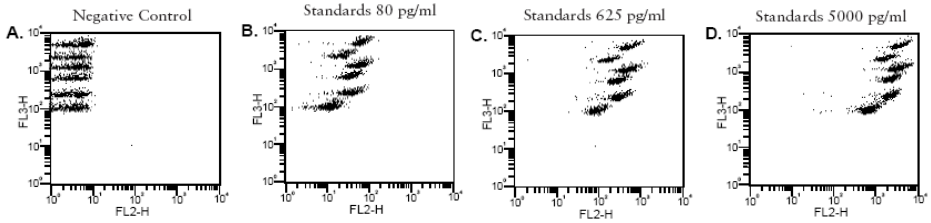




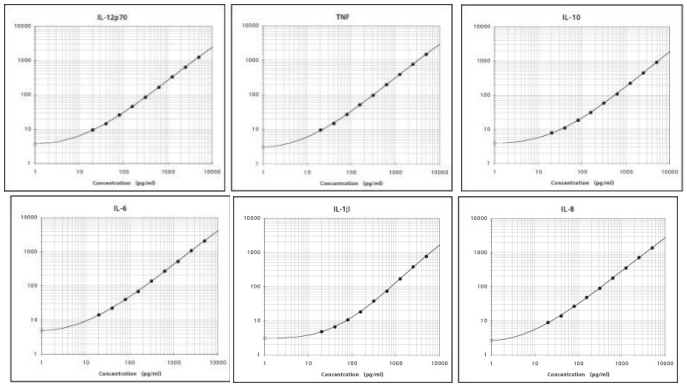


Standard Curves

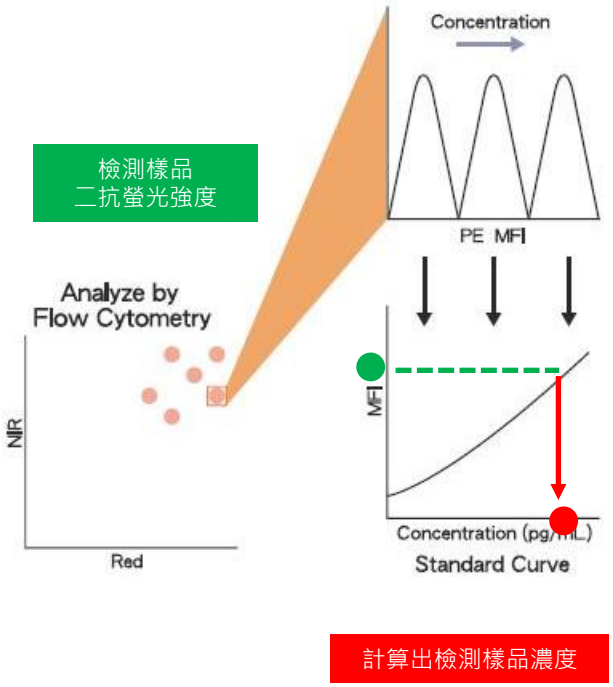
1. 以已知濃度樣品取得二抗平均螢光強度



2. 建立標準曲線

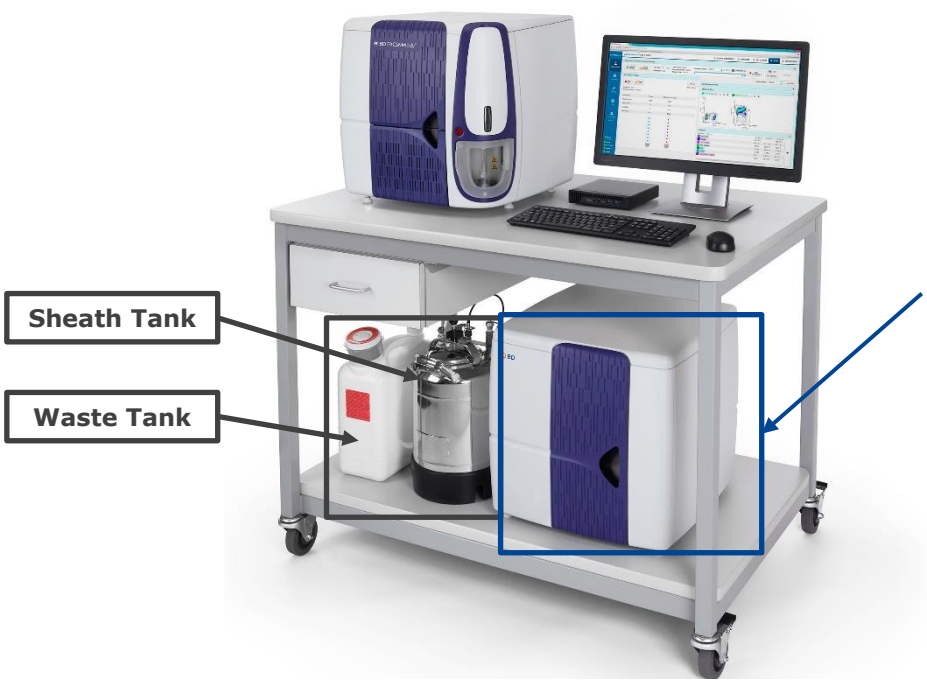


3. 計算檢測樣品細胞激素濃度

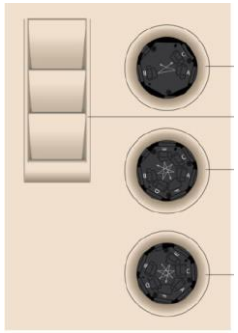


FACSMelody Overview

Fluidics Component

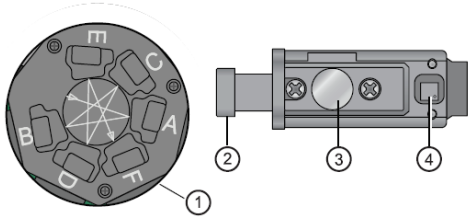


Optics Component



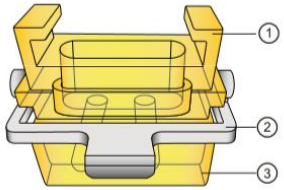
偵測器陣列

濾片-ID Chip:
自動偵測濾片移動與放置位置

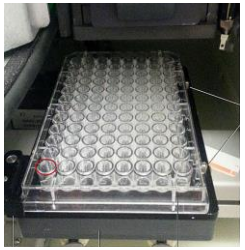


FACSMelody
-Sample Input / Sort Collection

- **Two-way sorting:** 1.5-, 2.0- and 5.0-mL tubes



- **One-way sorting:** 6-, 24-, 48-, 96- and 384-well plates, 96-well PCR tray



→**Temperature control**

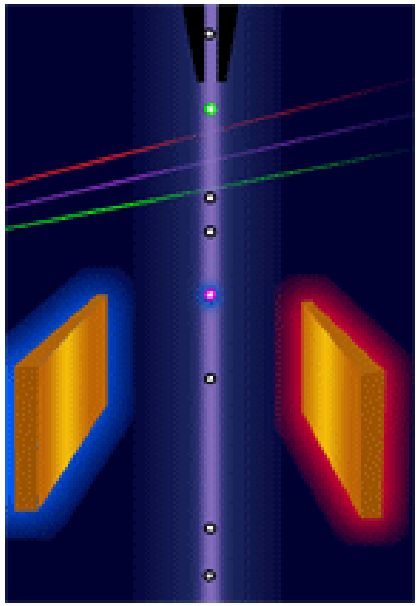
Temperature control:
4°C, 22°C, 37°C and 42°C or off

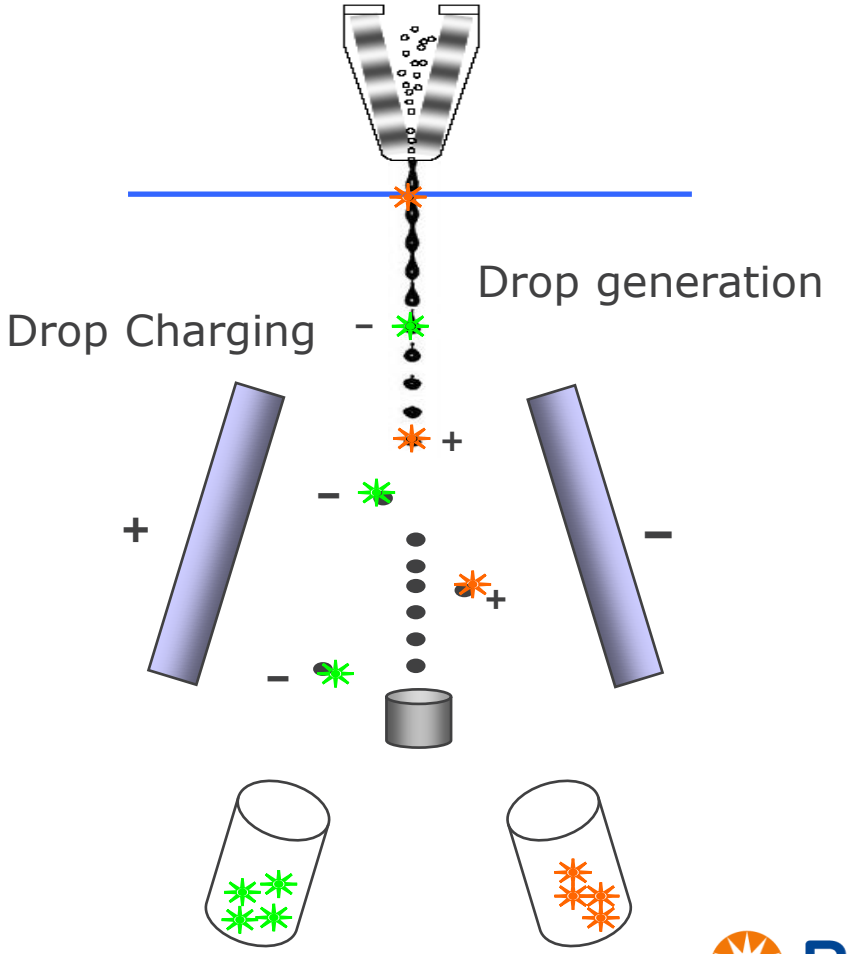
Sample agitation:
keep the sample constantly suspended

5.0-mL polystyrene or polypropylene tubes

Principle of FACS

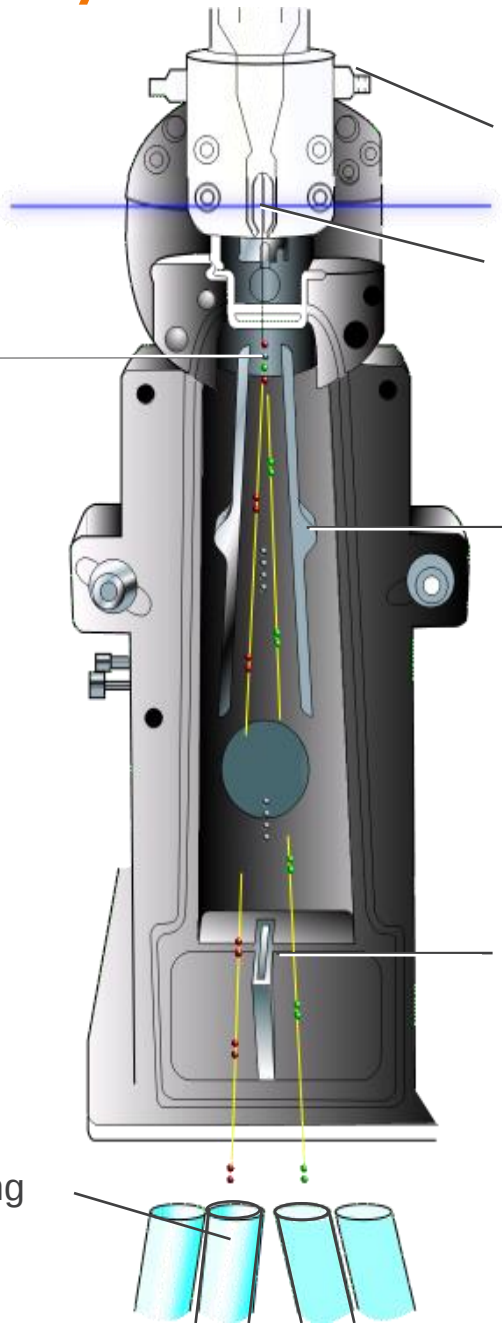
- The combination of 3 technologies:
  1. Fluorescence detection
  2. Blood cell counter
  3. Ink-jet technology





BD

Sorting Theory



2. Charge is applied via the stream-charging wire.

1. Sample generates light scatter and fluorescent signals; signals are analyzed.

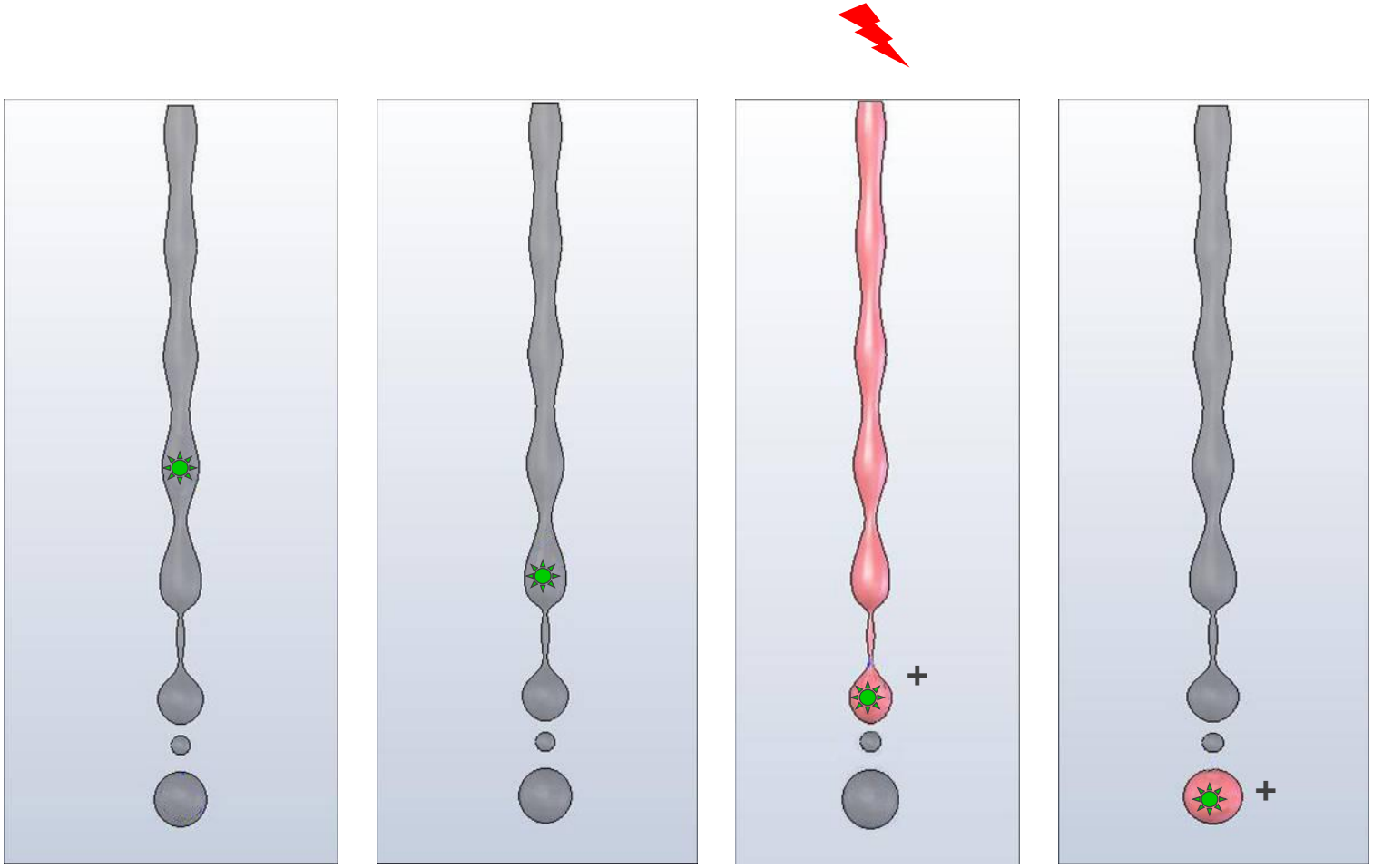
3. Charged droplets break off.

4. Deflection plates attract or repel charged droplet.

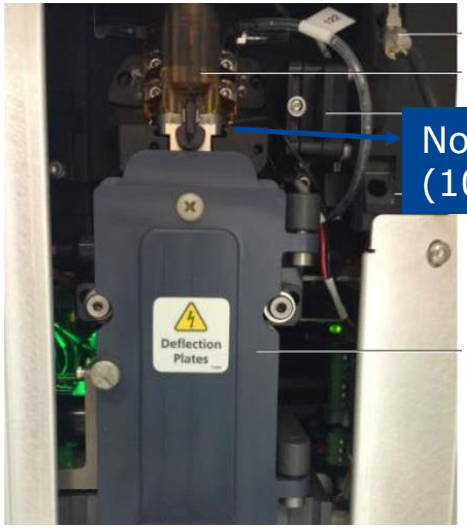
5. Uncharged droplets pass to waste.

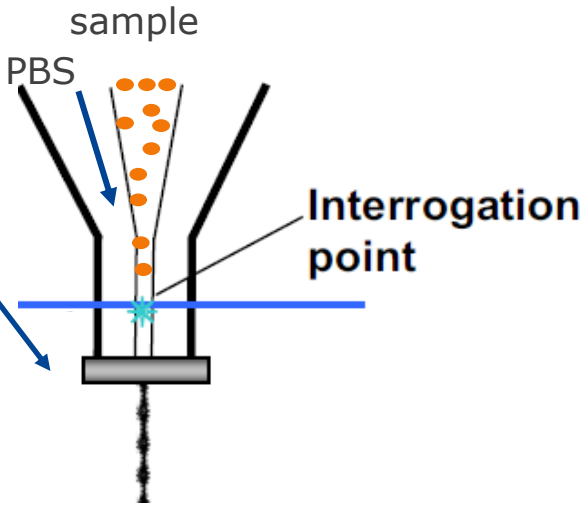
6. Charged drops containing particles of interest are collected.

Drop Charging



FACSMelody
-Flow Cell Structure





BD

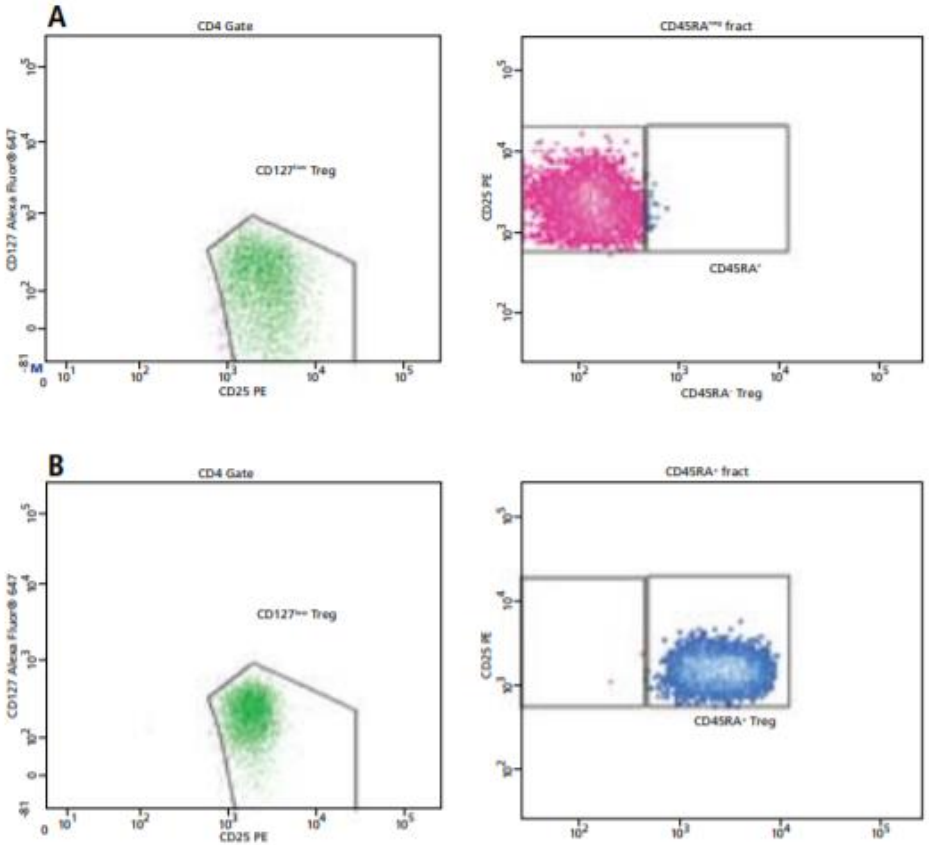
Sort mode

分選模式	功能
Yield	• 回收細胞數>純度的狀況下使用 • 具有目標細胞的液滴即會進行分選, 不考慮純度 • 適用於稀有細胞富集或想盡可能的不損失目標細胞時
Purity	• 純度>回收細胞數 • 可得到高純度的分選結果, 而犧牲部分目標細胞 • 分選速度越高, 分選效率越低, 目標細胞回收較少 • 適用於一般純度優先的分選, 建議準備1.5-2倍的起始理想細胞數量。
Singe Cell	**適用於盤式分選**, 希望單一個well中僅分選一顆細胞且純度優先的時候選擇。

- Cell Purity: Purity mode=Single Cell mode>Yield mode
- Recovery cell count: Yield>Purity>Single Cell

Sorting Cells By Surface Markers -Human Regulatory T-Cell Isolation

■ 分選後分析 **Phenotypic analysis of sorted Tregs**



Tube: CD45RAneg fract

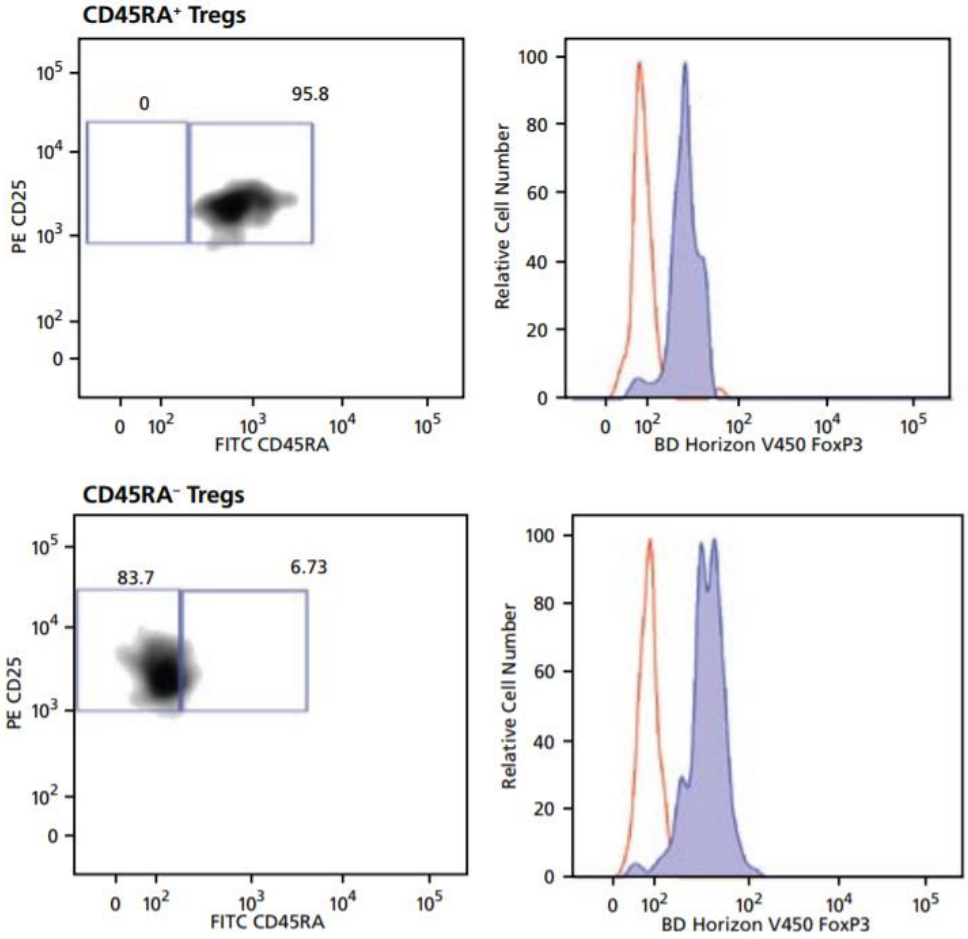
Population	#Events	%Parent	%Total
All Events	5,502	####	100.0
Lymphocyte Gate	5,172	94.0	94.0
Doublet Disc 1	5,156	99.7	93.7
Doublet Disc 2	5,150	99.9	93.6
CD4 Gate	5,127	99.6	93.2
CD25+CD127low Treg	4,997	97.5	90.8
CD45RA- Treg	4,962	99.3	90.2
CD45RA+ Treg	23	0.5	0.4

Tube: CD45RA+ fract

Population	#Events	%Parent	%Total
All Events	5,224	####	100.0
Lymphocyte Gate	4,912	94.0	94.0
Doublet Disc 1	4,898	99.7	93.8
Doublet Disc 2	4,898	100.0	93.8
CD4 Gate	4,890	99.8	93.6
CD25+CD127low Treg	4,840	99.0	92.6
CD45RA- Treg	3	0.1	0.1
CD45RA+ Treg	4,834	99.9	92.5

Sorting Cells By Surface Markers -Human Regulatory T-Cell Isolation

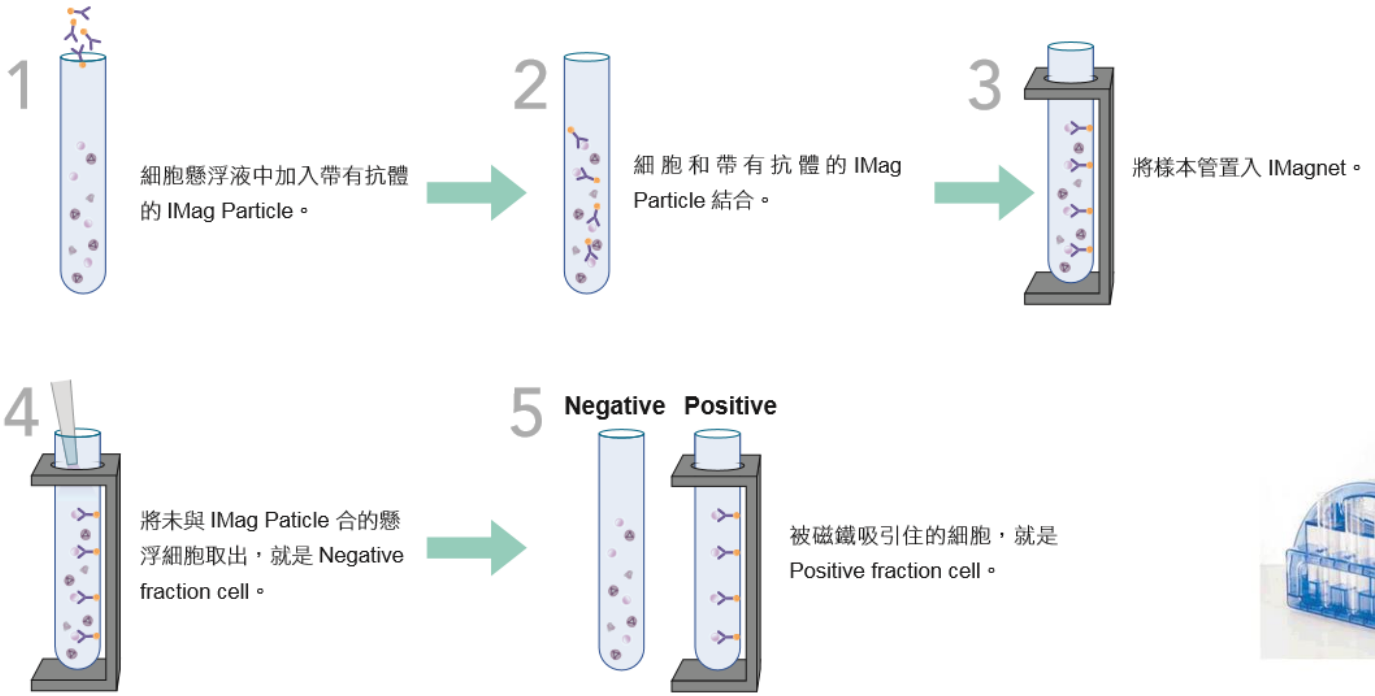
- 分選後，染**FoxP3確認Treg cell**
- **Representative FoxP3 staining of sorted Tregs**

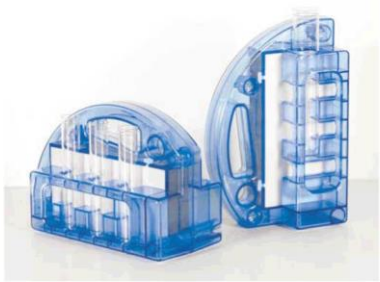


Sorting for Rare cell 稀有細胞分選

- 在分選稀有細胞群前最好能夠提升其所佔比例:
  - Bring the starting purity to > 5 %
  - Ficoll
  - Immune Panning
  - Magnetic Beads (Positive/Negative)
  - IMag

BD IMag 磁珠分選：
Positive or Negative selection or combination

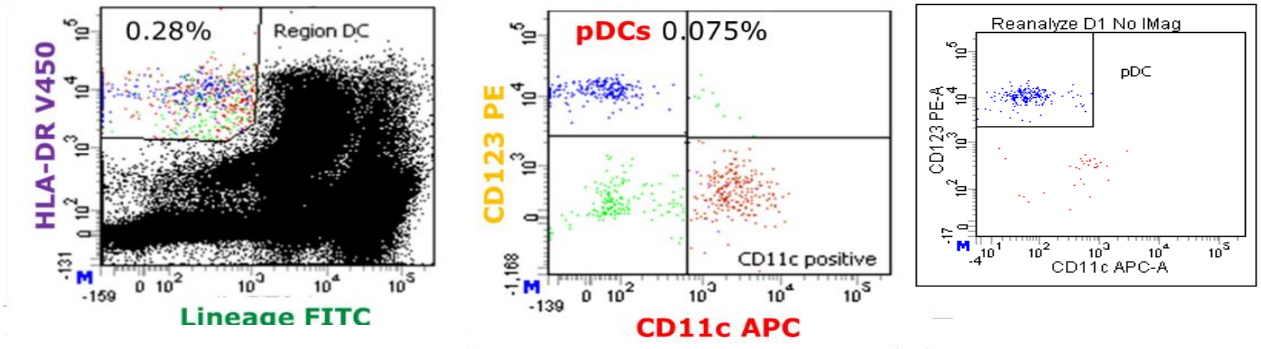




- Positive selection:
  去除上清，將試管移出磁場，分析被磁珠捕獲的細胞，即為目標細胞
- Negative selection:
  分析上清，目標細胞在上清液中

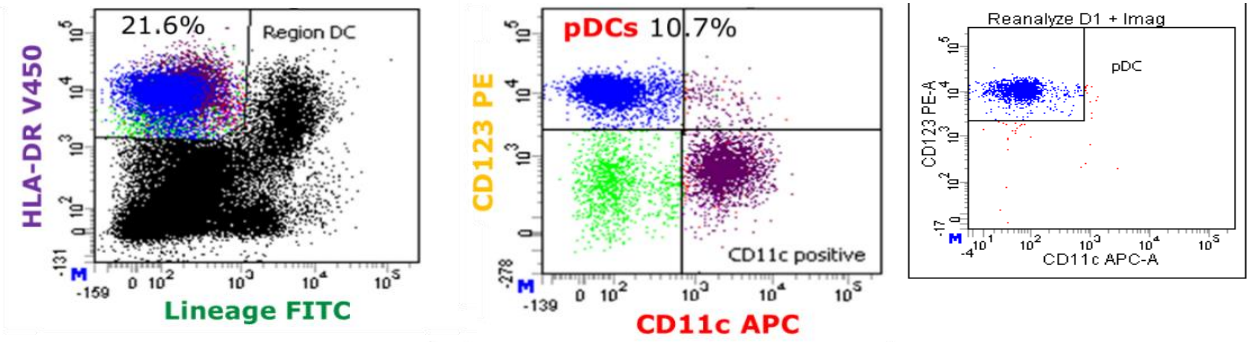
IMag increases purity and reduces time of sorting

w/o IMag enrichment



10^5 DCs
6.5 hours
74.4%

IMag enrichment



10^5 DCs
25 mins
95.3%

影響分選結果的因素

- 分選速度 (Event Rate)
- Drop drive frequency (Nozzle Size)
- % of the target population
- Sort mode

  Purity : High Purity and Recovery
  Yield: For Enrichment

樣品製備範例

- 所需準備之細胞量計算:
  - 若原始純度15%
  - 樣品濃度：$1X10^7$/ml
  - 低速分析速度：2000/s
  - 分選速度：300/s。
  - 每30 min可得約$5.4x10^5$細胞。
- 原始純度降低應調高樣品濃度。
- 原始純度低於5%則需先濃集處理。
- 建議準備所需量的1.5-2倍細胞
  (考慮到分析耗損, 分選過程中拋棄細胞, 離心損失細胞等)

樣品製備注意事項

- 高壓會使buffer的pH值下降, 進而影響細胞的存活. 所以建議於phenol red - free sample buffer中添加25mM HEPES可使環境的pH值維持一定值.
- sample buffer中的protein含量也會影響分選純度. 若是建議使用5% FBS, 則可改用0.5% - 2% BSA取代以獲得較好結果.
- Collection tube內置放適合細胞存活的cold culture media with higher concentration of FCS.
- Collection tube管壁請先coating 1 - 4% BSA overnight.
- 分選前，樣品先加入viable dye (例如 7-AAD)，以避免分選已死亡細胞。分選後，也應取出一些細胞加入7-AAD再上機確認細胞存活狀況，以改善分選過程中是否有任何疏失之處。
- 樣品必須經35 μm濾網以避免塞管

樣品製備注意事項

- 儘量多次低速離心以去除細胞碎片與小雜物
- 細胞直徑之考量
- 分選時間不應太長, 即分多管收集細胞. 建議測試不同的分選時間長短(10min, 20min, 或30min)以了解是否會影響細胞的活性與隨後的培養結果.

Thank you!