BD FACSMelody
兩向細胞分選儀

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Topic

- **Basic Concept of Flow Cytometry**
- **FACSMelody System Introduction**
- **Cell Sorting Theory**
- **Application Examples**
- **Sorting Tips**

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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.

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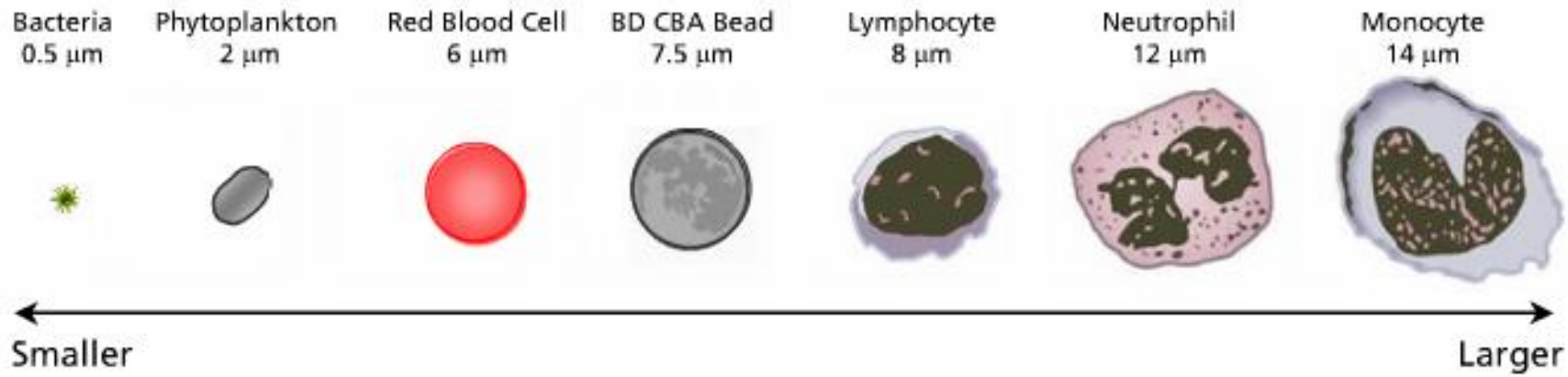
Particle Size

- Detection range: 0.5~50μm

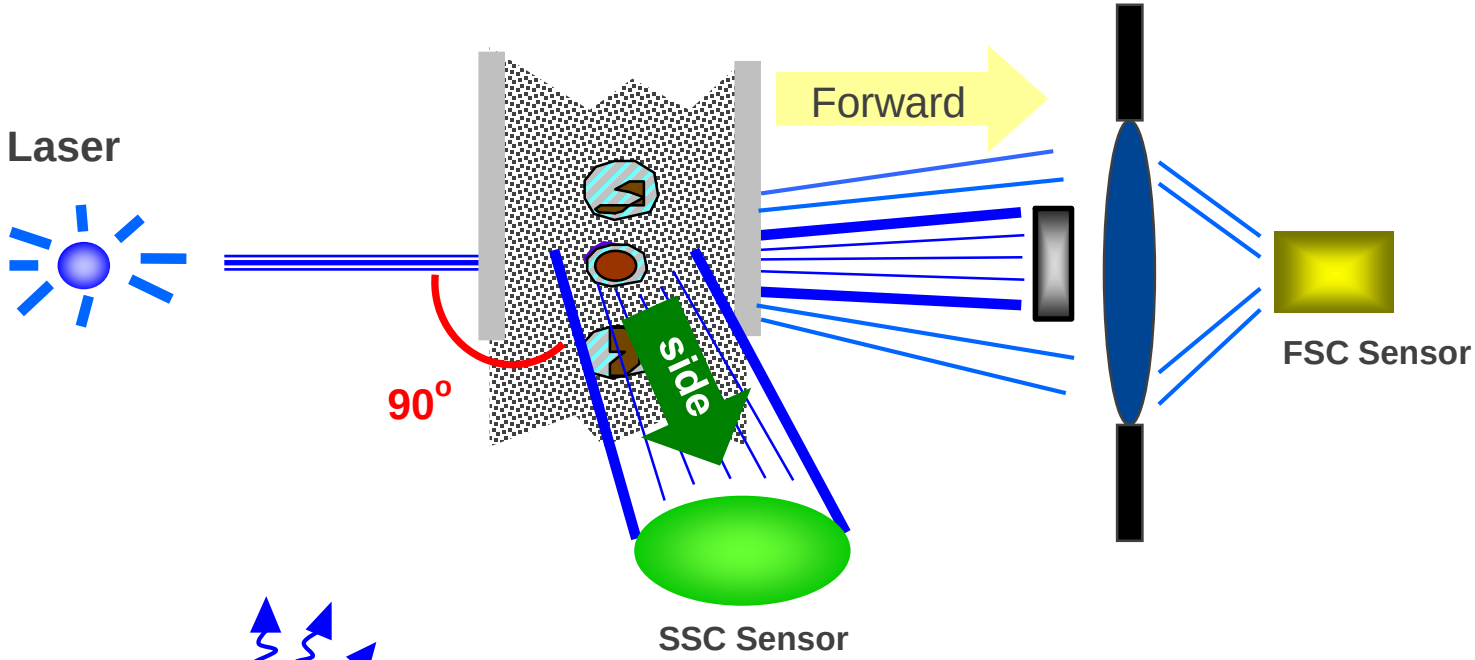


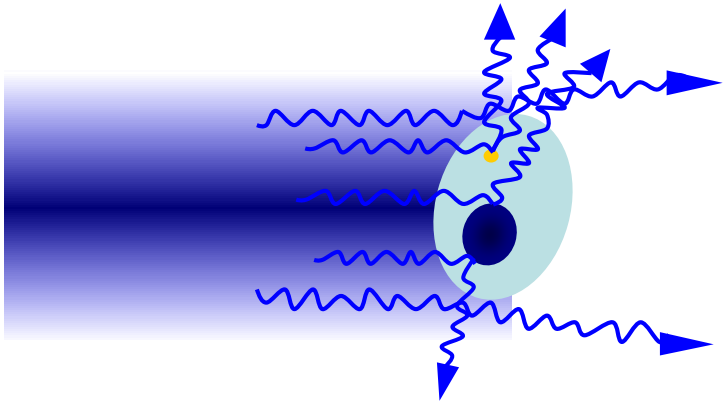
What Can a Flow Cytometer Tell Us About a Cell?

- 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 相對螢光強度

BD

Scatter Light





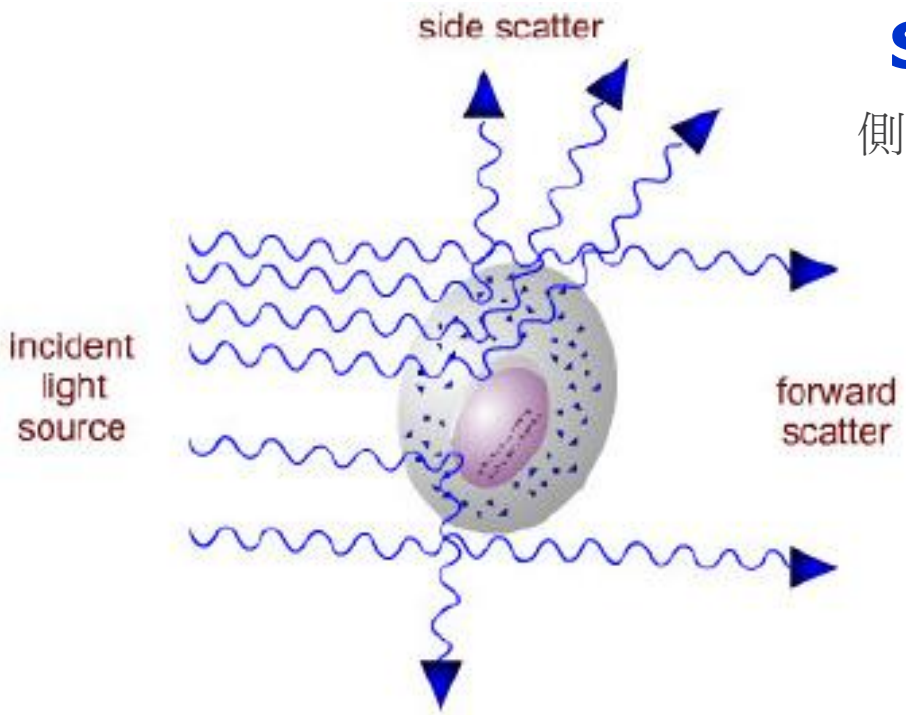
Forward Scatter—diffracted light

- **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**

Scatter Light



SSC

側向散射光

Internal
Complexity
Granularity

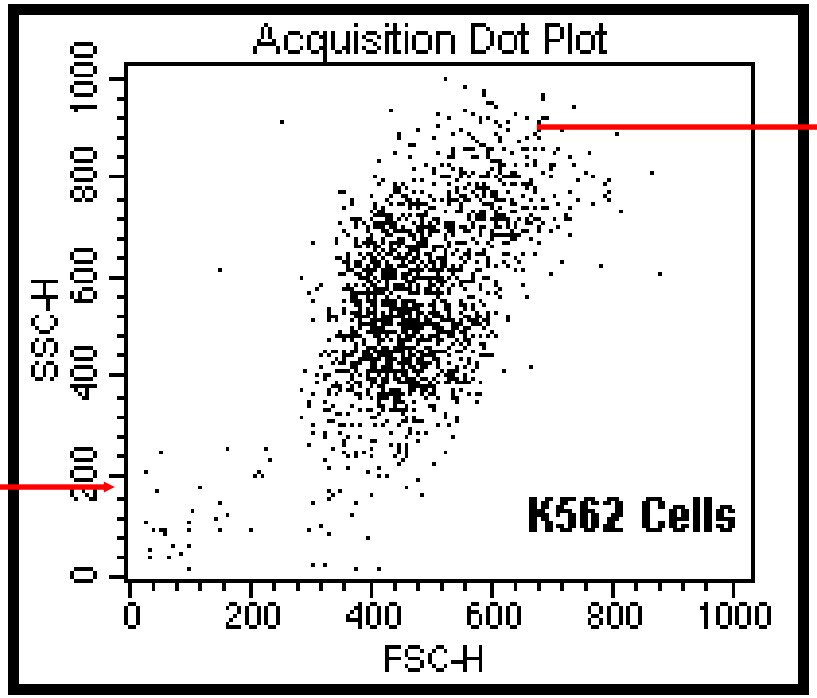
FSC

前向散射光

Size
Shape
Surface
Refractive index

BD

Cell Physical Property

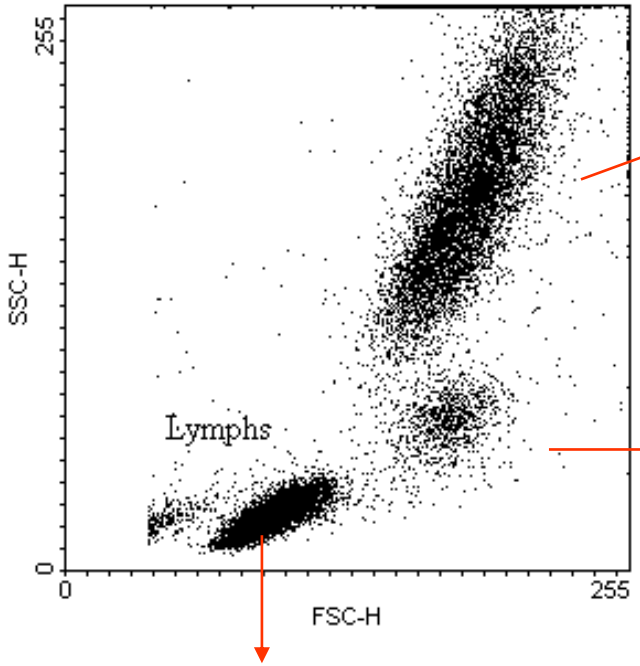


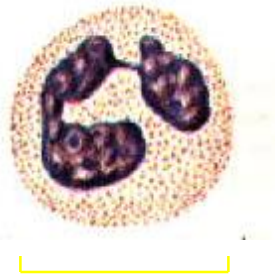
Aggregation

Cell debris

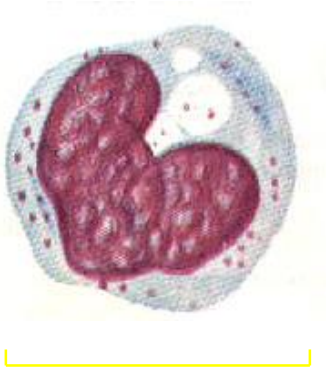
Cell Line

Lysed Human Blood

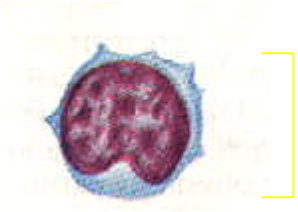




10 to 14 μm Granulocyte



>10 μm Monocyte

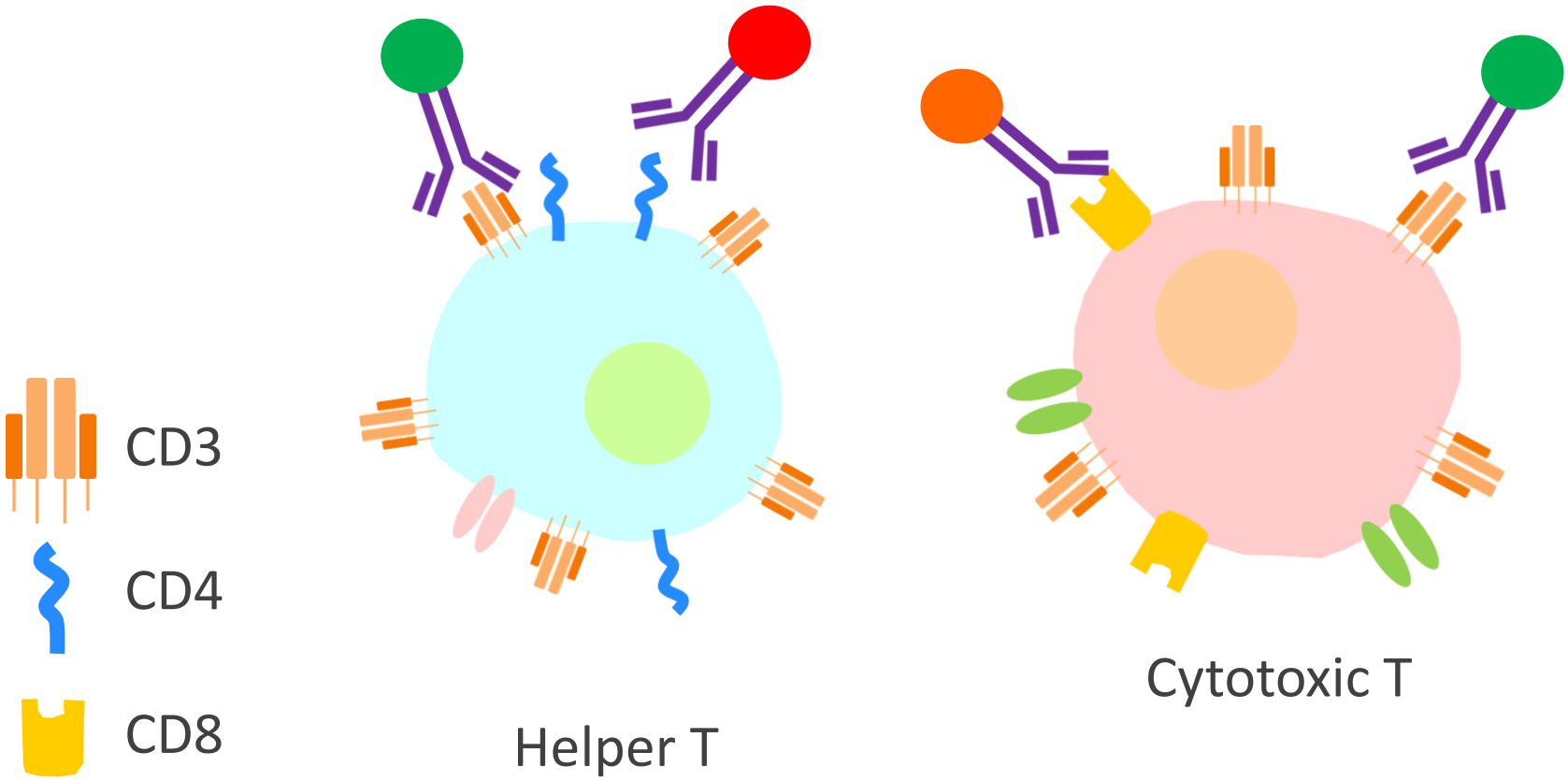


8 to 10 μm Lymphocyte

Peripheral blood

BD

Flow Cytometry Detection Principle



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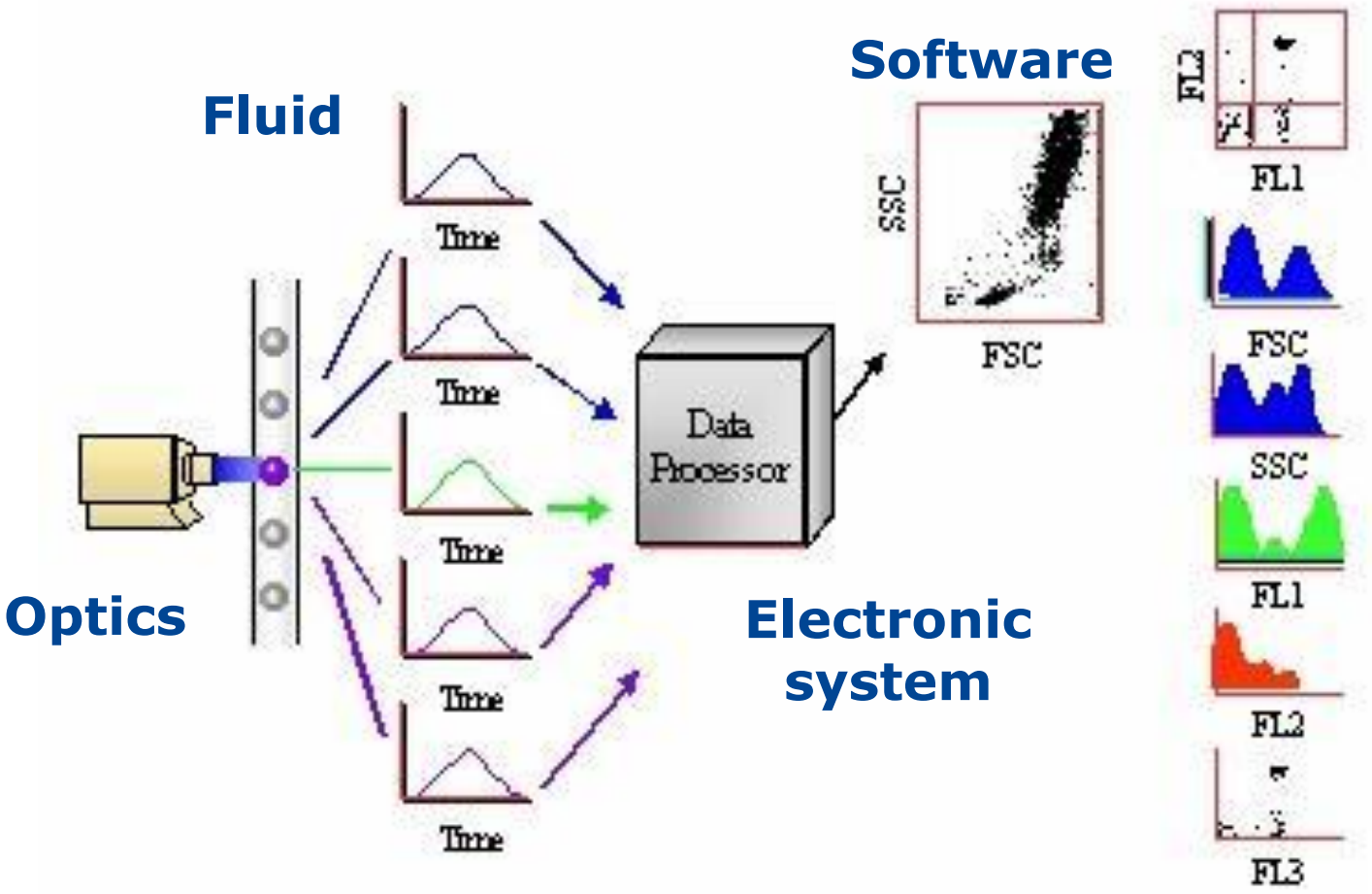
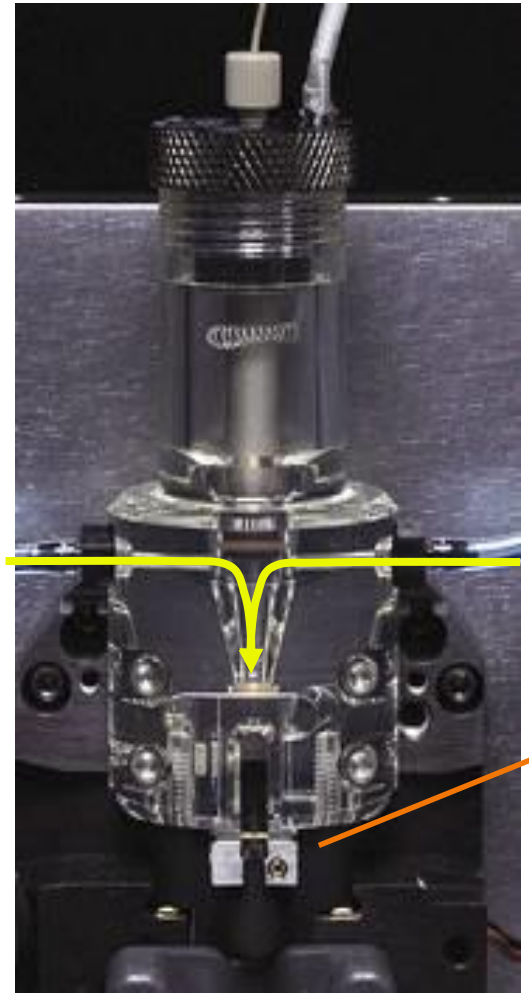
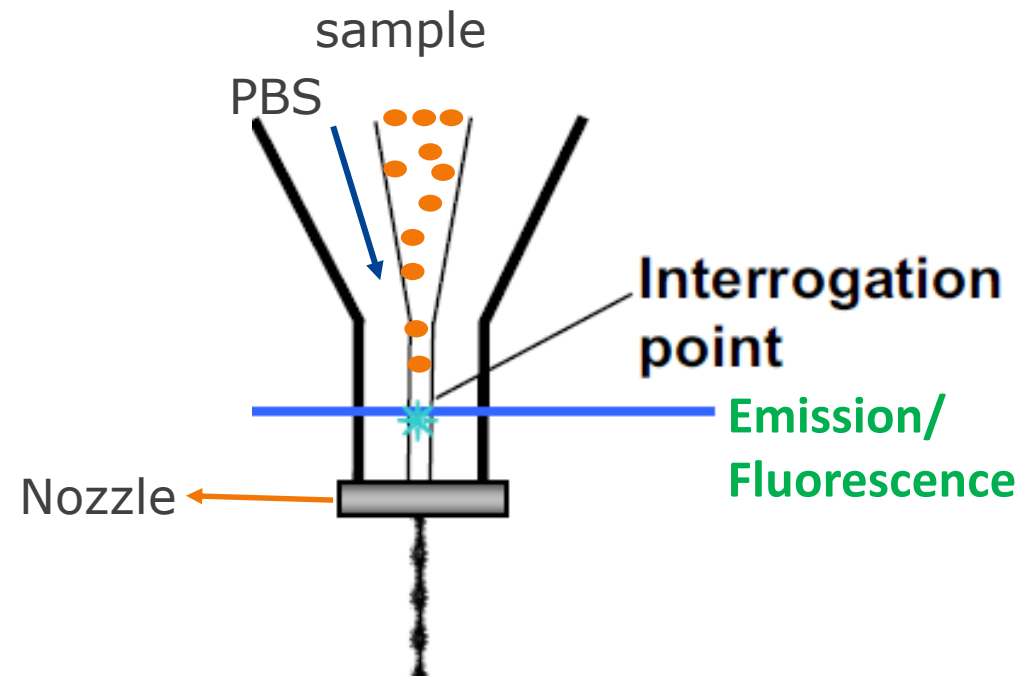
Flow Cytometry Overview



Flow Cell Structure of FACSMelody

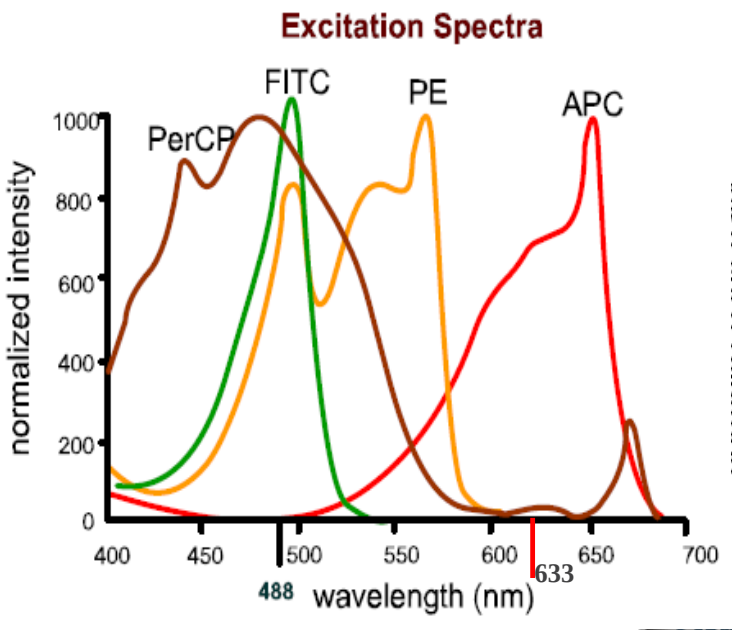


- Hydrodynamic focusing

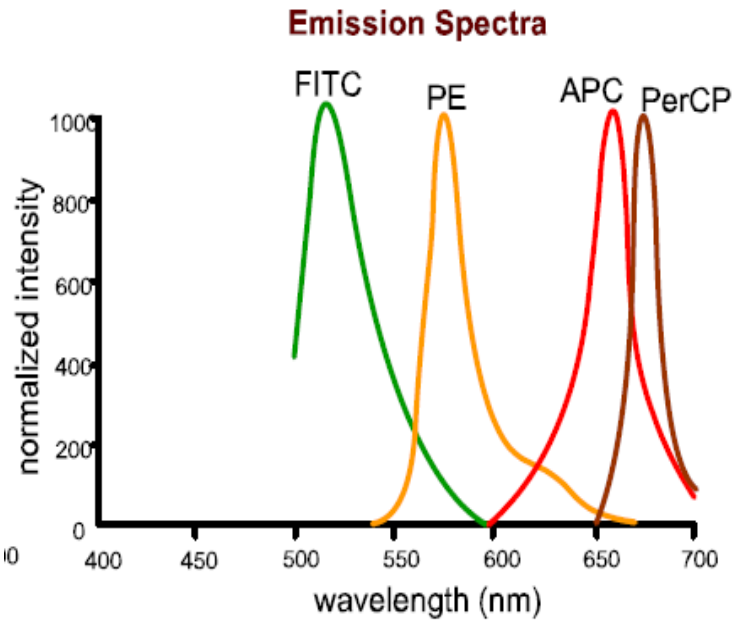


BD

Fluorescent dyes



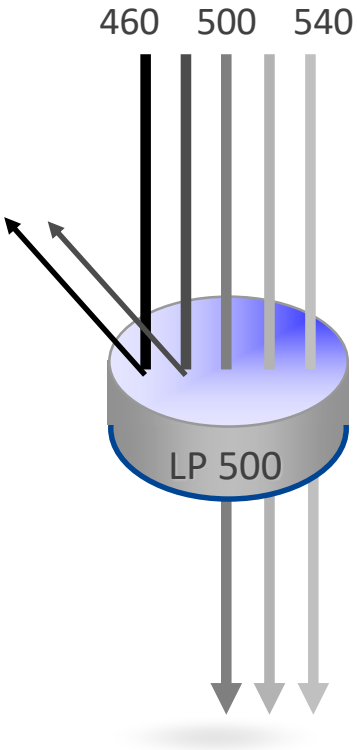
激發光譜- Excitation
用何雷射可激發此螢光



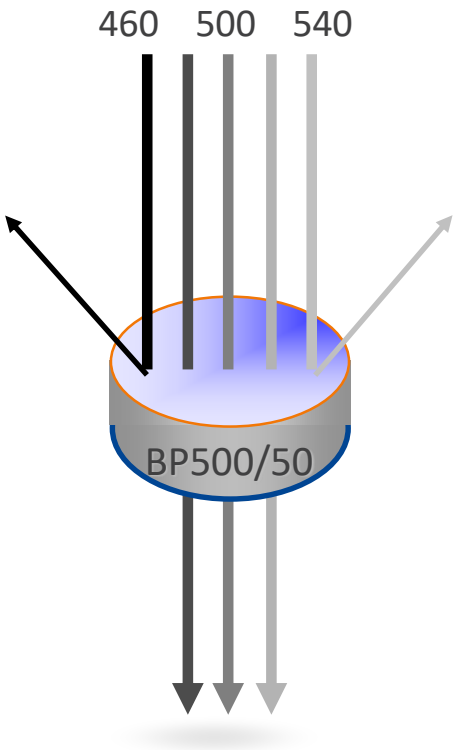
發散光譜-Emission
用何偵測器可收集此螢光訊號

Detectors and Filters

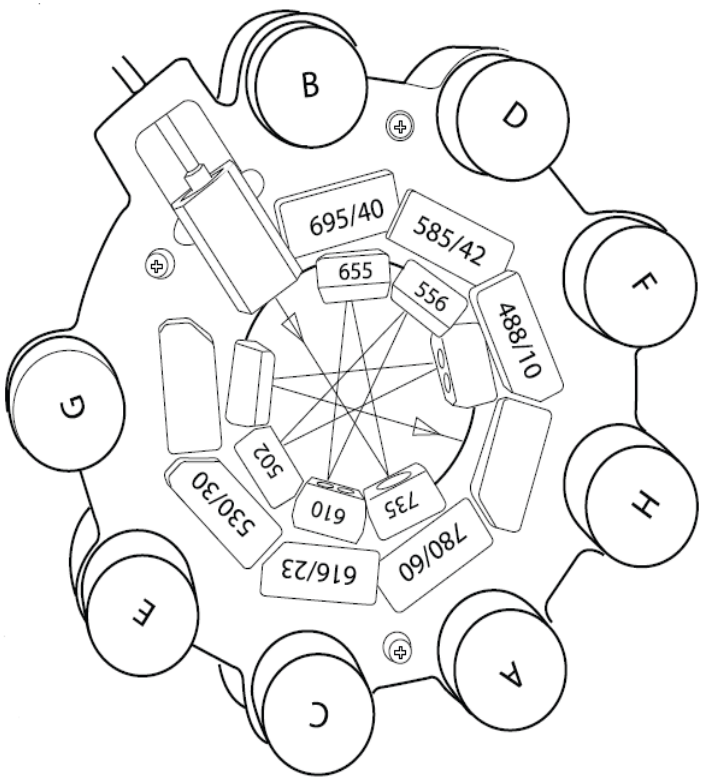
Longpass



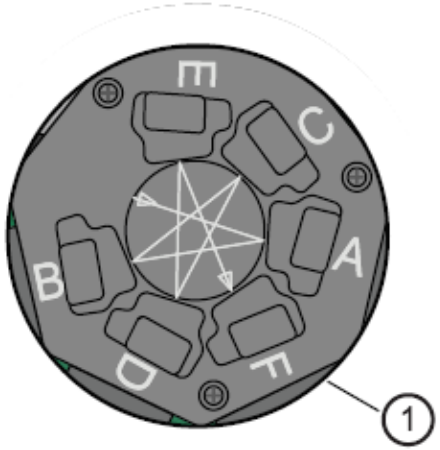
Bandpass

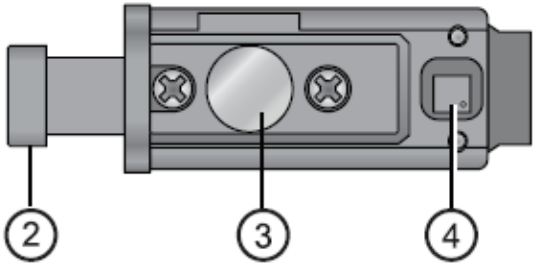


Octagon



Melody Optics Detector



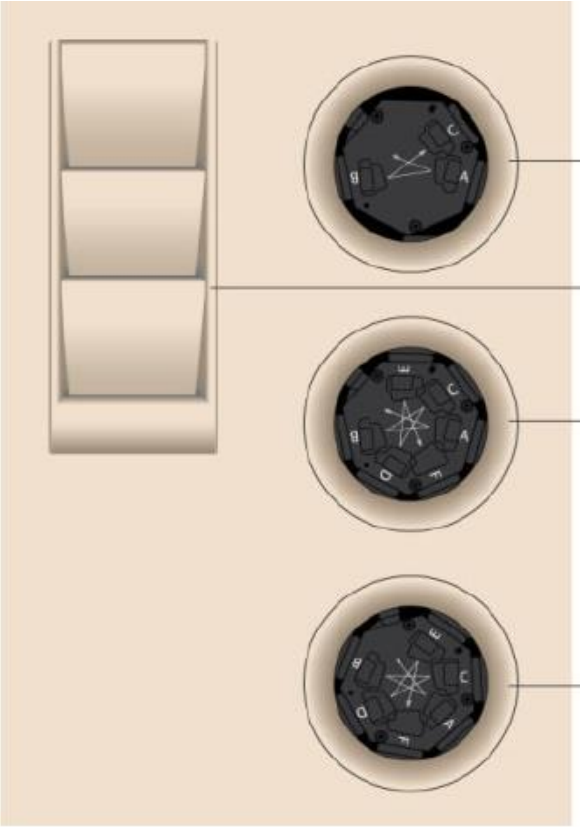


偵測器陣列

接收光源順序：
能量弱→強

反射傳遞：
能量折損較低

濾片
ID Chip：
自動偵測濾片移動與放置位置



偵測器陣列

BD Melody filter guide/Configuration

2 lasers: 488 nm Blue laser + 640 nm Red laser

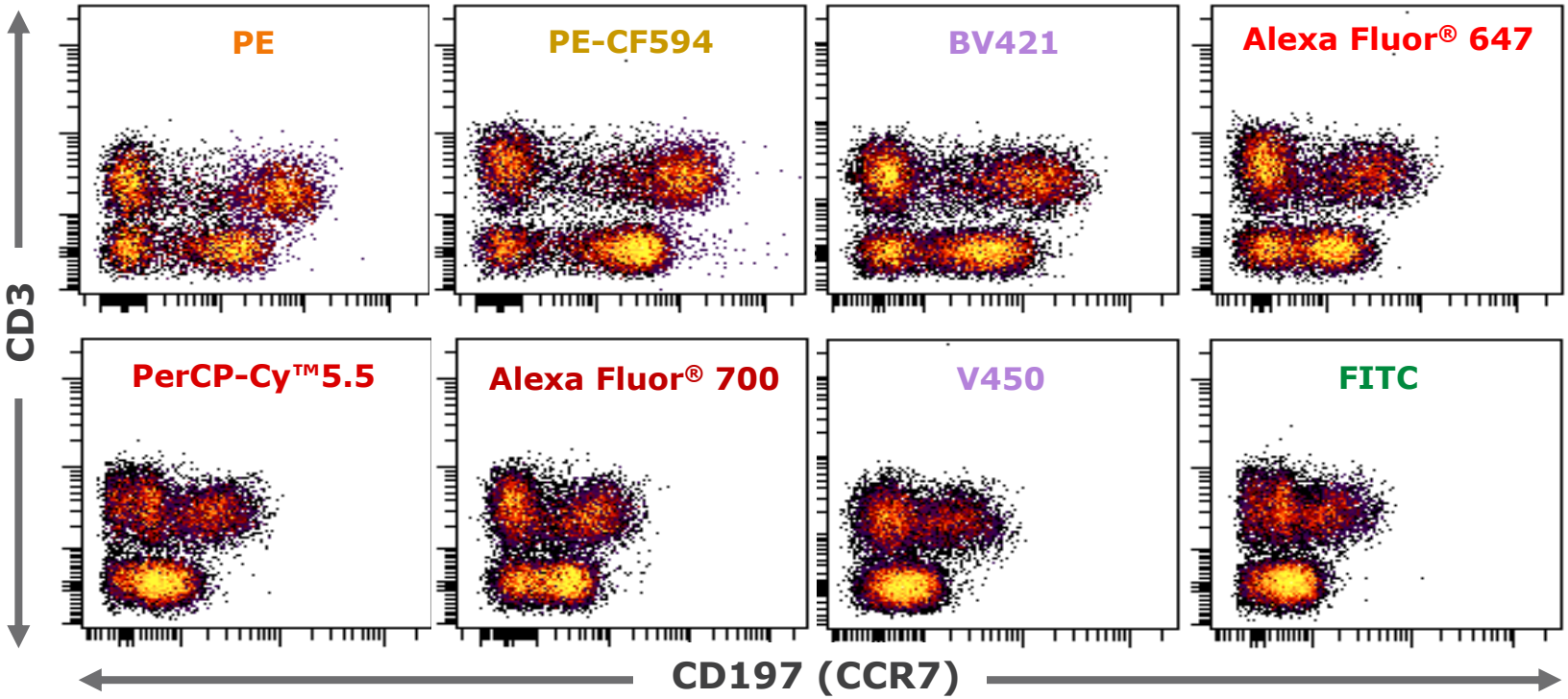
2-laser, 6-color (4-2) configuration

488 nm			
SSC	Side scatter	488/15	none
FITC, GFP, BD Horizon Brilliant Blue 515, Alexa Fluor® 488	Cell surface markers, fluorescent protein	527/32	507 LP
PE, PI	Cell surface markers, live/dead discrimination, cell cycle	586/42	560 LP
BD Horizon Brilliant Blue 700, PerCP, PerCP-Cy5.5, 7-AAD	Cell surface markers, live/dead discrimination, cell cycle	700/54	665 LP
PE-Cy7	Cell surface markers	783/56	752 LP
640 nm			
APC, Alexa Fluor® 647	Cell surface markers	660/10	660/10
APC-Cy7, APC-H7	Cell surface markers	783/56	752 LP

Choices of fluorochromes depends on the available instrument configuration and the total number of markers being used in an

BD

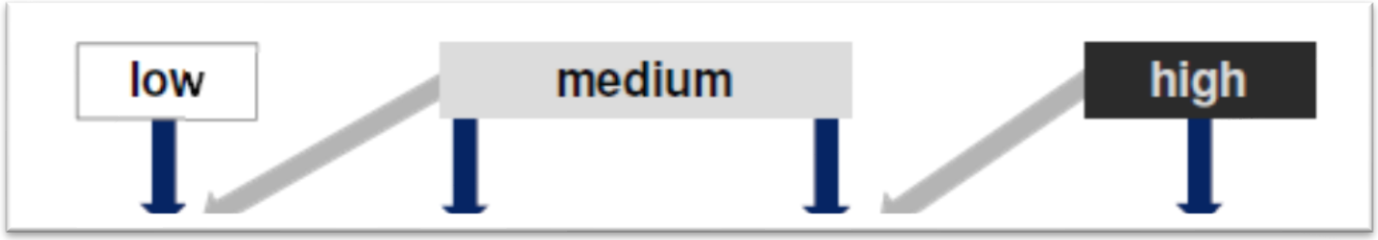
Fluorochromes Reveal Biology



- 根據實驗樣品的生物特性選擇合適的螢光能夠有較佳的實驗結果
- 使用強螢光染劑對於弱表達的抗原特別關鍵

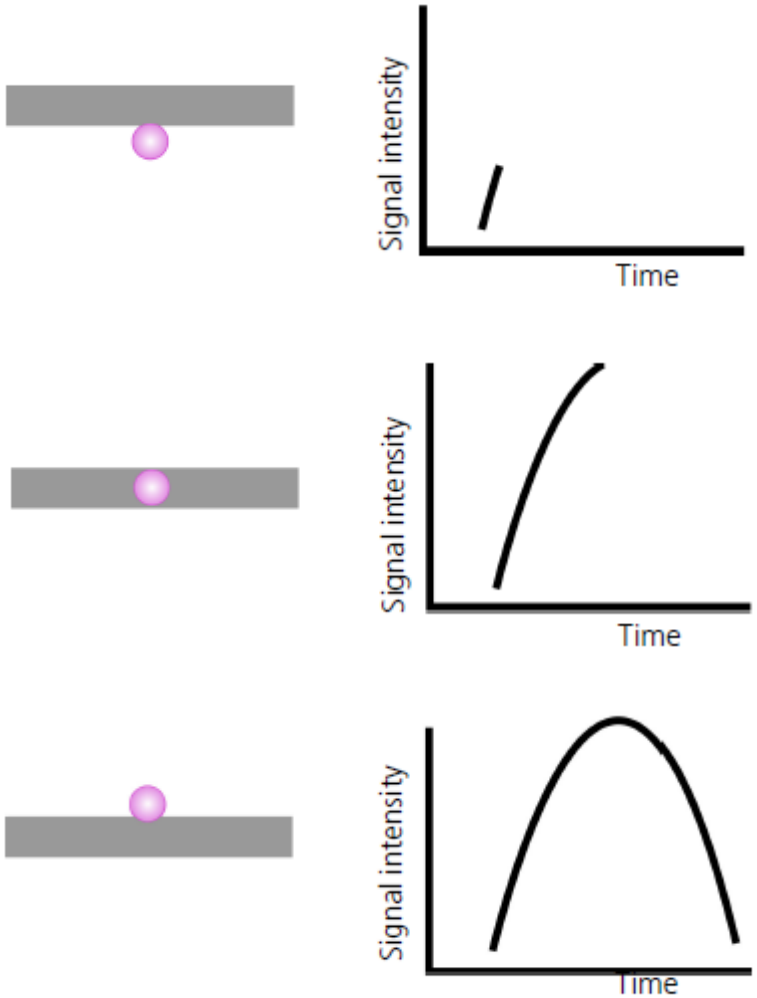
Fluorochrome/Antigen Combination

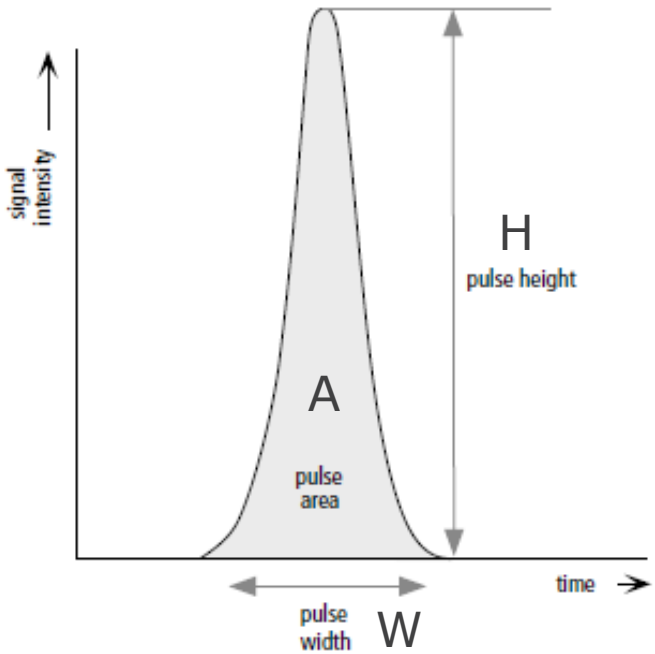
Antigen Density



Laser				
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

Electronics

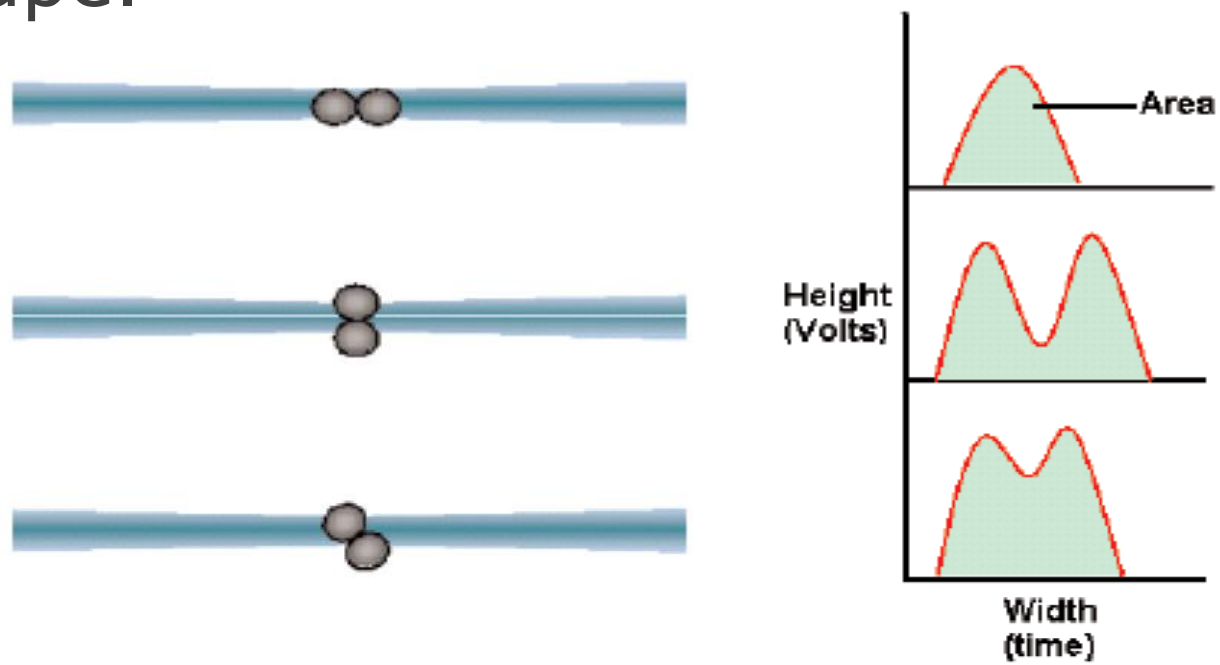




PMT偵測器將光學訊號轉換成電子訊號
H:電子訊號最高時數值
A:電子訊號總面積
W:電子訊號存在時間(細胞通過雷射時間)

Doublet Discrimination

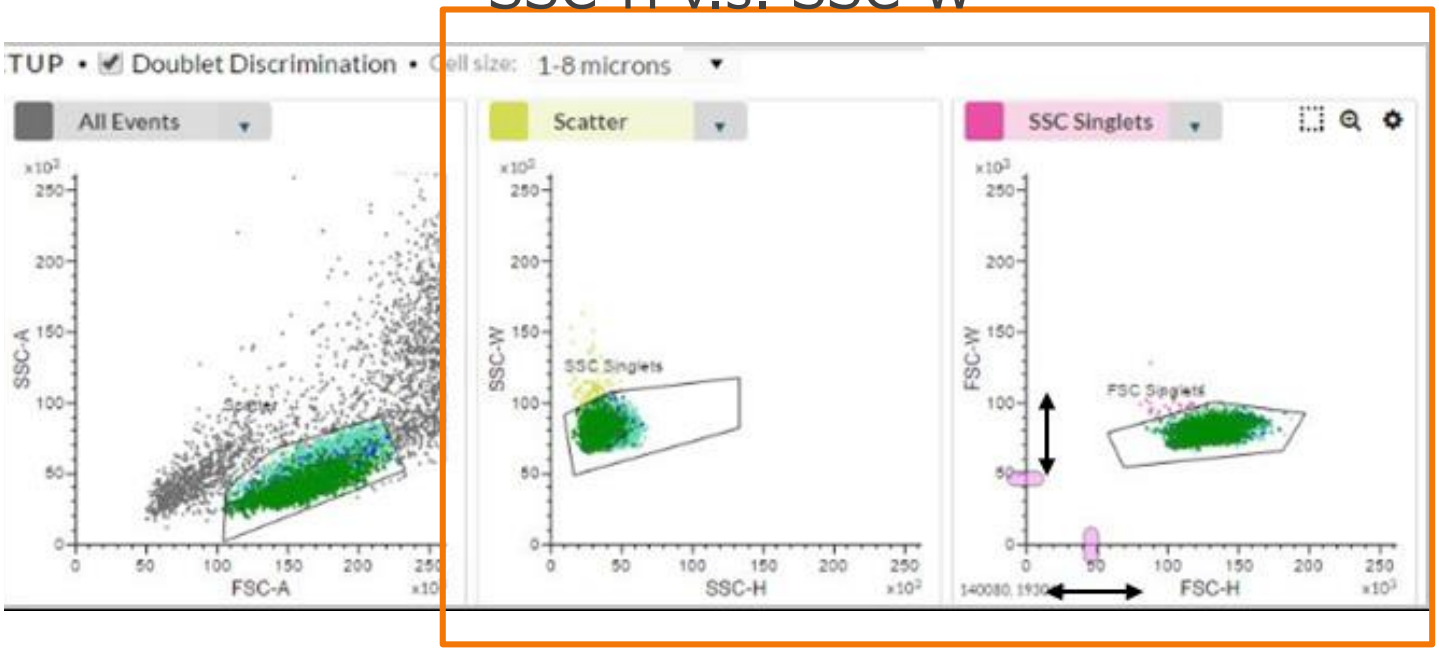
Doublets can be distinguished from singlets based on differences in voltage pulse shape.



Doublet Discrimination

The doublet discrimination gating template includes the following plots.

SSC-H v.s. SSC-W FSC-H v.s. FSC-W

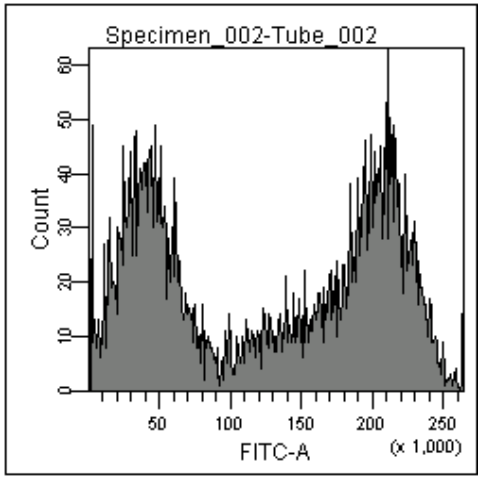


Data Storage

FCS: Flow Cytometry Standard
Melody:FCS 3.1

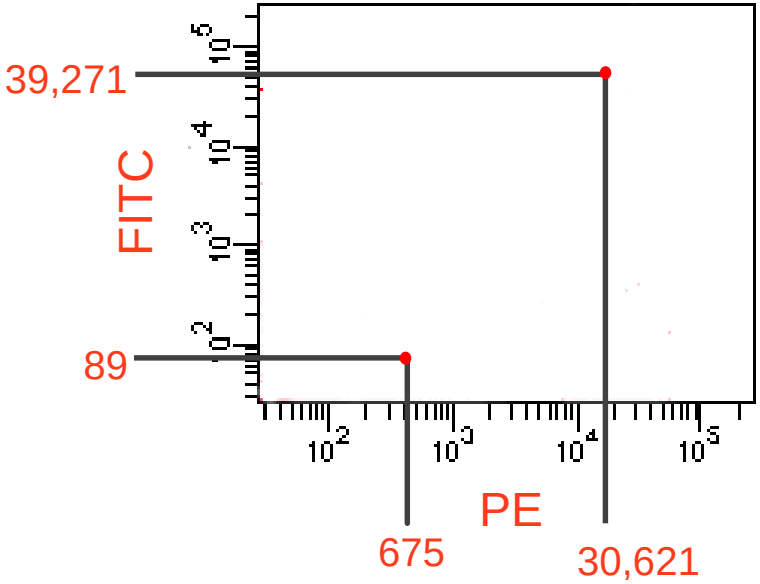
List-Mode Data

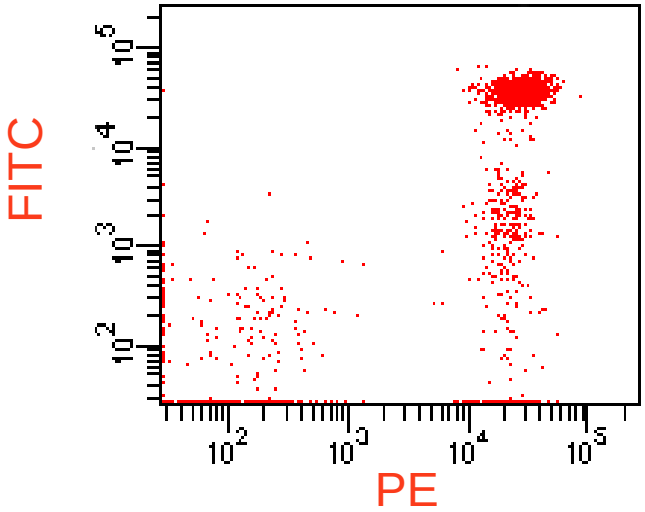
	Time	FSC	SSC	FITC	PE
Event 1	0	60	120	89	675
Event 2	10	160	65	39,271	30,621
Event 3	30	650	16	22,688	6,189



Histogram (1 parameter)

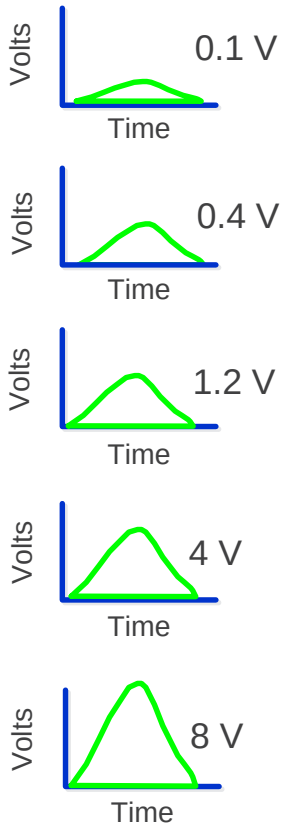
Dot Plot (2 parameters)

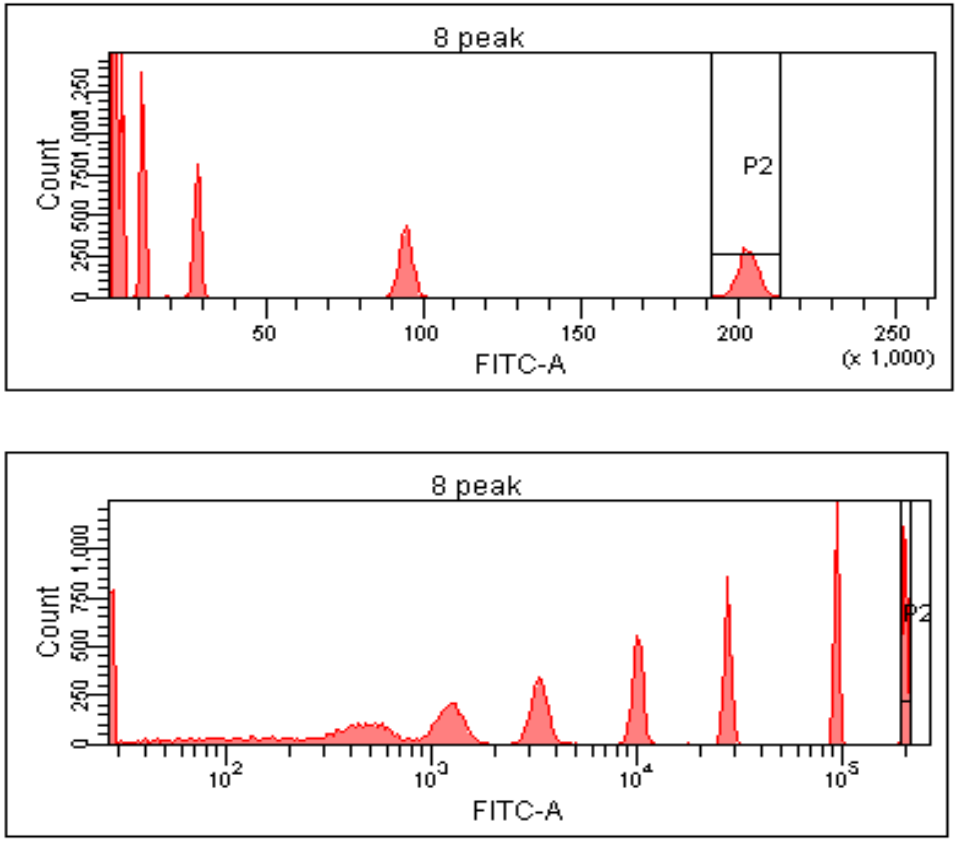




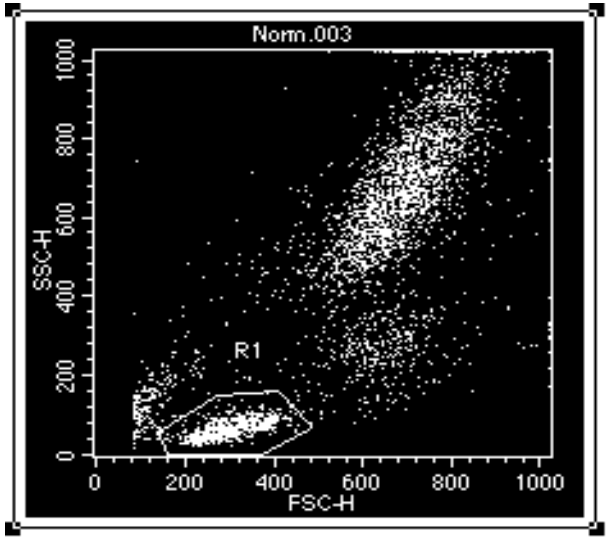
Data display- Linear v.s Log

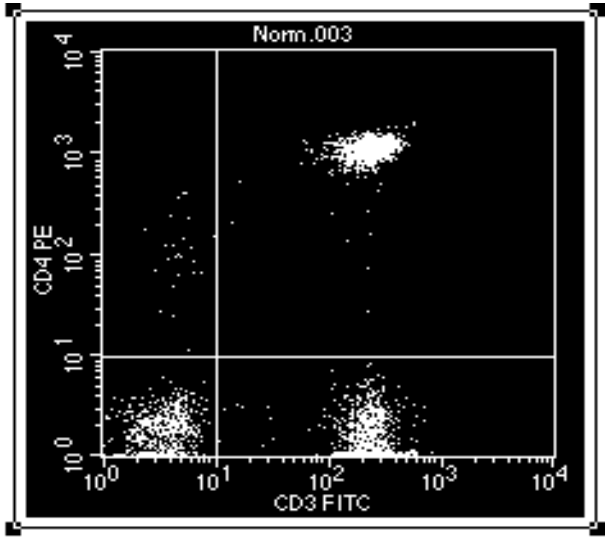
Voltage Pulses





Linear v.s. Log Amplification

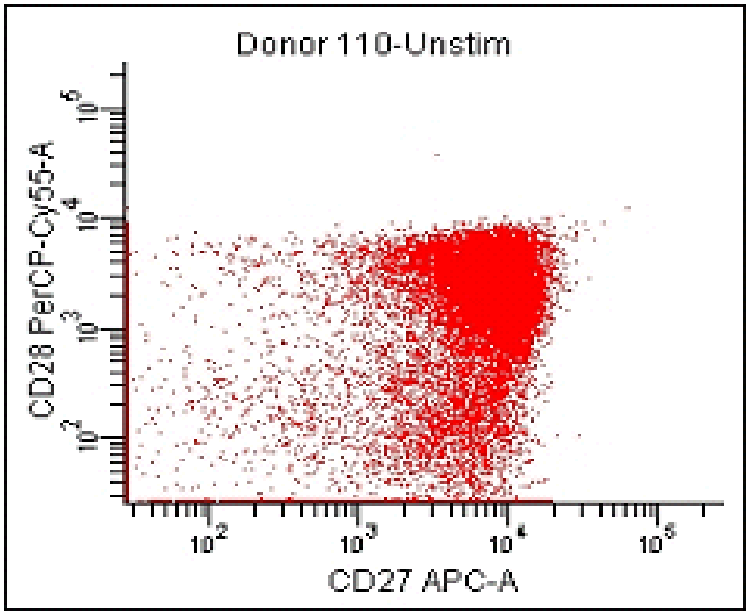




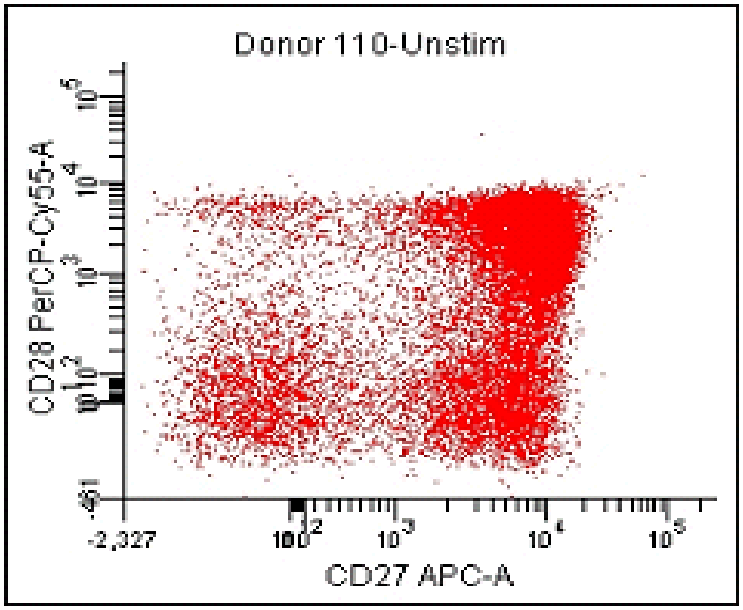
- **Linear** amplification is usually used for light scatter parameters and DNA analysis.
- **Log** amplification is used for fluorescence signals with a large dynamic range.

Log vs Biexponential Scaling

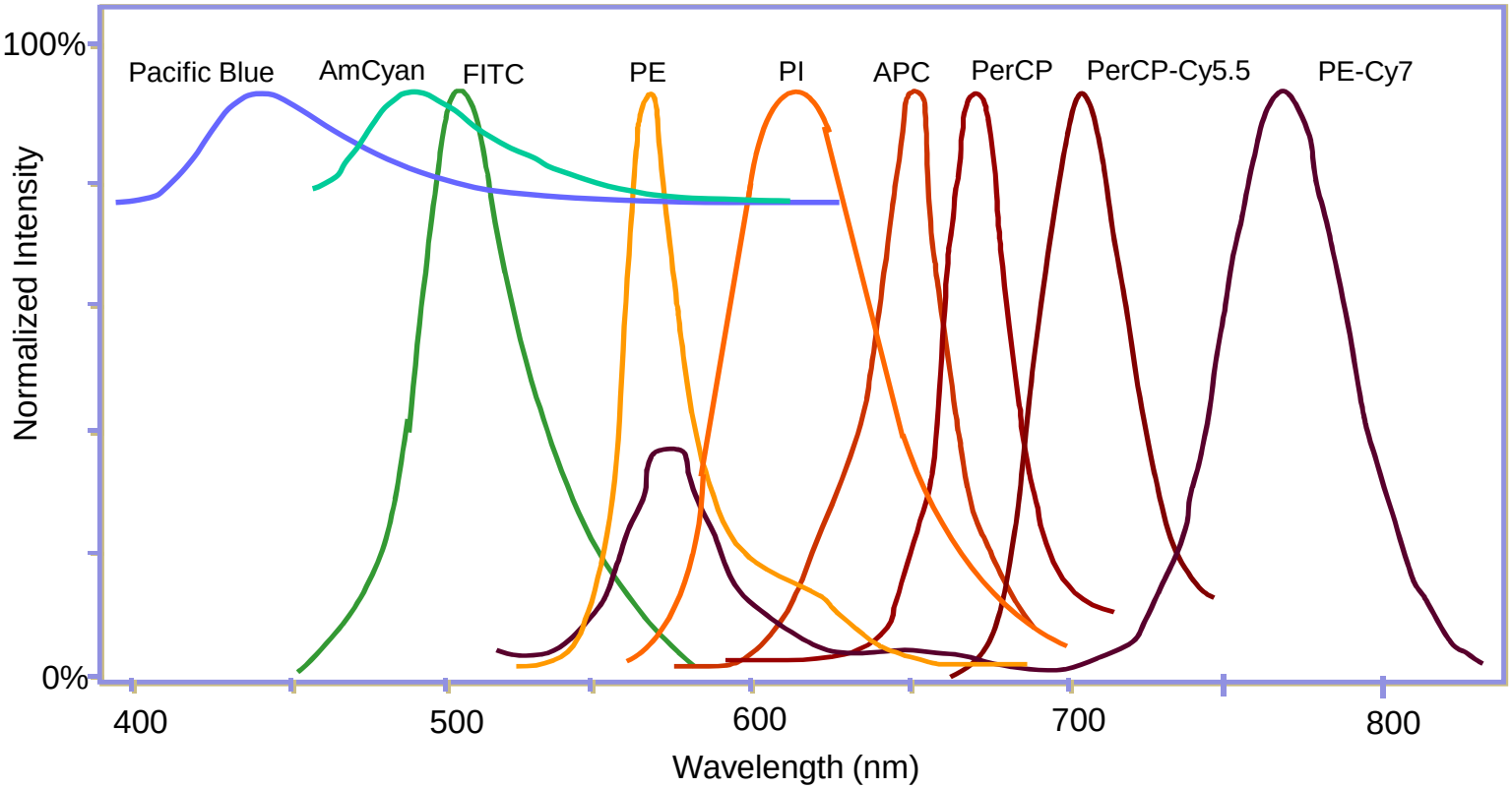
log scaling



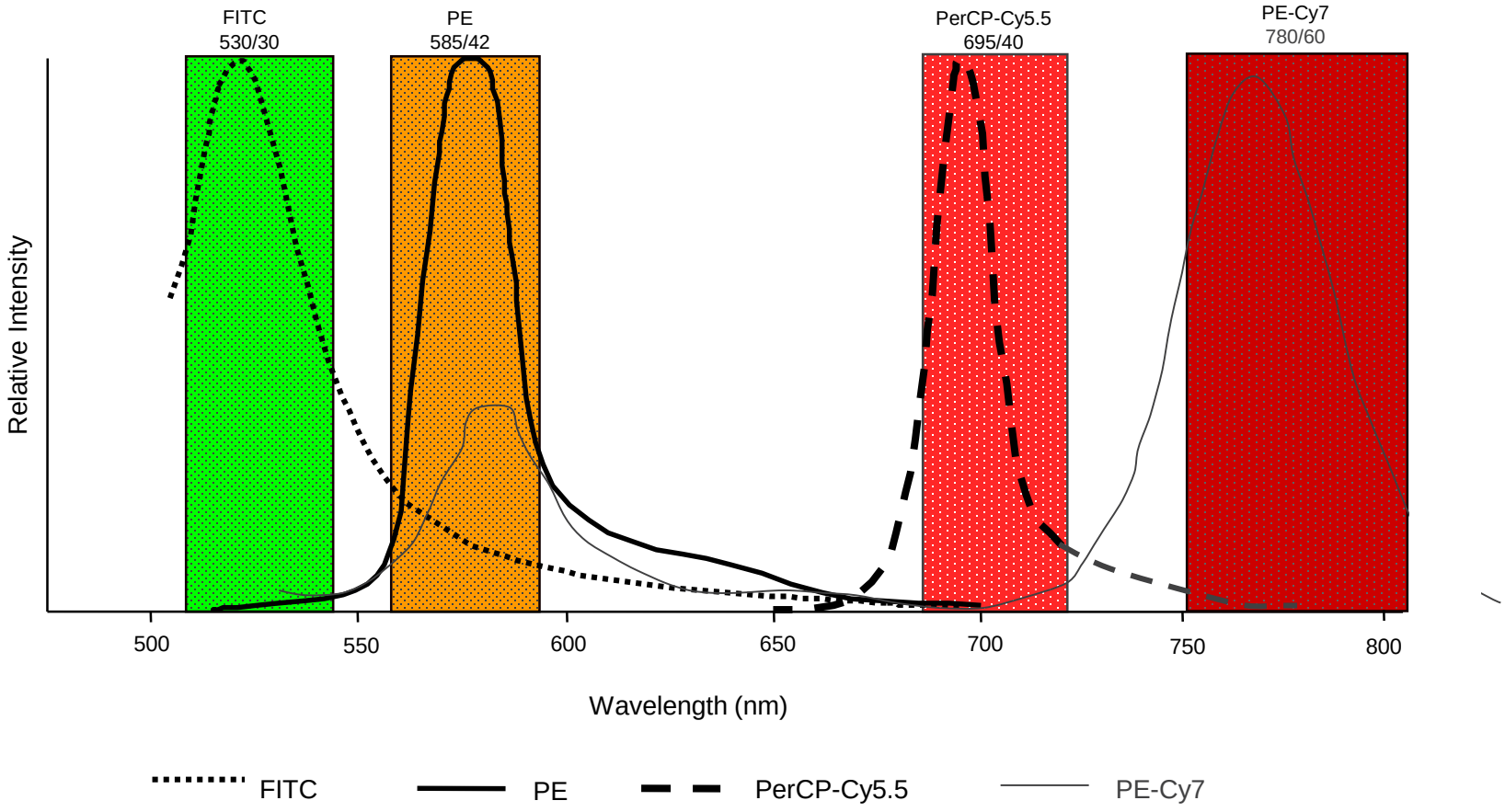
biexponential scaling



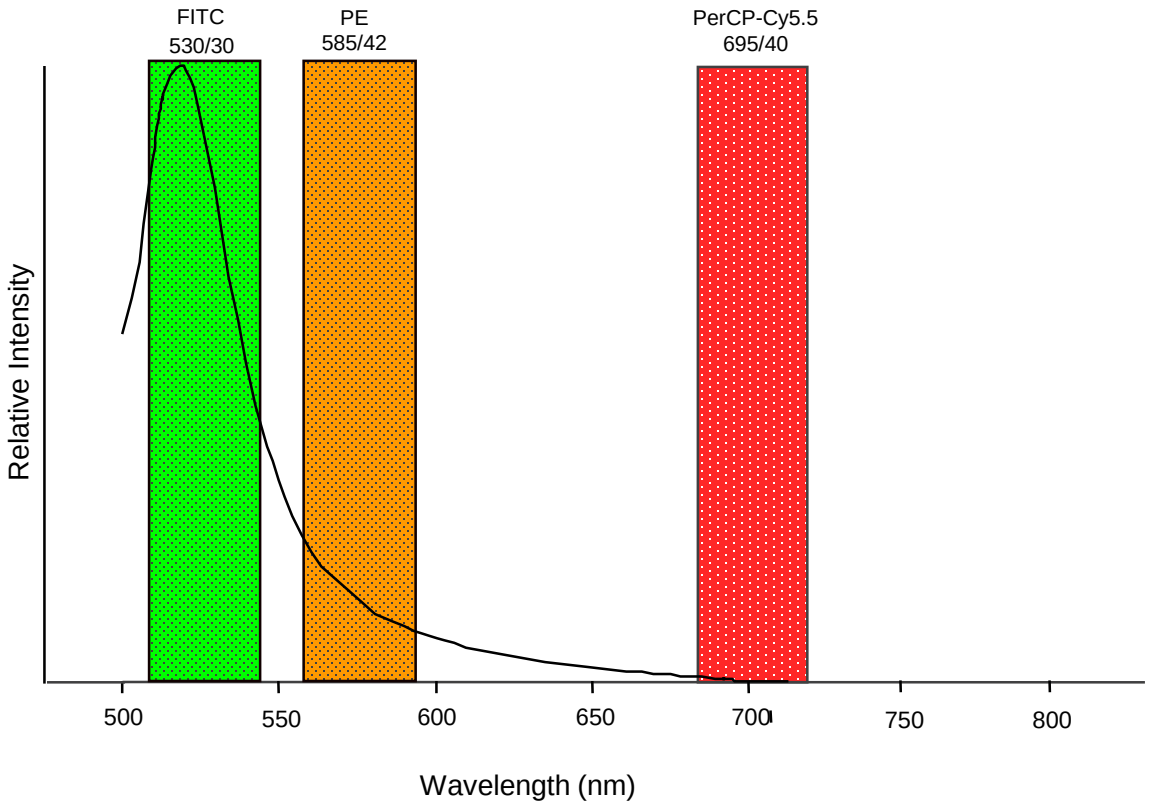
Spectral Overlap–Compensation Theory

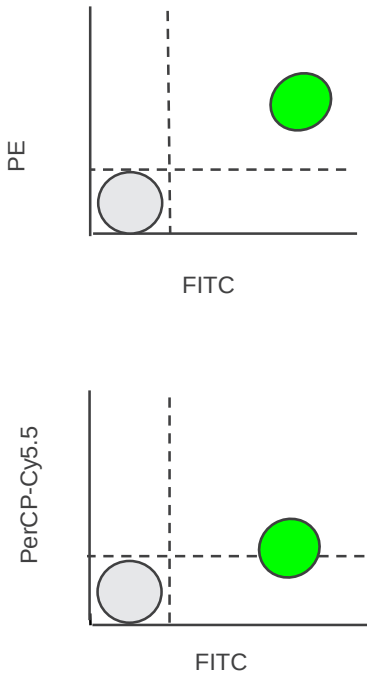


Spillover


FITC
530/30
PE
585/42
PerCP-Cy5.5
695/40
PE-Cy7
780/60
Relative Intensity
500
550
600
650
700
750
800
Wavelength (nm)
FITC
PE
PerCP-Cy5.5
PE-Cy7

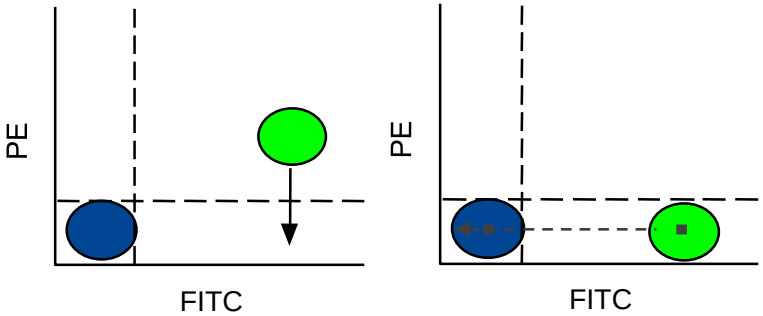
FITC Spillover



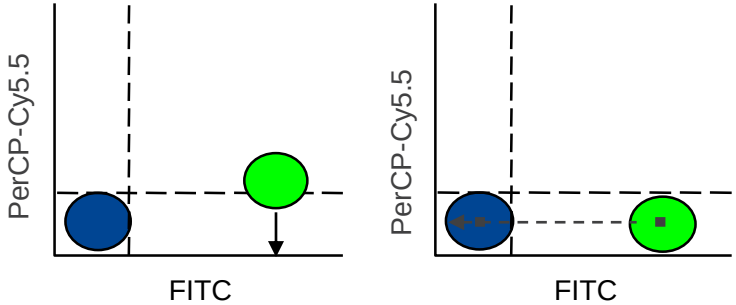


FITC Compensation

PE-%FITC

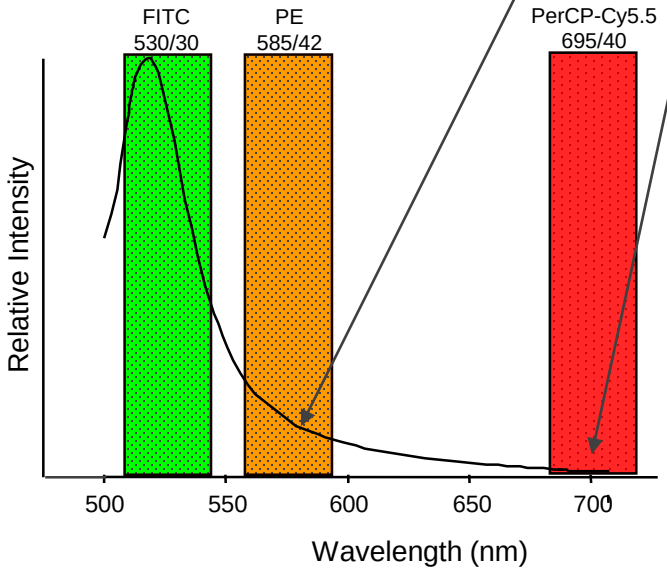


PerCP-Cy5.5-%FITC



To lower cluster, increase value.

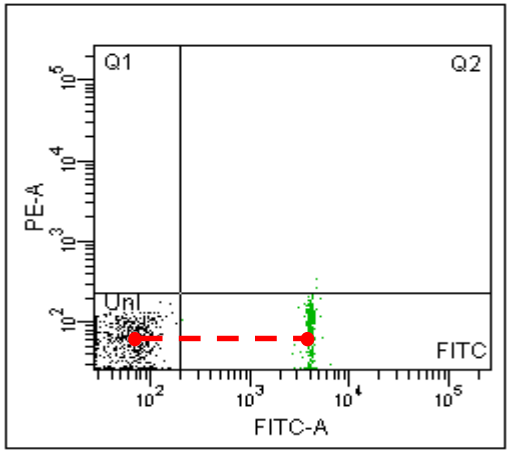
	Fluorochrome	- % Fluorochrome	Spectral Overlap
•	PE	FITC	20.10
•	PerCP-Cy5-5	FITC	0.90



Compensation Examples

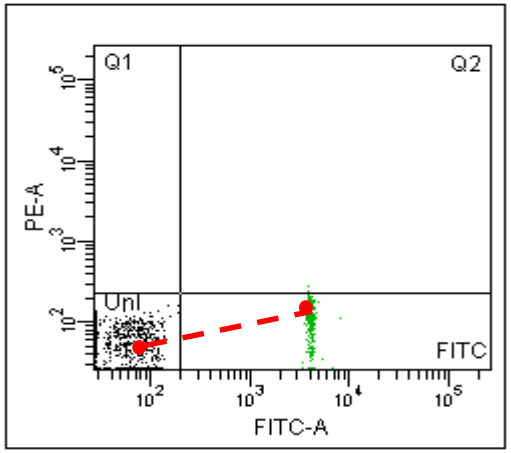
Incorrect Compensation

Correct Compensation



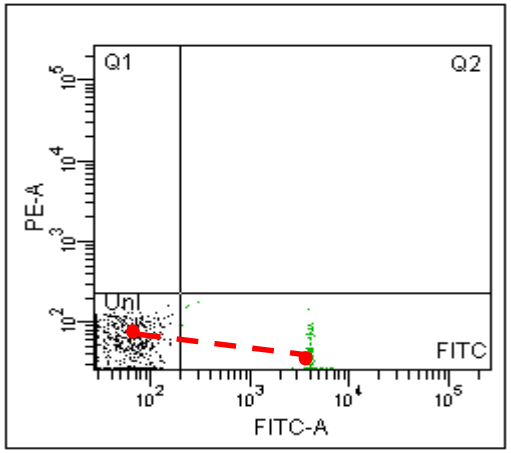
Population	PE-A Mean
Unl	64
FITC	69

Undercompensation



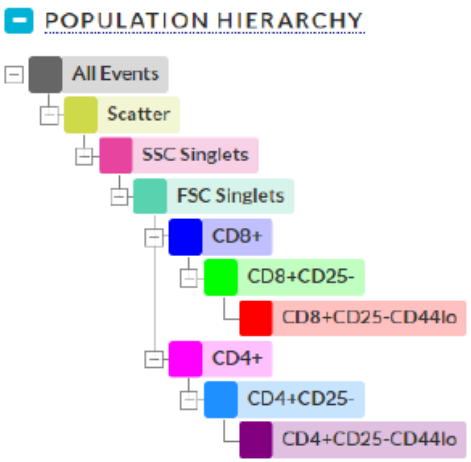
Population	PE-A Mean
Unl	61
FITC	96

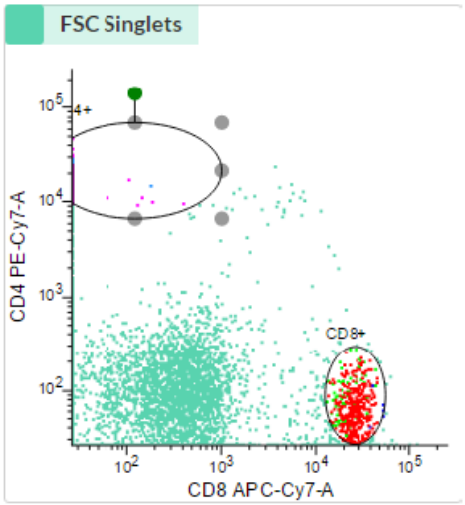
Overcompensation

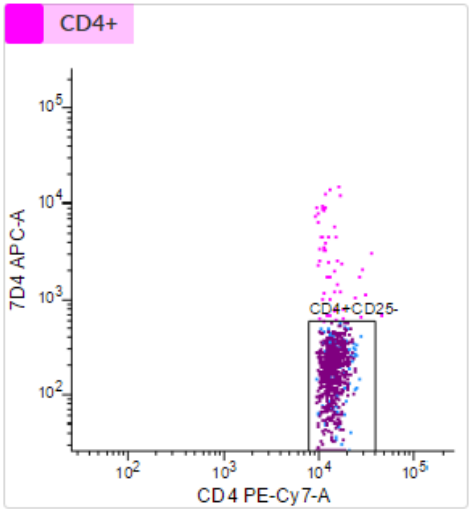


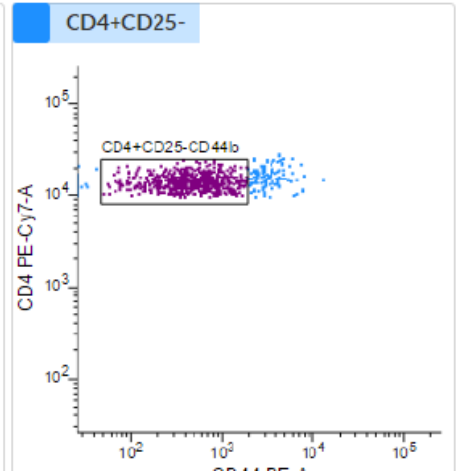
Population	PE-A Mean
Unl	62
FITC	-1

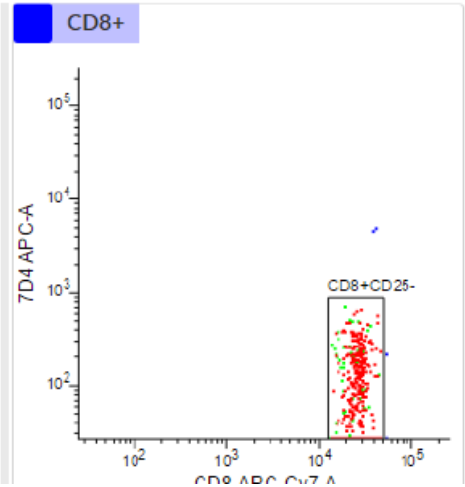
Data Analysis

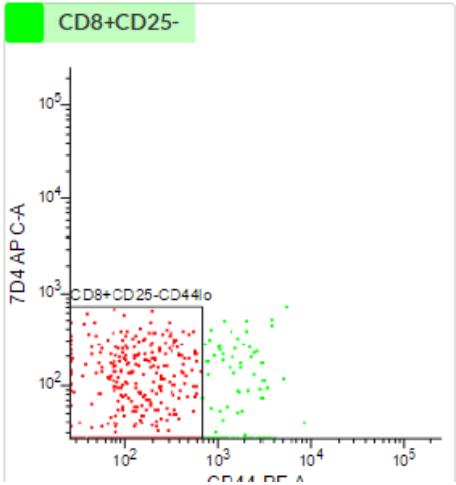












Topic

- **Basic Concept of Flow Cytometry**
- **FACSMelody System Introduction**
- **Cell Sorting Theory**
- **Application Examples**
- **Sorting Tips**

Sorting theory

1.樣品經過雷射產生散射光, 螢光等訊號被軟體分析.

2. 透過液滴給電電線給電

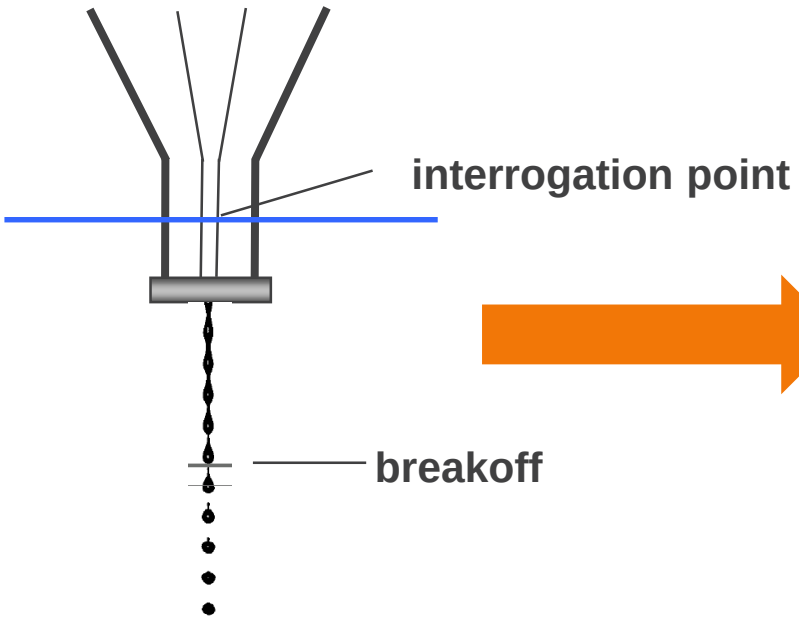
3. 細胞經過斷點時該滴液滴被打電

4. 電極板吸引帶電的液滴(含目標細胞)

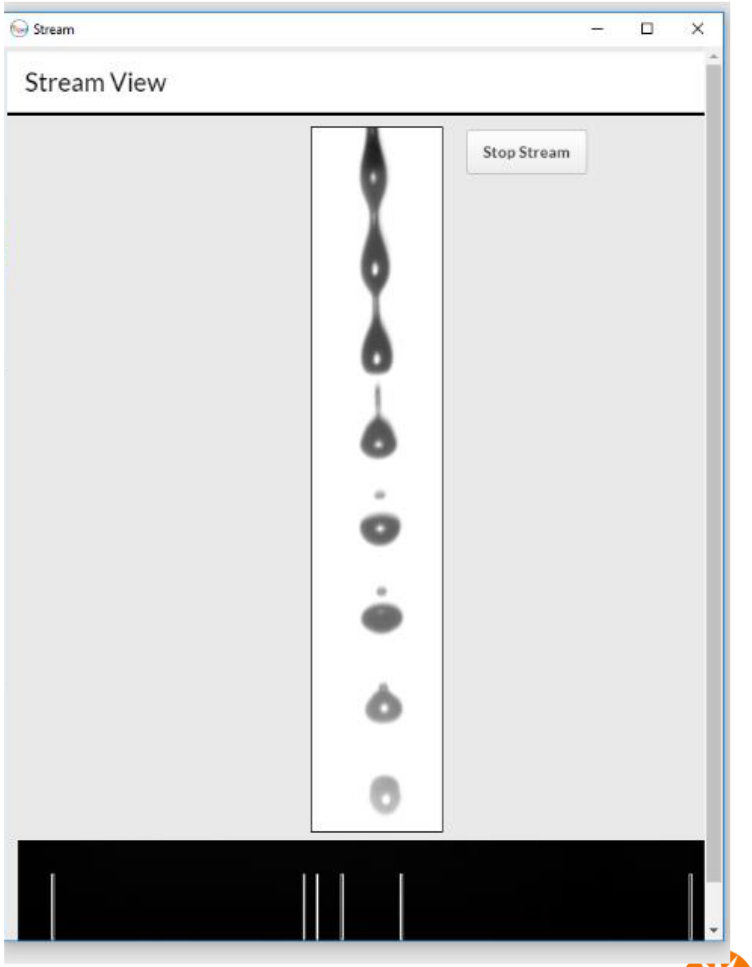
5. 未帶電之液滴進入廢液抽屜

6. 帶有目標細胞之液滴偏折後進入收集裝置

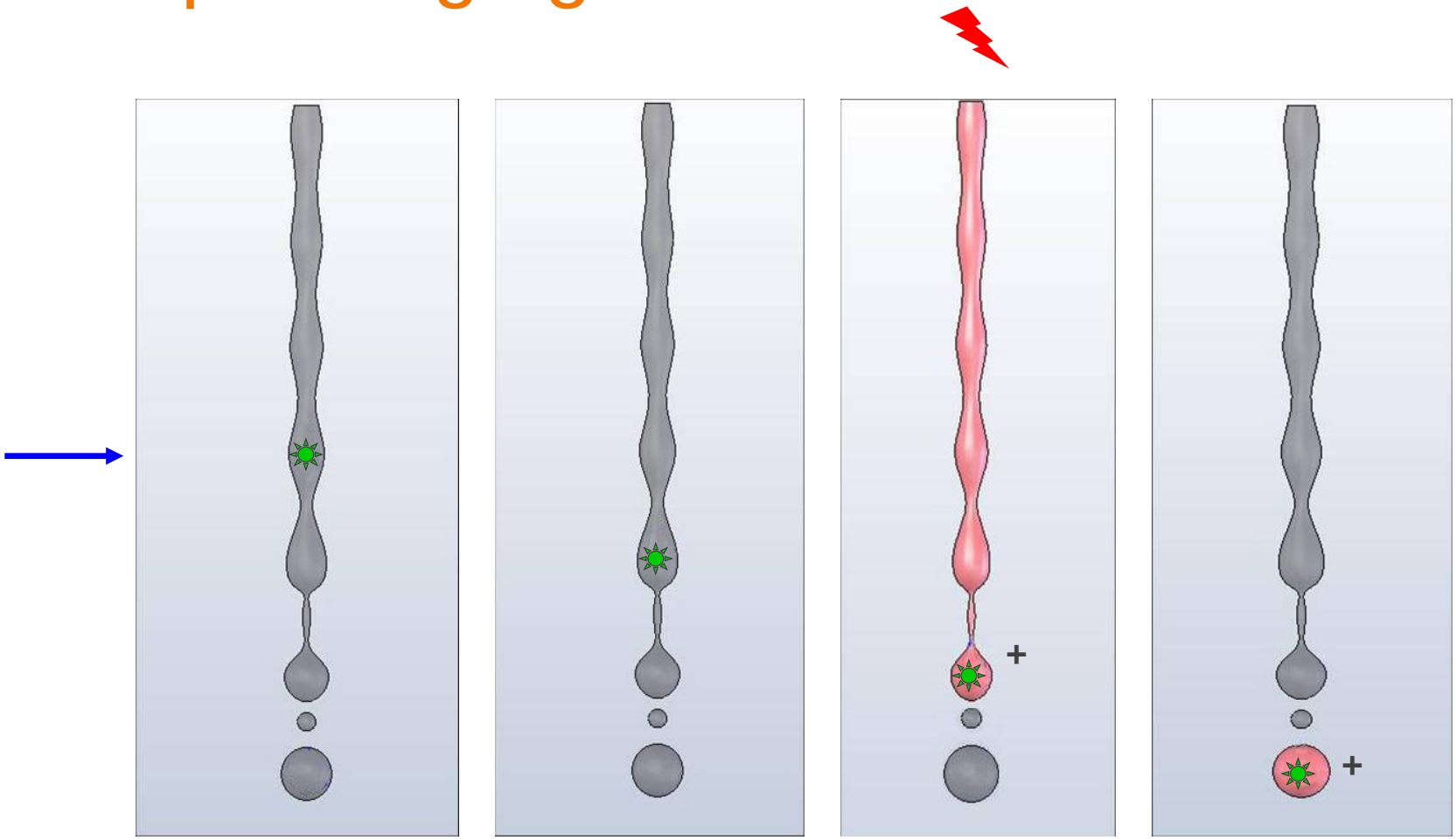
Drop Formation



Stream View



Drop Charging

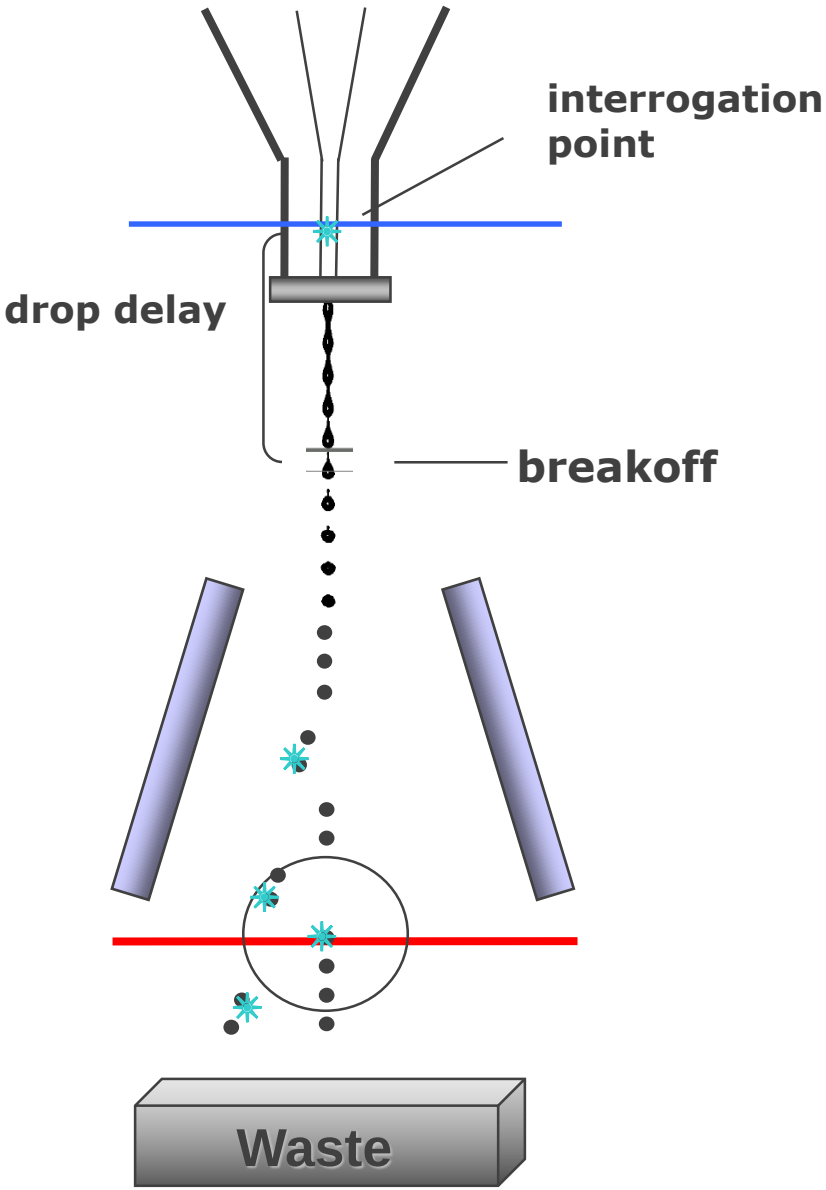


Time

Drop Delay

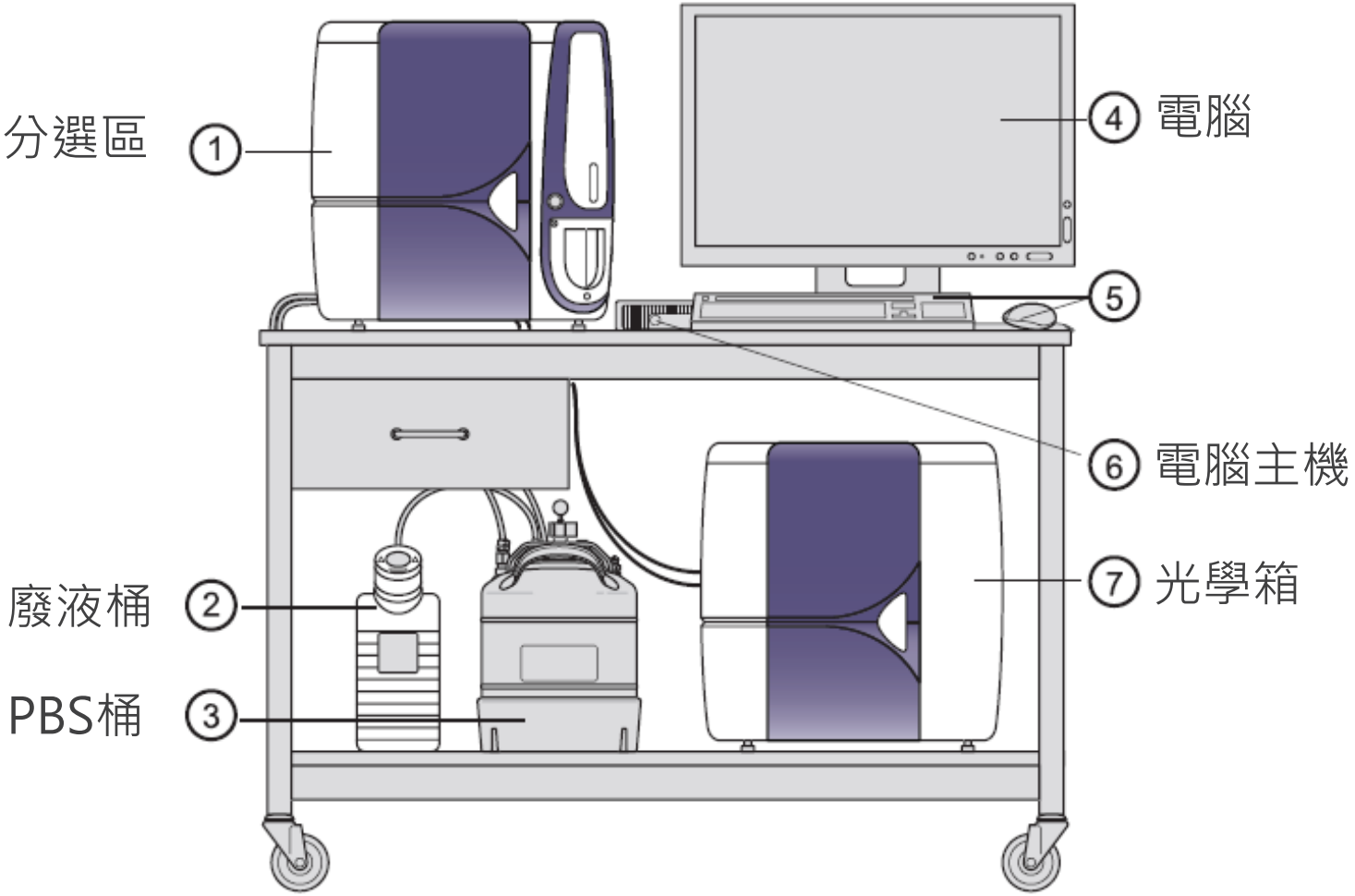
BD FACS™ Accudrop technology

- Accudrop beads
- Diode laser
- Camera
- Optical filter

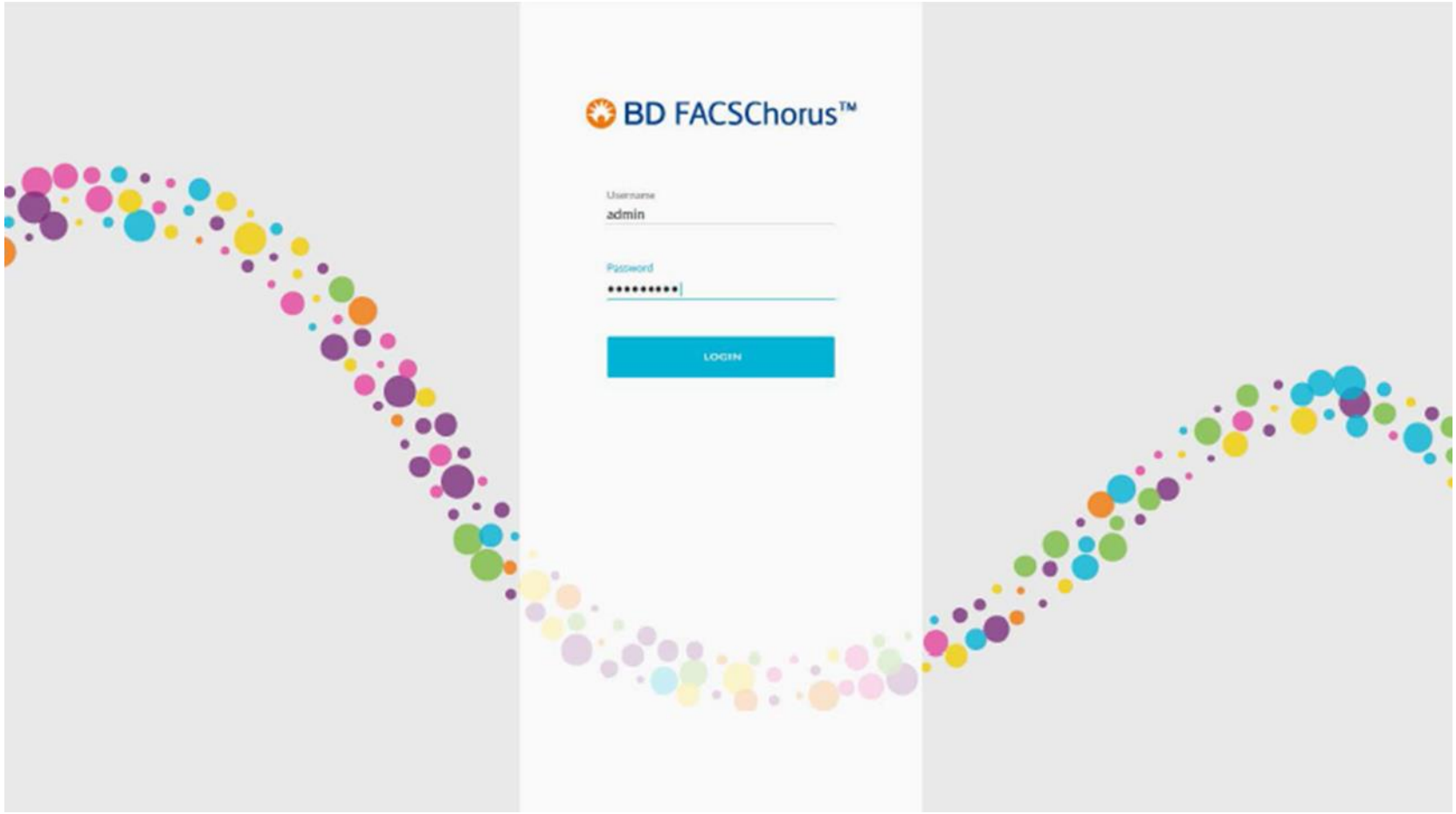


BD

FACSMelody 基本組成



FACSChorus



Fluidics Startup

1 Fluidics Startup | 2 Cleaning | 3 Sort Nozzle | 4 Cytometer Setup (CS&T) | 5 Drop Delay

Cytometer Connection	Sheath Tank	Waste Tank
Connected	5 hr 10 min remaining	OK

Last Shutdown: 08/24/2016 11:54 PM Type: Daily

Last Fluidics Startup: 08/25/2016 1:59 PM Type: Daily

Run Daily Fluidics Startup | Run Extended Fluidics Startup | Skip

BD

Cleaning

1 Fluidics Startup 2 Cleaning 3 Sort Nozzle 4 Cytometer Setup (CS&T) 5 Drop Delay

Select the cleaning that you want to run.

Prepare for Aseptic Sort

Cleans the sheath and sample paths with bleach, DI water, and ethanol.

Last Run: N/A

Flow Cell Clean

Cleans the sample path and fills the flow cell with DI water. Run this procedure when poor optical performance indicates that additional cleaning is needed.

Last Run: 08/22/2016 7:44 PM

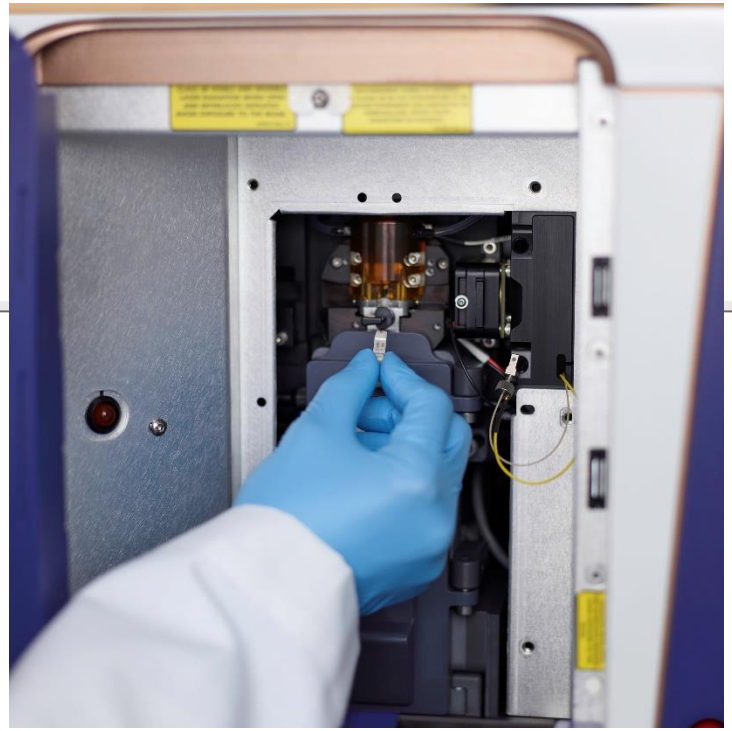
Skip

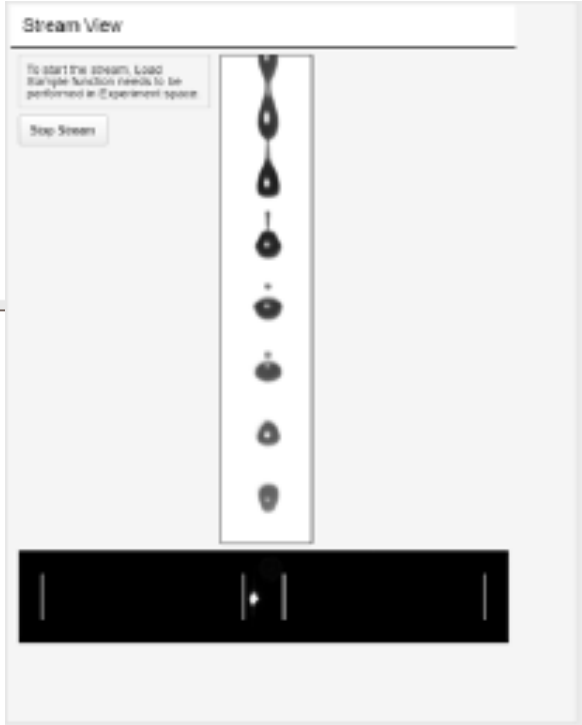
BD

Sort Nozzle

1 Fluidics Startup 2 Cleaning 3 Sort Nozzle 4 Cytometer Setup (CS&T) 5 Drop Delay

Insert the sort nozzle.





Drop Delay

1 Fluidics Startup 2 Cleaning 3 Sort Nozzle 4 Cytometer Setup (CS&T) 5 Drop Delay

Run Drop Delay daily before you perform any experiments.

Drop Delay Last Run: 08/25/2016 4:14 PM

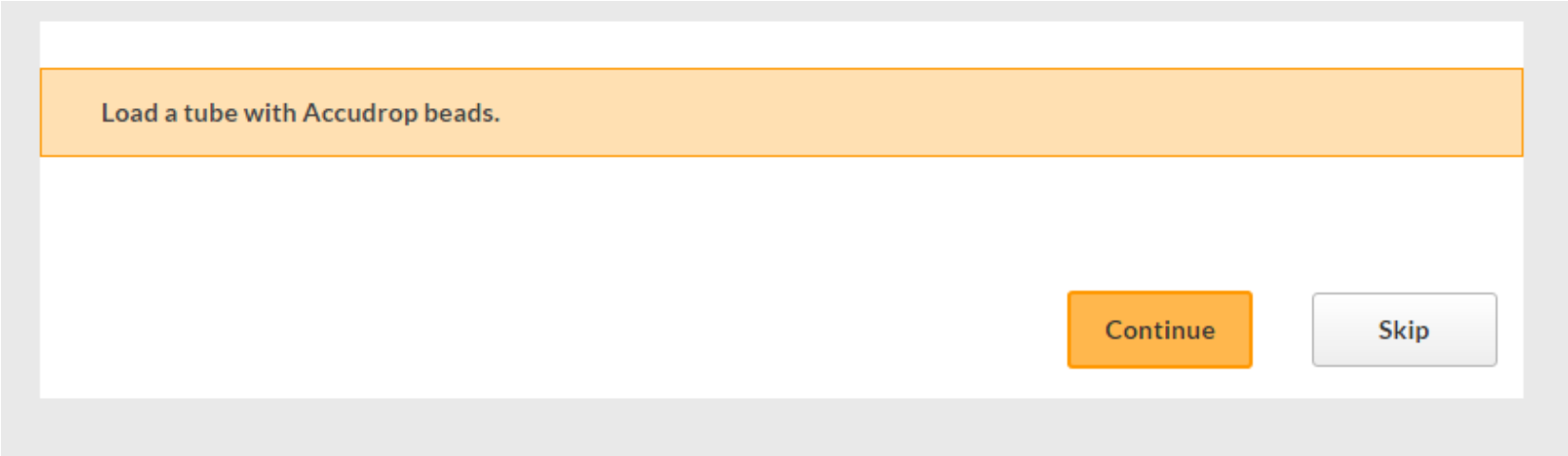
Status: Passed

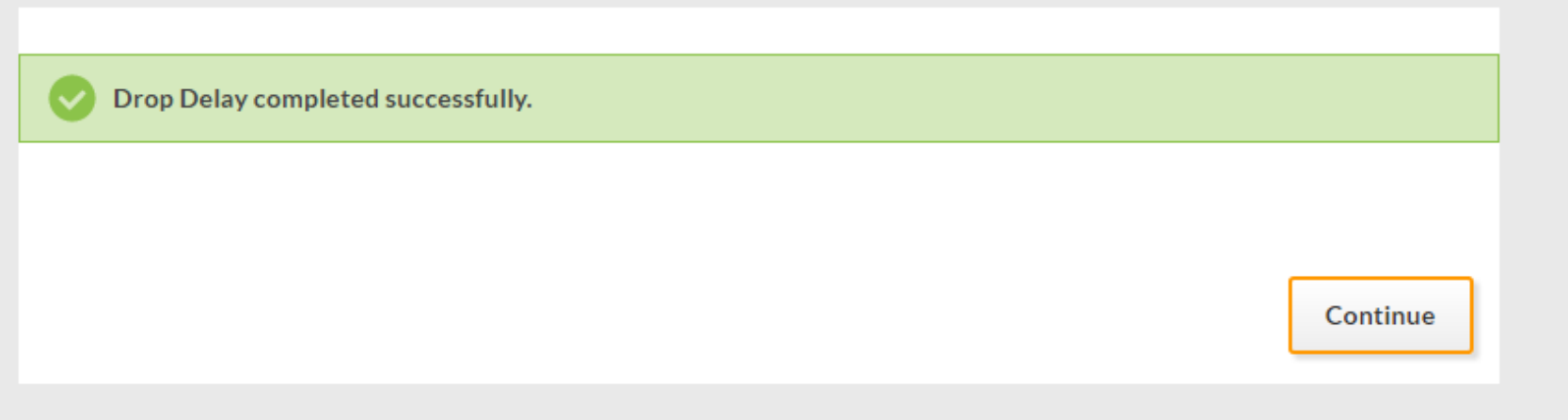
Run Drop Delay Skip

BD

Drop Delay

Beads solution: 0.5ml PBS +0.5-1 drop accudrop beads





Stream View

BD FACSChorus

Experiments » T reg 3 color

1 DESIGN EXPERIMENT | 2 VIEW DATA | 3 SET UP SORT | 4 SORT | 5 VIEW REPORTS

Experiments
Cytometer
Service
Help
John Smith
Log Out

Select Sort Report: Sort Report 4/22/2016 2:15 PM
Export Report

Sort Report 4/22/2016 2:15 PM

Cytometer Info

User Name: Simplexity Admin
Experiment Name: T reg 3 color
Application Name: BD FACSHarmony
Application Version: 1.0.0.0

Sort Details

Sort Mode: Purity
Sort Device: Tubes 5.0mL
Total Events: 1,037,579
Processed Events: 100.0%
Sort Status: Completed
Nozzle Size: 100 micron
Pressure: 25.00 PSI
Drop Frequency: 34.0 KHz
Start Date Time: 04/22/2016 02:16PM
End Date Time: 04/22/2016 02:24PM

Sort Statistics

Tube	Population	Target Count	Sort Count	Sort Rate	Efficiency	Time
1	T reg	50,000	50,000	89	93%	9m 17s
2	CD4+ CD127+ CD25-	50,000	50,000	89	93%	9m 17s

Cytometer Settings

Fluorochrome	PMT Voltages
FSC	150
SSC	522

Threshold:FSC@18000

Population Hierarchy

All Events
Scatter
SSC Singlets
FSC Singlets
CD4
T reg
CD4+ CD127+ CD25-

Stream View

Stop Stream

Sheath 13.7 hours
Waste
Connected
System
Stream

BD

Proper Sorting Rate

Recommodation Cell size <30um

Nozzle size	Default Pressure(PSI)	Drop Frequency	理想分選速度 (event rate)
100um	23psi	34KHz	3400-8500event/s

- 建議細胞大小介於1/6-1/3噴嘴尺寸間
- 分選合適速度應不超過drop frequency的 1/10-1/4
- EX: Drop frequency 34KHz
  34K/4=8500 event /s

BD

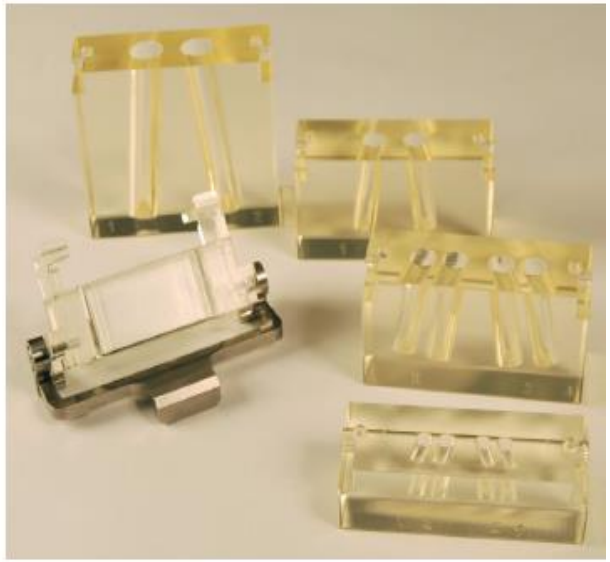
Sort mode

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。

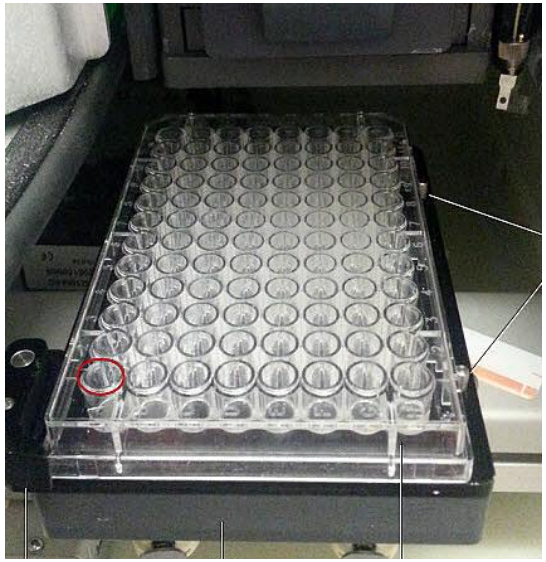
- Cell Purity:
  Purity mode=Single Cell mode>Yield mode
- Recovery cell count:
  Yield>Purity>Single Cell

Sort Layout

- Devices
  - 2 way (1.5, 2.0 and 5.0 mL tubes)
  - Multiwell Plates
    - (6-, 24-, 48-, 96-, and 384-well plates)
    - (96-well PCR tube strip)
  - Slides (3 x 9 grid)



Tube holders



選配ACDU

BD

Topic

- **Basic Concept of Flow Cytometry**
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- **Application Examples**
- **Sorting Tips**

Application Examples

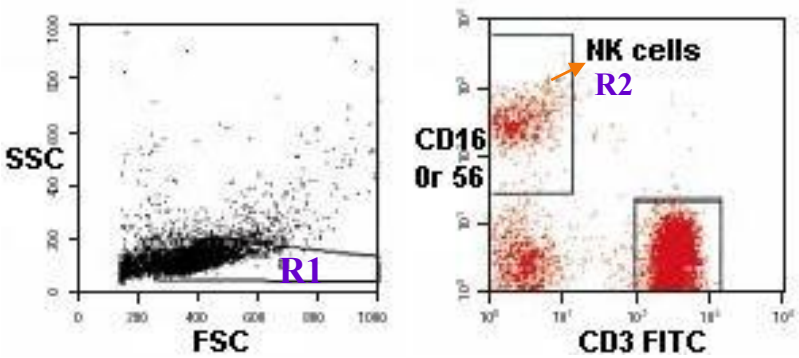
- Life or death 死活
- Morphology　細胞型態
- Surface antigens 表面抗原
- Gene expression 基因表現
- Cell functions 細胞功能
- Others 其他

BD

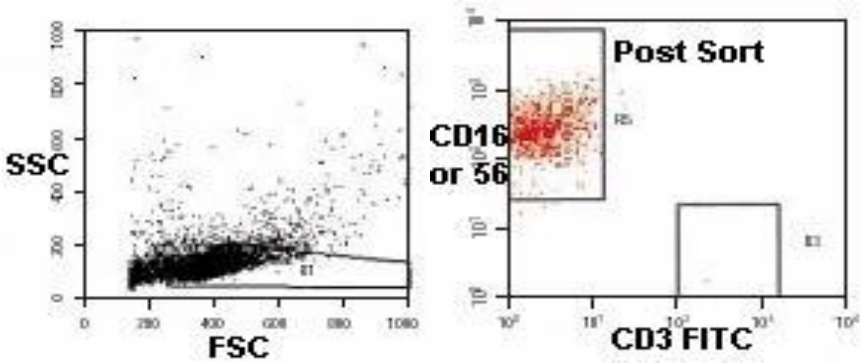
Sorting Cells By Surface Markers

- **Sorting NK Cells**
  - CD3 FITC to exclude T cells
  - $CD56^{+}CD16^{+}$ PE to include all NK Cells.

分選前的分析

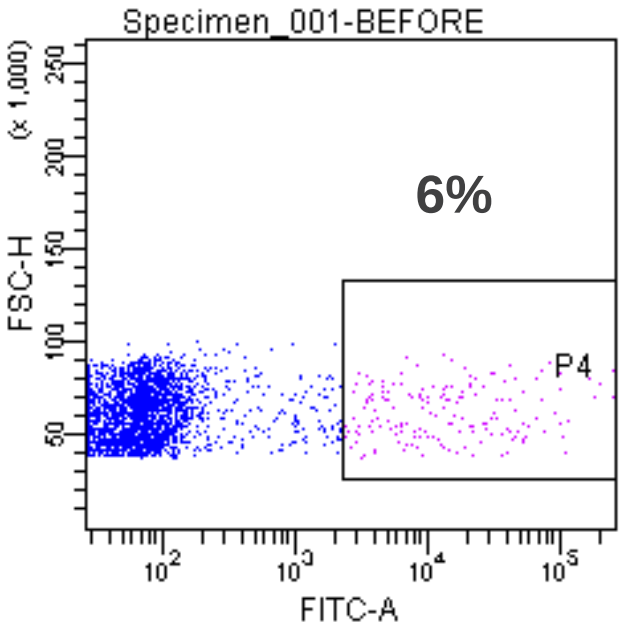


分選後結果

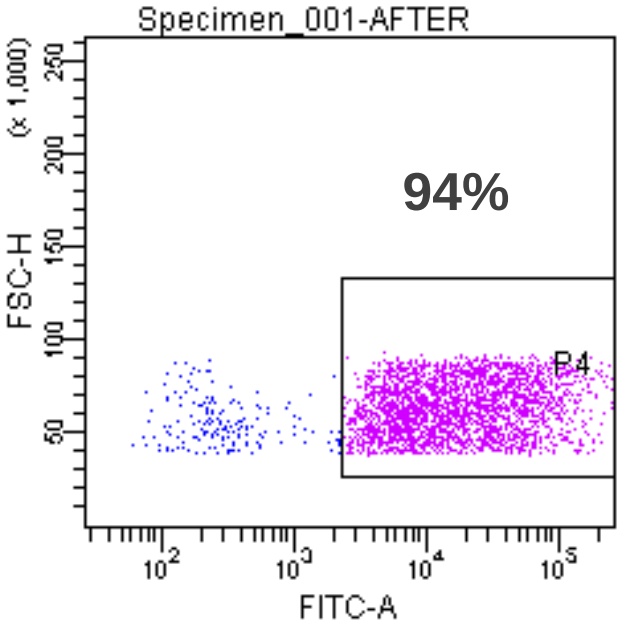


Sorting for GFP+ Cell Line

Before Sorting

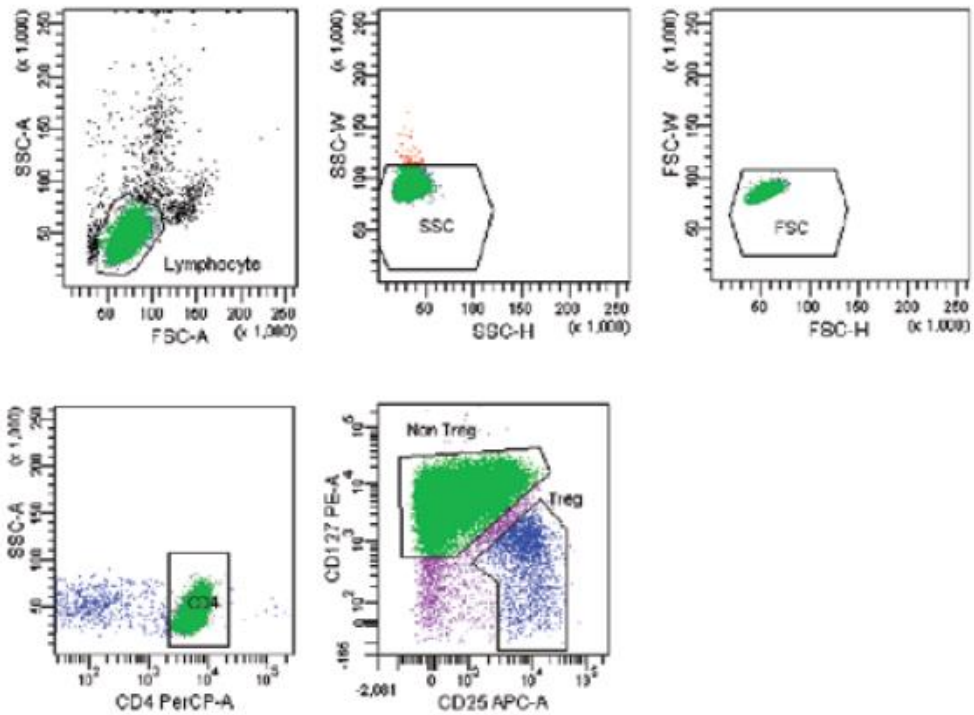


After Sorting



CD4/CD25+ Treg

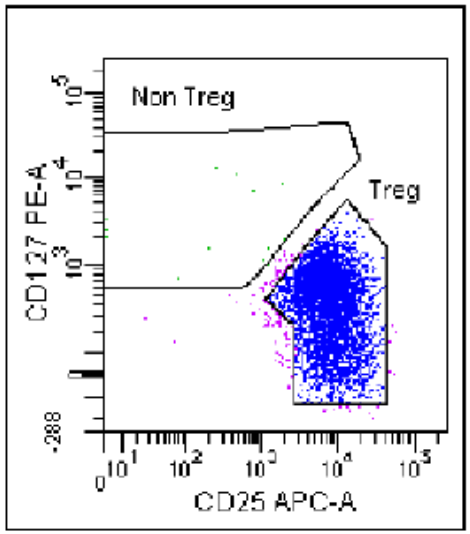
- $CD4^{+}$
- $CD25^{dim/+}$
- $CD127^{-/dim}$

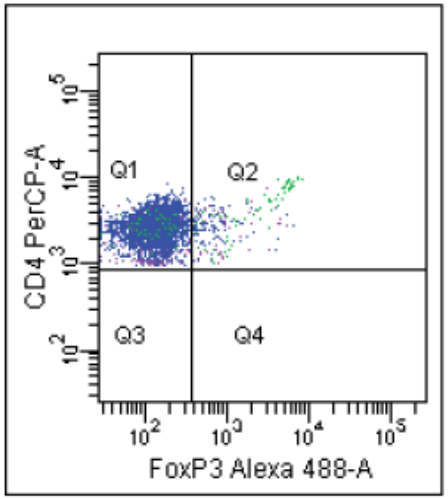


Tube: Pre-sort

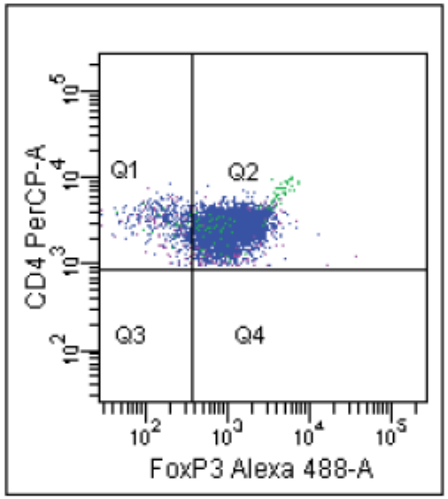
Population	#Events	%Parent	%Total
All Events	50,000		100.0
Lymphocyte	49,060	98.1	98.1
SSC	48,990	99.9	98.0
FSC	48,990	100.0	98.0
CD4	48,107	98.2	96.2
Treg	2,019	4.2	4.0
Non Treg	44,602	92.7	89.2

Sorting for Treg





Panel A: Isotype Control



Panel B: FoxP3

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

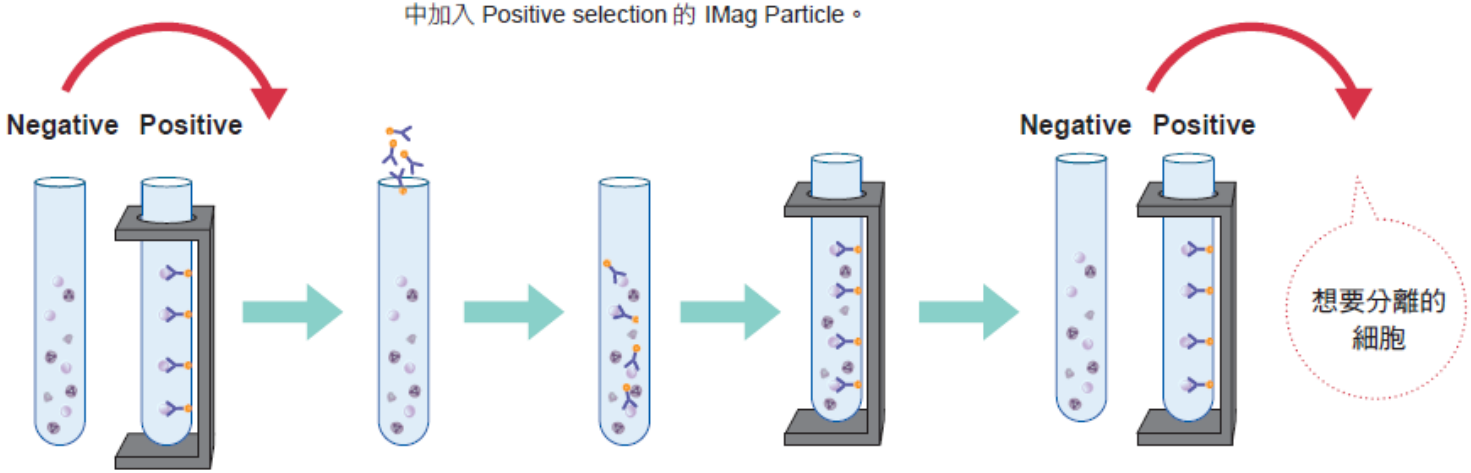
BD IMag 磁珠分選,
Positive or Negative selection or combination

Positive Selection ＆ Negative Selection Combination

Positive selection 和 Negative selection 合併使用的方式，可以用來分離帶有兩個特殊標誌的細胞，例如：調節性 T 細胞（帶有 CD4 及 CD25)。

首先，進行 Negative selection。

接著，前一步驟的 negative fraction 細胞懸浮液中加入 Positive selection 的 IMag Particle。



Human

Cat. No.	Name	Contents	Size
558142	Regulatory T Lymphocyte Separation Set - DM	CD8, CD11b/Mac-1 (CR3), CD16, CD19, CD36, CD41a, CD56, CD123, CD235a, γ δ TCR biotinylated antibody cocktail, CD25 APC, BD IMag™ SAv particles, and BD™ IMag anti-APC particles	1 x 10^9 cells

＊另有 Mouse Treg cell 分離組合，請洽騰達行當區業務。

BD IMag

磁珠大小介於兩種之間
結合以上兩者的優點

1. 操作簡單，可直接在 PS 試管中完成。
2. 不影響後續細胞培養
3. 分離後可直接用流式細胞儀檢測。
4. 分離速度快，純度及回收率高。
5. 成本低。

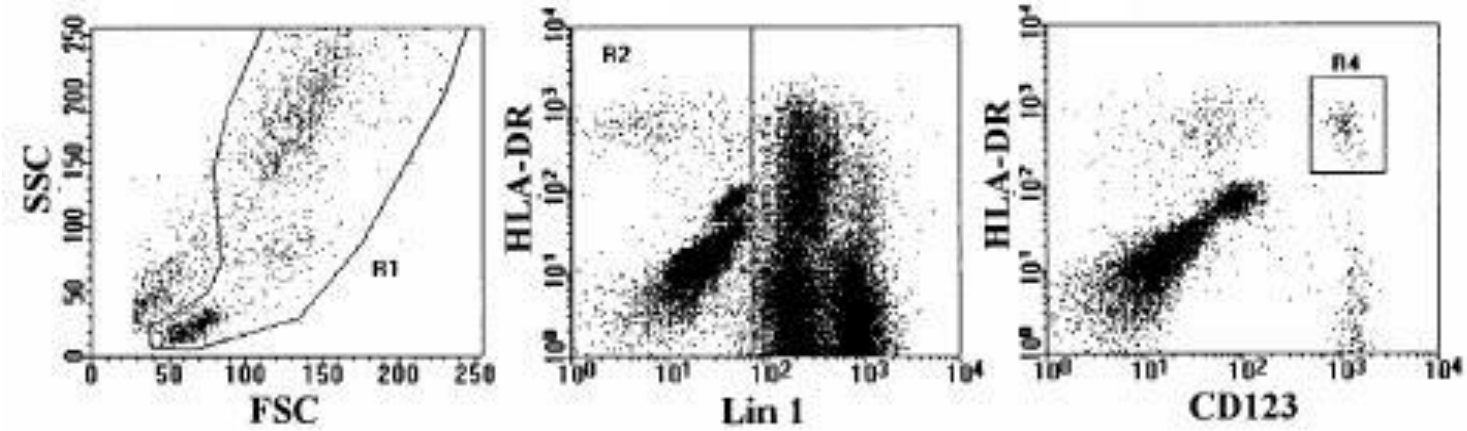
BD

Rare Cell Sorting

Parameter	CD123+ DCs	CD11c+ DCs	Basophils
Lin 1	-	-/+	-
Anti-HLA-DR	++	+++	-
CD123	+++	+	+++
CD11c	-	+++	+

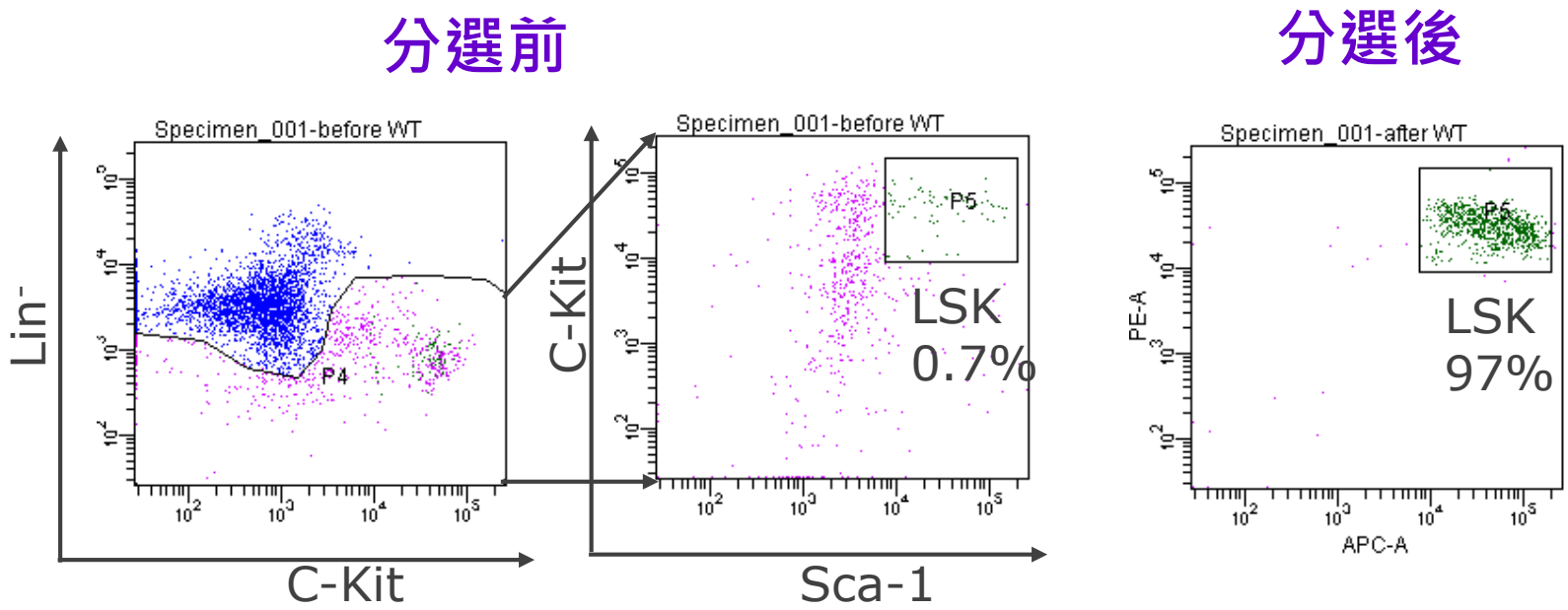
Immunophenotype of CD11c, CD123 DCs and baso
(fluorescence intensities)

- **4-Color Staining**
  - Lineage Cocktail FITC
    - CD3, 14, 16, 19, 20, 56
  - CD123 (IL-3Rα) PE
  - HLA-DR PerCP
  - CD11c APC
  - Controls
  - FACLysing Solution



BD

Three Color-staining for Defining and Sorting Mouse LSK Bone Marrow Stem Cell



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期望分選結果

- 分選細胞純度高達 95% 以上
- 分選細胞應仍保持存活度
- 分選細胞可保無菌

BD

分選結果之評估

分選10,000顆細胞，其細胞原始純度為10%
-理想的分選結果為1000顆細胞
-收集管內共計有960顆細胞
-分選後分析符合分選條件的細胞共計950顆
-儀器顯示分選細胞總數為975顆

- 純度(Purity)：
  符合分選條件的物件數目/物件總數　950/960 = 99%

- 回收率(Recovery)：
  實際分選標的細胞總數/儀器計算總數　950/975 = 97%

- 產率(Yield)：
  實際分選標的細胞總數/理想分選總數　950/1000 = 95%

BD

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

BD

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)

BD

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% FCS, 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

BD

樣品製備注意事項

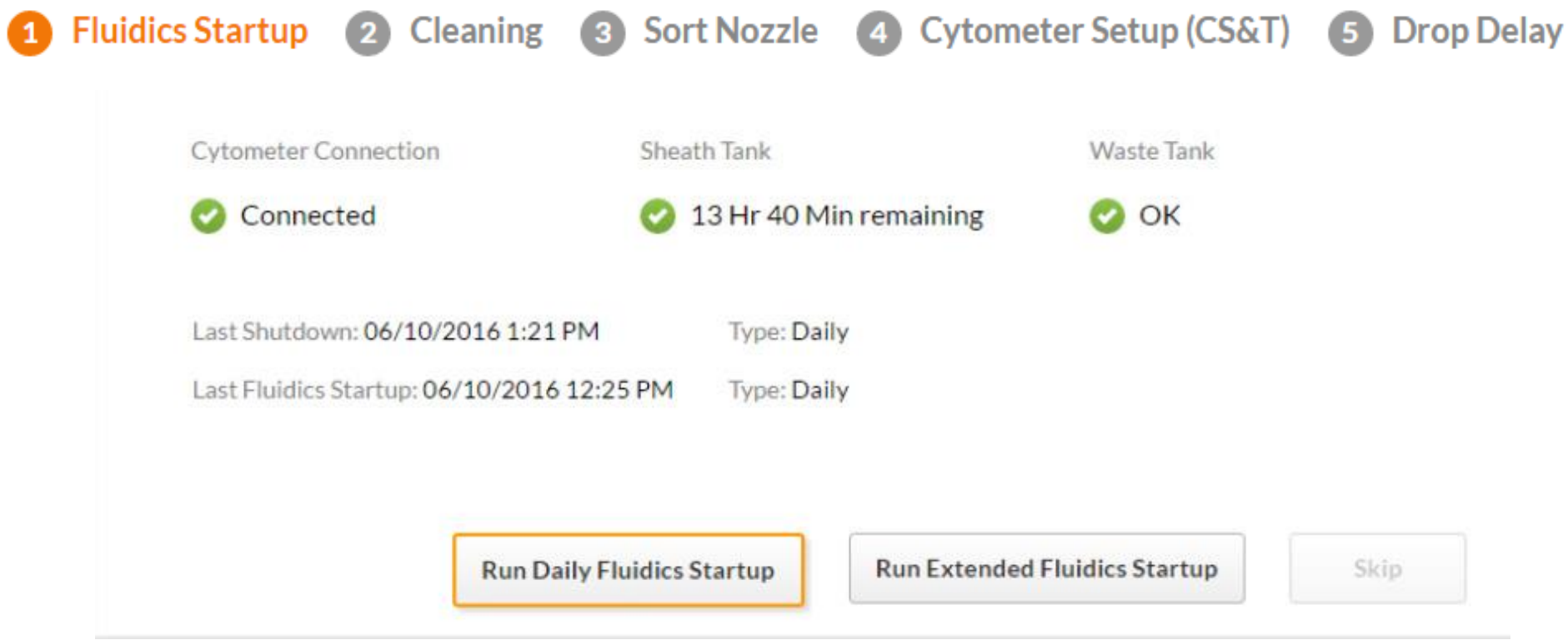
- 儘量多次低速離心以去除細胞碎片與小雜物
- 細胞直徑之考量
- 分選時間不應太長, 即分多管收集細胞. 建議測試不同的分選時間長短(10min, 20min, 或30min)以了解是否會影響細胞的活性與隨後的培養結果.
- 應使用NA/LE(no azide, low endotoxin) reagent標識細胞. 若是無法找到此類試劑, 則細胞分選下來後應離心清洗多次以去除NA及endotoxin. 離心條件:4°C, 1000rpm.
- 單一clone細胞之培養較mixed population不易, 主要是適合細胞生長所需環境與營養因子太複雜. 以傳統培養hybridoma clones來說, 通常是需要加入feeder cells或是conditioned media. 可試著使用conditioned media加入culture media(5%, 10%或20%)中來加強培養環境.

BD

開機啟動

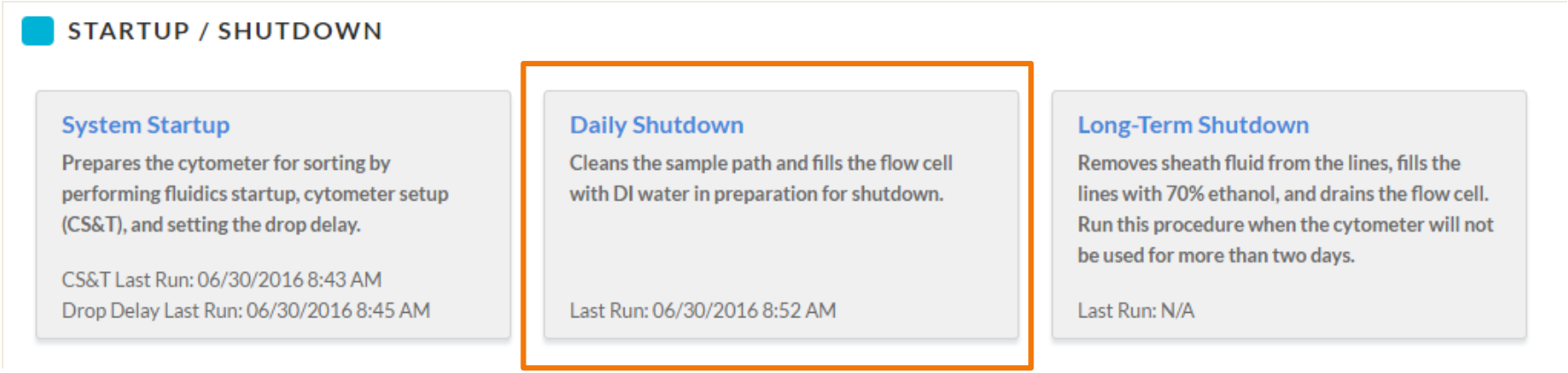
開啟電腦電源>儀器電源>空氣幫浦電源>開啟軟體連線

軟體連線後>Run Daily Fluidics Startup



關機清洗

- 任意實驗中上樣Rinse→Clean→ddH20
- 使用5ml上樣管裝3ml以速度11至少各洗10分鐘
- 執行液流關閉(Cytometer>Daily shutdown)



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關機清洗

- 以超音波震盪清洗拆下來的噴嘴(Nozzle)
- 液流關閉完成>放置一管clean solution=ddH2O
- 洩壓sheath tank, 補充PBS
- 清空廢液桶
- 將所有物品物歸原位, 清潔電極板等(去離子水, 酒精)
- 儲存數據
- 關閉儀器電源, 電腦電源, 空氣幫浦電源

常用耗材表

Tube

Cat#	耗材名稱	Size	用途
352235	Falcon 5 mL Polystyrene test tubes, 12X75mm with cell-strainer cap	25/ bag 500/box	含分選前去除細胞多聯體或凝集塊之濾網**5ml**上樣管
352054	Falcon 5 mL Polystyrene test tubes, 12X75mm capped	125/bag (Sterile)	上機用樣品管或分選收集管。含蓋子
352058	Falcon 5 mL Polystyrene test tubes, 12X75mm capped	25/ bag (Sterile)	上機用樣品管或分選收集管。含蓋子
352052	**Falcon 5 mL Polystyrene test tubes, 12X75mm uncapped**	**125/bag (Sterile)**	上機用一般樣品管。無蓋

with cell-strainer cap

With cap

常用耗材表

Beads

Cat#	耗材名稱	size	用途
661414	CS&T RUO Beads (RUO)	50 tests	**QC beads for Melody**
661612	**Accudrop Beads**	**25 tests**	計算**drop delay**(液滴延遲)分選設定用。**7.5-µm particles. Every particle contains a fluorophore that is excited at 660 nm and emits at 780 nm**

Buffer and Detergent

Cat#	耗材名稱	用途
342003	BD FACSFlow Sheath Fluid 20L	Commercial PBS buffer
352052	Round-Bottom Tube	12x75mm Flow Tube, 5ml 上樣/收集管
340345	BD FACS Clean Solution 5L	Clean solution, 10% filtered bleach, 儀器清潔用
340346	BD FACS Rinse Solution 5L	Detergent that could clean for Nucleic Acid and protein remains, 儀器清潔用排除殘餘核酸與蛋白質

資源:

FACSMelody 線上操作影片位置

http://www.bdbiosciences.com/us/s/training/e-learning

Courses	Course Information and Links
Introduction to Flow Cytometry 	• Overview (05:01) • Fluidics (3:30) • Optics (07:48) • Electronics (07:00) • Optical Measurement (09:48) • Data Analysis (08:54) • Sorting (04:25) CDs are also available for purchase. To order, call 877.232.8995, prompt 1, and request part number 651474.
BD FACSMelody™ Online Training Program* *additional content available in the e-learning library	• Getting Started (03:53) • System Startup (06:40) • Design Experiment (03:50) • View Data (06:25) • Set Up and Sort (06:50) • Daily Shutdown (03:15) • Loading the Collection Devices (06:10)