Basics of Flow Cytometry & Attune NxT

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20250918

Delivering Growth – in Asia and Beyond.

Authorized Distributor

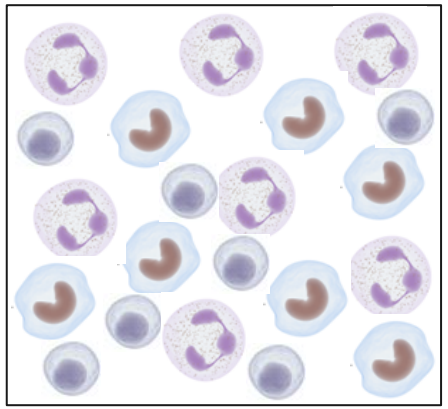
thermo scientific invitrogen

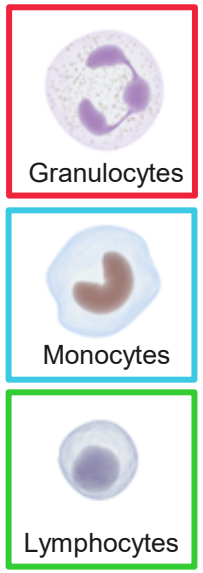
Basics of Flow Cytometry

Flow Cytometer – a different kind of “microscope”

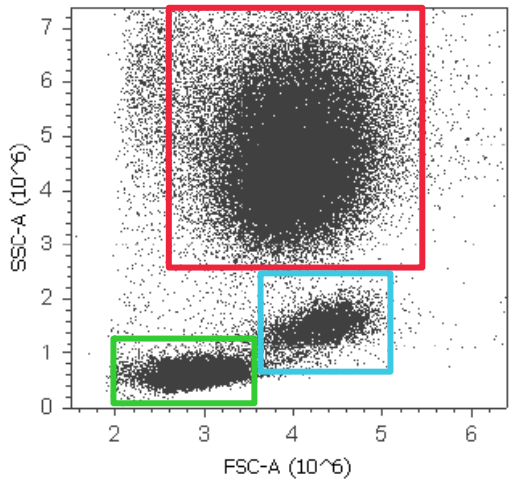
Microscope

Lysed human whole blood





Flow Cytometer



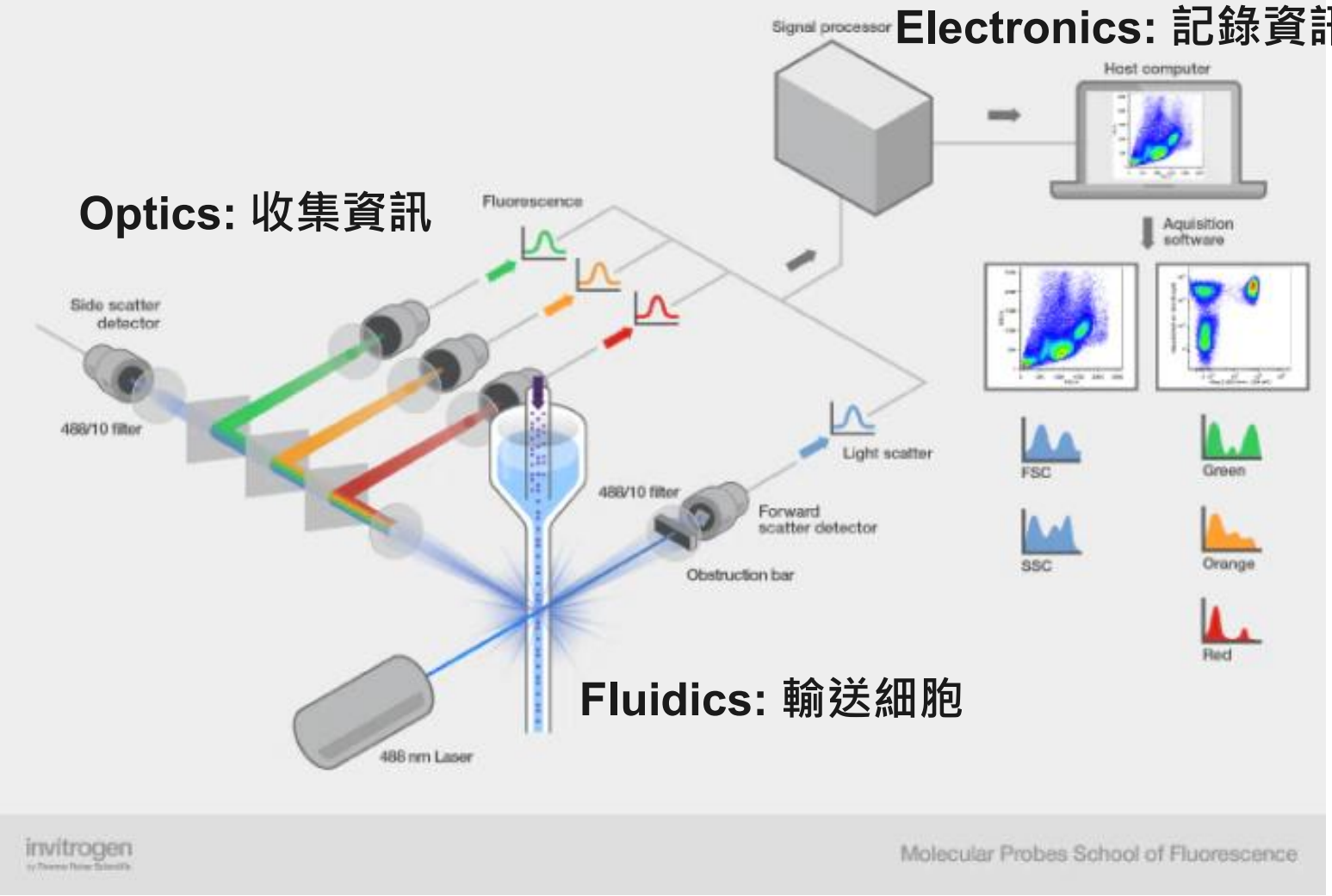
Advantage

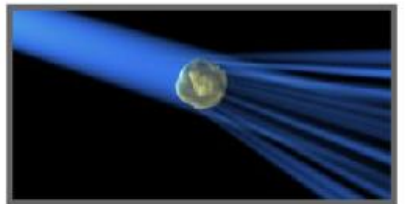
- Quantitative
- Rare population
- Multiple phenotype

Disadvantage

- Lost structure information of tissue/cell

Flow Cytometer Components



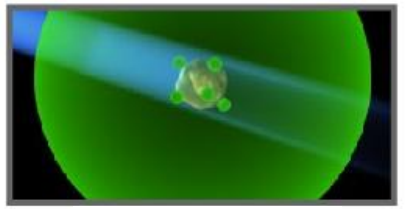


Size

Forward scatter (FSC)

Complexity

Side scatter (SSC)

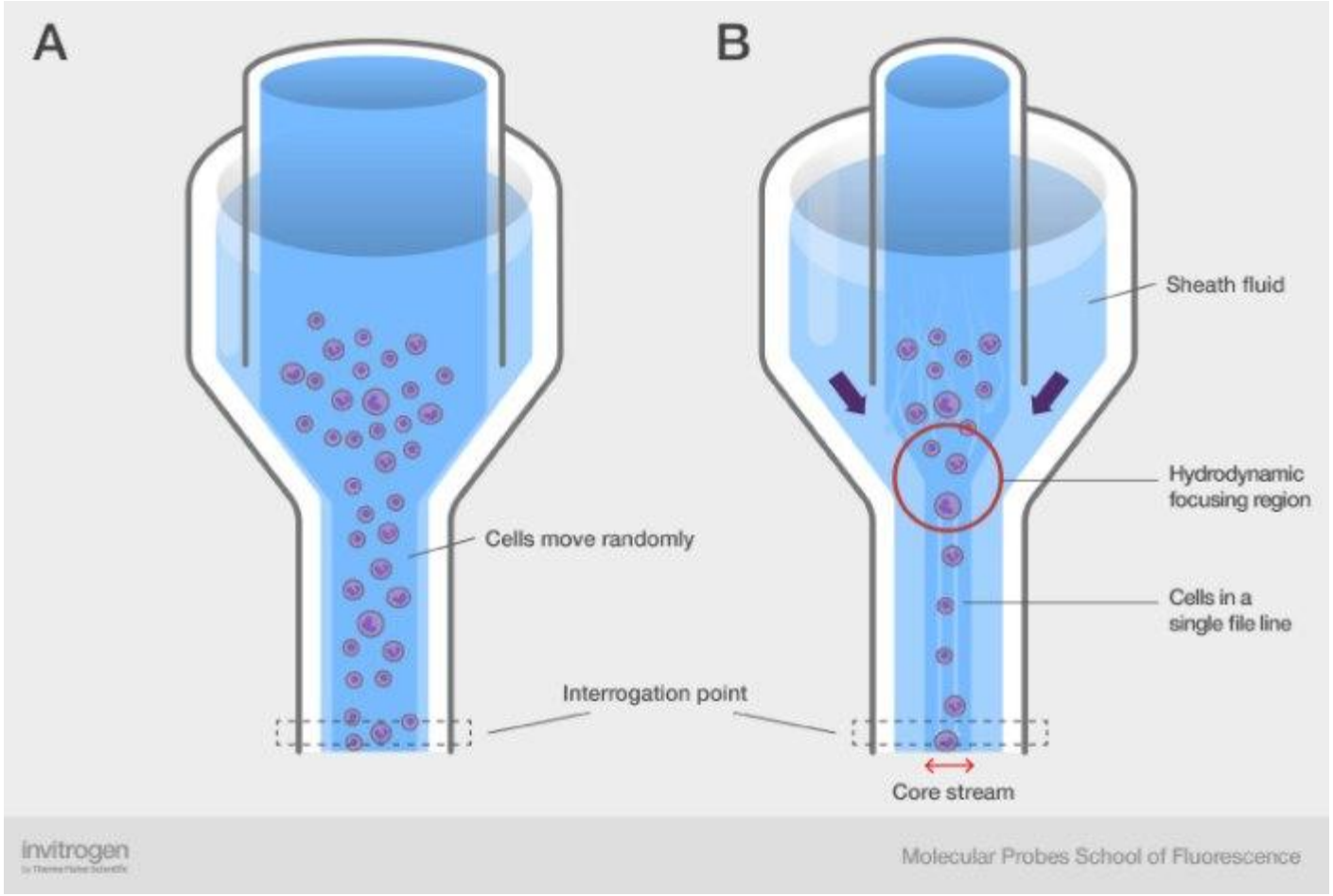


Phenotype

Fluorescence

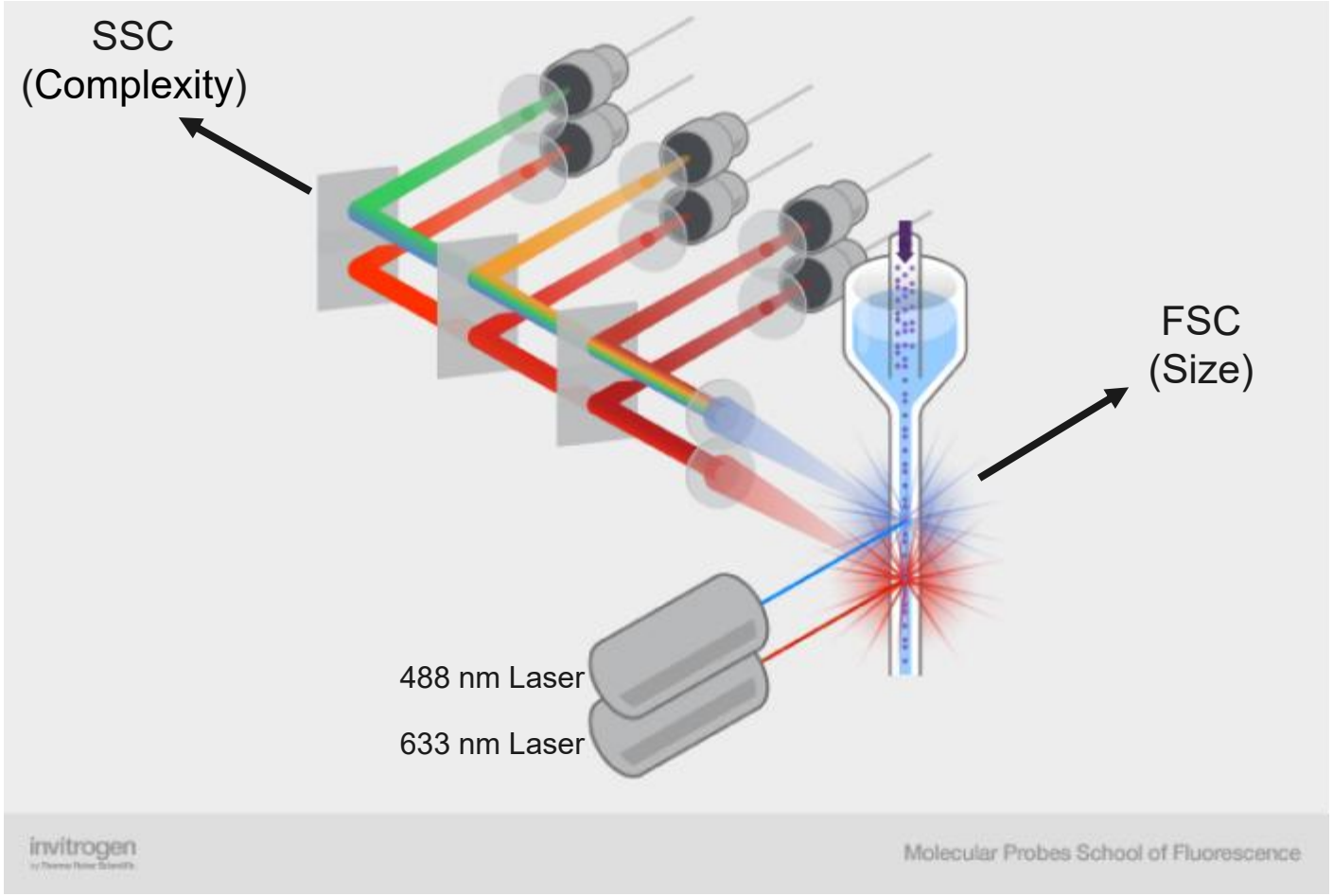
Fluidics: 輸送細胞

The **fluidics system** of a flow cytometer is responsible for transporting sample from the sample tube to the flow cell.



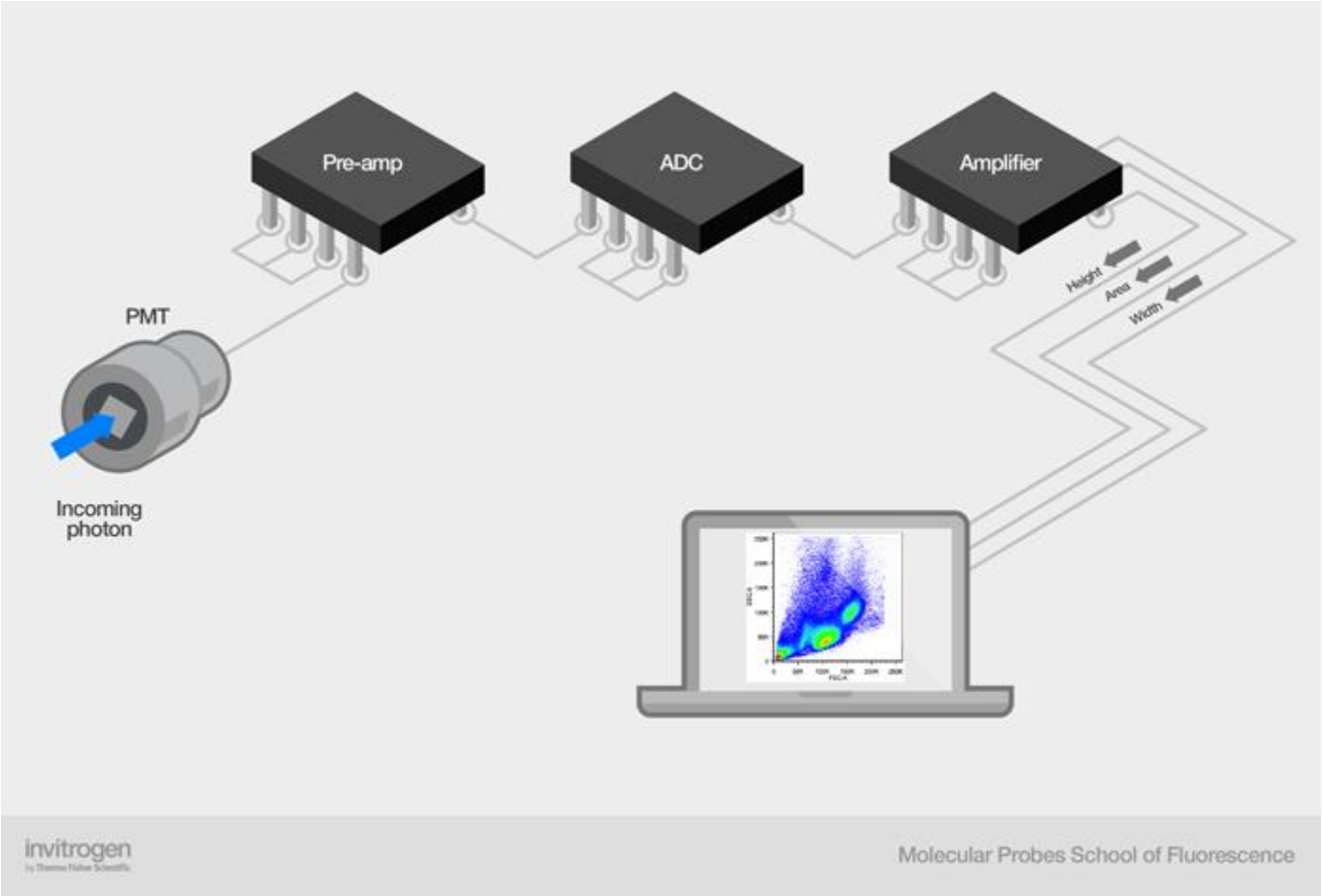
Optics: 獲取資訊

The components of the **optical system** include excitation light sources, lenses, and filters used to collect and move light around the instrument and the detection system that generates the photocurrent.

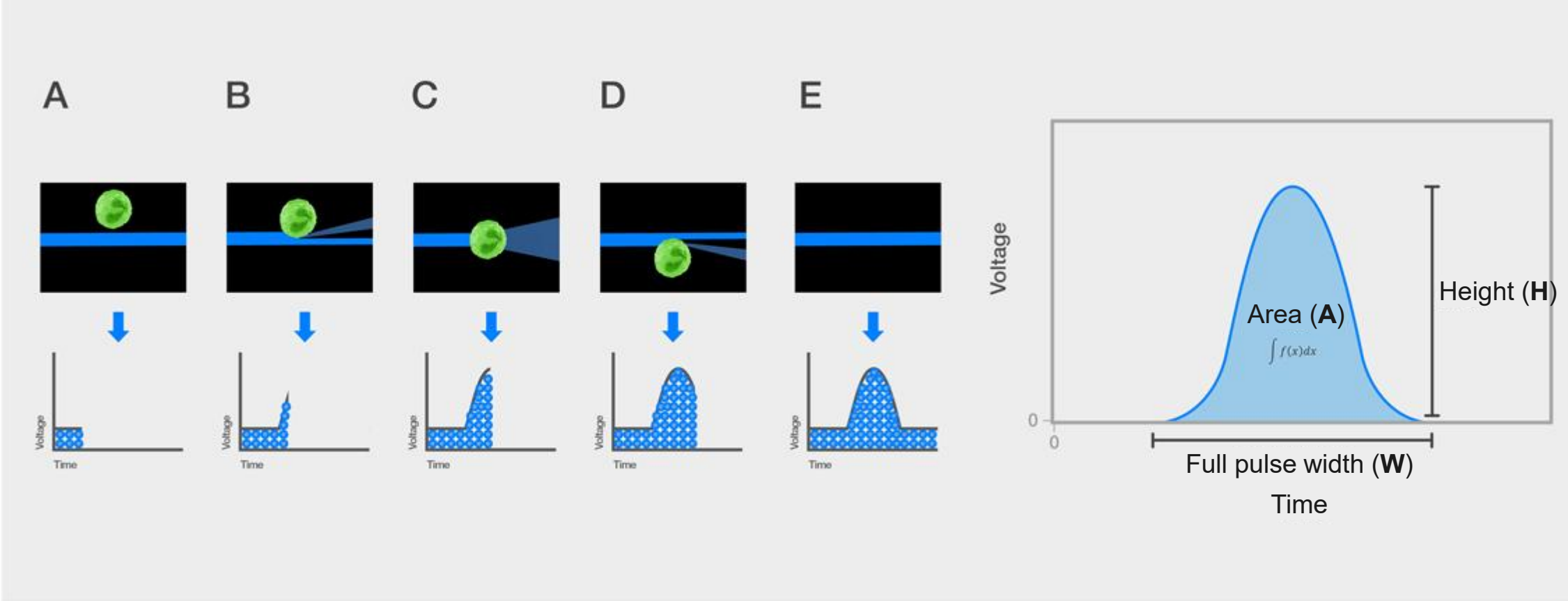


Electronics: 記錄資訊

The **electronics** are the brains of the flow cytometer. Here, the photocurrent from the detector is digitized and processed to be saved for subsequent analysis.



Signal Pulse



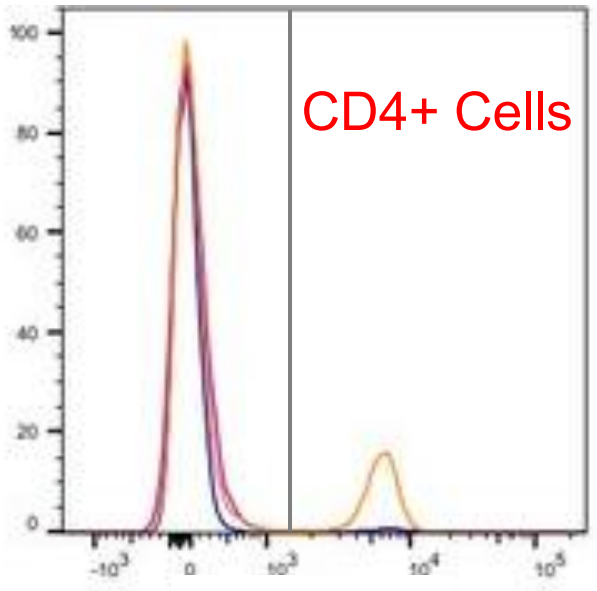
Data of Flow Cytometry

File format: FCS3.0, FCS3.1

Cell	FSC	SSC	FITC	PE	APC	…
1	91.3	27.8	62.0	78.9	83.4	73.1
2	93.0	44.9	73.8	47.7	19.2	29.0
3	39.5	75.7	23.3	68.3	49.2	53.7
4	76.5	3.9	12.3	76.1	72.5	70.0
5	98.8	92.8	63.2	52.3	24.2	11.4
6	48.6	46.5	93.7	52.9	74.8	87.0
7	87.7	29.2	4.1	6.9	48.7	57.7
8	54.4	26.5	68.1	72.1	12.7	80.1
9	91.5	80.8	63.8	71.6	15.0	89.9
…	19.8	63.9	69.4	46.7	43.9	25.7

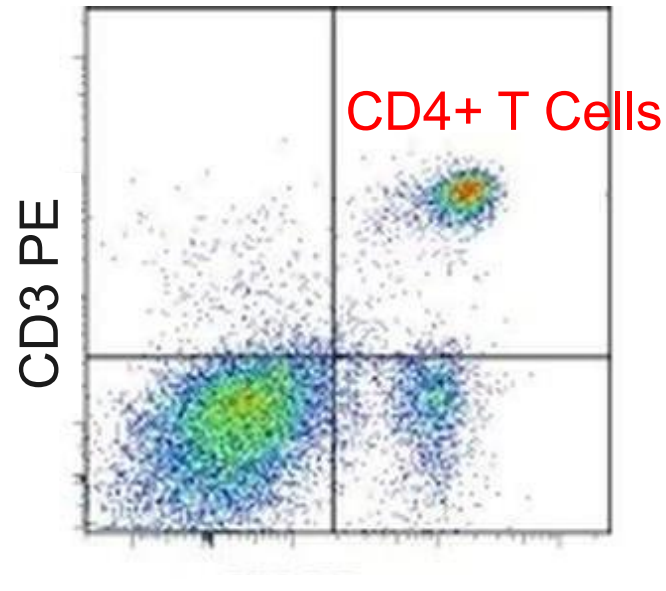
Flow Cytometry Standard (FCS)
https://isac-net.org/page/Data-Standards

1 marker



Histogram

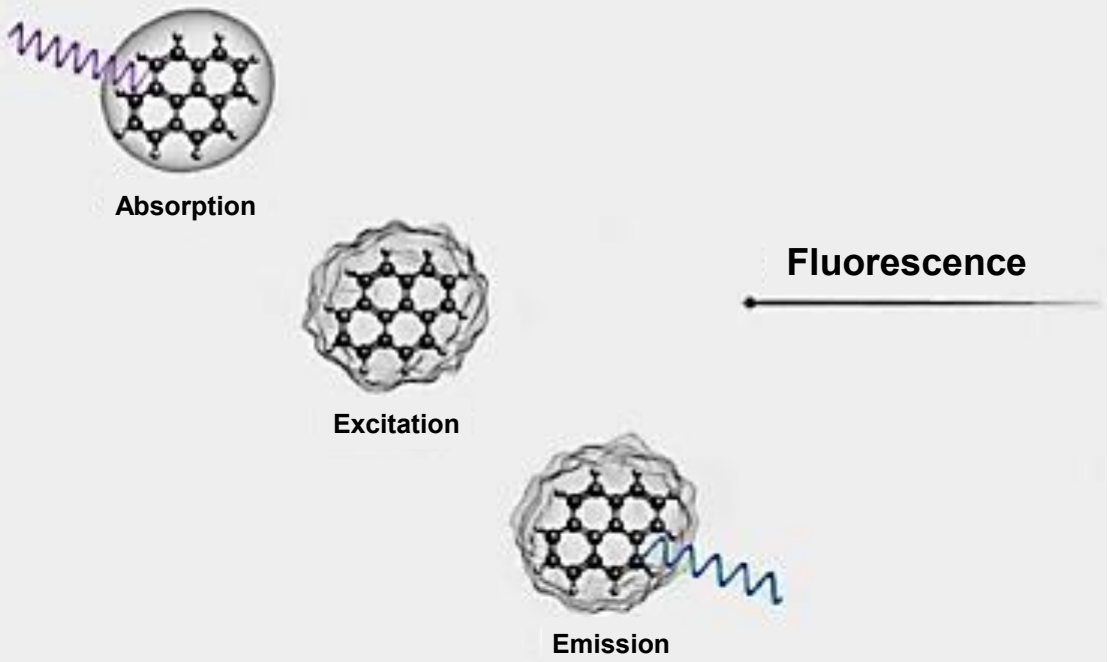
2 markers



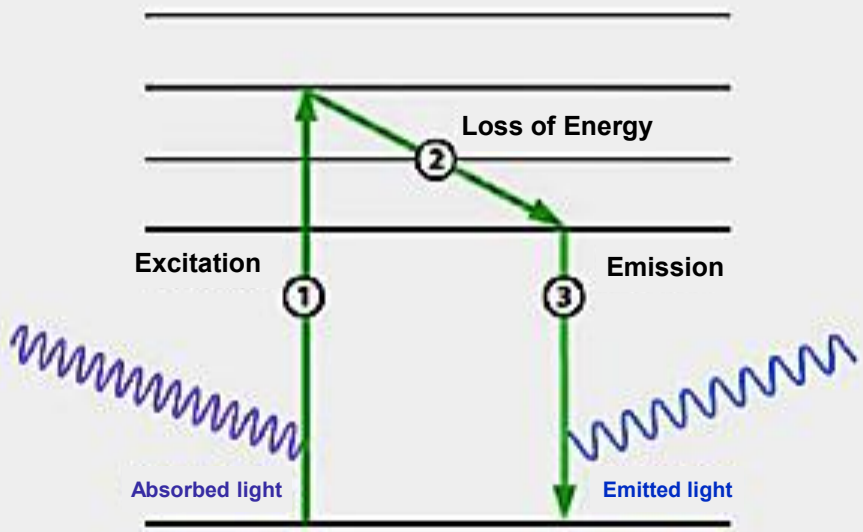
Dot plot

Fluorescence

Definition of Fluorescence

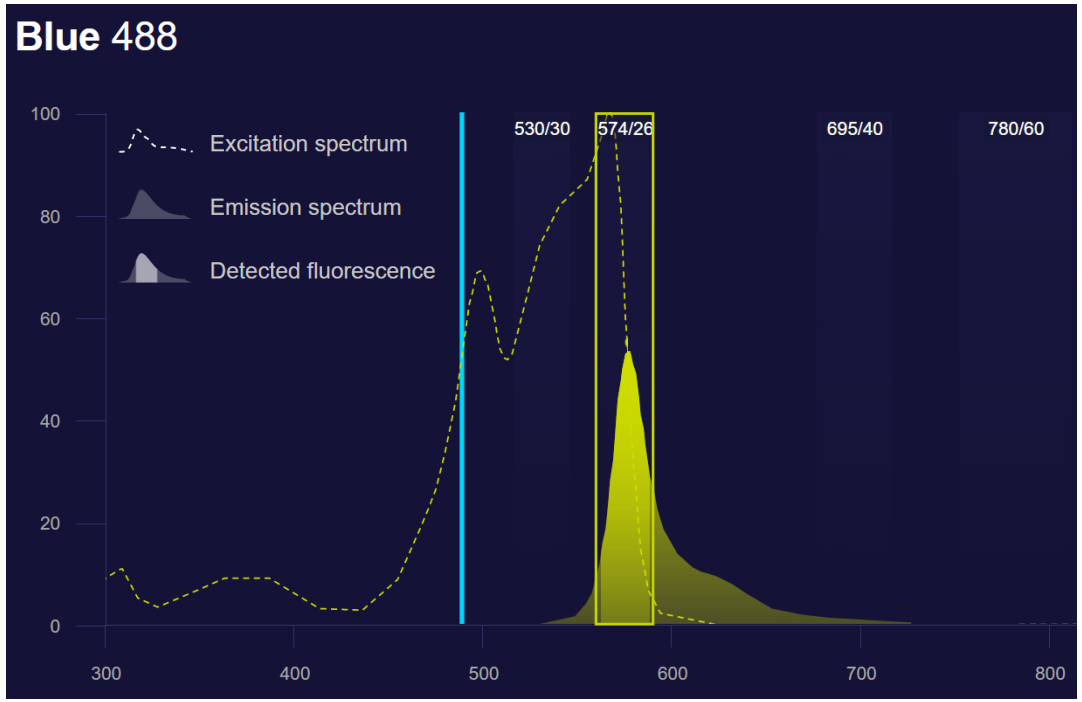


Jablonski Diagram Summary

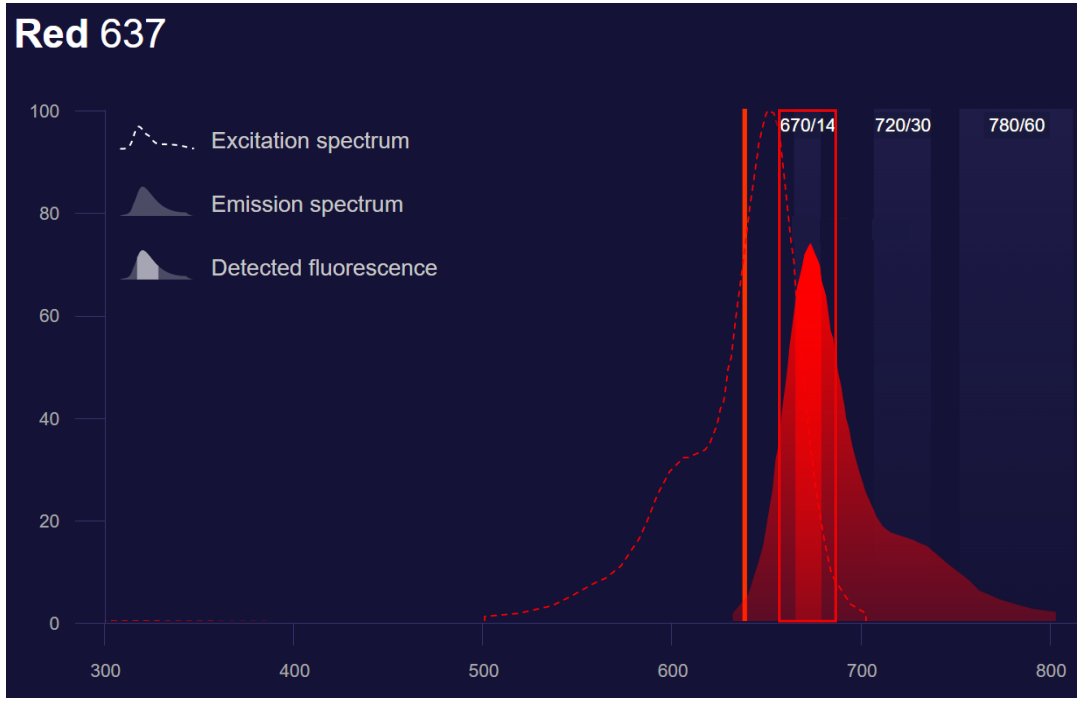


Channels for Fluorochrome

Fluorochrome: PE
Channel: Ex 488, Em 574/26

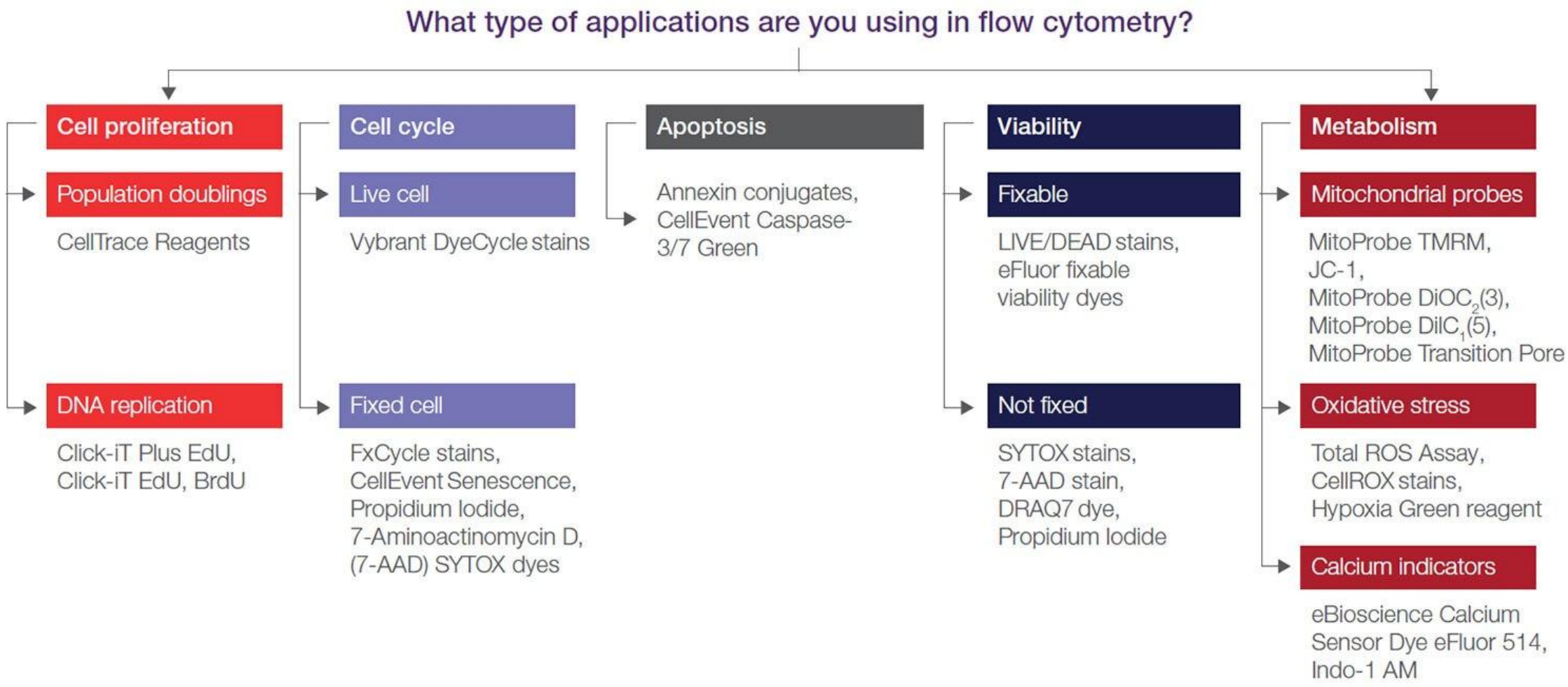


Fluorochrome: APC
Channel: Ex 637, Em 670/14



https://www.thermofisher.com/order/fluorescence-spectraviewer#!/

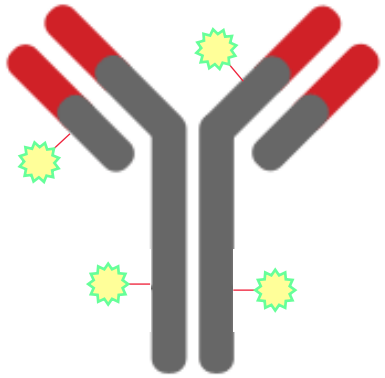
Fluorescent Reagents



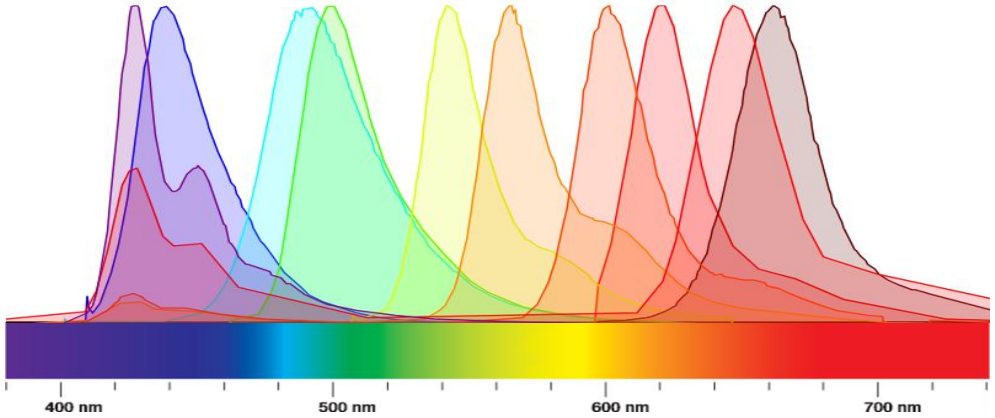
https://www.thermofisher.com/tw/en/home/life-science/cell-analysis/flow-cytometry/flow-cytometry-assays-reagents.html

Fluorescent Antibody

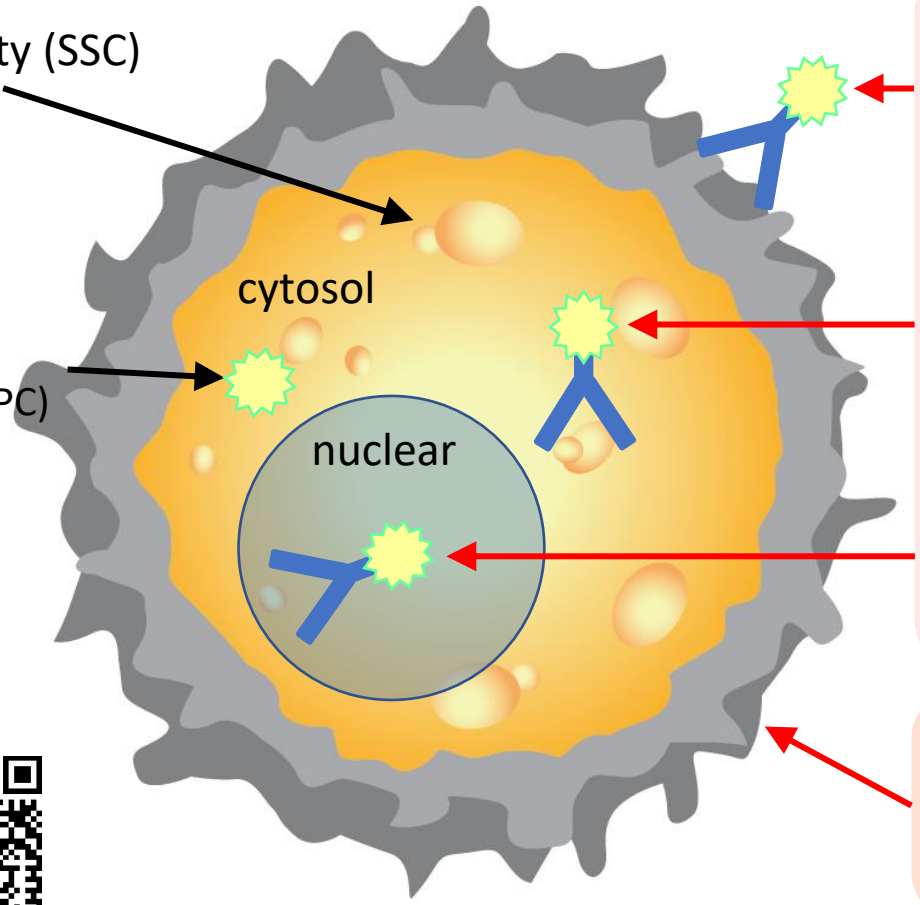
Antibody: Specificity 專一性



Fluorescence: Identity 辨識度



Cell Characteristics by Flow Cytometry



胞内螢光
(e.g. GFP, PE, APC)

胞外染色
Surface staining
(e.g. CD markers)

免疫分型
Immunophenotyping

胞内染色
Cytosolic staining
(e.g. cytokines)

核内染色
Nuclear staining
(e.g. transcription factors)

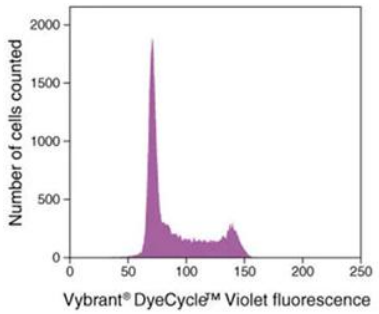
Intracellular staining

細胞功能試劑
Cell function assays
(e.g. viability, proliferation, cell cycle, apoptosis, metabolism)

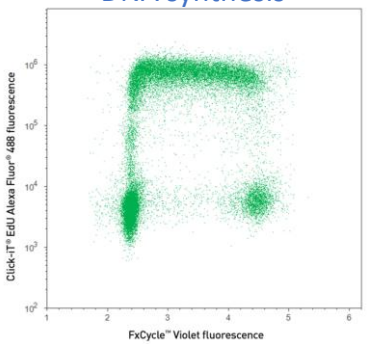
https://www.thermofisher.com/us/en/home/life-science/cell-analysis/flow-cytometry.html

Applications of Flow Cytometry

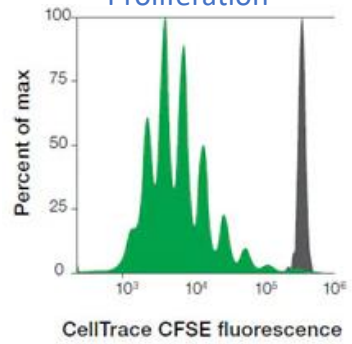
細胞週期
Cell cycle



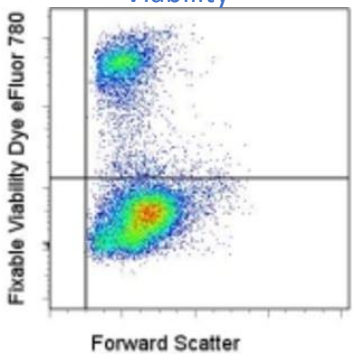
DNA合成
DNA synthesis



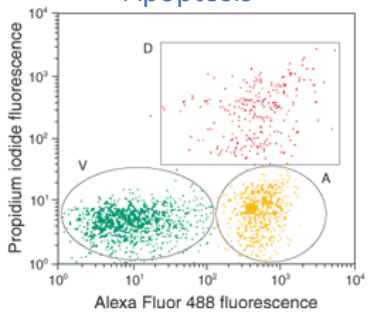
細胞增長
Proliferation



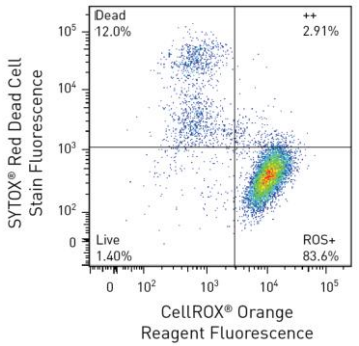
細胞存活
Viability



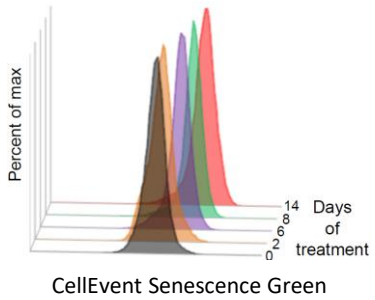
細胞凋亡
Apoptosis



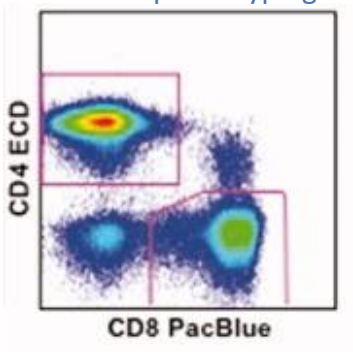
氧化壓力
Oxidative Stress



細胞老化
Senescence

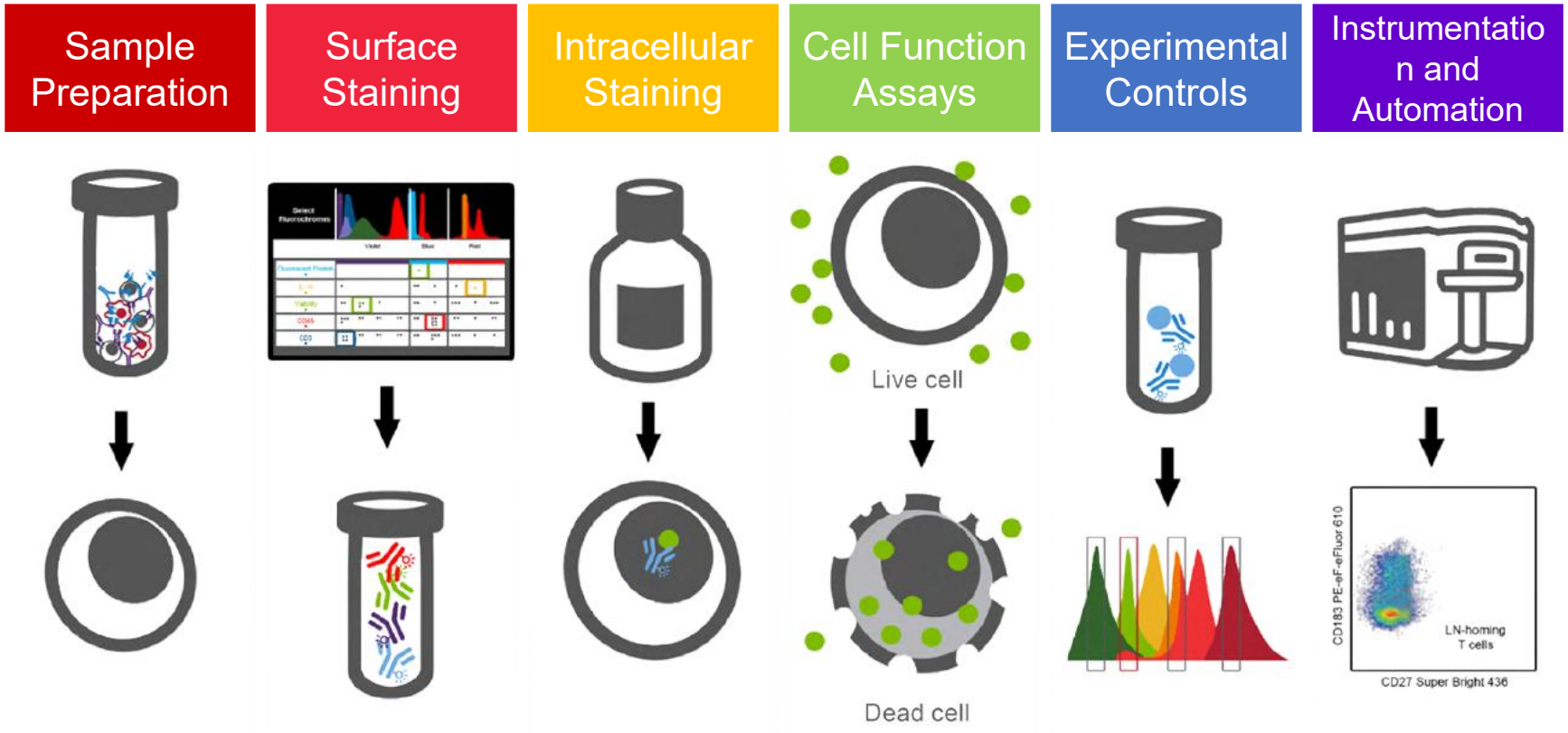


免疫分型
Immunophenotyping

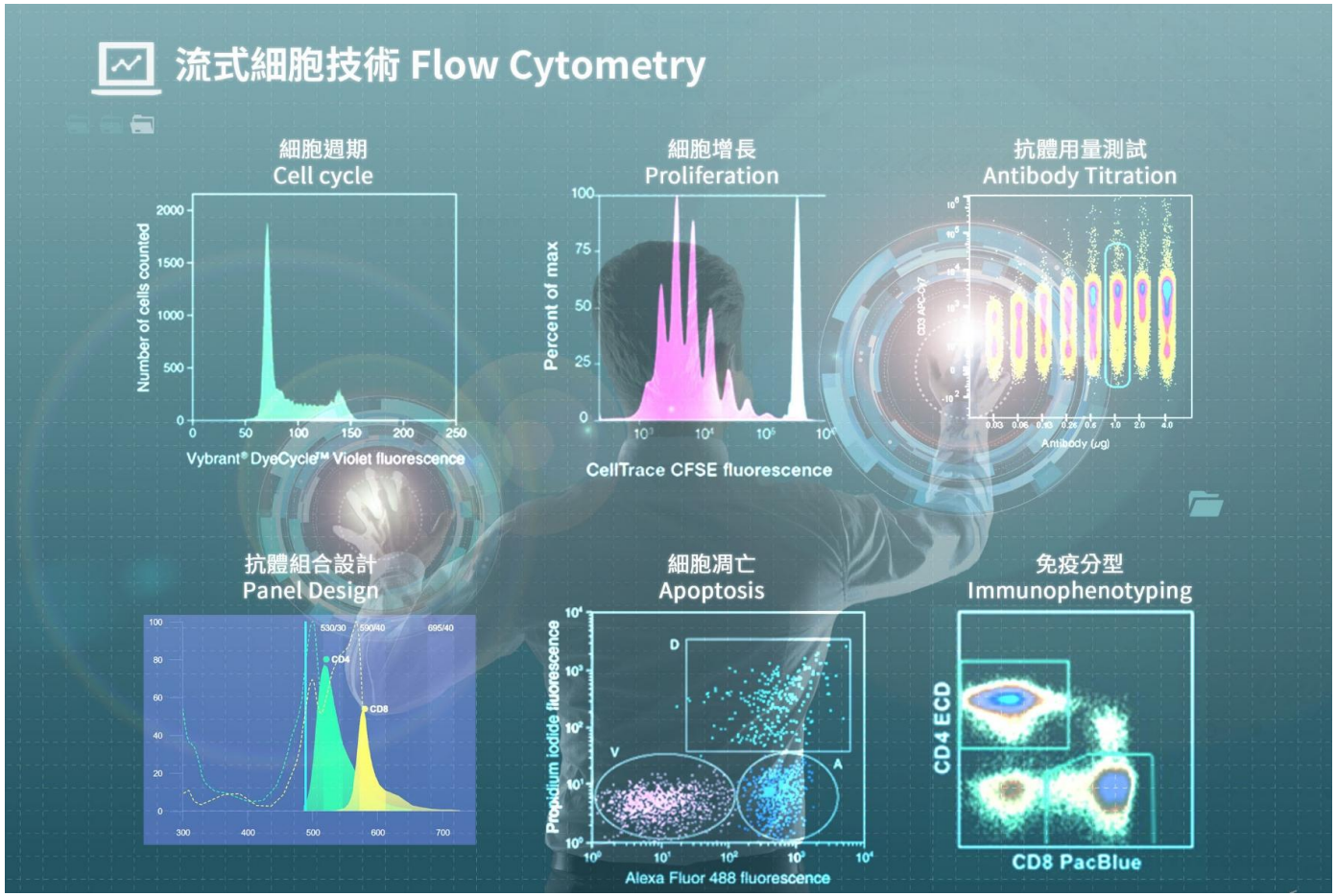


Flow Cytometry Solutions from Thermo Fisher Scientific

DKSH



Let Us be Your Partner in Flow Cytometry!



Thermo Fisher Products

- Invitrogen
- eBioscience
- Molecular Probes

Custom Service

歡迎與當區業務

美卉/思螢

聯絡取得相關訊息~

Workflow of Flow Cytometry with Attune NxT

Evolving with the Growing Needs of the Flow Cytometrist

DKSH

2015 → 2021 → Present

Reliable workhorse instrumentation

More information needed per cell

Increasing panel size, rarer populations

Invitrogen™ Attune™ NxT Flow Cytometer

Up to 14 fluorescent channels

Efficient, flexible, transformative

Invitrogen™ Attune™ CytPix™ Flow Cytometer

Up to 14 fluorescent channels with **brightfield imaging**

Two data sets, one step, zero doubt

Invitrogen™ Attune™ Xenith™ Flow Cytometer

51 fluorescent channels and **spectrally enabled**

Understand your cells on a whole new level

Attune NxT Acoustic Focusing Flow Cytometer

Small in size, big in performance

Flat-Top Laser
平頂雷射均勻激發細胞，
提供穩定且高解析度的分析結果

Acoustic Focusing
專利聲波輔助流體動力聚焦技術，
大幅提升最高分析流速，同時依舊
維持高解析度

Autosampler
可選配自動上樣機**CytKick (MAX)**，
盤式上樣更省時方便

Syringe Pump
針筒幫浦定量上樣體積，
可絕對計數細胞濃度

Flow Cytometry Workflows

- Panel Design – Flow Cytometry Panel Builder
- Antibody Titration
- Controls

實驗規劃

- Sample preparation
- Cell staining

樣本製備與染色

- Flow Cytometer Start Up
- Select **Channels**
- Setup **Workspace** (***Cell > Singlet***, gating strategies, controls for threshold setup)
- Setup **Collection Panel**
- Setup **PMT** (signal min. from unstained control, signal max. from positive sample)
- Setup **Compensation** (single stained control for all fluorophore)
- Analyze Samples
- Data Export (FCS 3.0 or higher)
- Flow Cytometer Shutdown

上樣分析流程

- Data Analysis

數據分析

Principles of Panel Design

1. Know your flow cytometer (***channels***).
2. Identify ***markers*** of interest (literatures for gating strategy; Immune cell guide).
3. Know the ***spectrum of fluorophores*** and minimize spillover.
4. Brighter fluorophores for lower-expressed markers, and vice versa.
5. Use spectrally similar fluorophores for different cell subpopulations.

Attune NxT Configuration, CGU

FSC: Forward scatter
SSC: Side scatter

Excitation Laser	Emission Filter (nm)	Channel	Common Fluorophores
Blue-488 nm	530/30	BL1	FITC, Alexa Fluor 488, GFP, YFP
	590/40	BL2	PE-Texas Red, PE-Alexa Fluor 610, PI
	695/40	BL3	PerCP, PerCP-Cy-5.5, PE-Cy-5.5, PE-Alexa Fluor 700
Red-637 nm	670/14	RL1	APC, Alexa Fluor 647
	720/30	RL2	Alexa Fluor 680, Alexa Fluor 700, APC-Alexa Fluor 700
	780/60	RL3	APC-Alexa Fluor 750, APC-Cy7
Yellow-561 nm	585/16	YL1	PE, Alexa Fluor 568, PI
	620/15	YL2	Alexa Fluor 594, PE-Texas Red, PE-Alexa Fluor 610, m-Cherry
	695/40	YL3	TRI-COLOR, PE-Cy-5, PE-Cy-5.5, PE-Alexa Fluor 700, PerCP-Cy-5.5, PerCP
	780/60	YL4	PE-Alexa Fluor 750, PE-Cy-7, Qdot 800
Violet-405 nm	440/50	VL1	Pacific Blue, Alexa Fluor 405
	512/25	VL2	Pacific Green, Qdot 525
	603/48	VL3	Pacific Orange, Qdot 605
	710/50	VL4	Qdot 705

Flow Cytometry Panel Builder

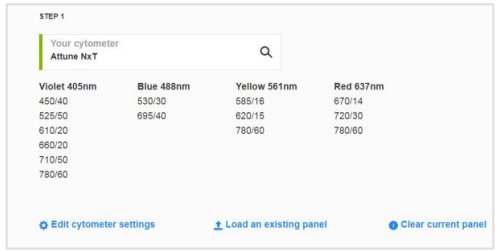
Step 1: Cytometer
機器規格

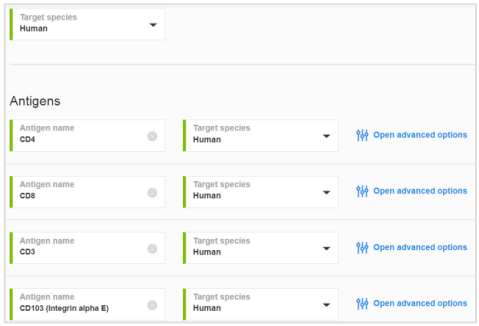
Step 2: Antigens
設定抗原

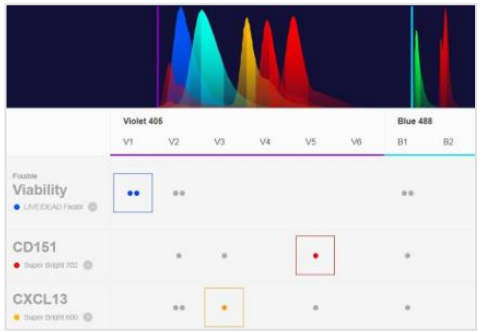
Step 3: Fluorochromes
配置螢光

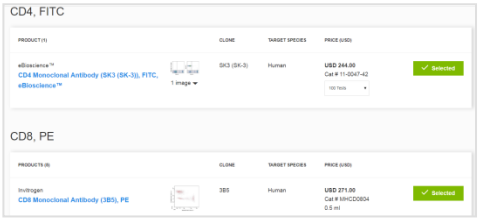
Step 4: Products
選擇產品

Step 5: Summary
輸出規劃



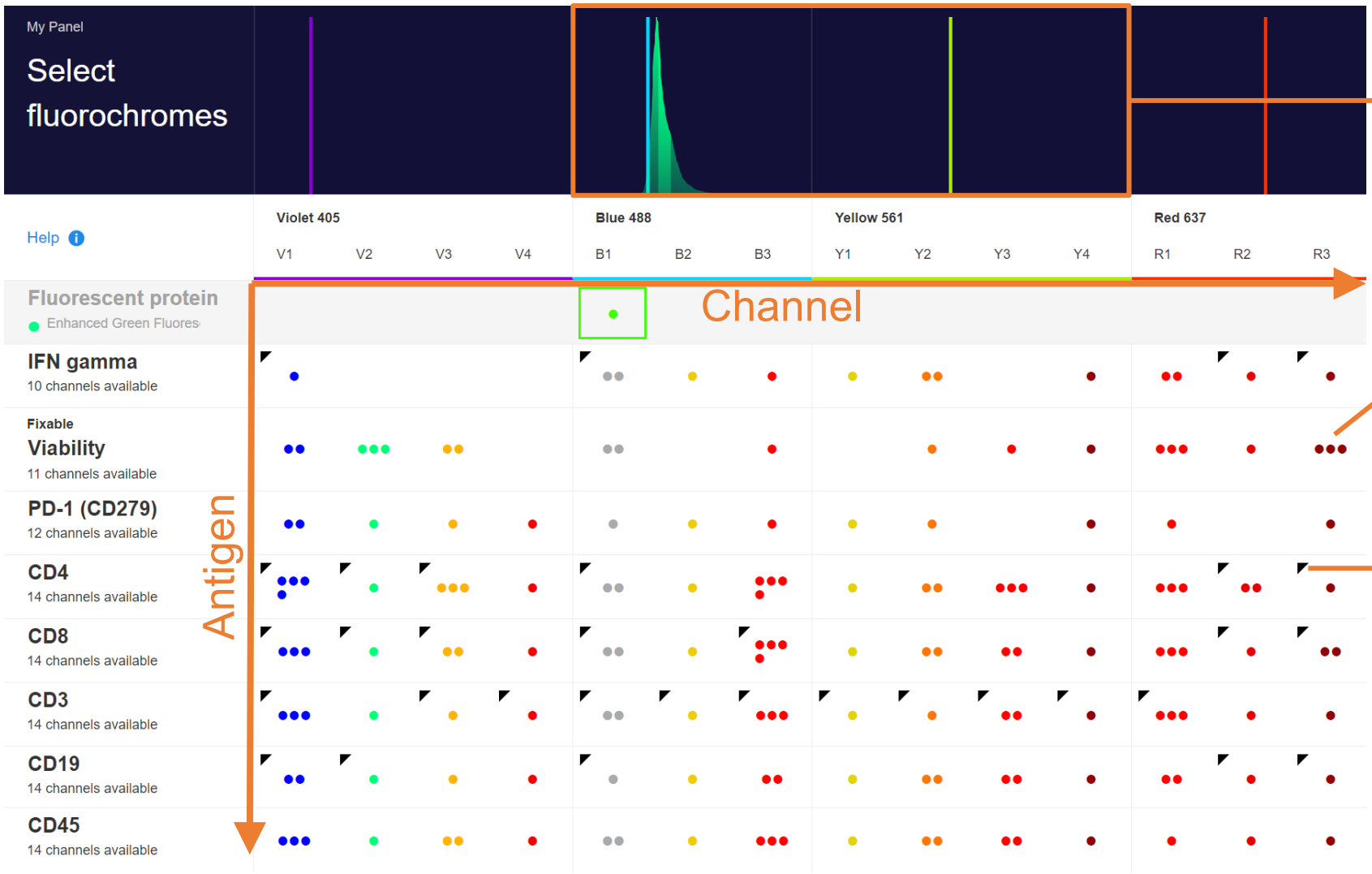






https://www.thermofisher.com/order/panel-builder/#!/

Flow Cytometry Panel Builder



SpectraViewer

Spot
Fluorochromes for each channel

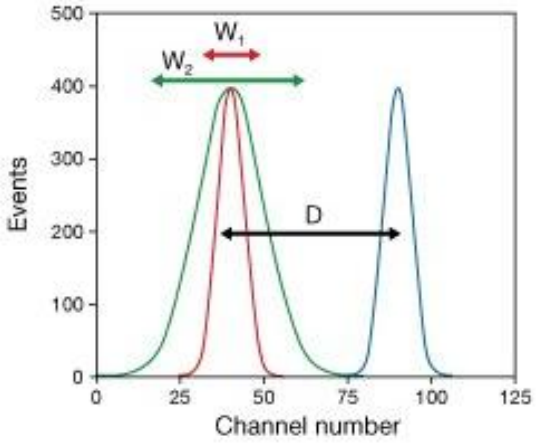
Flag
Recommended channel

- Expression level of marker
- Brightness of fluorochrome

Antibody Titration

- Use antibodies at the **right concentration**
- Antibody **batch dependent**
- **Reduce background** and increase signal to noise ratio
- **Reduce cost** of antibodies

1. Setup target cell type, protocol, and cytometer configurations
2. Label cells with serial dilution of antibodies
3. Examine **Stain Index** to find optimized antibody concentration



Stain Index = D/W

Where:

D is the difference between positive and negative peak medians.

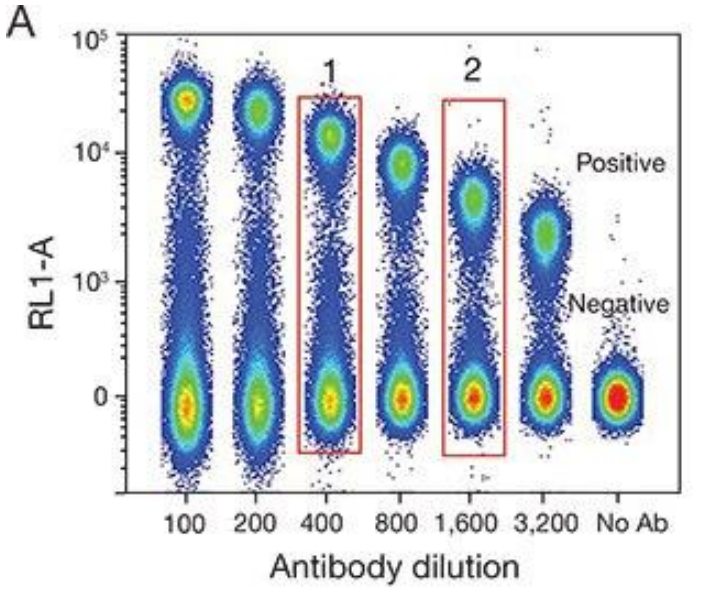
W is the spread of the negative peak and is equal to 2 x rSD.

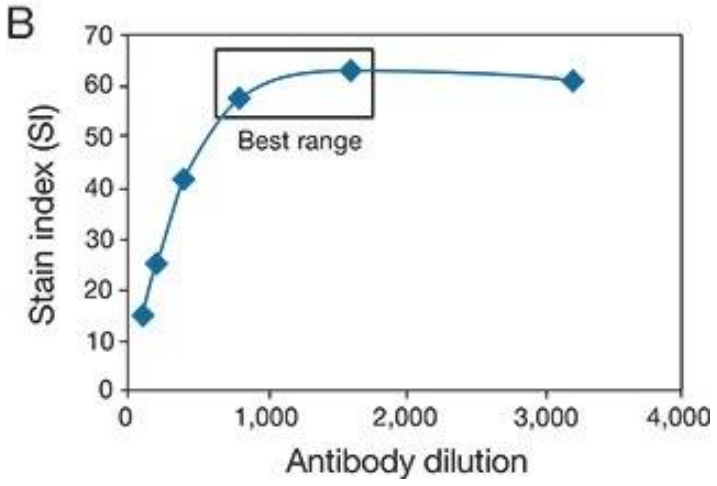
rSD is the robust standard deviation.

Signal-to-noise ratio = MFI (positive cells) / MFI (negative cells)

Where:

MFI is the median fluorescence intensity.



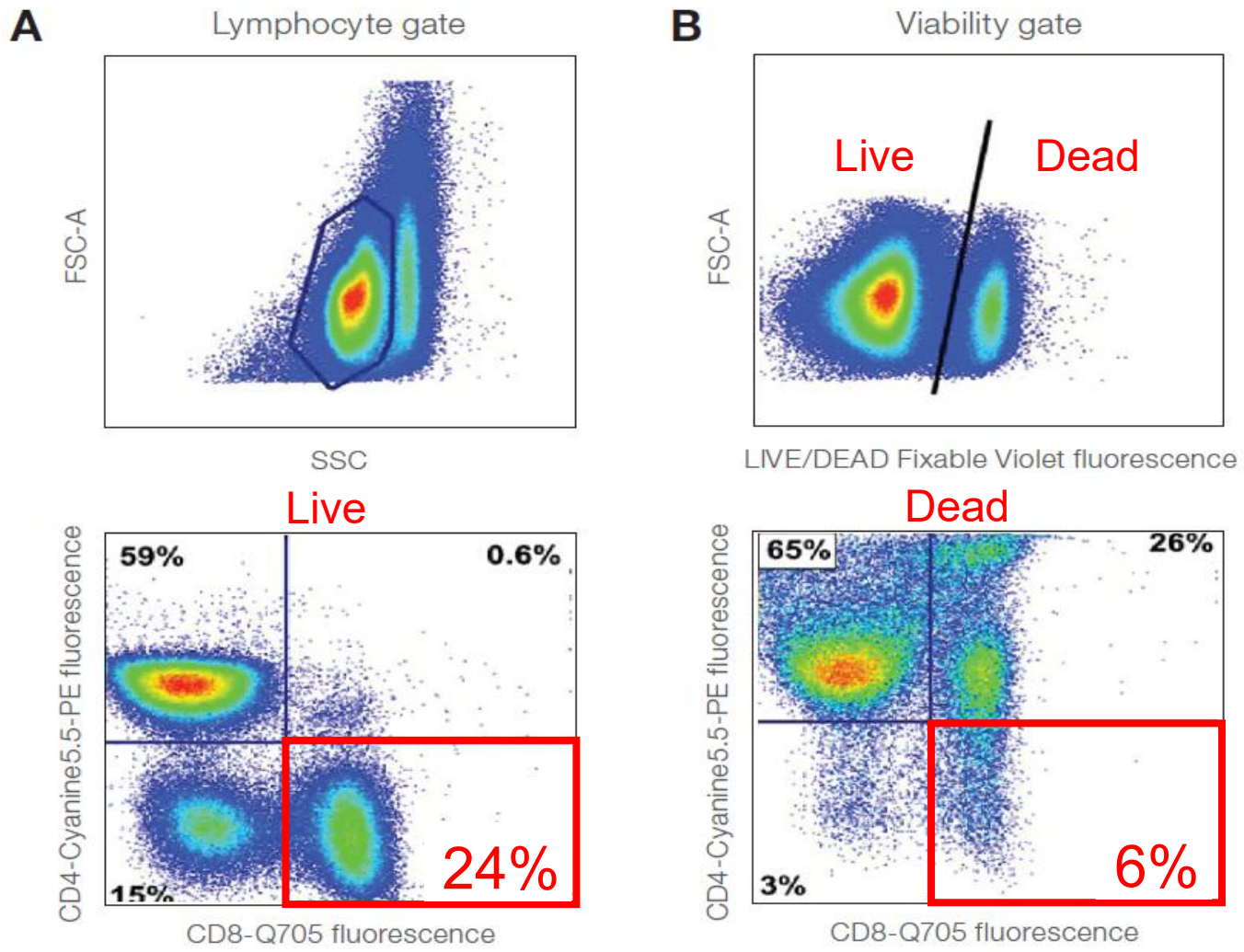


https://www.thermofisher.com/tw/zt/home/references/newsletters-and-journals/bioprobes-journal-of-cell-biology-applications/bioprobes-79/best-practices-multiparametric-flow-cytometry.html

Put Viability Dye into Consideration - Dead Cell Exclusion

DKSH

Dead cells adds significant staining *artifacts* to analysis.



Perfetto et al. (2006) J Immunol Methods 313:199

Flow Cytometry Controls

Single stained control for compensation

Negative control: 判斷訊號背景值

1. Unstained control
2. Isotype control
3. Fluorescence minus one (FMO) control for multicolor panel
4. FMO + isotype control

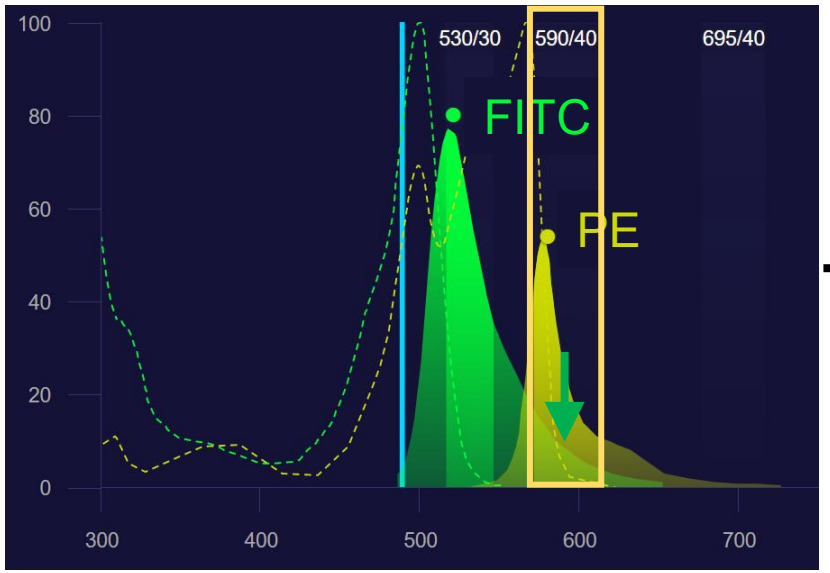
Positive control: 確認實驗流程正確，可以得到預期訊號

Flow Cytometry Controls – Single Stained for Compensatio

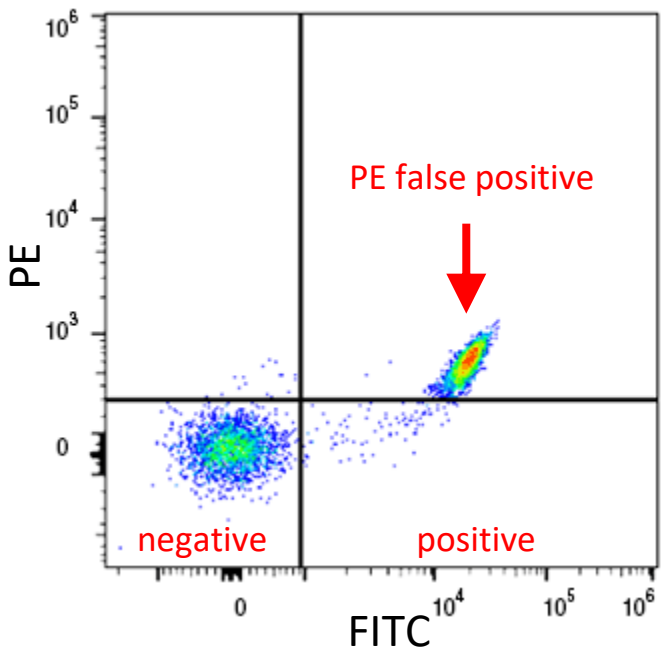
DKSH

Compensation is the mathematical method used to correct the emission overlap from one ***fluorophore*** into the emission channel of another ***fluorophore***.

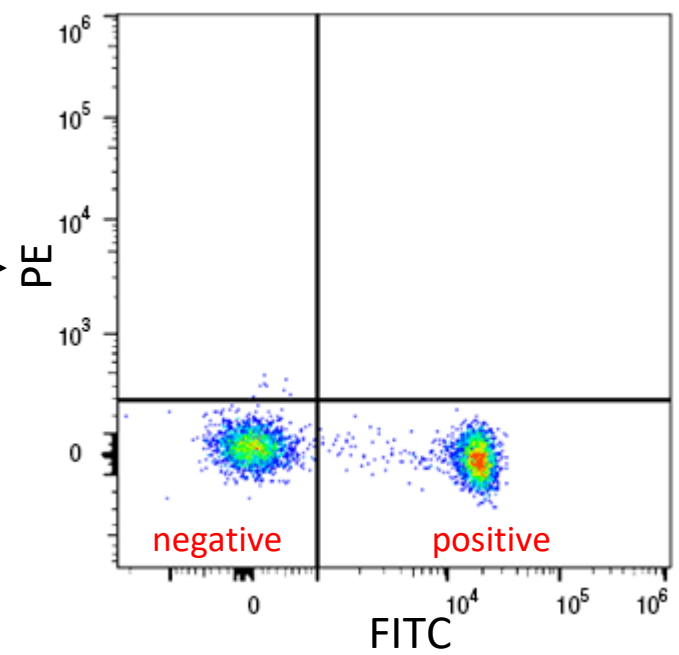
Spillover



FITC single stain



Post compensation



When to Use Compensation Beads

Cat.#01-3333-41
UltraComp eBeads™ Plus Compensation Beads

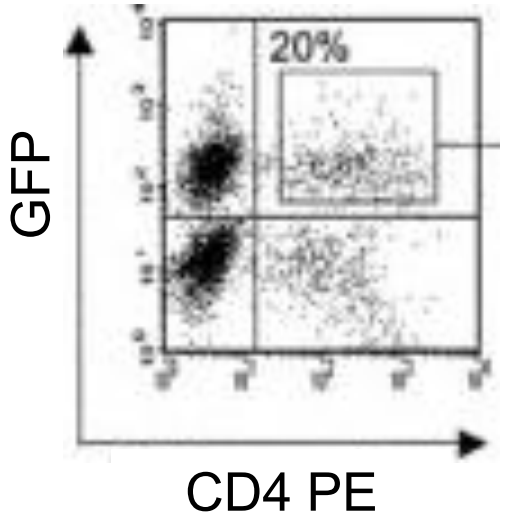
Intracellular fluorescence

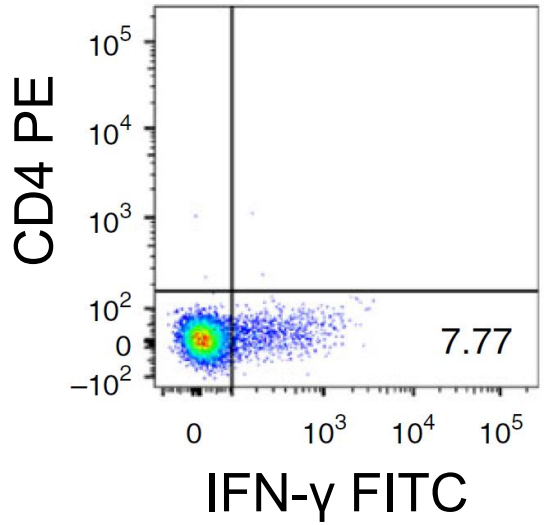
Poorly expressed markers

Limited amount of sample

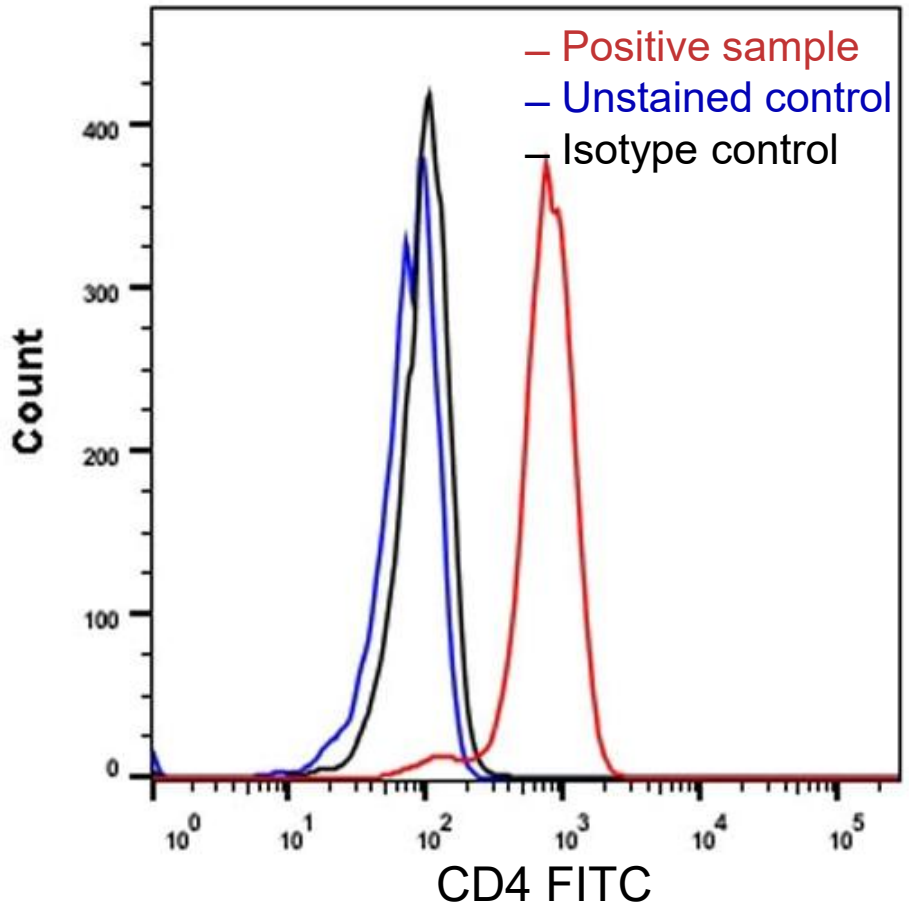
Large multicolor panel

Standardization





Flow Cytometry Controls – Unstained and Isotype Control

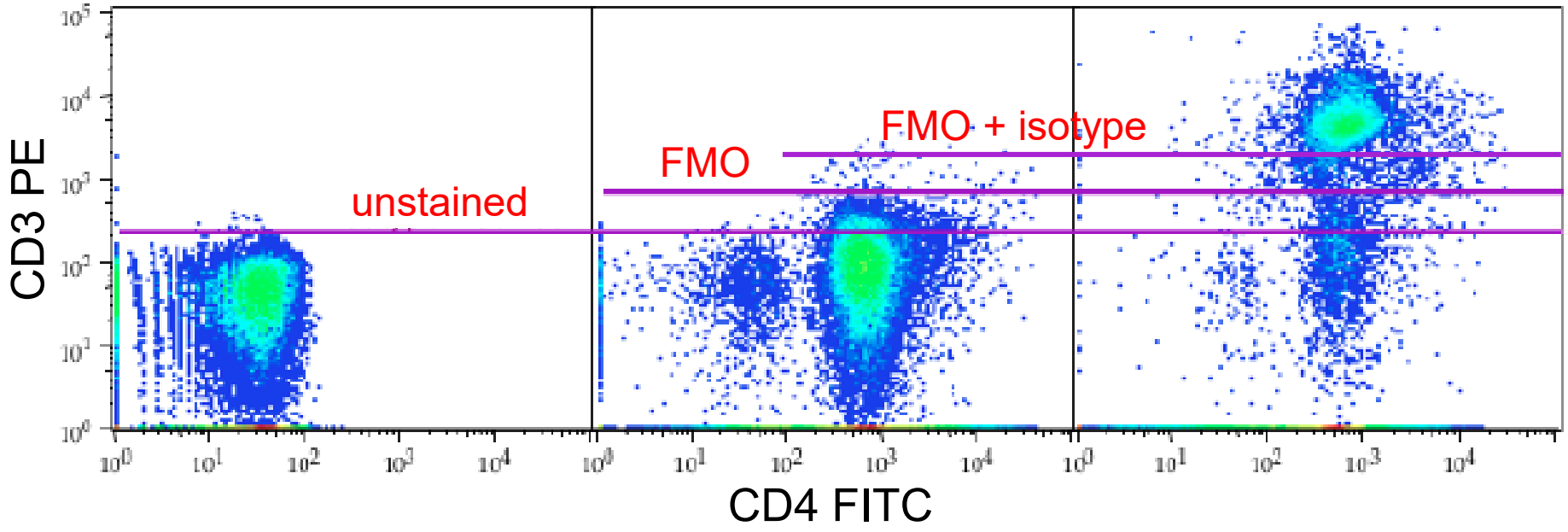


- CD4 Monoclonal Antibody (RM4-5), FITC
  Expression System: Rat IgG2a kappa
- Recommended Isotype Control:
  Rat IgG2a kappa Isotype Control (eBR2a), FITC

Isotype control for non-specific binding background

Flow Cytometry Controls – FMO control

	Unstained Control	FMO control	Fully Stained
FITC	-	CD4	CD4
PE	-	- + isotype Ab	CD3
PerCP	-	CD8	CD8
APC	-	CD45	CD45



Flow Cytometry Workflows

- Panel Design – Flow Cytometry Panel Builder
- Antibody Titration
- Controls

實驗規劃

- Sample preparation
- Cell staining

樣本製備與染色

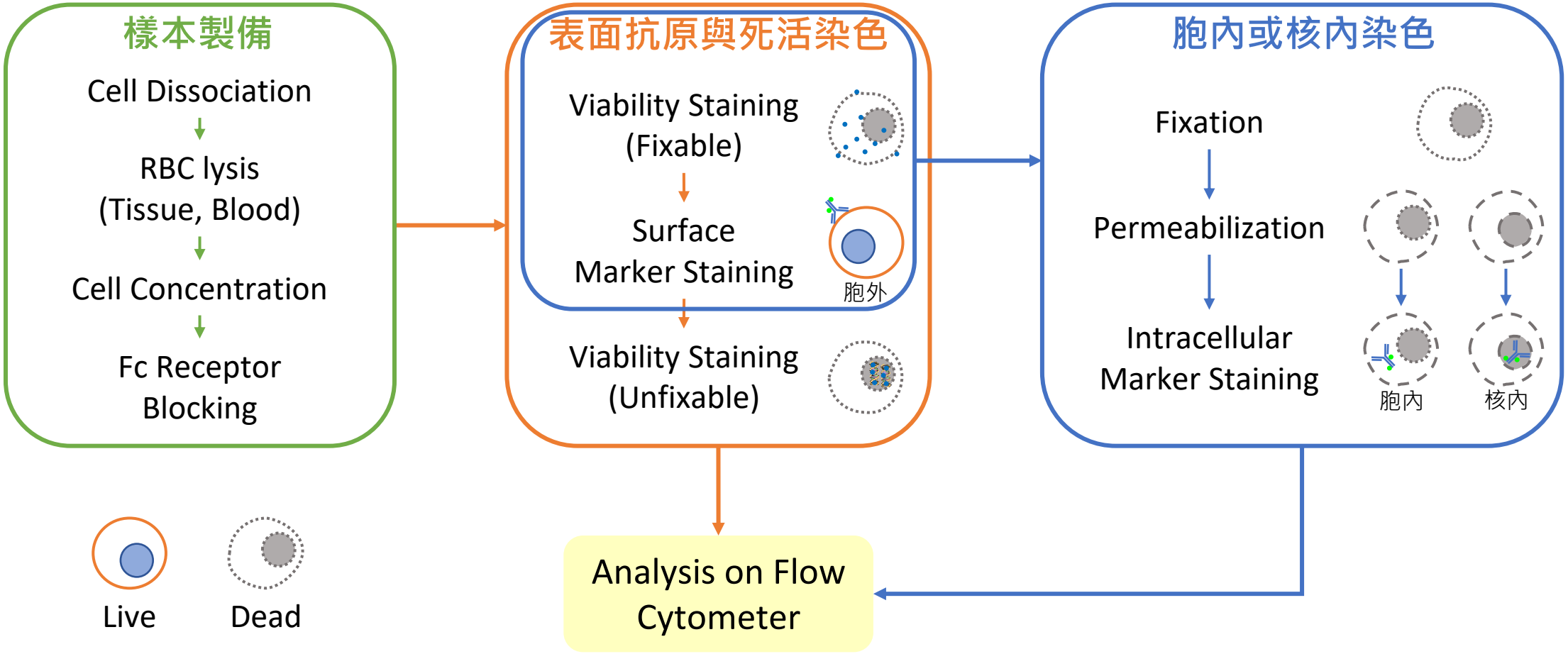
- Flow Cytometer Start Up
- Select **Channels**
- Setup **Workspace** (***Cell > Singlet***, gating strategies, controls for threshold setup)
- Setup **Collection Panel**
- Setup **PMT** (signal min. from unstained control, signal max. from positive sample)
- Setup **Compensation** (single stained control for all fluorophore)
- Analyze Samples
- Data Export (FCS 3.0 or higher)
- Flow Cytometer Shutdown

上樣分析流程

- Data Analysis

數據分析

Immunophenotyping with Flow Cytometry



Cell Preparation for Flow Cytometry Protocols

- Cell preparation for flow cytometry protocols
  - Protocol A: Tissue Culture Cells
  - Protocol B: Lymphoid Tissue
  - Protocol C: Non-lymphoid Tissue
  - Protocol D: Isolation of PBMC from Whole blood

- Worthington Tissue Dissociation Guide
  The Worthington Tissue Dissociation Guide provides a useful summary and guide of the various methods that can be used for tissue dissociation.

Cell Staining Protocols

- Viability Dye Staining
  - Protocol A: Staining Dead Cells with Propidium Iodide or 7-amino-actinomycin D (7-AAD)
  - Protocol B: Staining Live Cells with Calcein Dyes
  - Protocol C: Staining Dead Cells with Fixable Viability Dyes (FVD)

- Staining cell surface targets protocols
  - Protocol A: Cell Suspensions
  - Protocol B: Human Lysed Whole Blood

- Staining Intracellular Antigens protocols
  - Protocol A: Two-step protocol: intracellular (cytoplasmic) proteins
  - Protocol B: One-step protocol: intracellular (nuclear) proteins
  - Protocol C: Two-step protocol for Fixation/Methanol

Flow Cytometry Workflows

- Panel Design – Flow Cytometry Panel Builder
- Antibody Titration
- Controls

實驗規劃

- Sample preparation
- Cell staining

樣本製備與染色

- Flow Cytometer Start Up
- Select **Channels**
- Setup **Workspace** (***Cell > Singlet***, gating strategies, controls for threshold setup)
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- Setup **PMT** (signal min. from unstained control, signal max. from positive sample)
- Setup **Compensation** (single stained control for all fluorophore)
- Analyze Samples
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- Flow Cytometer Shutdown

上樣分析流程

- Data Analysis

數據分析

Q & A

Attune NxT上樣分析流程

1. 檢查機器外觀(Fluid bottles and connections, Syringe, SIP)，緩衝液是否充足，廢液是否過多。
2. 開啟Attune NxT與電腦電源。
3. 啟動Attune NxT分析程式，登入使用者帳號 (operator: 執行Performance Test)。
4. 執行**Startup** (約5分鐘)。

5. 設定**Experiment**
6. 勾選**Channels**，以及欲觀察的A，H，W數值。
7. 設定**Workspace**: *Cell* (FSC-A, SSC-A) > *Singlet* (SSC-A, SSC-H) > *Chart for markers*。
8. 設定**Collection Panel**: 吸取樣本體積，分析流速，數據蒐集目標
9. 調整**PMT voltage**: 以unstained樣本觀察各channel背景值，以正式染色樣本觀察各channel最大值，調整各channel PMT voltage。
10. 調整**Compensation**: 使用大於一種螢光顏色時，上樣單染樣本以利軟體進行自動Compensation。
11. 依序上樣: 其他controls以及正式染色樣本。
12. 輸出實驗結果: atx原始數據檔案，FCS3.1檔案，excel檔案，與PDF報告。

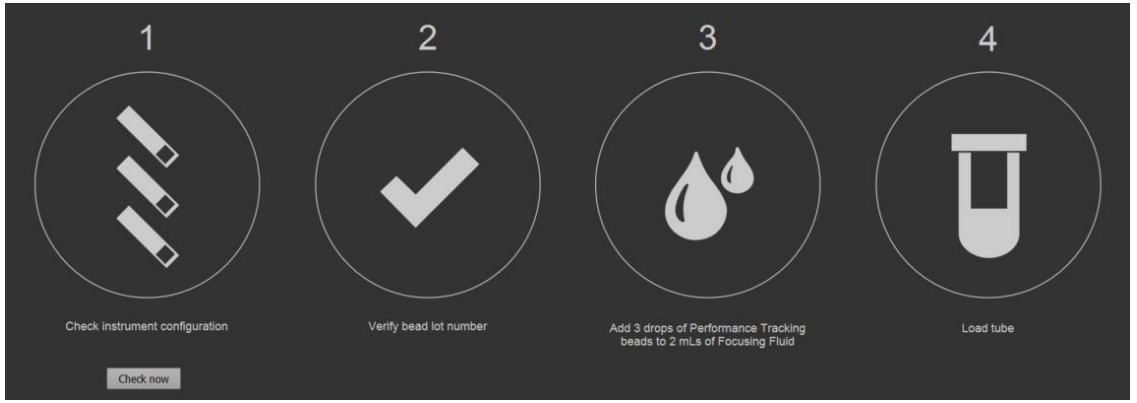
13. 執行**Shutdown** (約40分鐘)。
14. 關閉Attune NxT程式，關閉電腦與Attune NxT電源。
15. 清空廢液桶。

Main Manu


Attune™ Acoustic Focusing Cytometer
Performance Test
New Experiment
Import Experiment
New Experiment from Template
Log Out

Performance Test

Cat.#4449754
Attune™ Performance Tracking Beads



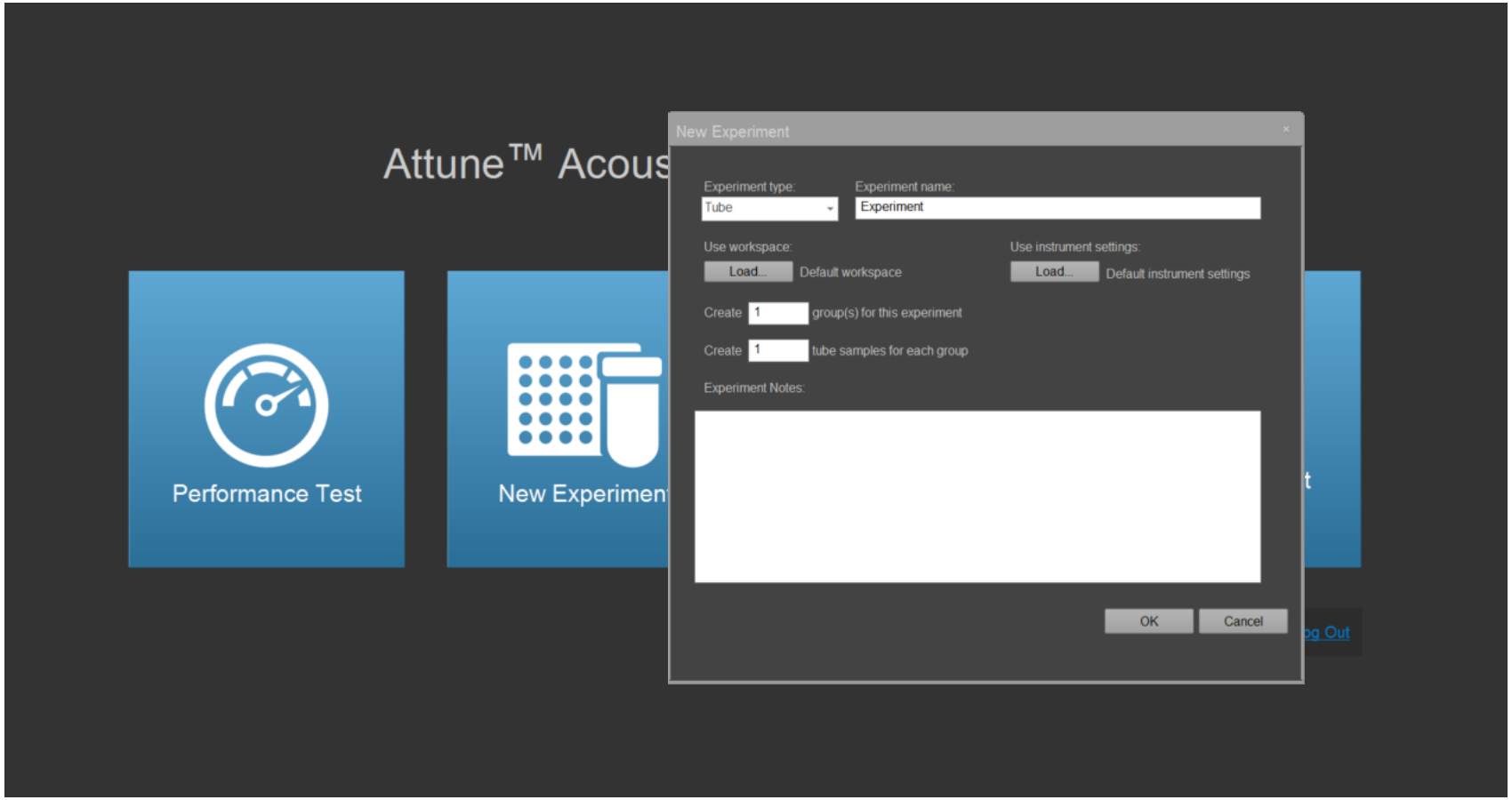
Performance Test Results

Performance test successful

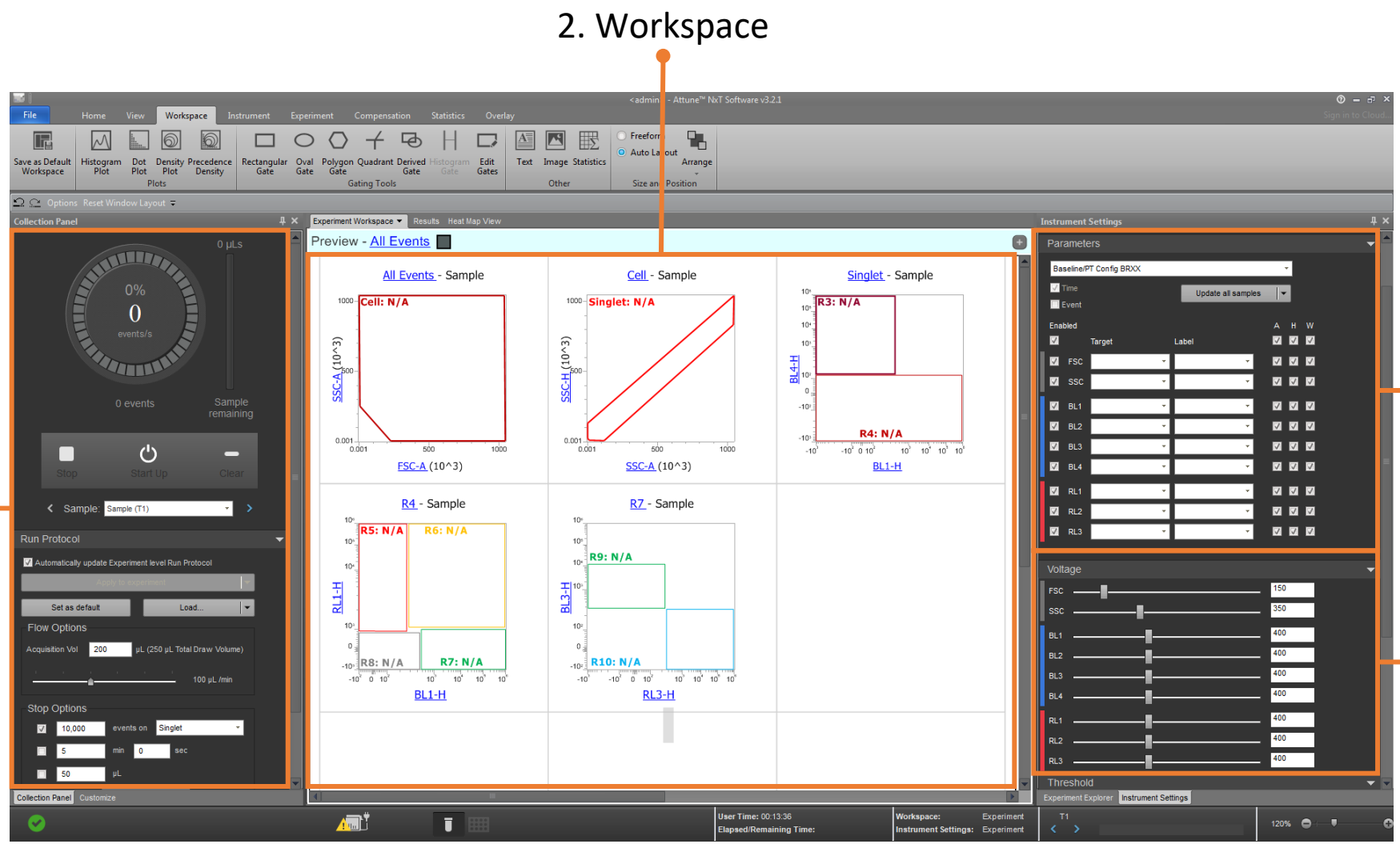
Baseline 1759476 - 4/3/2017 6/8/2017 6:41:55 PM

Channel	PMTV	Delta PMTV	Target MFI	MFI	Robust %CV	Qr	Background	Linearity	ASF	Laser Delay	Result
FSC	395	-4	300,000	302,628	2.23 %	0.000	0	0.000	1.06	1100	✓
SSC	360	0	300,000	290,538	2.94 %	0.000	0	0.000	1.06	1100	✓
BL1	417	-2	300,000	301,217	1.49 %	0.060	132	1.000	1.06	1100	✓
BL2	352	-3	300,000	305,144	1.17 %	0.054	177	1.000	1.06	1100	✓
BL3	437	-6	300,000	301,522	2.44 %	0.039	22	0.999	1.06	1100	✓
RL1	380	-4	300,000	300,287	3.58 %	0.003	13	0.981	1.03	1415	✓
RL2	371	-3	300,000	306,141	3.54 %	0.000	43	0.947	1.03	1415	✓
RL3	392	-3	300,000	304,672	3.57 %	0.004	23	0.948	1.03	1415	✓
VL1	322	1	300,000	296,147	1.35 %	0.018	1350	0.999	1.08	655	✓
VL2	374	1	300,000	296,768	1.66 %	0.022	419	0.996	1.08	655	✓
VL3	374	-1	300,000	302,433	2.57 %	0.030	76	1.000	1.08	655	✓
VL4	423	-3	300,000	300,499	3.19 %	0.004	264	0.999	1.08	655	✓
YL1	375	-2	300,000	306,149	1.55 %	0.117	94	0.999	1.01	329	✓
YL2	402	0	300,000	294,123	2.81 %	0.072	27	1.000	1.01	329	✓
YL3	406	-2	300,000	296,638	4.00 %	0.008	147	0.996	1.01	329	✓
YL4	467	-2	300,000	299,562	4.51 %	0.003	306	0.993	1.01	329	✓

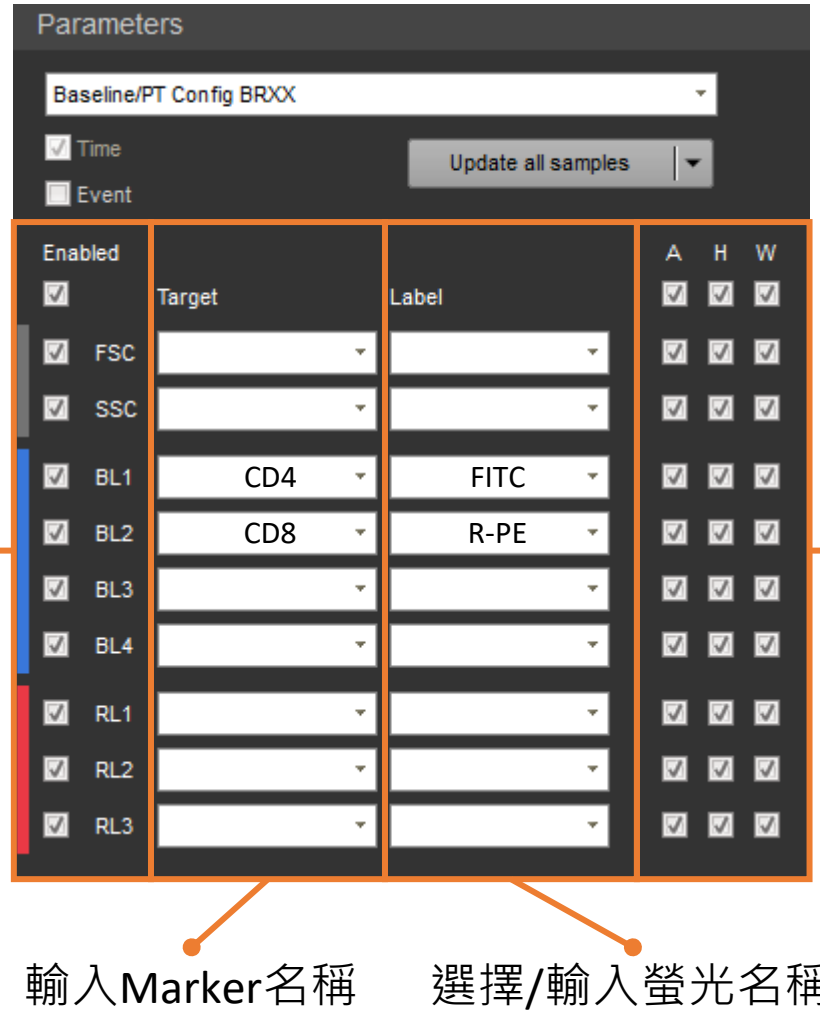
Main Manu – New Experiment



設定儀器參數


2. Workspace
1. Channel
3. Collection Panel
4. PMT V.
Collection Panel
Experiment Workspace
Results
Heat Map View
Instrument Settings
Preview - All Events
All Events - Sample
Cell - Sample
Singlet - Sample
R4 - Sample
R7 - Sample
Parameters
Voltage
Threshold
Run Protocol
Flow Options
Stop Options

設定儀器參數 – 1. Channels



勾選預計觀察的Channels

勾選各Channel預計收集的數值

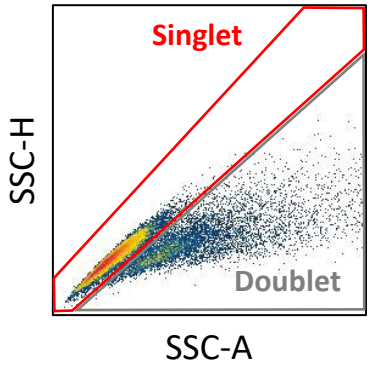
輸入Marker名稱

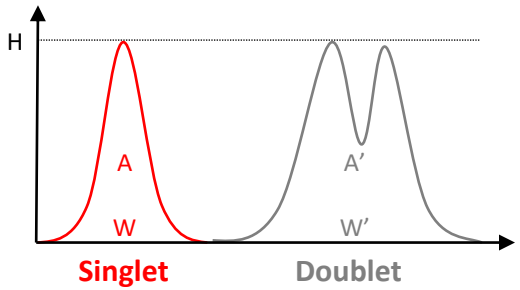
選擇/輸入螢光名稱

設定儀器參數 – 2. Workspace

最基本的兩個圖:

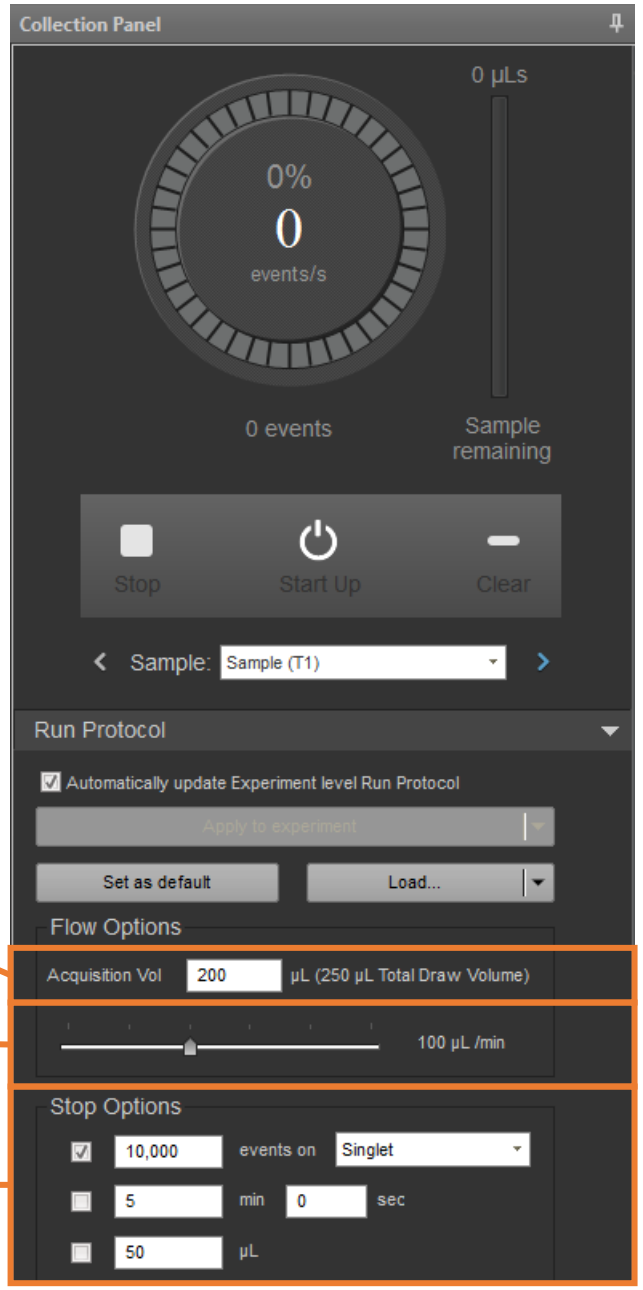
1. **圈選細胞位置**: FSC-A vs SSC-A
2. **圈選單顆細胞**: SSC-A vs SSC-H





https://expert.cheekyscientist.com/6-flow-cytometry-gating-tips-that-most-scientists-forget/

設定儀器參數 – 3. Collection Panel

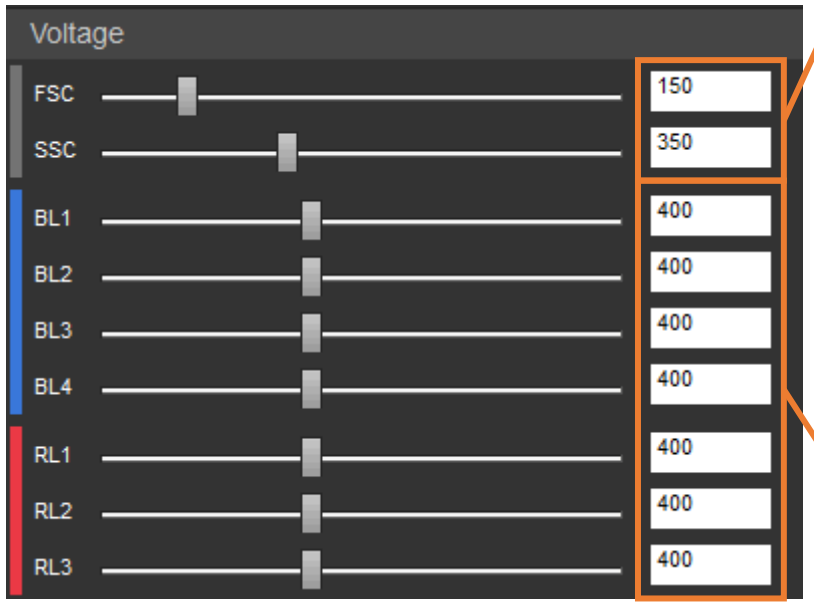


吸取樣本體積
(確認細胞足夠達到蒐集目標)

分析流速

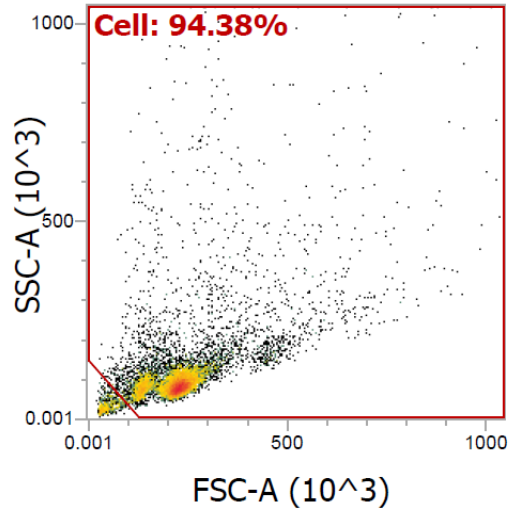
數據蒐集目標

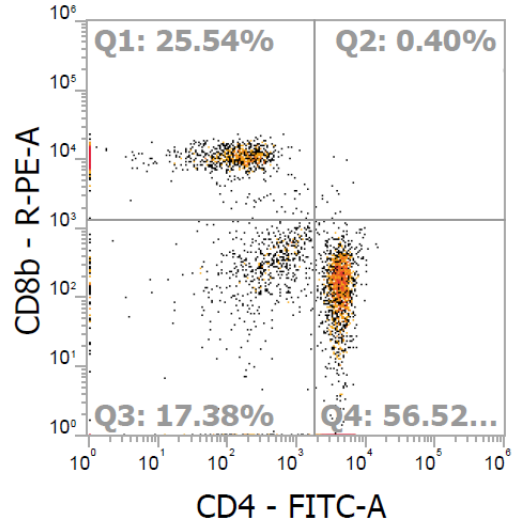
設定儀器參數 – 4. PMT Voltage



一般哺乳類動物細胞(~ 10 µm)建議從FSC (150)以及SSC (350)開始測試，再根據結果調整以利觀察主要群體

以unstained樣本調整訊號最小值
以正式染色樣本調整訊號最大值

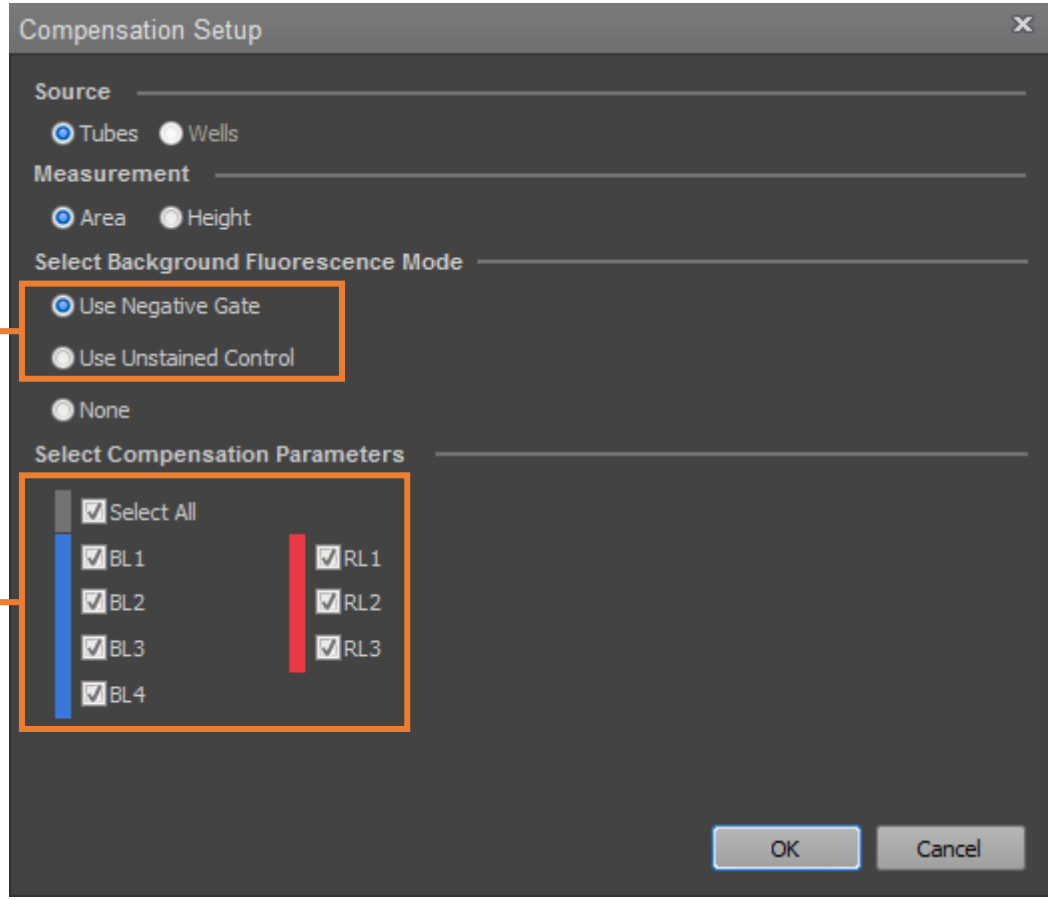




設定儀器參數 – Compensation

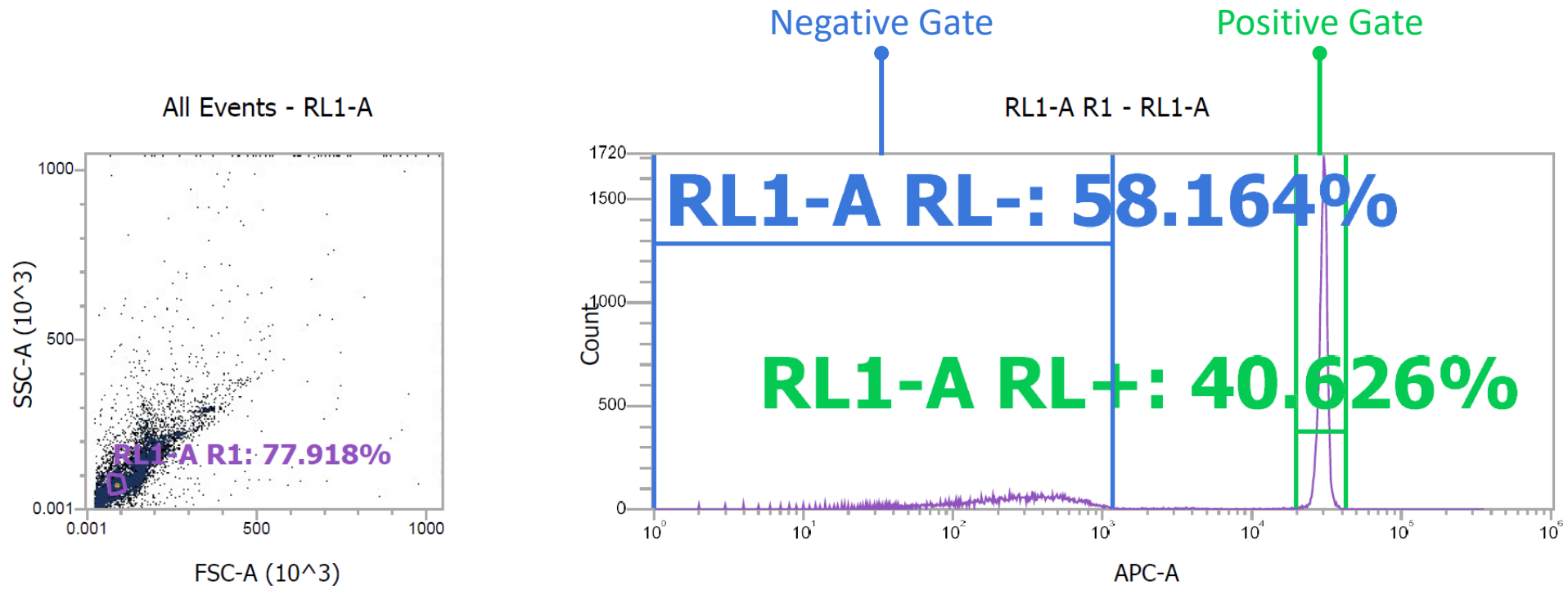
選擇螢光背景值的判斷模式

勾選需要進行compensation的channels



Note: Cells for negative and positive signal must have the *same level of background fluorescence*.

設定儀器參數 – Compensation: Use Negative Gate

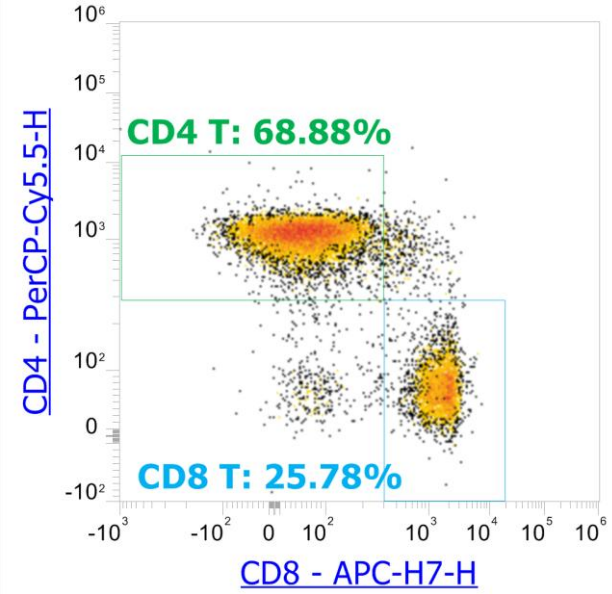


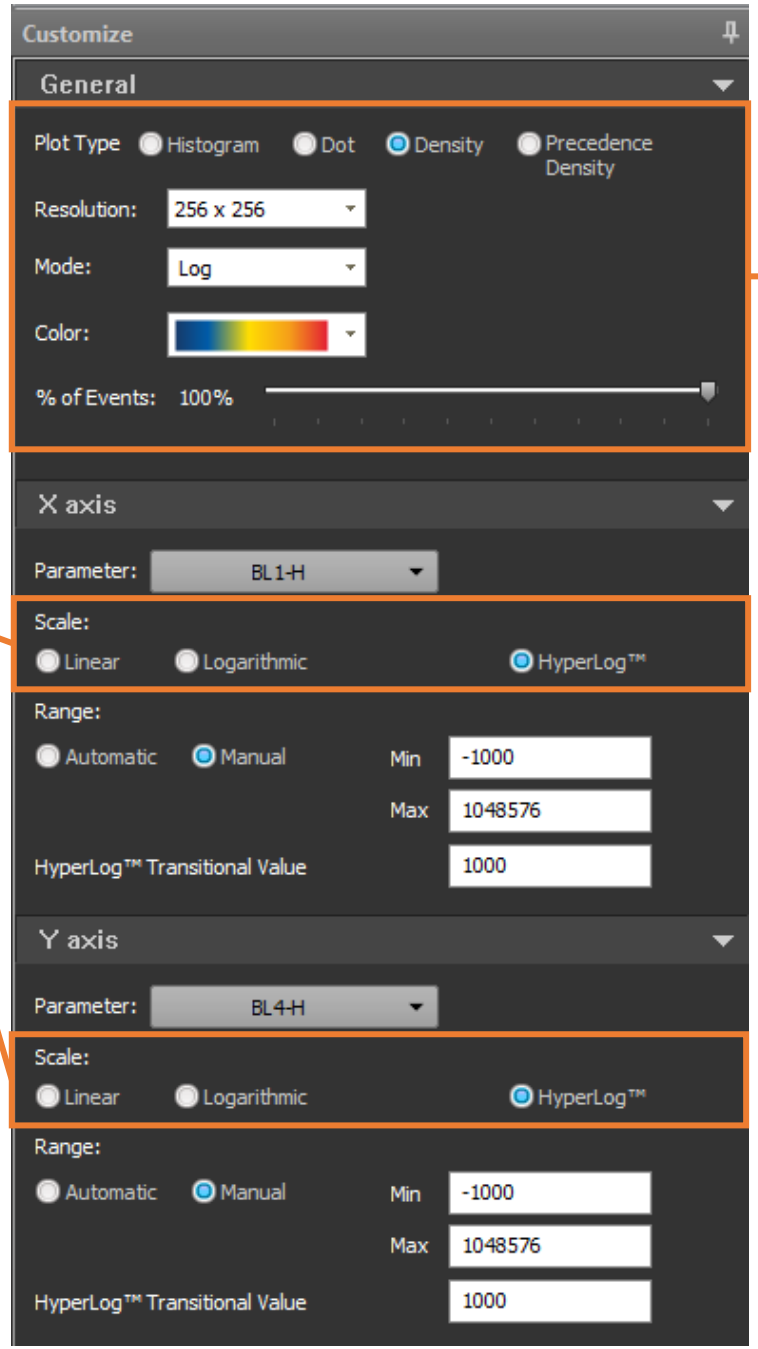
記錄Compensation controls之前確認:

1. 已調整好各channel的PMT voltage
2. 已設定好R1，Negative，以及Positive Gates

設定儀器參數 – Customize

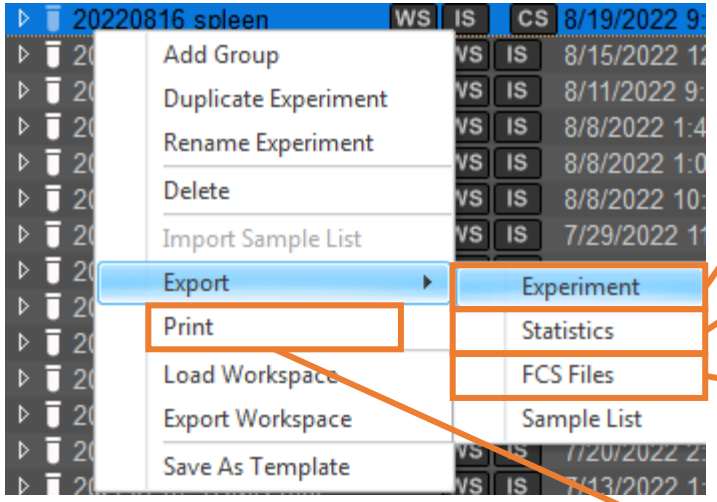
多色螢光實驗進行compensation後，數值軸建議使用**HyperLog**，以正確呈現過小的數值





Workspace Chart類型選擇與參數調整

輸出實驗結果



Experiment (*.atx): 完整原始實驗數據檔案

Statistics (*.csv): 可使用excel開啟的數據檔案

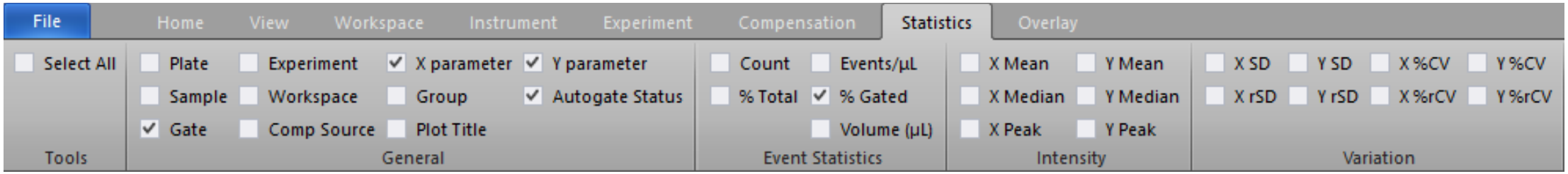
FSC Files (*.fcs): Flow Cytometry通用數據檔案，可使用第三方分析軟體開啟

Print (*.pdf): Compensation與Workspace圖檔與統計表格

Data Analysis

調整分析流程，留下高品質數據 (單顆細胞，排除死細胞)
規劃良好的controls以協助分析結果
決定統計數值的呈現方式 (影響數據蒐集目標的設定)

- % Total
- % Gated
- Events/µL (細胞濃度)
- MFI (mean fluorescence intensity)



清洗功能與錯誤排除

Function	狀況
Rinse	清洗樣本管路
Sanitize SIP	清洗樣本管路與上樣針SIP 不同使用者之間避免樣本互相干擾 使用易沾黏管路的樣本
Deep Clean	清洗樣本管路與flow cell
Debubble	系統偵測到氣泡，清除樣本管路與flow cell氣泡
Unclog	無訊號，樣本管路可能阻塞管路時
Decontamination	儀器管理員進行定期保養

狀況無法排除時，問題回傳:

1. System log
2. Print screen

操作注意事項

1. Attune NxT可分析的最大細胞尺寸約為**50 µm**，因此樣品必須先過濾去除細胞塊或組織塊，例如以40或70 µm Cell Strainer 進行過濾。
2. 樣品建議調整濃度為 **1 x 10^6 cell/ml**。細胞濃度過高，易造成管路阻塞；過稀則增加上機時間。上樣體積建議最少為 **500 uL**。
3. 分析實驗檢體前，可透過空跑緩衝液(例如PBS)以確認儀器管路的乾淨程度；若出現過多雜質訊號時可先透過清潔功能清洗管路。
4. 上機過程中若儀器出現錯誤訊息，或運作時發出明顯的異常聲音，請聯絡儀器管理員協助確認問題。

Attune NxT手冊

Attune™ Cytometric Software

USER GUIDE

For data acquisition and analysis using the Attune™ NxT and Attune™ CytPix™ Flow Cytometers

Publication Number MAN0026553

Revision B.0

https://downloads.thermofisher.com/Attune_v6.0.1/MAN0026553-RevB-AttuneCytometricSW-UG-EN-27Apr2023.pdf

USER GUIDE

invitrogen

Attune™ NxT Acoustic Focusing Cytometer

Catalog Numbers A24858, A24859, A24860, A24861, A24862, A24863, A24864, A28993

Publication Number 100024235

Revision C.0

https://assets.thermofisher.com/TFS-Assets/LSG/manuals/100024235_AttuneNxT_HW_UG.pdf

其他工具

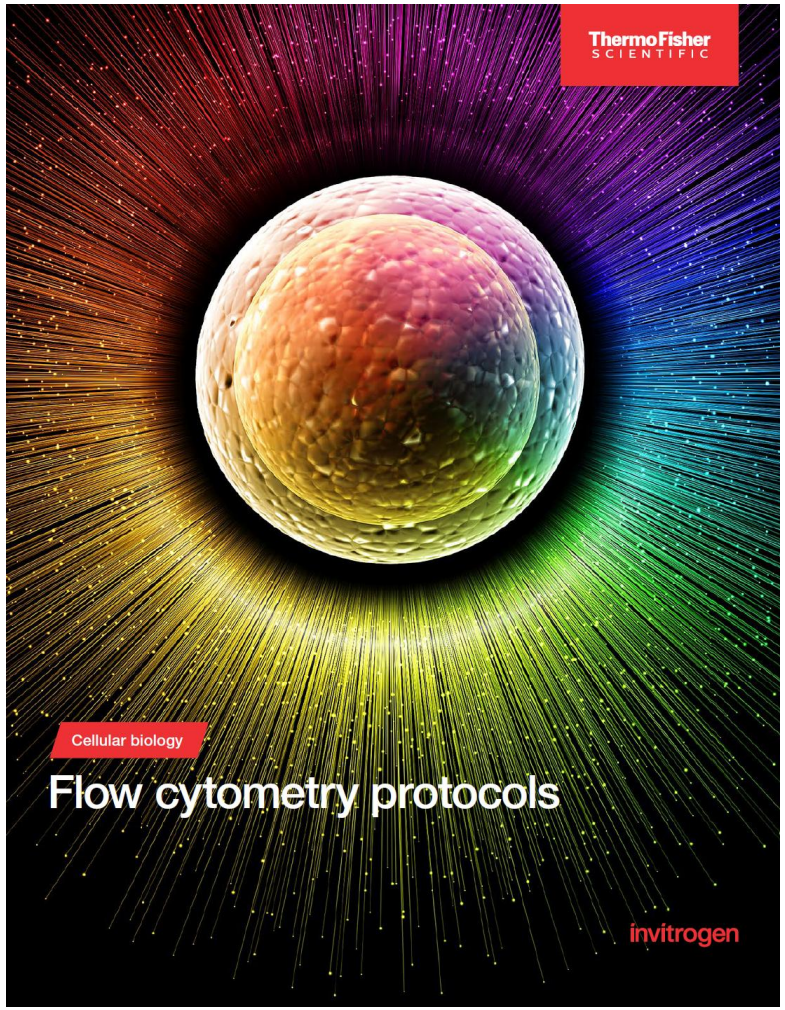
Human and mouse antigens

ThermoFisher SCIENTIFIC

https://assets.thermofisher.com/TFS-Assets/LSG/brochures/immune-cell-guide.pdf

其他工具

http://assets.thermofisher.com/TFS-Assets/BID/brochures/flow-cytometry-capabilities-guide-brochure.pdf



https://www.thermofisher.com/tw/zt/home/global/forms/flow-cytometry-protocols-handbook.html

https://www.taqkey.com/attunenxt-flow-cytometrt/

Thank you

edward.chang@dksh.com