TAQKEY SCIENCE | 20 YEARS ANNIVERSARY

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Flow Cytometry Applications with Attune NxT

20220908

Taqkey Science

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Before We Start…

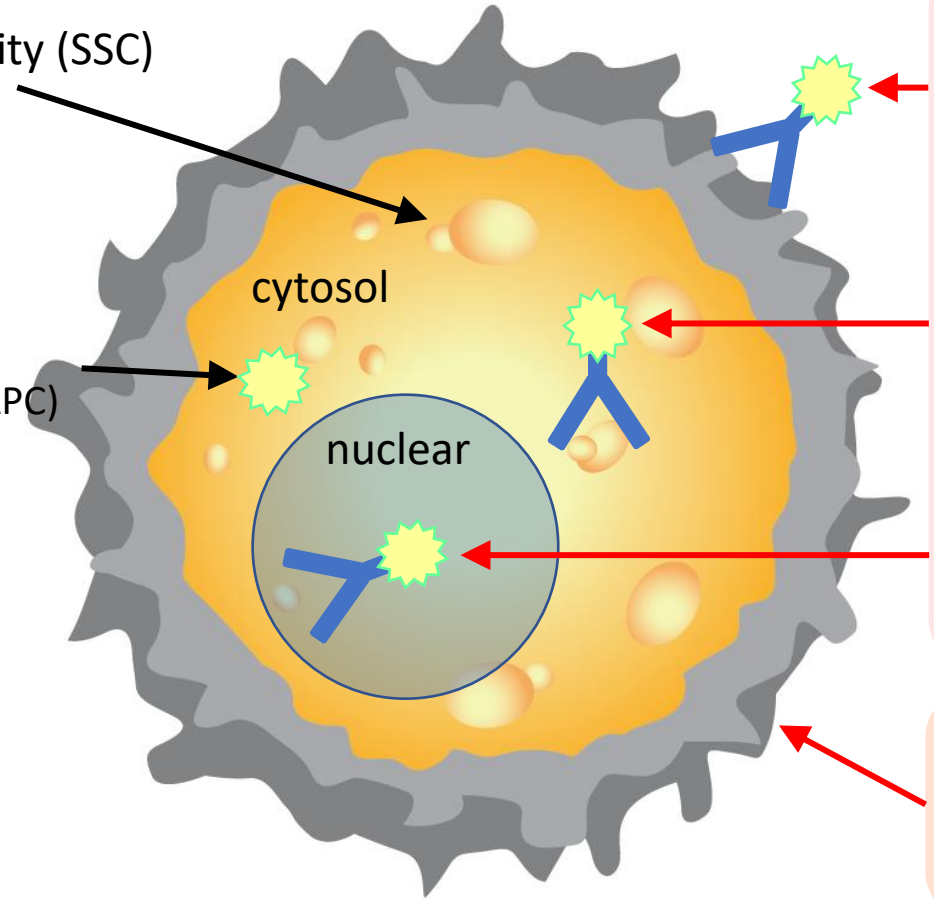
1. 從paper看到的flow cytometry實驗結果，如何在Attune NxT上分析?

2. 從paper看到的flow cytometry試劑或抗體，能否直接在Attune NxT上使用?

3. 我用的螢光試劑(抗體)，要用Attune NxT的哪個channel觀察訊號?

4. 如何從頭規劃一組在Attune NxT上使用的多色flow cytometry panel?

Cell Characteristics by Flow Cytometry



胞內螢光
(e.g. GFP, PE, APC)

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

免疫分型
Immunophenotyping

胞內染色
Cytosolic staining
(e.g. cytokines)

核內染色
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)

Attune NxT Configurations, NTOU

FSC: Forward scatter
SSC: Side scatter

Excitation Laser	Emission Filter (nm)	Channel	Common Fluorophores	Fluorescent Proteins/Compounds
Blue-488 nm	530/30	BL1	Alexa Fluor 488, FITC	eGFP, Emerald, eYFP
	574/26	BL2	Alexa Fluor 546, PE(**phycoerythrin**), Nile Red(N)	eYFP, mCitrine, Venus
	695/40	BL3	PE-Alexa Fluor 700, PE-Cy5.5, PerCP, PerCP-Cy5.5	***chlorophyll**
	780/60	BL4	PE-Cy7, PE-Alexa Fluor 750	
Red-637 nm	670/14	RL1	APC, Alexa Fluor 647	**phycocyanin**
	720/30	RL2	Alexa Fluor 680, Alexa Fluor 700, APC-Alexa Fluor 700	
	780/60	RL3	APC-Alexa Fluor 750, APC-Cy7	

Flow Cytometry Procedures

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

實驗規劃

- Sample preparation
- Cell staining
- 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

- Data Analysis

Principles of Panel Design

1. Identify ***markers*** of interest (literatures for gating strategy; Immune cell guide).

2. Know your flow cytometer (***channels***).

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.

Flow Cytometry Panel Builder

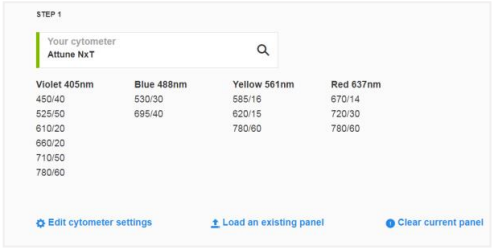
Step 1: 機器規格

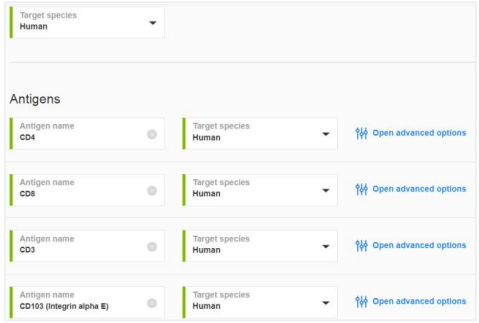
Step 2: 設定抗原

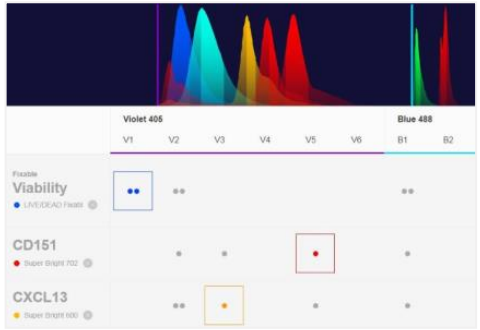
Step 3: 配置螢光

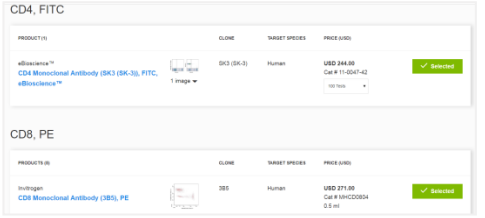
Step 4: 選擇產品

Step 5: 輸出規劃





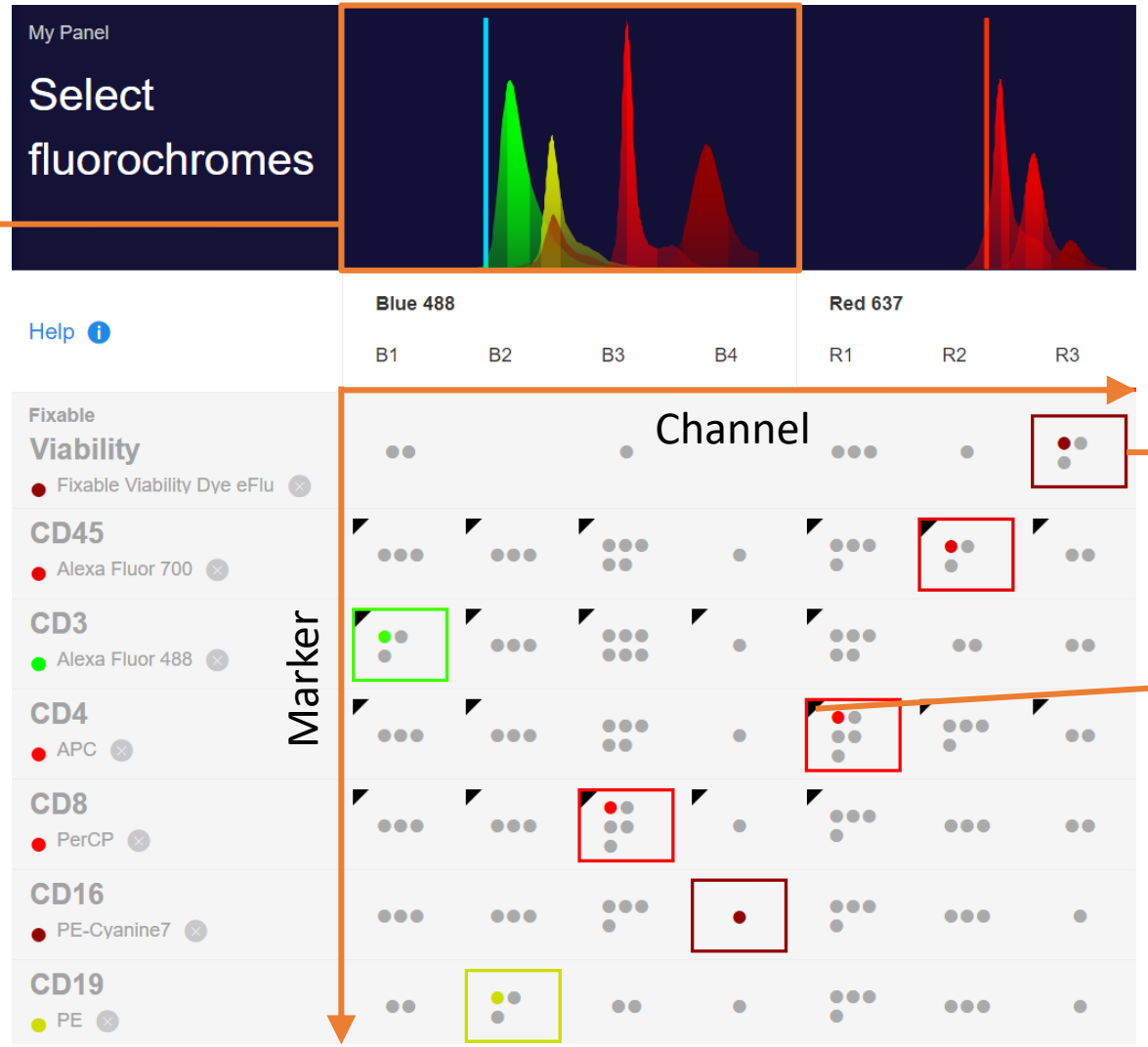




https://www.thermofisher.com/order/panel-builder/#!/

Flow Cytometry Panel Builder

SpectraViewer



圓點
每個channel可選擇的螢光染劑種類

黑旗
建議的螢光通道

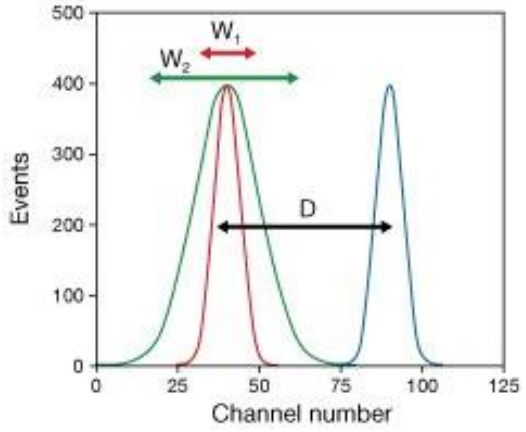
建議邏輯

- 蛋白質表現量
- 螢光染劑的亮度

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.

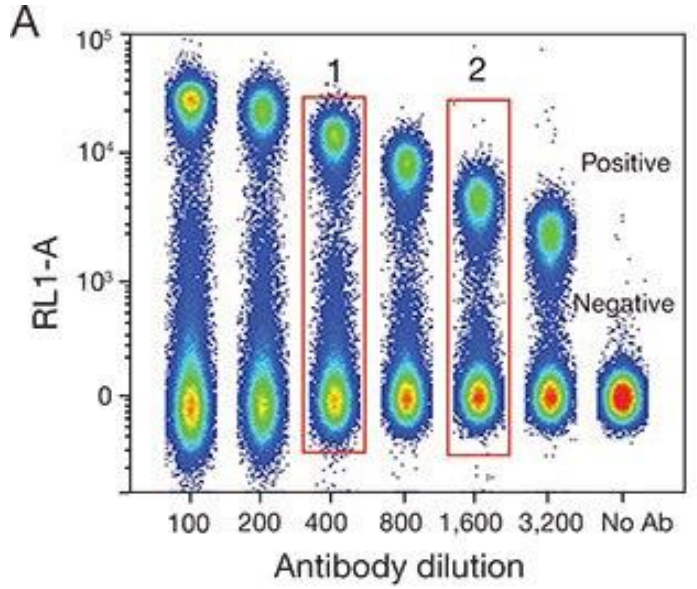
W is the spread of the negative peak and is equal to 2 x rSD.

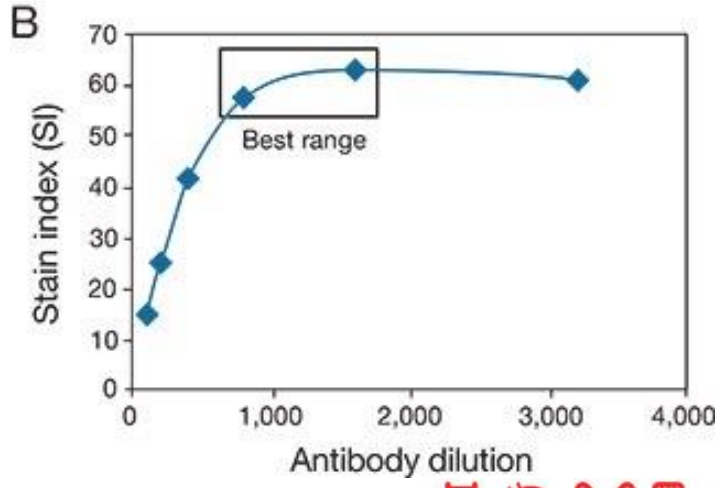
rSD is the robust standard deviation.

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

Where:

MFI is the median fluorescence intensity.

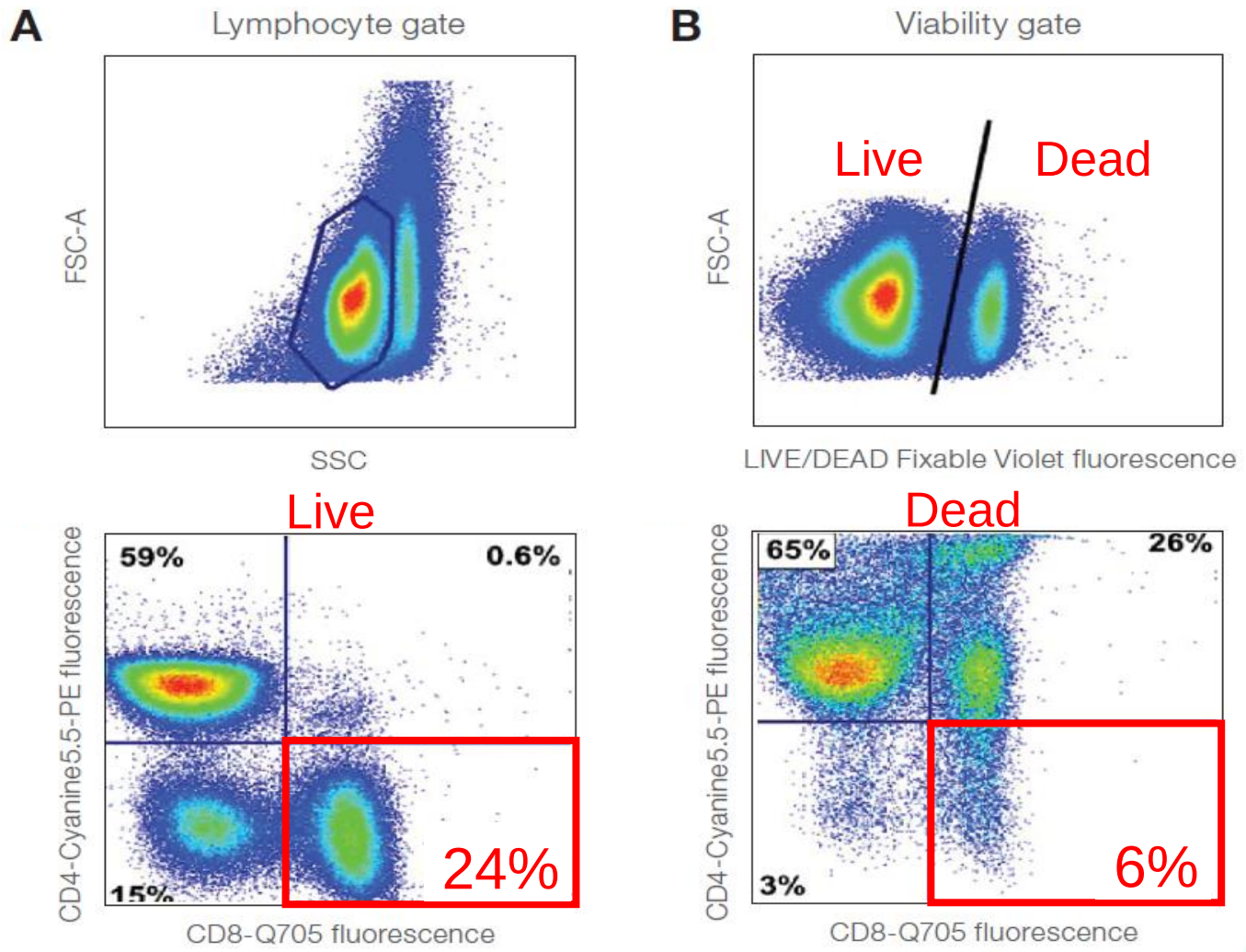




https://www.thermofisher.com/tw/zt/home/references/newsletters-and-journals/bioprobes-journal-of-cell-biology-applications/bioprobes-79/best-practices-multiparametric-flow-cytometry.html

Put Viability Dye into Consideration - Dead Cell Exclusion

Dead cells adds significant staining *artifacts* to analysis.



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

Flow Cytometry Controls

- **Single stained control:** 螢光校正 compensation

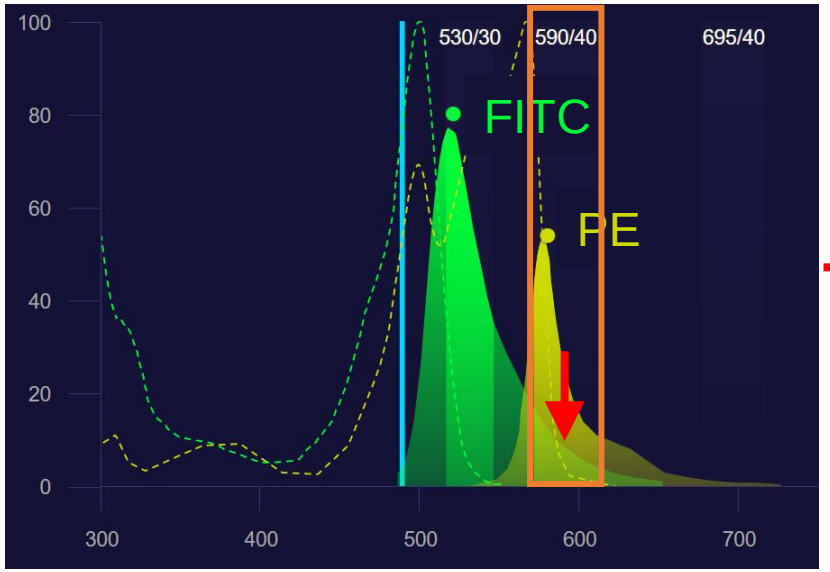
- **Negative control**: 判斷訊號背景值
  1. Unstained control
  2. Isotype control
  3. Fluorescence minus one (FMO) control for multicolor panel
  4. FMO + isotype control

- **Positive control**: 確認實驗流程正確，可以得到預期訊號

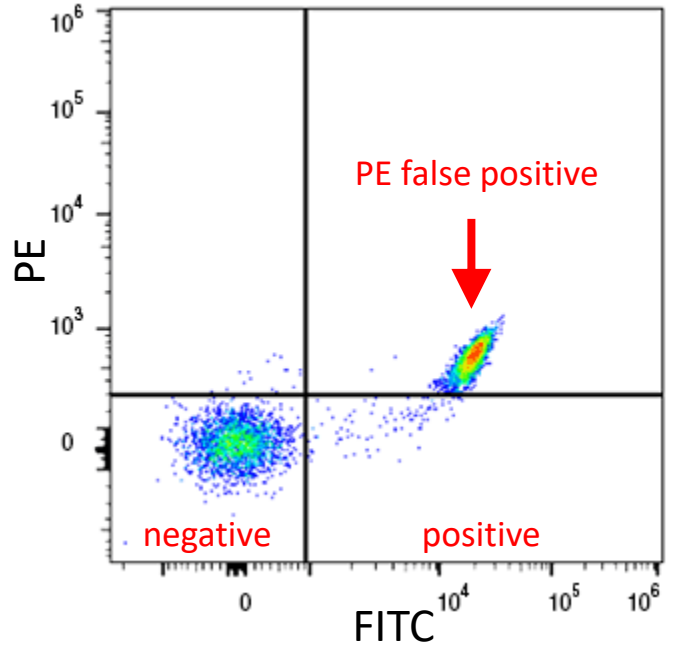
Flow Cytometry Controls – Single Stained for Compensation

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

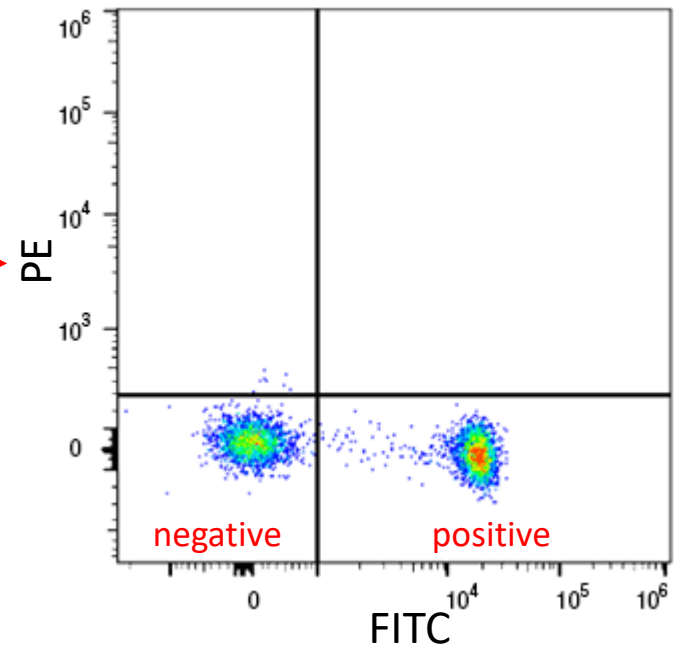
Spillover



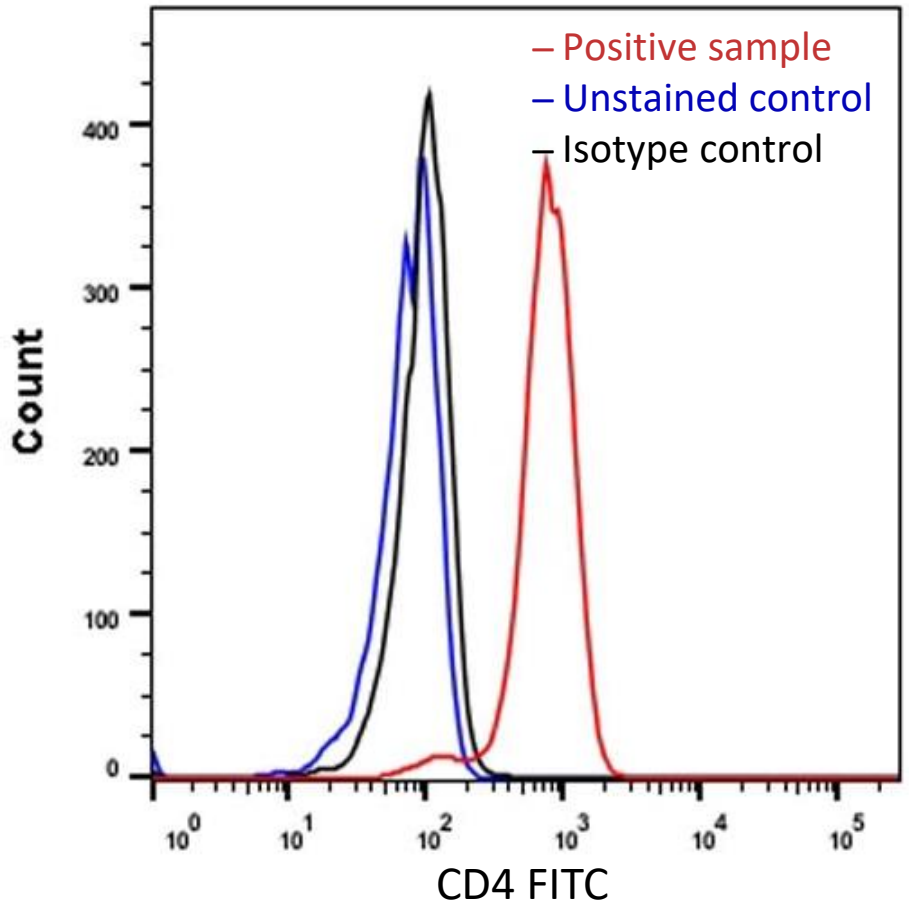
FITC single stain



Post compensation



Flow Cytometry Controls – Unstained and Isotype Control

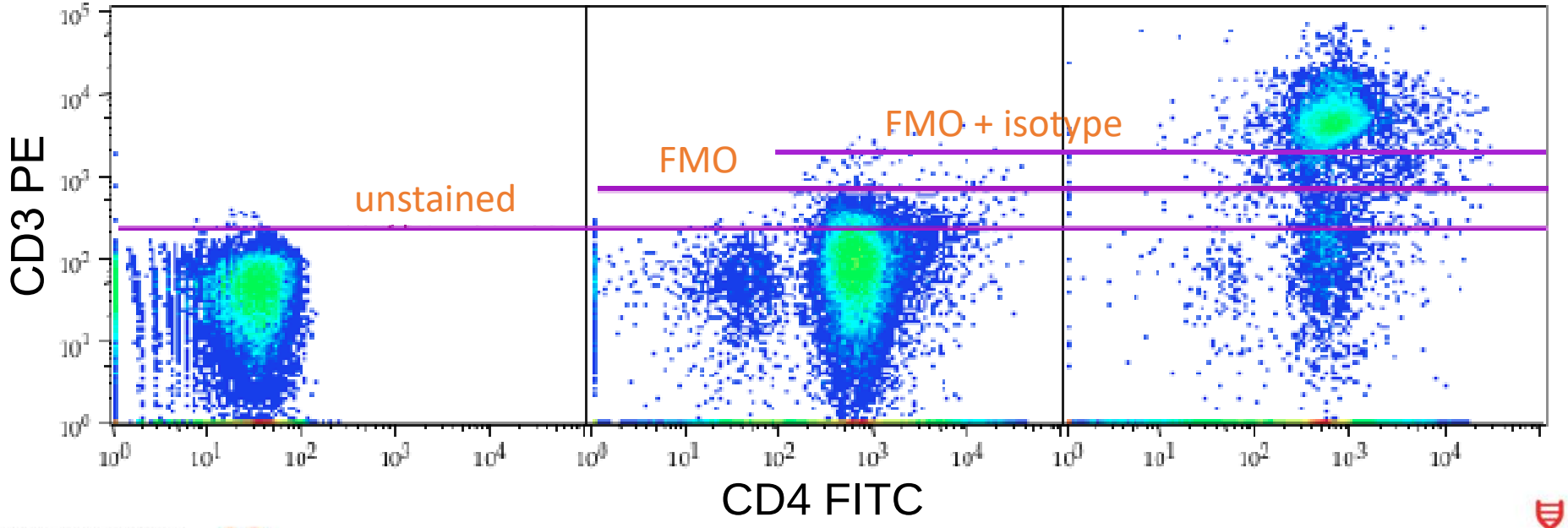


- CD4 Monoclonal Antibody (RM4-5), FITC
- Expression System
  Rat IgG2a kappa
- Recommended Isotype Control
  Rat IgG2a kappa Isotype Control (eBR2a), FITC

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 Procedures

- Panel Design – Flow Cytometry Panel Builder
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- Sample preparation
- Cell staining

樣本製備與染色

- Flow Cytometer Start Up
- Select **Channels**
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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 (http://www.worthington-biochem.com/tissuedissociation/) 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

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- Analyze Samples
- Data Export (FCS 3.0 or higher)
- Flow Cytometer Shutdown

上樣分析流程

- Data Analysis

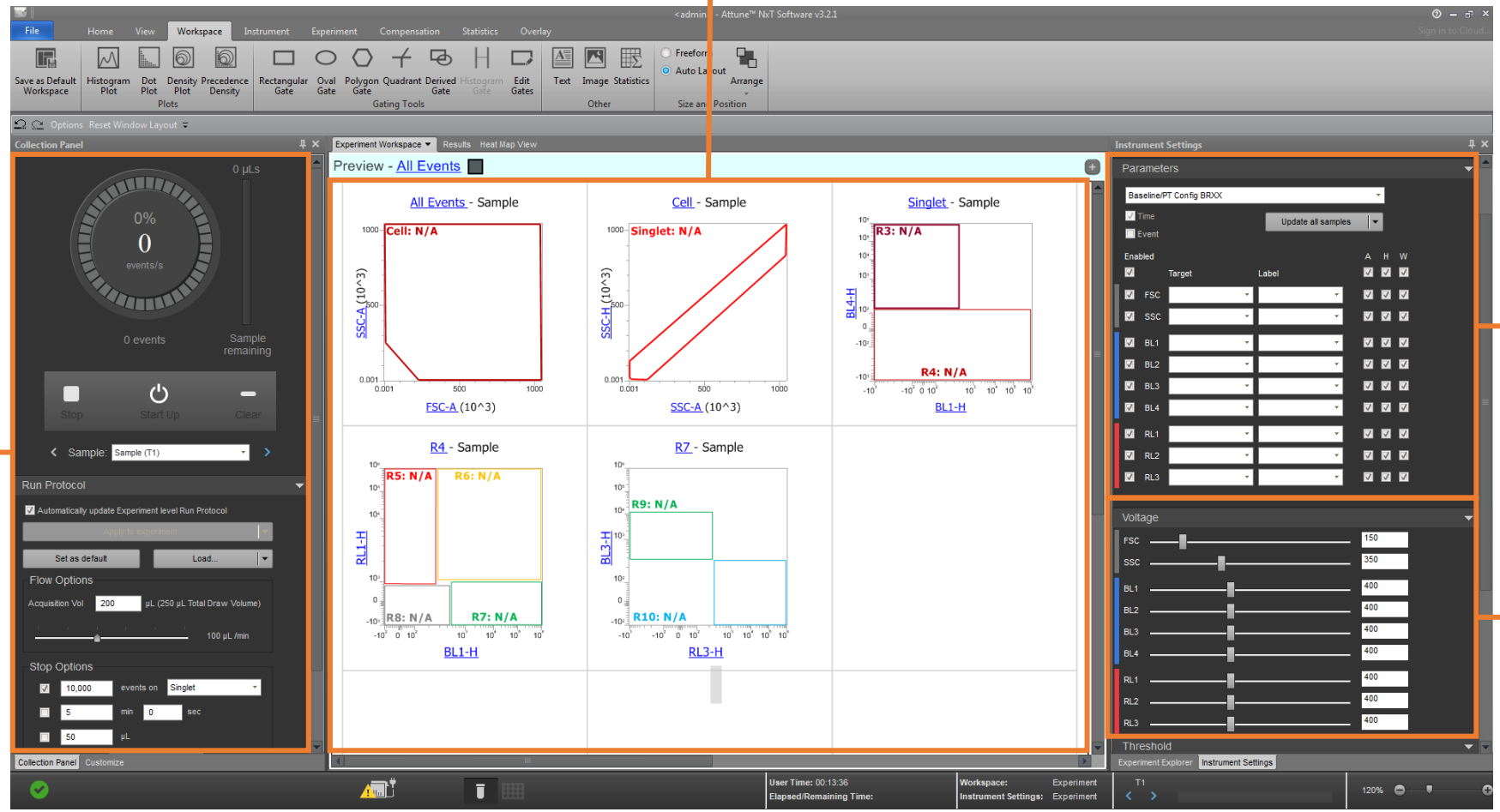
Attune NxT上樣分析流程

1. 檢查機器外觀，緩衝液是否充足，廢液是否過多。
2. 開啟電腦與Attune NxT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. 勾選**Channels**，以及欲觀察的A，H，W數值。
6. 設定**Workspace**: *Cell* (FSC-A, SSC-A) > *Singlet* (SSC-A, SSC-H) > *Chart for markers*。
7. 設定**Collection Panel**: 吸取樣本體積，分析流速，數據蒐集目標
8. 調整**PMT**: 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。
9. 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。
10. 依序上樣: 其他controls以及正式染色樣品。
11. 輸出實驗結果: atx原始數據檔案，FCS3.1檔案，excel檔案，與PDF報告。

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

設定儀器參數

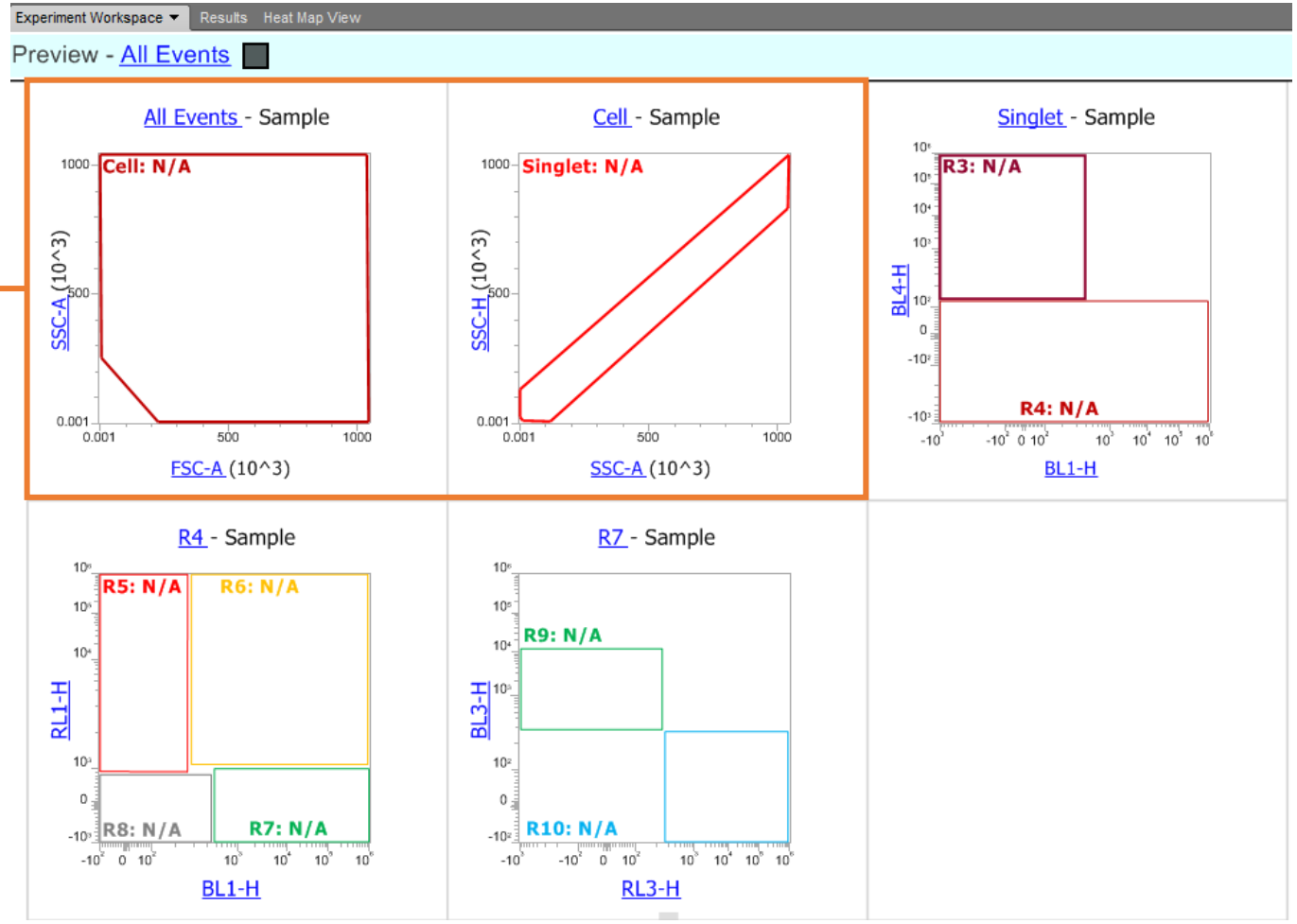

2. Workspace
1. Channel
3. Collection Panel
4. PMT
Attune™ NxT Software v3.2.1
Collection Panel
Experiment Workspace
Results
Heat Map View
Instrument Settings
Preview - All Events
All Events - Sample
Cell - Sample
Singlet - Sample
R4 - Sample
R7 - Sample
Parameters
Voltage
Threshold
Run Protocol
Flow Options
Stop Options

設定儀器參數 – 1. Channels

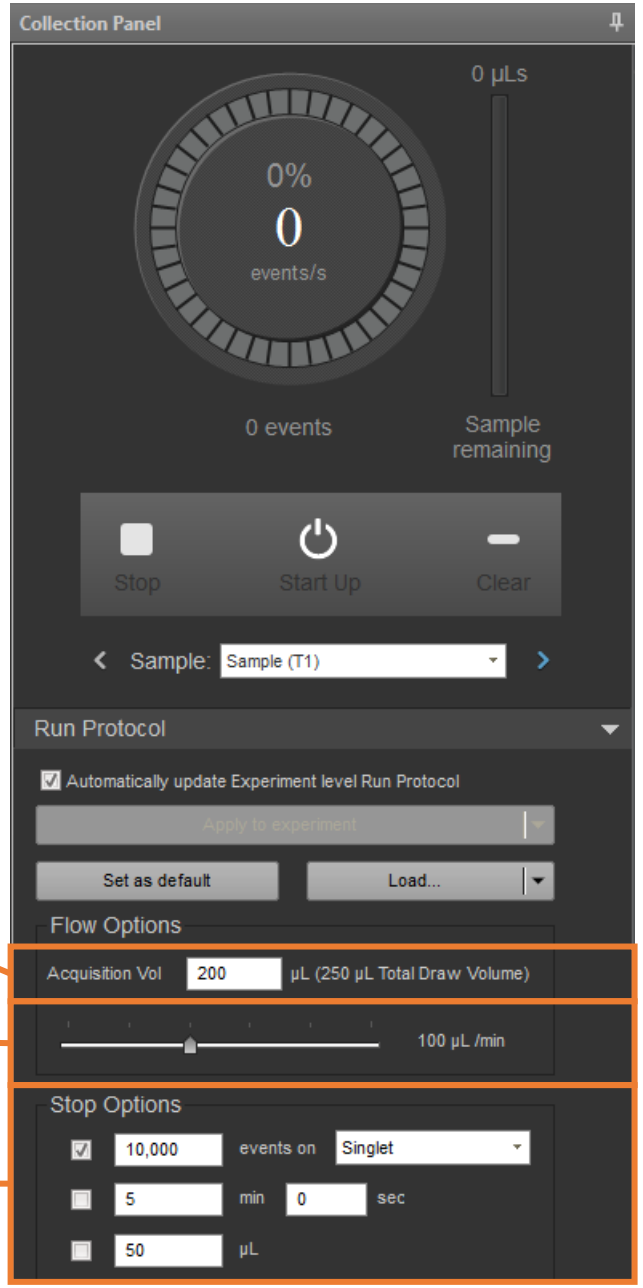
設定儀器參數 – 2. Workspace

最基本的兩個圖:

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



設定儀器參數 – 3. Collection Panel

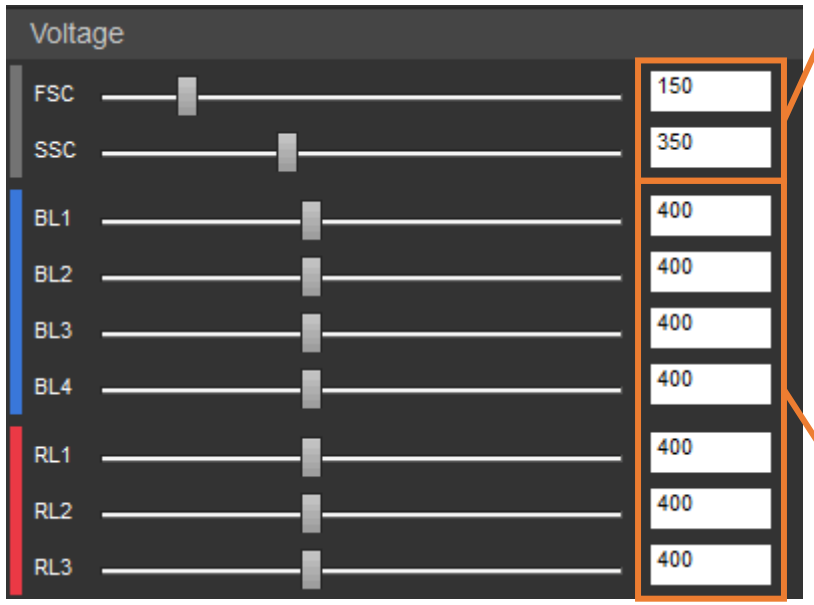


吸取樣本體積

分析流速

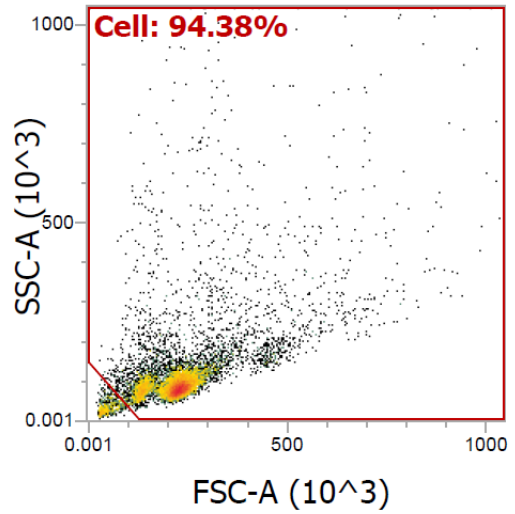
數據蒐集目標

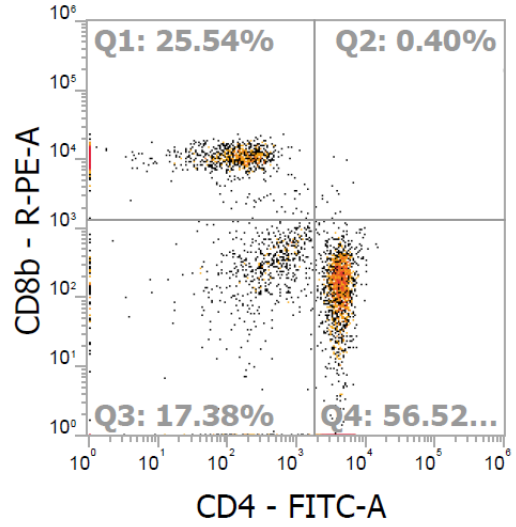
設定儀器參數 – 4. PMT



一般細胞建議從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樣本觀察訊號背景值
以正式染色樣本觀察訊號最大值

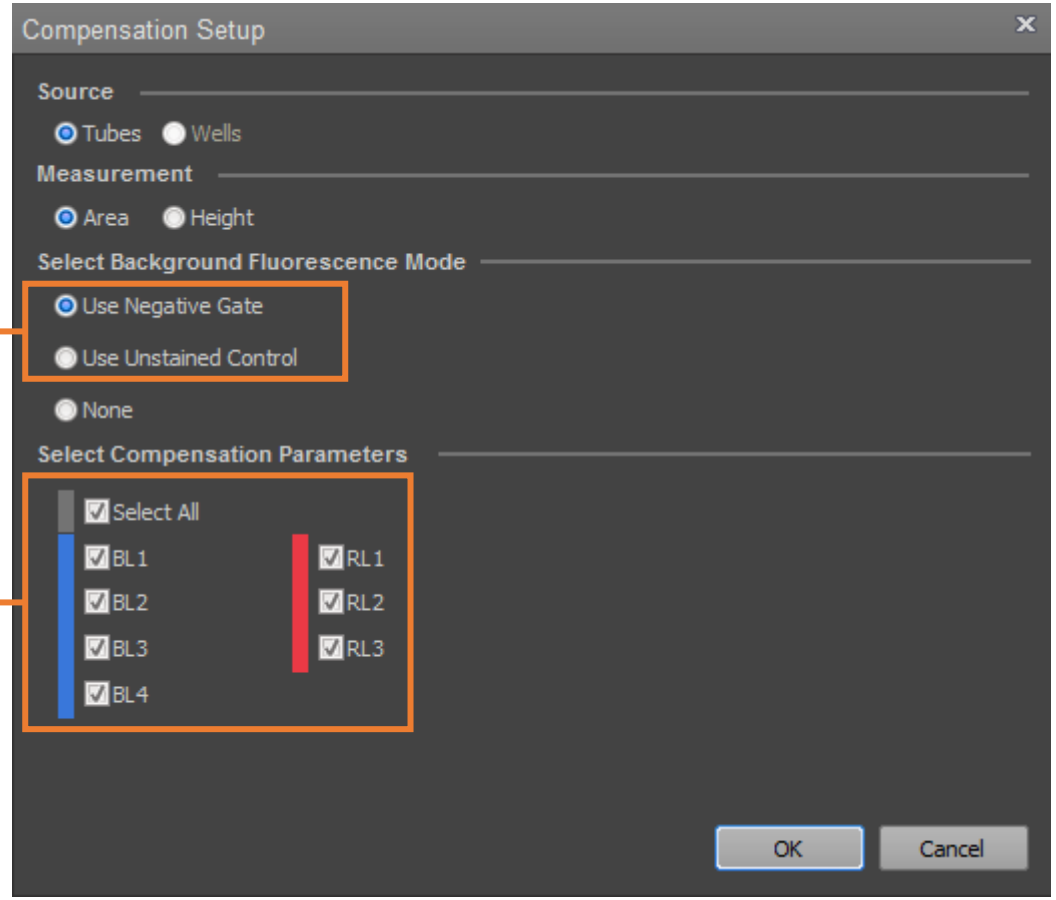




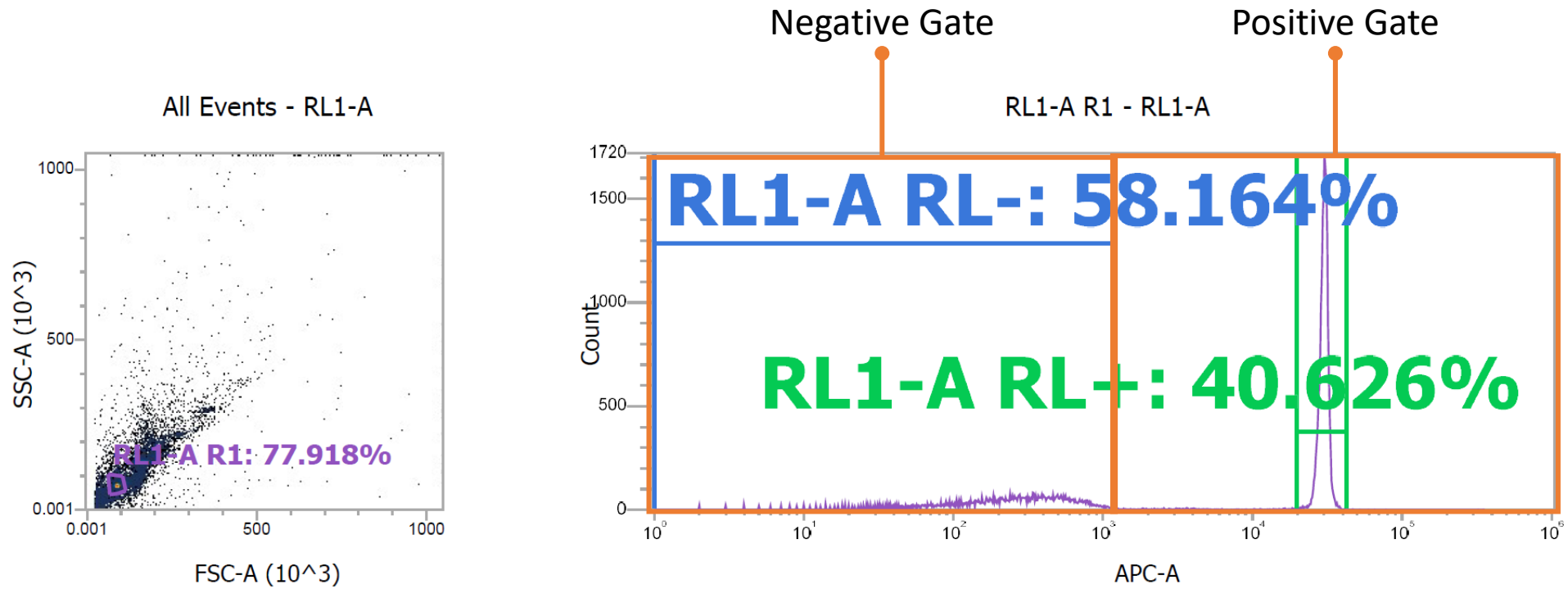
設定儀器參數 – Compensation

選擇與positive cells背景螢光較相近的negative cells

勾選需要進行compensation的channels



設定儀器參數 – Compensation: Use Negative Gate



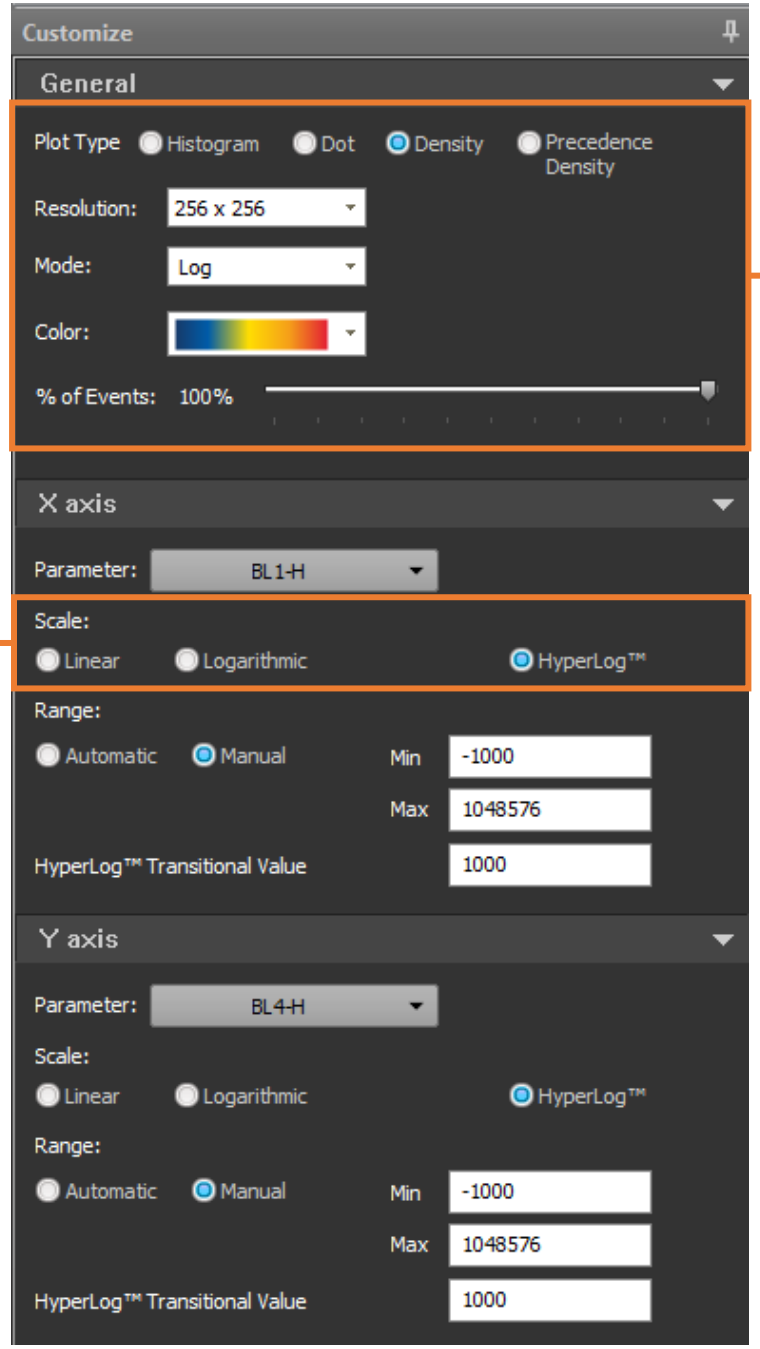
記錄Compensation controls之前先確認:

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

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

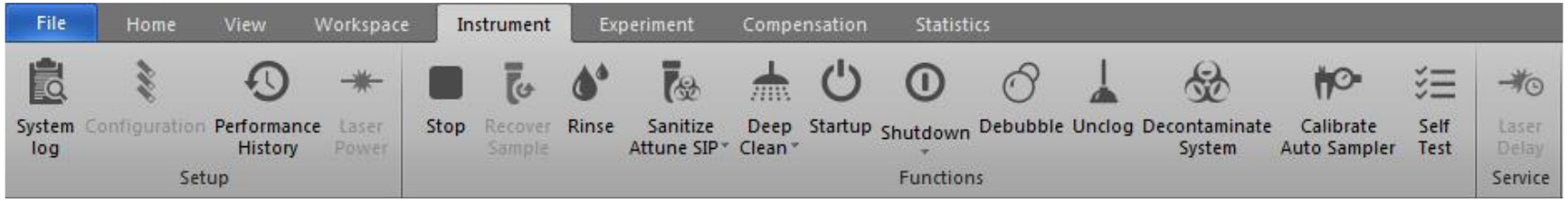
設定儀器參數 – Chart

多色螢光實驗進行compensation後，建議使用HyperLog數值軸，以使極小的數值仍可呈現在圖上



Workspace Chart類型選擇與參數調整

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，樣本管路可能塞管時
Decontamination	儀器管理進行定期保養

狀況無法排除時，問題回傳:

1. System log
2. Print screen

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 A.0

http://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0026553_Attune_Cytometric_SW_UG.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

https://www.thermofisher.com/tw/zt/home/life-science/cell-analysis/flow-cytometry/flow-cytometers/attune-nxt-flow-cytometer/resources.html

其他工具

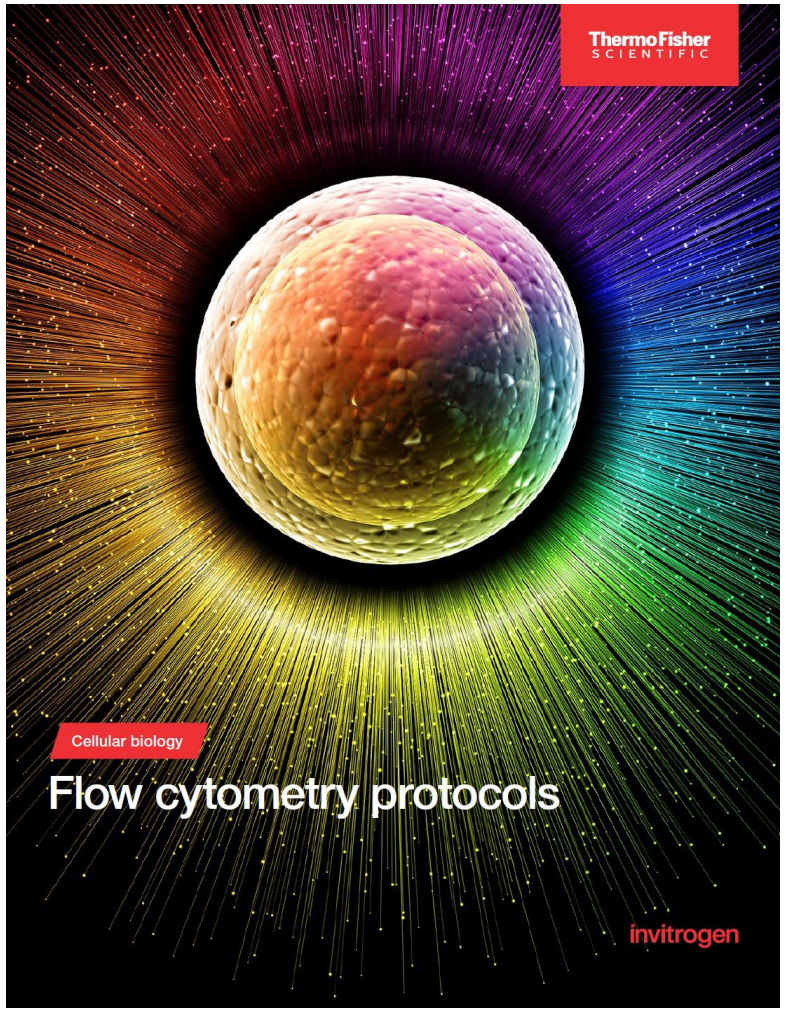
Human and mouse antigens

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

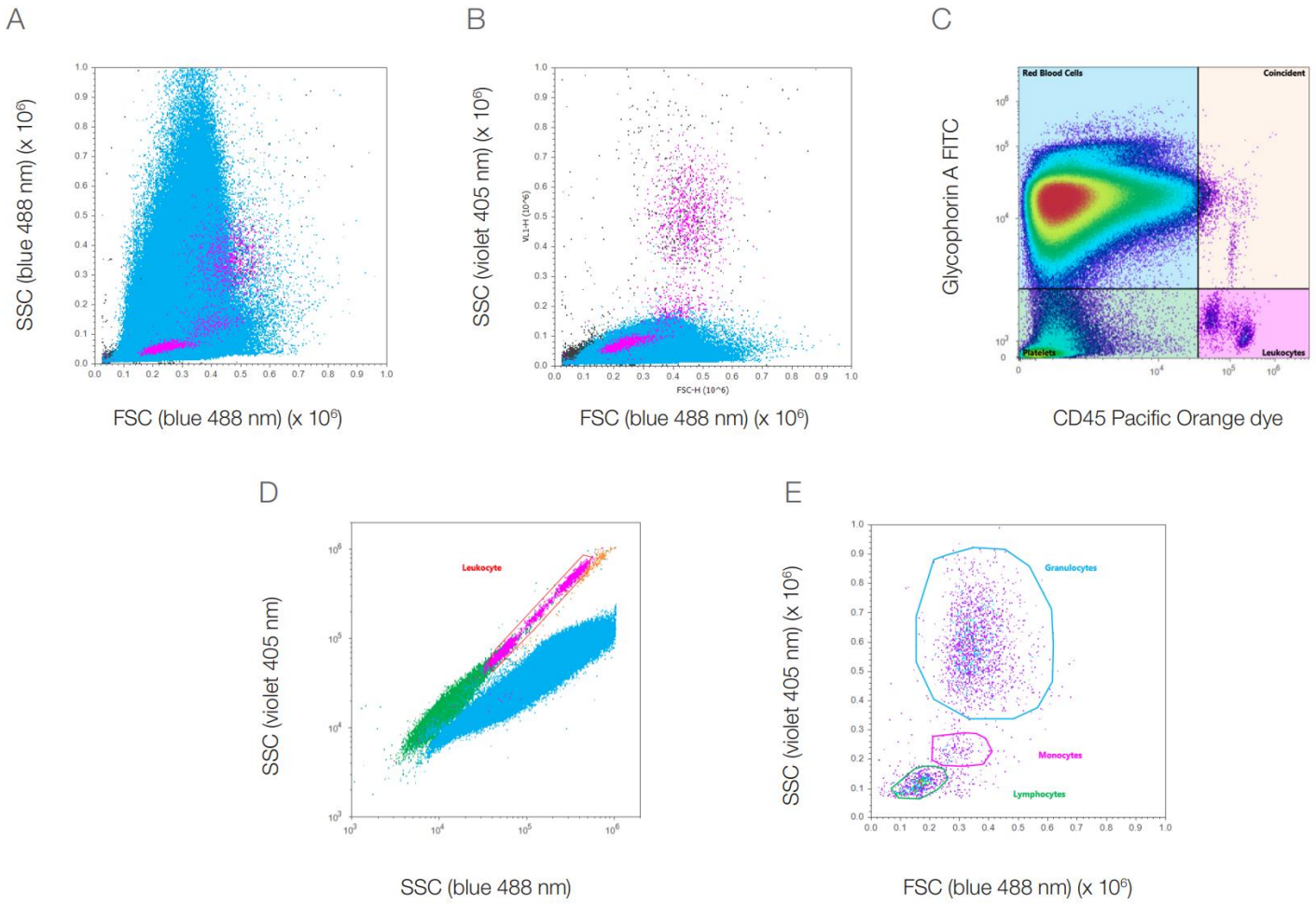


https://www.thermofisher.com/tw/zt/home/global/forms/flow-cytometry-protocols-handbook.html

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

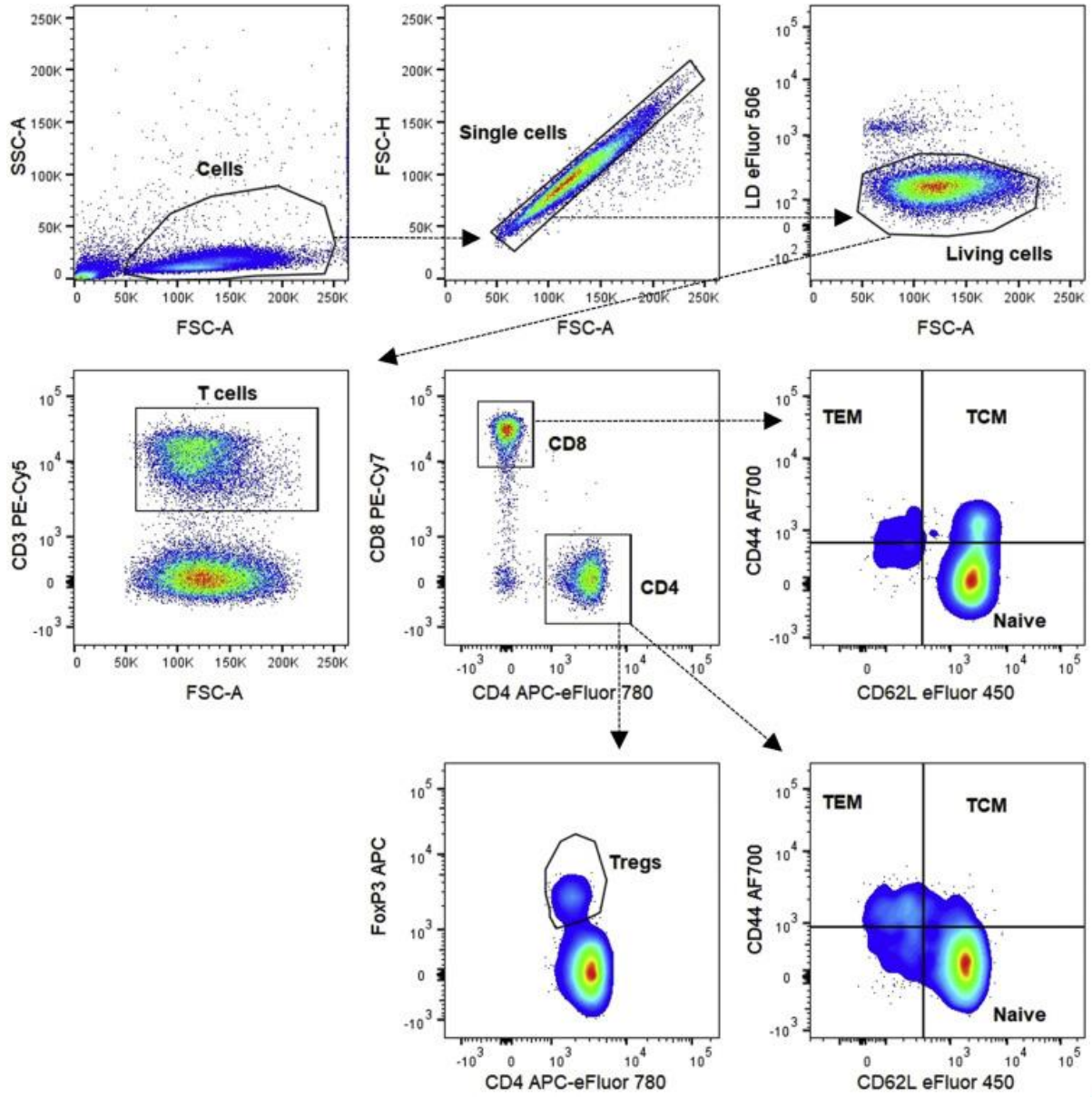
Applications

No-Lyse, No-Wash Whole Blood Analysis



http://assets.thermofisher.com/TFS-Assets/BID/Application-Notes/detection-leukocytes-attune-nxt-appnote.pdf

Immunophenotyping



Gating strategy for T cell activation and Treg cells in mouse blood.

https://www.sciencedirect.com/science/article/pii/S2666166721000587

Phytoplankton 浮游植物

- 可以觀察的參數:
  1. FSC, SSC
  2. Auto-fluorescence (phycoerythrin, phycocyanin, chlorophyll-a and b, carotenoid,…)
  3. DNA content (SYBR,…)
  4. Viability (PI,…)
  5. Concentration
  …

➢ Filter samples through appropriate size mesh to prevent blocking the cytometer nozzle.

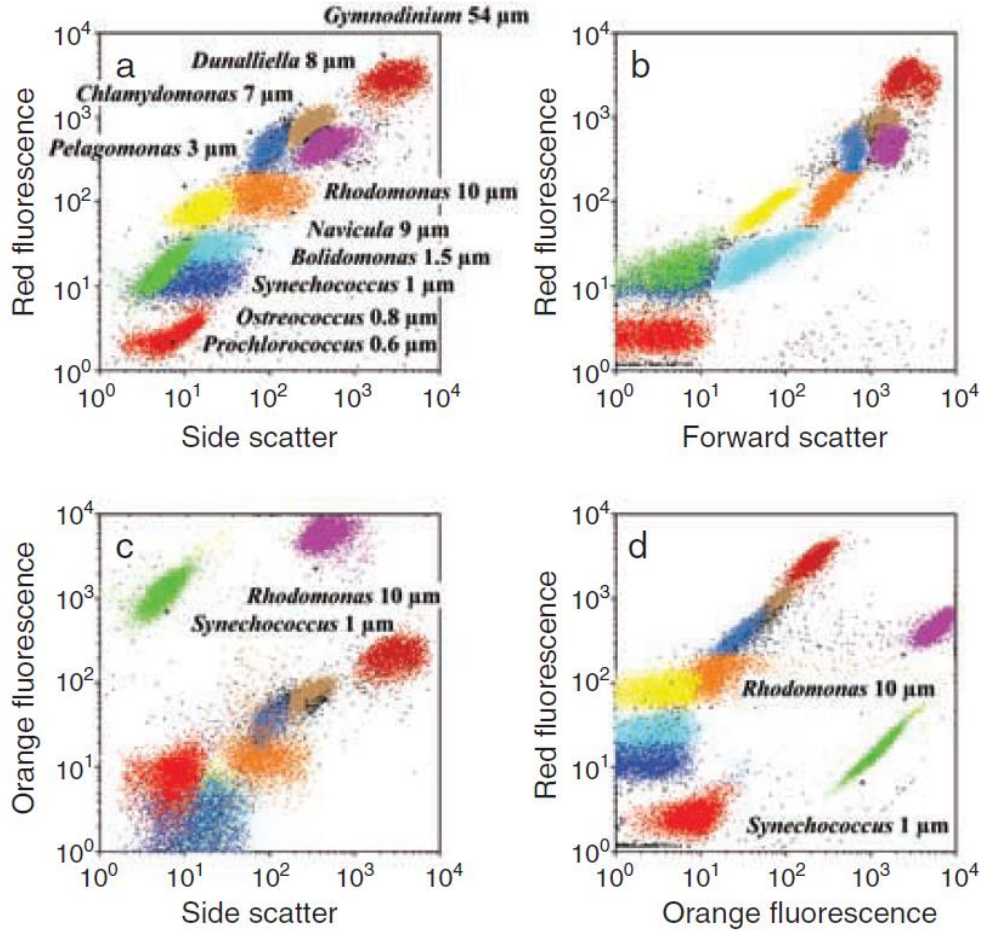
➢ Comparison of fluorescence against SSC is useful.

Phytoplankton – Populations

Phycoerythrin (BL2)
Chlorophyll (BL3)

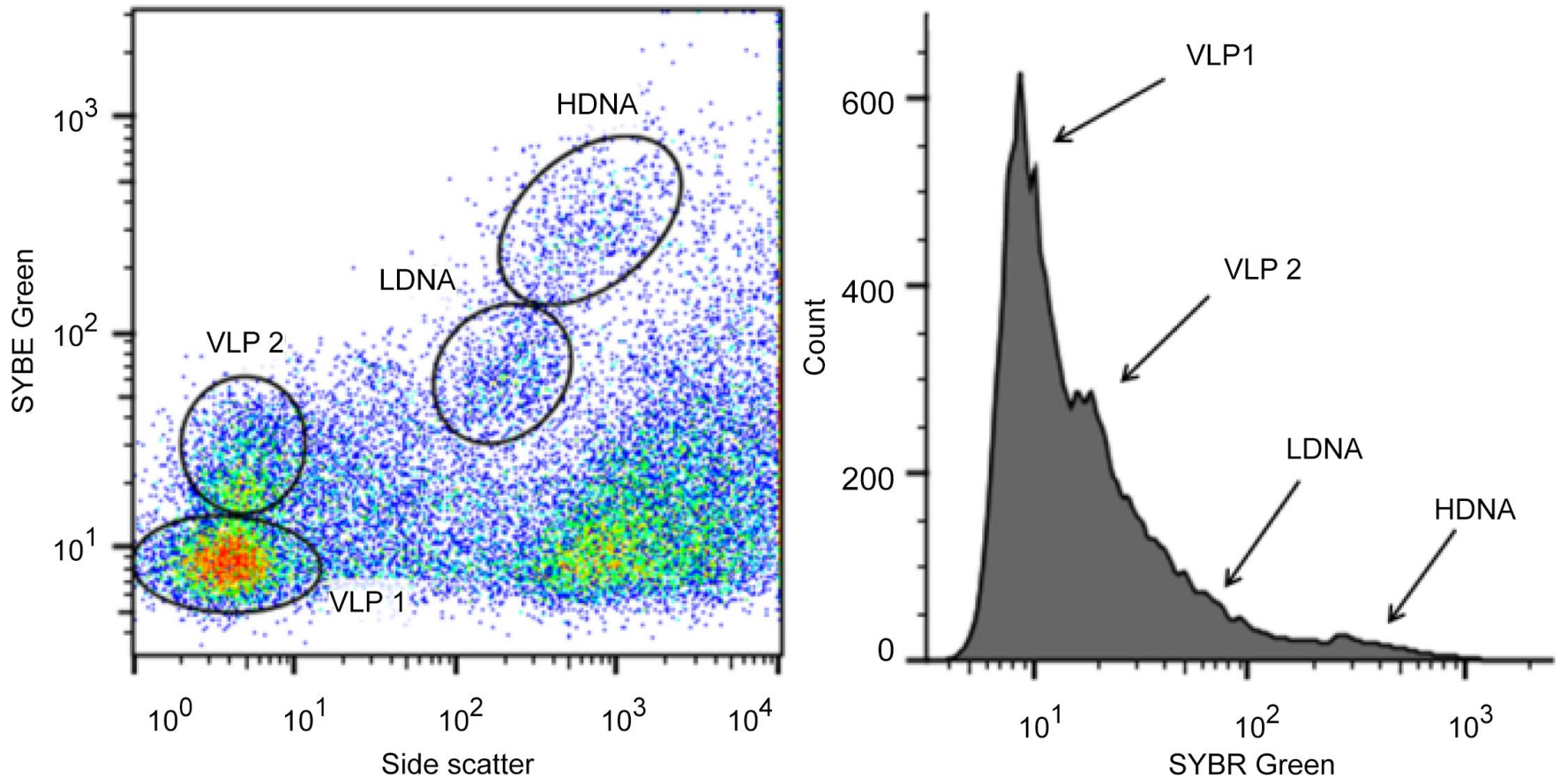
TABLE 17.1 Cultures referred to in this chapter. The RCC column corresponds the reference number of the culture in the Roscoff Culture Collection (http://www.sb-roscoff.fr/Phyto/collect.html).

RCC	Class	Taxon	Size (μm)
1	Chlorophyceae	*Chlamydomonas* sp.	7
6	Chlorophyceae	*Dunaliella tertiolecta* Butcher	8
22	Chrysophyceae	*Picophagus flagellatus* Guillou et Chrétiennot-Dinet	2
29	Cyanophyceae	*Synechococcus* sp.	1
80	Bacillariophyceae	*Navicula transitans* Cleve	9
89	Dinophyceae	*Gymnodinium sanguineum* Hirasaka	60
100	Pelagophyceae	*Pelagomonas calceolata* Andersen et Saunders	3
116	Prasinophyceae	*Ostreococcus tauri* Courties et Chrétiennot-Dinet	0.8
238	Bolidophyceae	*Bolidomonas mediterranea* Guilou et Chrétiennot-Dinet	1.5
286	Pelagophyceae	*Ankylochrysis lutea* Billard	6
350	Cryptophyceae	*Rhodomonas baltica* Karsten	10
407	Cyanophyceae	*Prochlorococcus* sp.	0.6



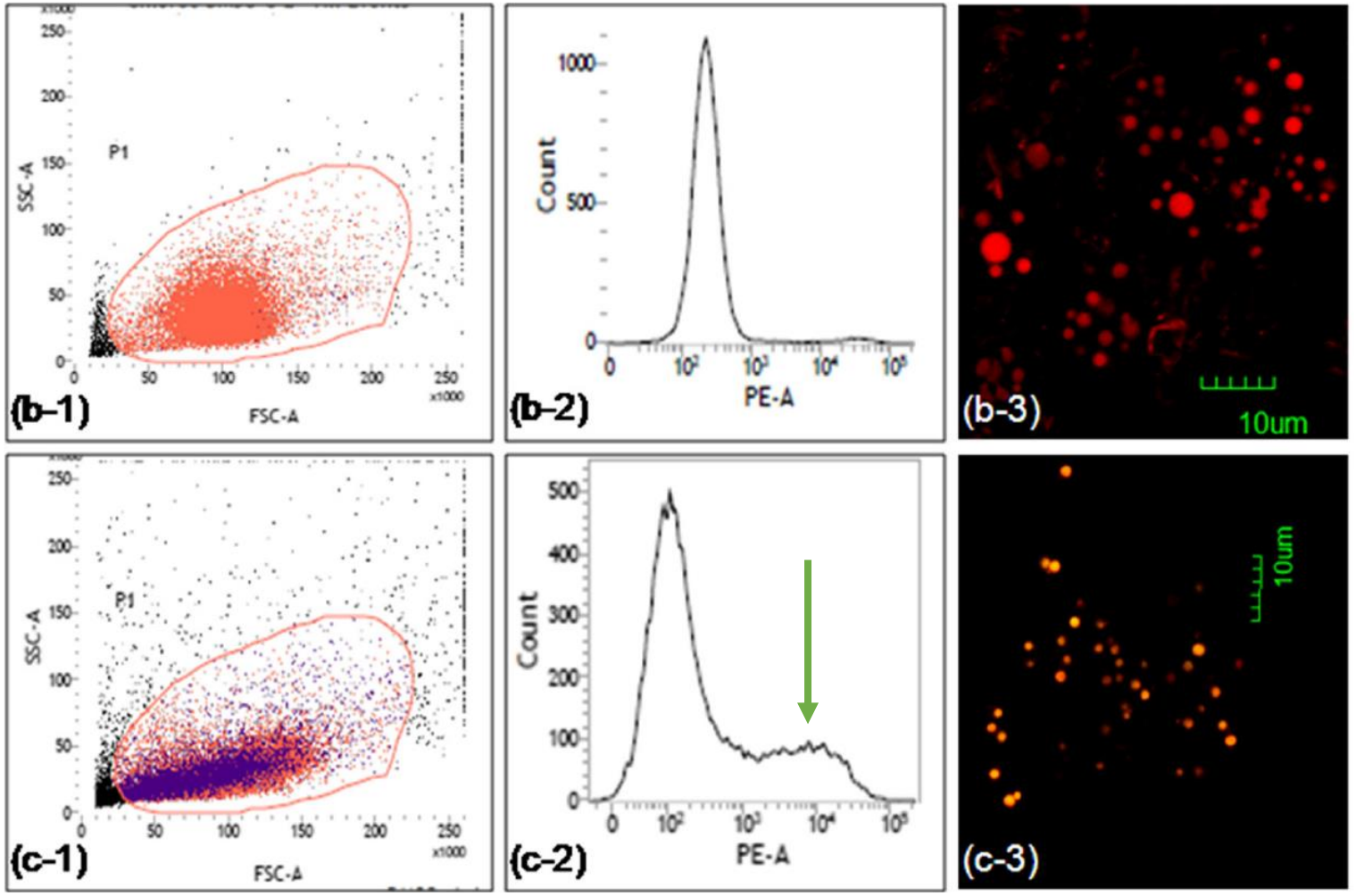
https://www.elsevier.com/books/algal-culturing-techniques/andersen/978-0-12-088426-1

Phytoplankton – DNA content



https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0146984

Detection of Neutral Lipid in Green Microalgae

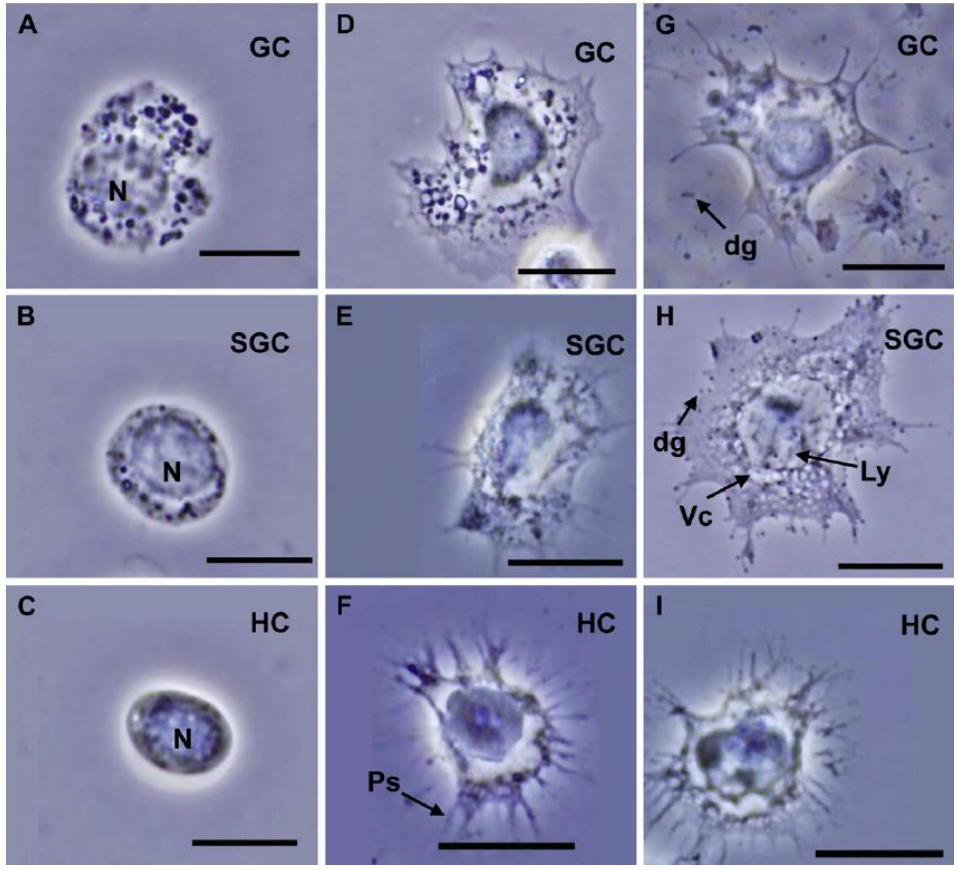


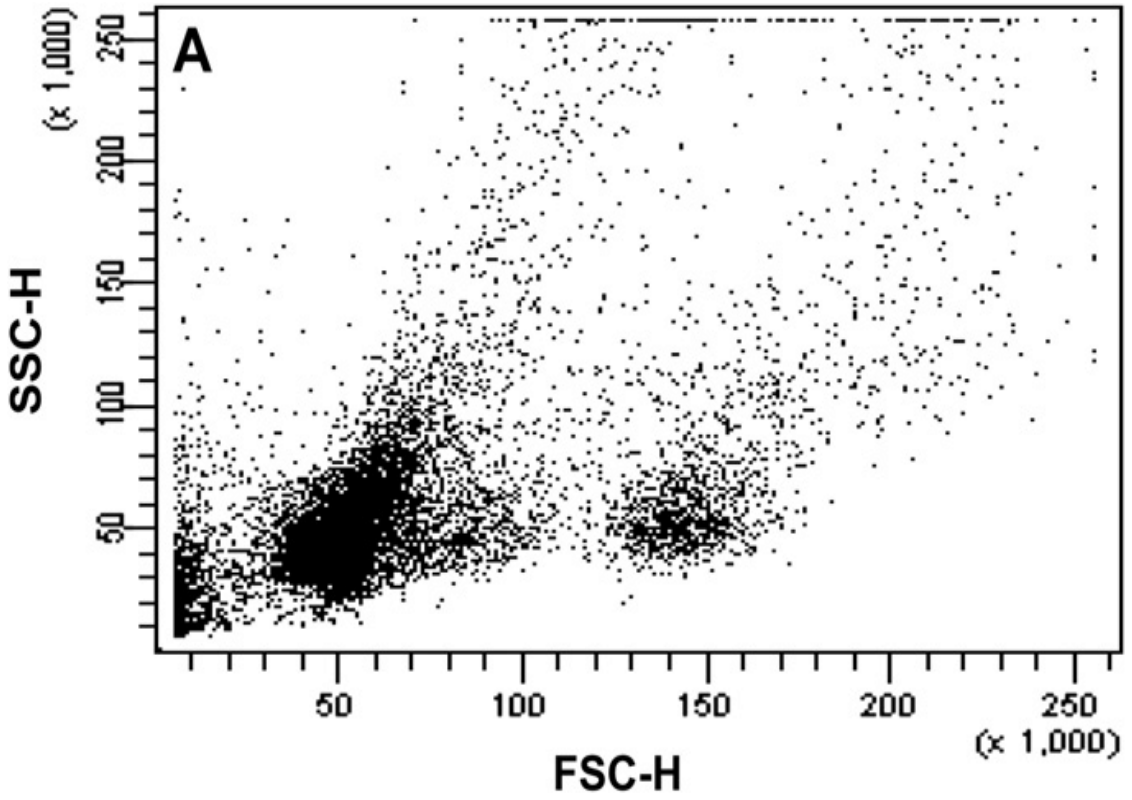
Chlorophyll

Chlorophyll +
Nile Red

https://annalsmicrobiology.biomedcentral.com/articles/10.1007/s13213-014-0937-5

White Shrimp Whole Blood, *Litopenaeus vannamei*

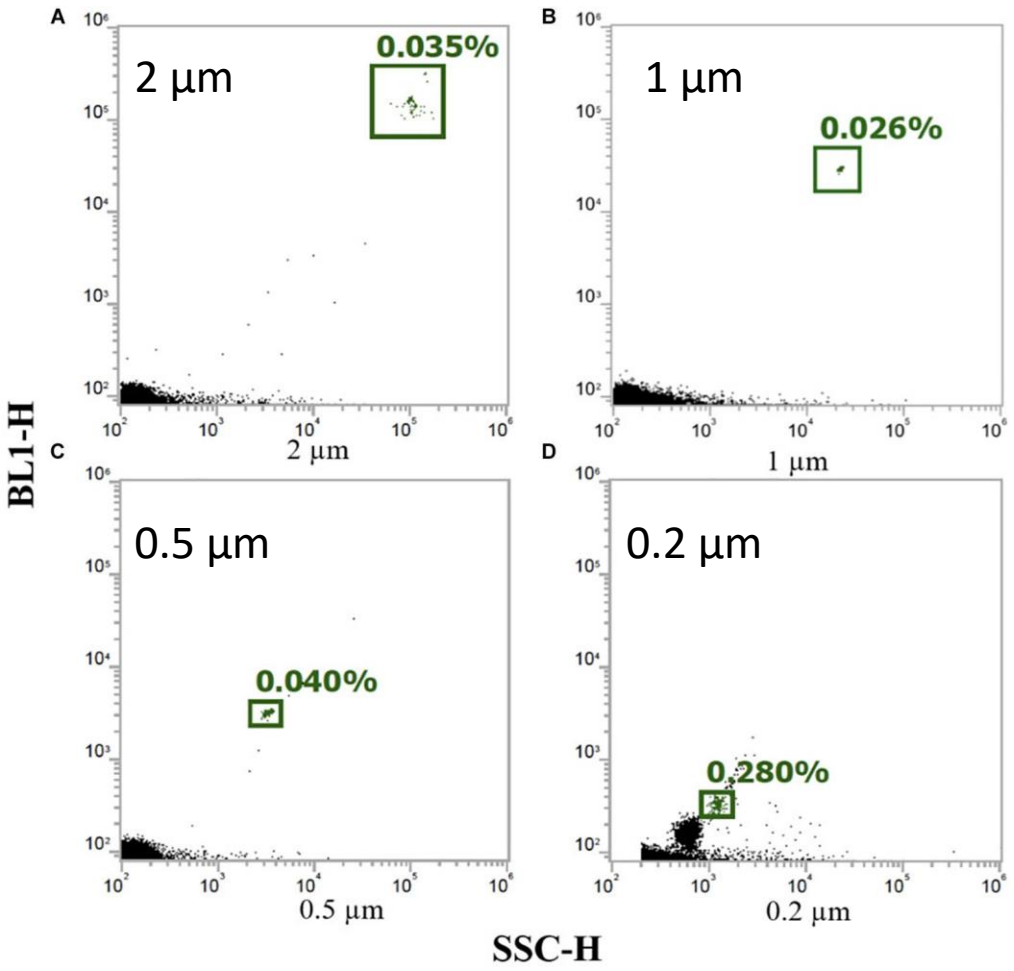


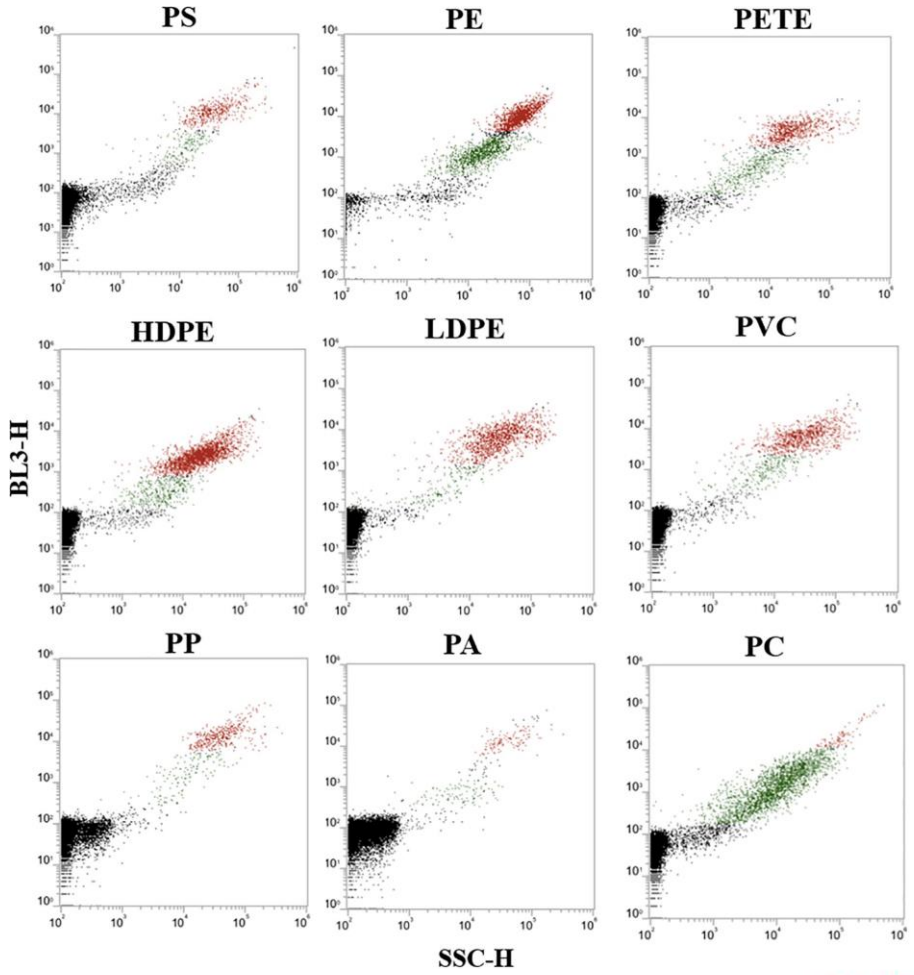


granular cell (GC), semi- granular cell (SGC), hyaline cell (HC)

https://www.sciencedirect.com/science/article/abs/pii/S1050464812004184

Microplastics in Liquid Samples

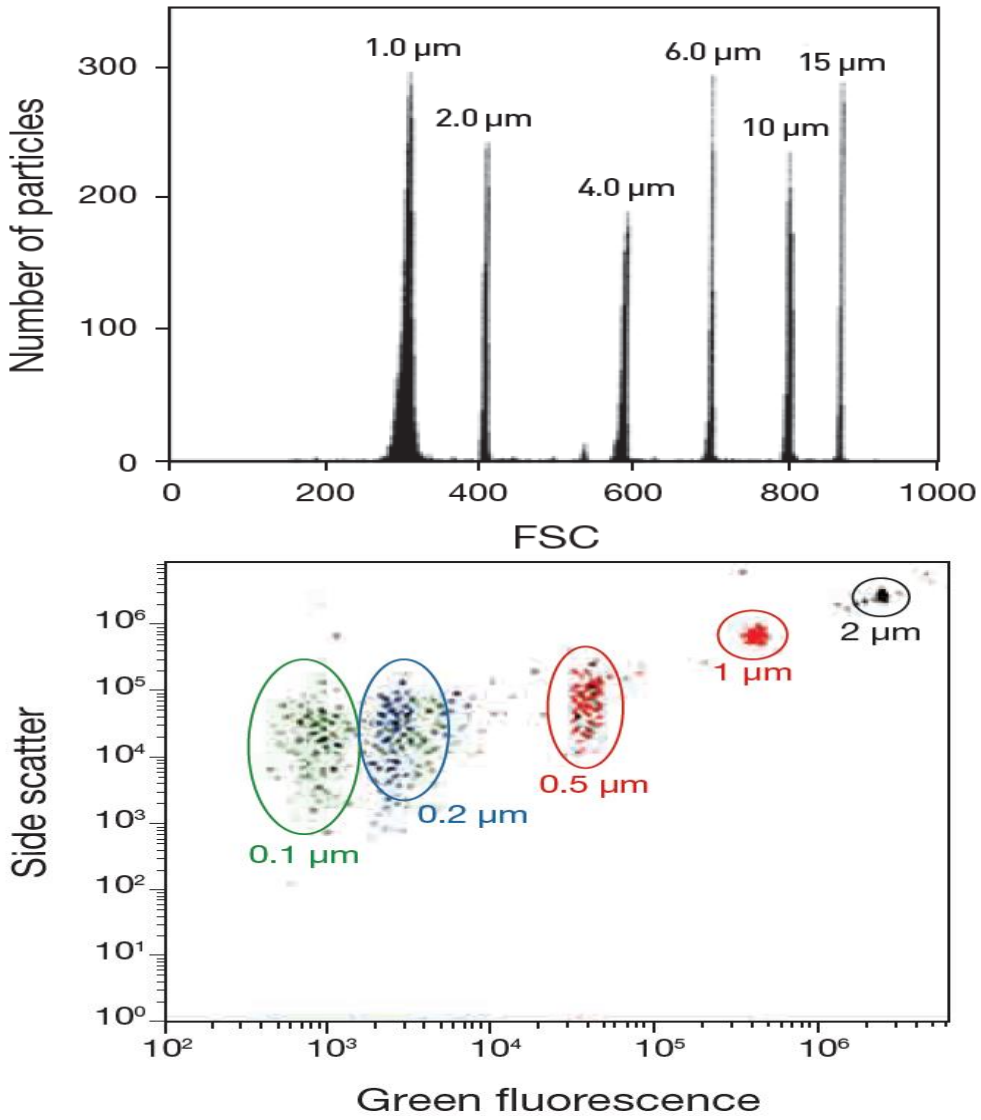




https://www.frontiersin.org/articles/10.3389/fmars.2020.552688/full

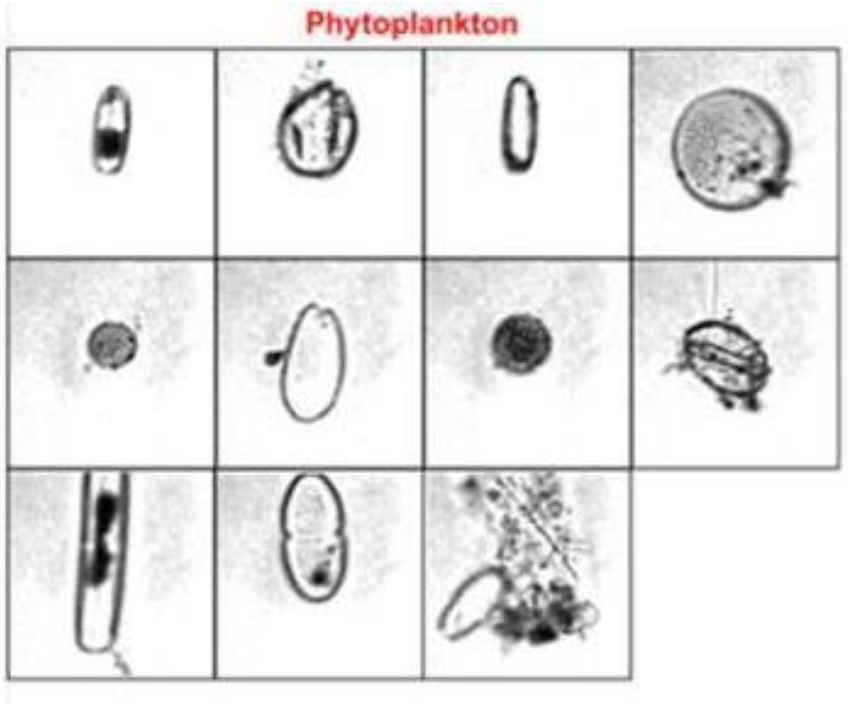
Size Reference Beads

	Size calibration	
Product	Flow Cytometry Size Calibration Kit	Flow Cytometry Sub-micron Particle Size Reference Kit
Use	Size reference	Size reference
Emission	No fluorescence	Green fluorescence
Bead size	6 sizes, 1.0–15 µm range	6 sizes, 0.02–2.0 µm range
Cat. No.	F13838	F13839



Attune CytPix

flow cytometer with high-speed ***brightfield camera***



pond water, Thermo Fisher, UK TSS/FAS

海洋中心 流式細胞儀委託服務要點

收費標準:

分析方式	廠牌型號	開機費（元）	校內（元）	校外（元）
分析	Thermo Attune	500	400/ hr	1,200/ hr
	BD FACSAria	800	500/ hr	1,500/ hr
分選	BD FACSAria	1,000	800/ hr	2,400/ hr

*如未滿半小時者以半小時計；超過半小時不滿一小時以一小時計費。

分析儀送檢須知:

1. 樣品濃度 : 約 **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**。
2. 樣品必須先過濾去除細胞塊或組織塊，如以 Cell Strainer (BD #352340，with **40 µm** nylon mesh)進行過濾。若因未過濾的樣品上機而造成機器堵塞及損壞，使用者需負賠償之責任。
3. 請先自行完成染色及固定等流程，若欲以多重螢光染色進行分析，請提供未經染色(或染有isotype)及單染之樣品，以便做檢測前校正。

管理員: **郭翊慧**小姐 分機#5289 **林瑩祝**小姐 分機#5285

http://www.ceo.ntou.edu.tw/ezfiles/48/1048/img/444/427683278.pdf

Q & A

Thank you

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