Attune NxT

NTHU, BMES

20210811

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Attune NxT Configurations

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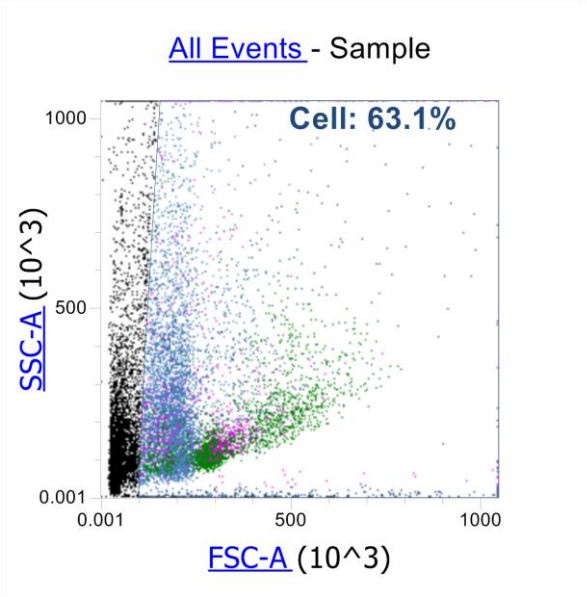
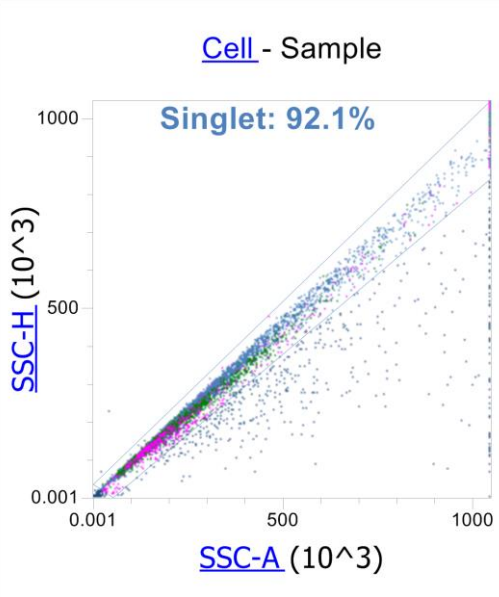
Excitation Laser	Emission Filter (nm)	Channel	Common Fluorophores	Fluorescent Proteins
Blue-488 nm	530/30	BL1	Alexa Fluor 488, FITC, GFP	eGFP, Emerald, eYFP
	574/26	BL2	Alexa Fluor 546, PE	eYFP, mCitrine, Venus
	695/40	BL3	PE-Alexa Fluor 700, PE-Cy5.5, PerCP, PerCP-Cy5.5	
	780/60	BL4	PE-Cy7, PE-Alexa Fluor 750	
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	

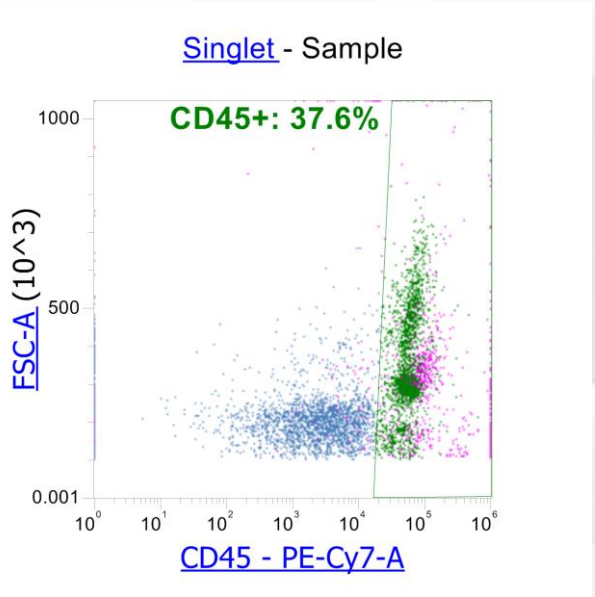
Panel Design

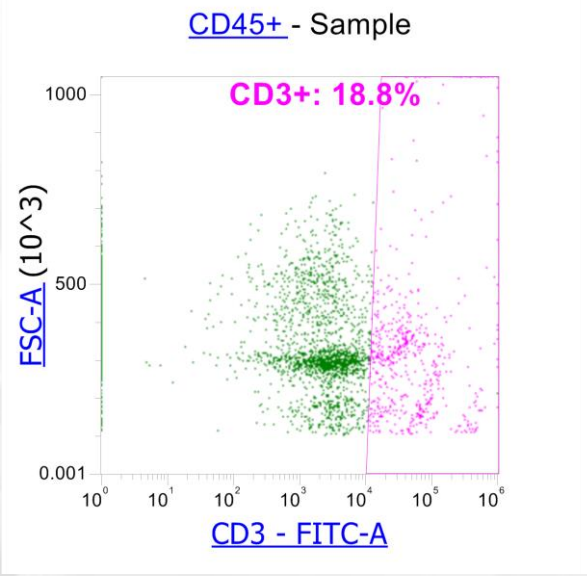
- anti-mouse CD3-FITC BL1 530/30
- anti-mouse CD4-PE BL2 574/26
- anti-mouse CD8-APC RL1 670/14
- anti-mouse CD45-PE-Cy7 BL4 780/60
- Fixable Viability Dye eF780 RL3 780/60

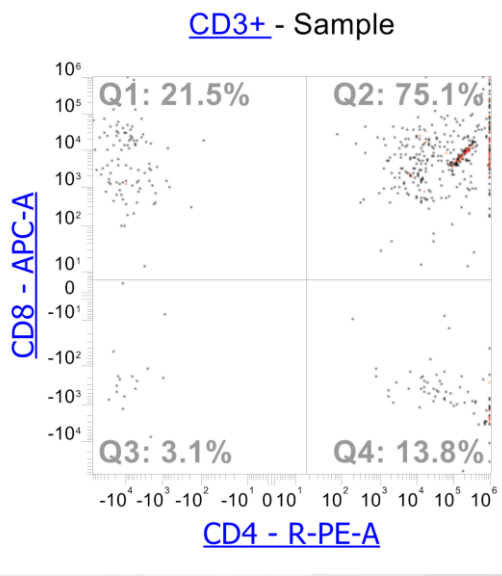
Analysis procedure: Cell > Singlet > **Live >** CD45+ > CD3+ > CD4, CD8











1. Antibody titration
2. Viability
3. Controls

Flow Cytometry Procedures

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

- Sample preparation
- Cell staining
- Flow Cytometer Start Up
- Setup **PMT** (signal min. from unstained control, signal max. from positive sample)
- Setup **Compensation** (single stained control for all fluorophore)
- Setup **Workspace** (***Cell > Singlet > Live***, gating strategies, controls for threshold setup)
- Analyze Samples
- Data Export (FCS 3.0)
- Flow Cytometer Shutdown
- Data Analysis

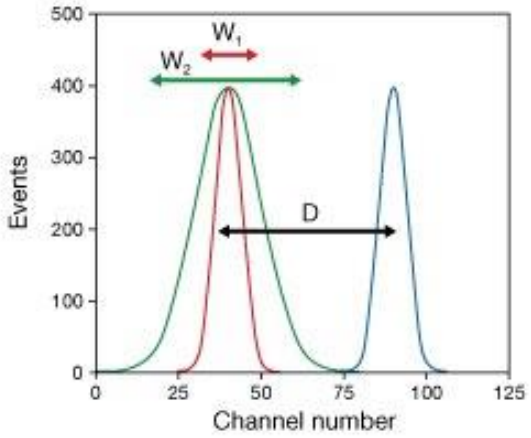
Panel Design – Flow Cytometry Panel Builder

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

1. Understand your flow cytometer (channels) and spectral overlap of fluorophores.
2. Brighter fluorophores for lower-expressed markers, and vice versa.
3. Use fluorophores that are spectrally similar for different cell subpopulations that will be gated and analyzed separately.

Resolution Sensitivity of Fluorochrome in Flow Cytometry

- Stain index S = D/W
- Spillover affects resolution sensitivity



Stain Index = D/W

Where:

D is the difference between positive and negative peak medians.

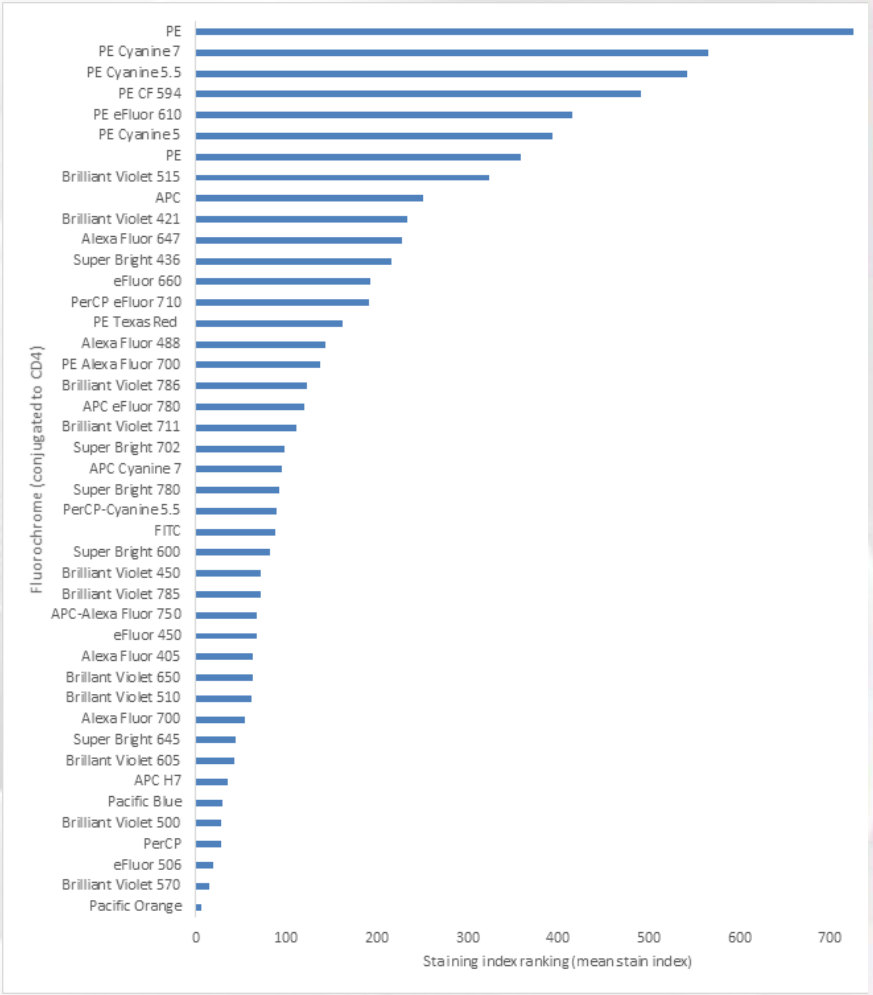
W is the spread of the negative peak and is equal to 2 x rSD.

rSD is the robust standard deviation.

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

Where:

MFI is the median fluorescence intensity.



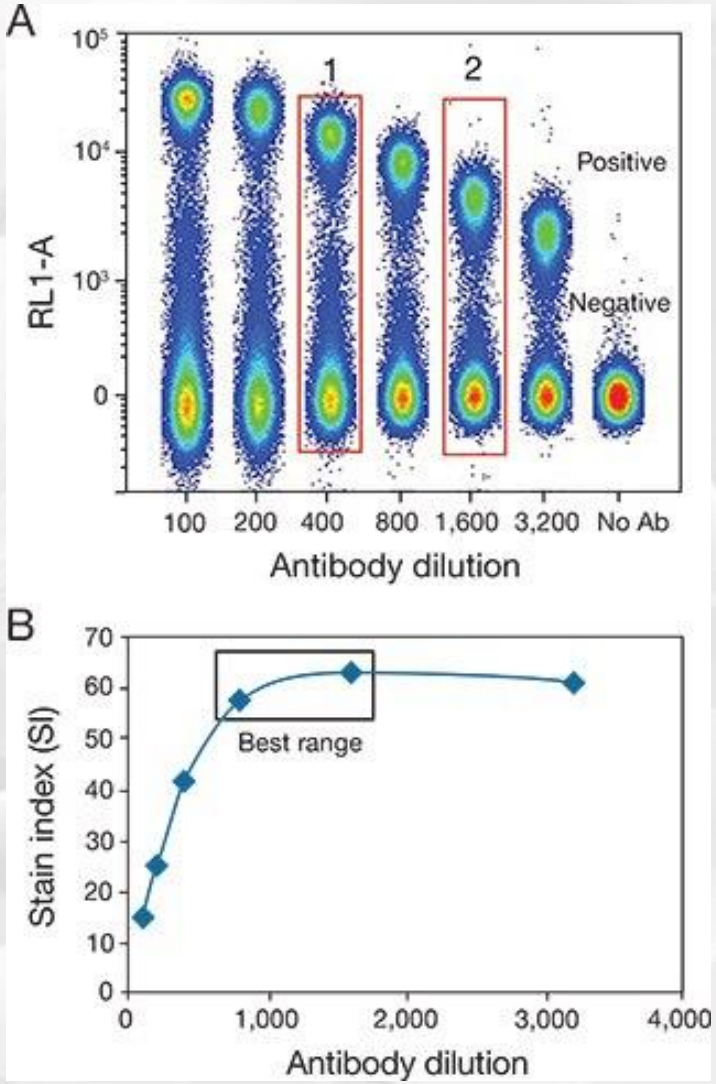
Average stain index of fluorochrome conjugated to CD4 and analyzed on the Attune NxT.

https://www.thermofisher.com/tw/zt/home/references/newsletters-and-journals/bioprobes-journal-of-cell-biology-applications/bioprobes-71/bioprobes-71-flow-cytometry-panel-design.html

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



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www.TAQKEY.com

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.

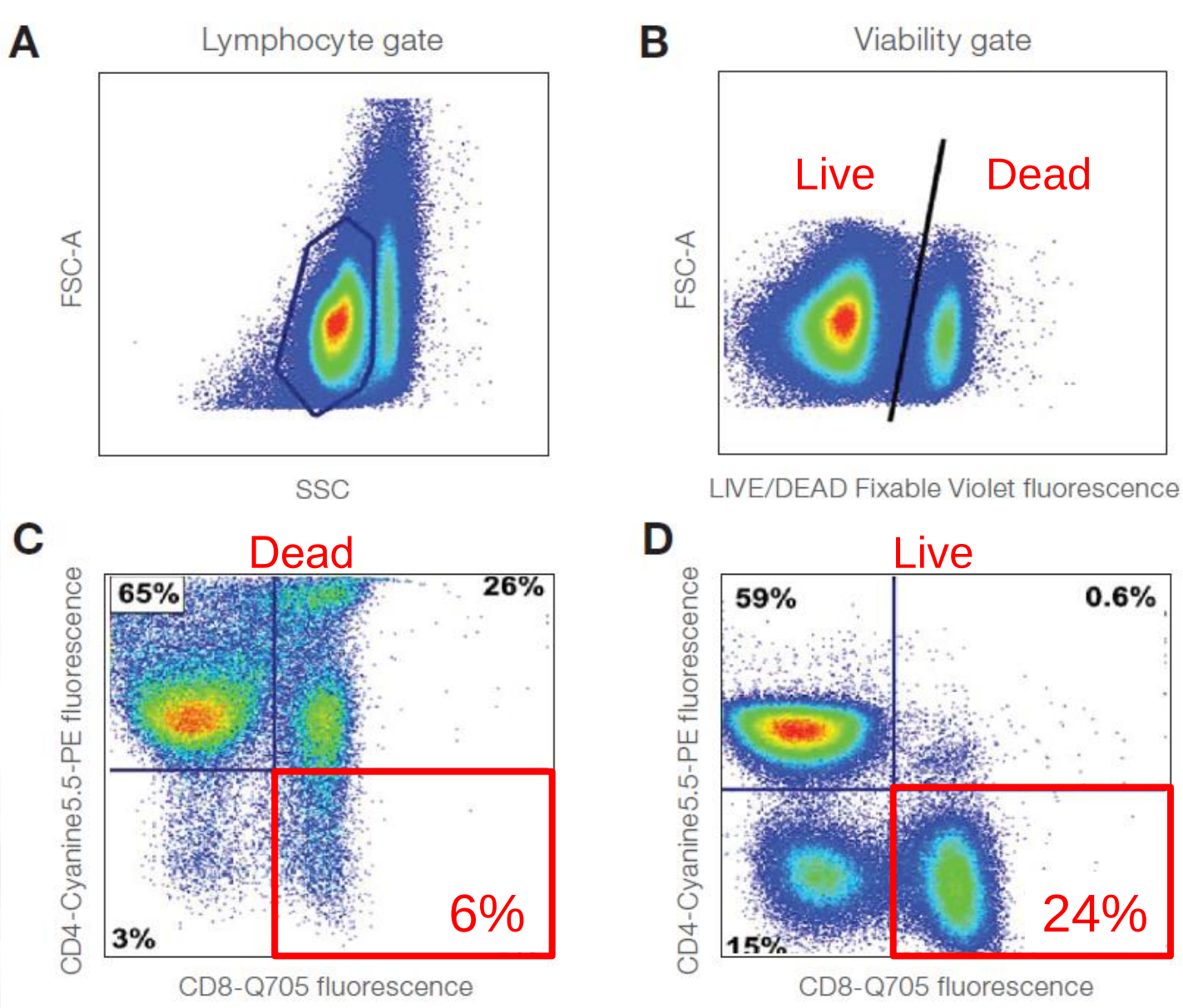
https://www.thermofisher.com/tw/zt/home/references/protocols/cell-and-tissue-analysis/protocols/cell-preparation-flow-cytometery.html

Cell Staining

- 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

Dead Cell Exclusion

Dead cells significantly affect results.



Flow Cytometry Controls

- Single stained control for compensation

- Negative control

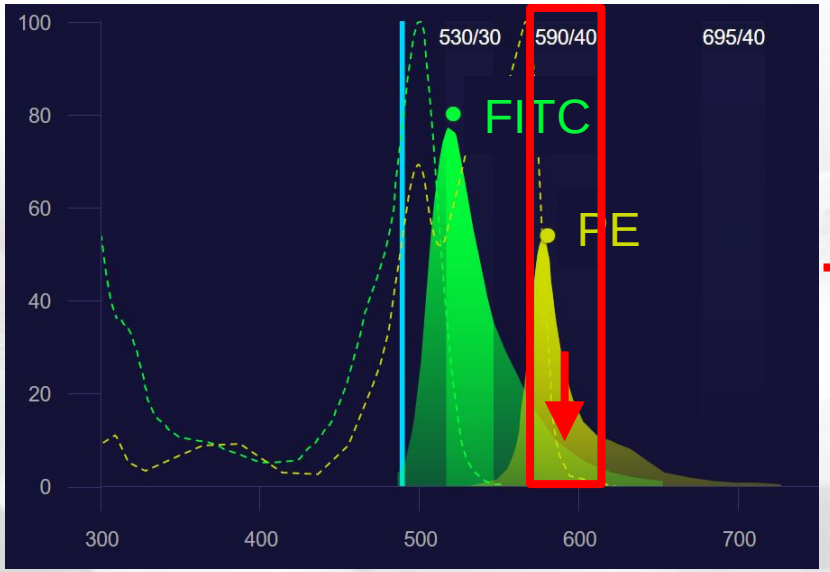
1. Unstained
2. Isotype control
3. Fluorescence minus one (FMO) control for multicolor panel
4. FMO + isotype control

- Positive control

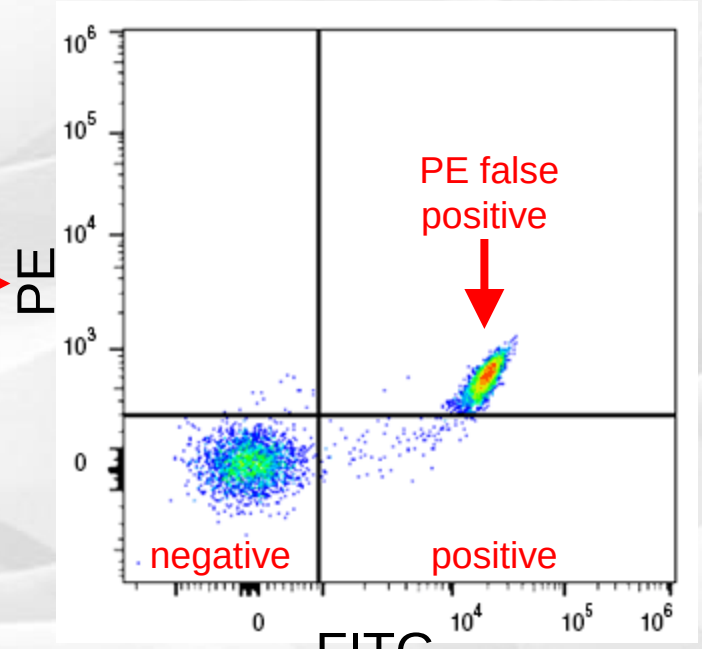
Flow Cytometry Controls – Single Stained for Compensation

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

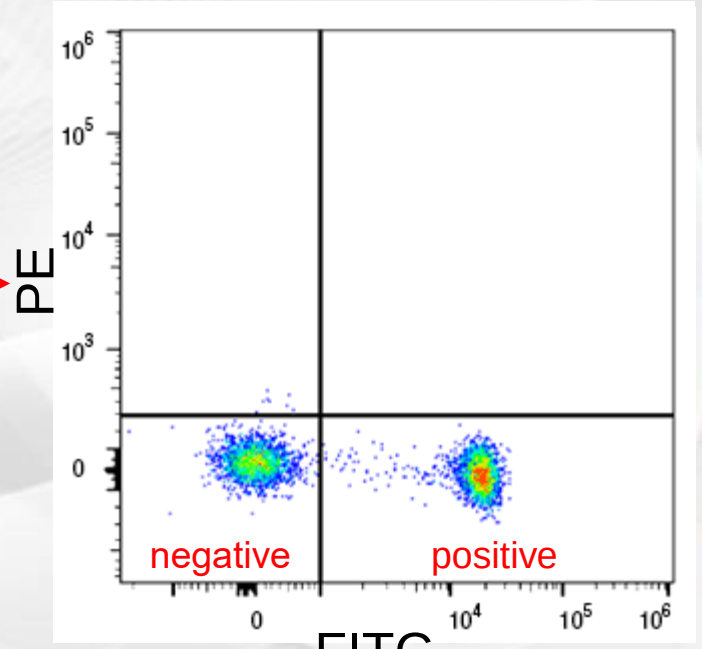
Spillover



FITC single stain

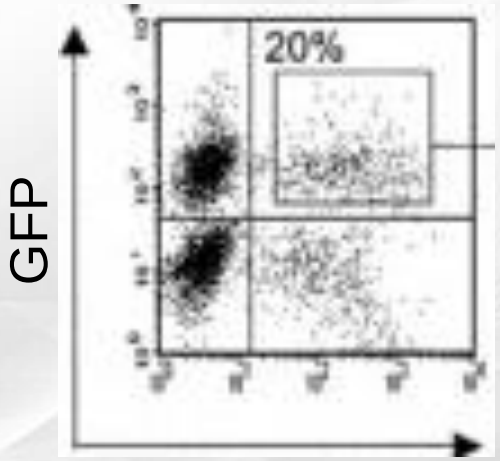


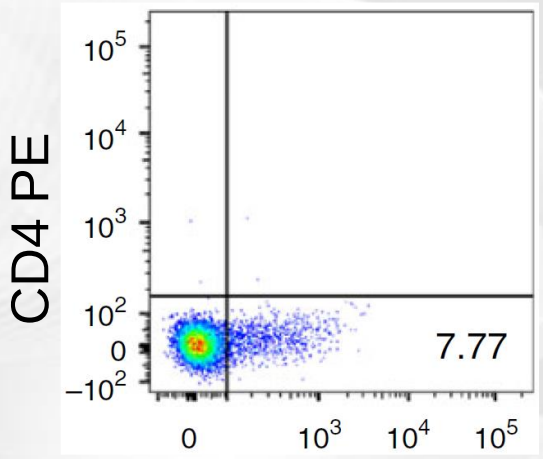
Post compensation



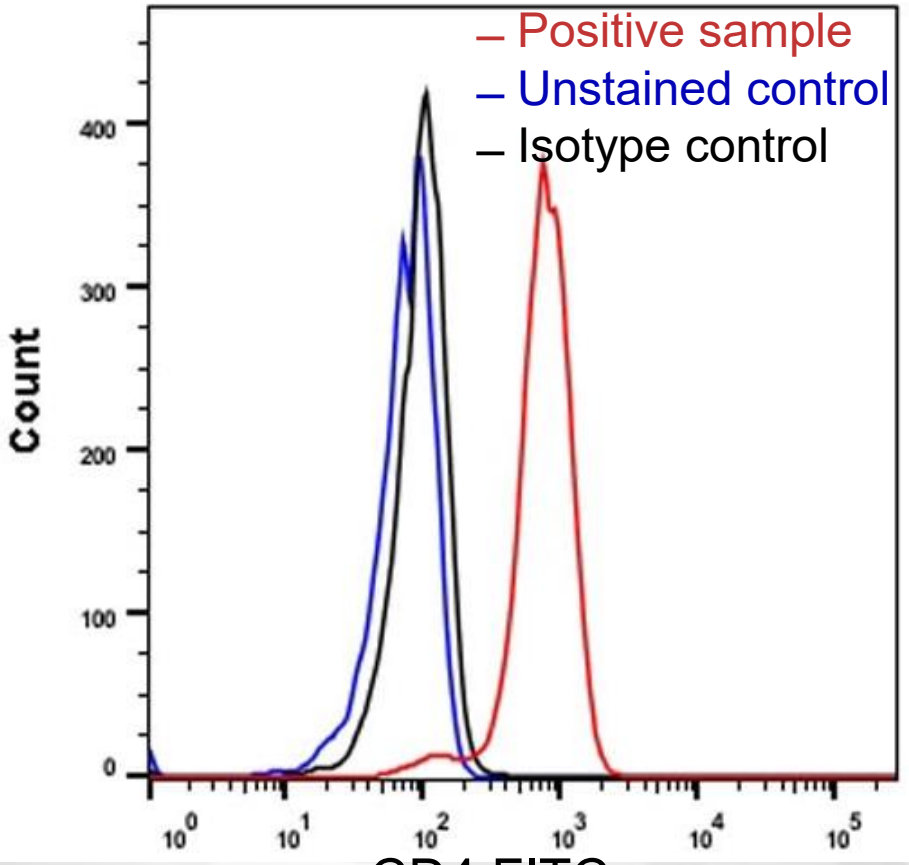
When to Use Compensation Beads

- Fluorescence in cells
- Poorly expressed markers
- Limited amount of sample
- Large multicolor panel
- Standardization





Flow Cytometry Controls – Unstained and Isotype Control

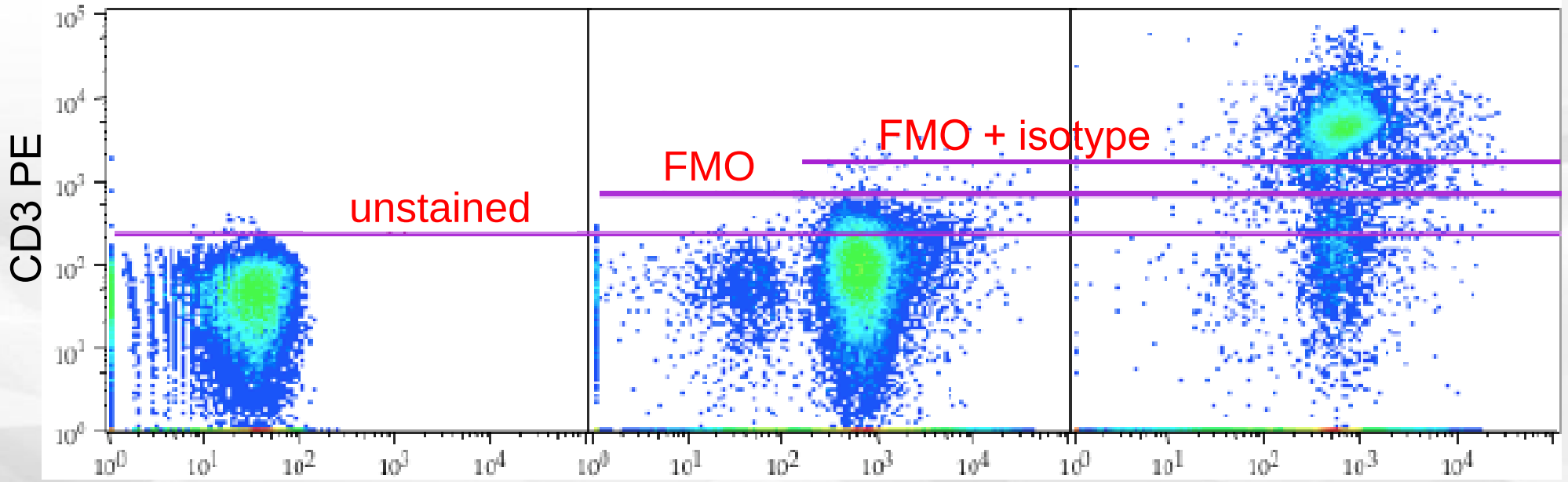


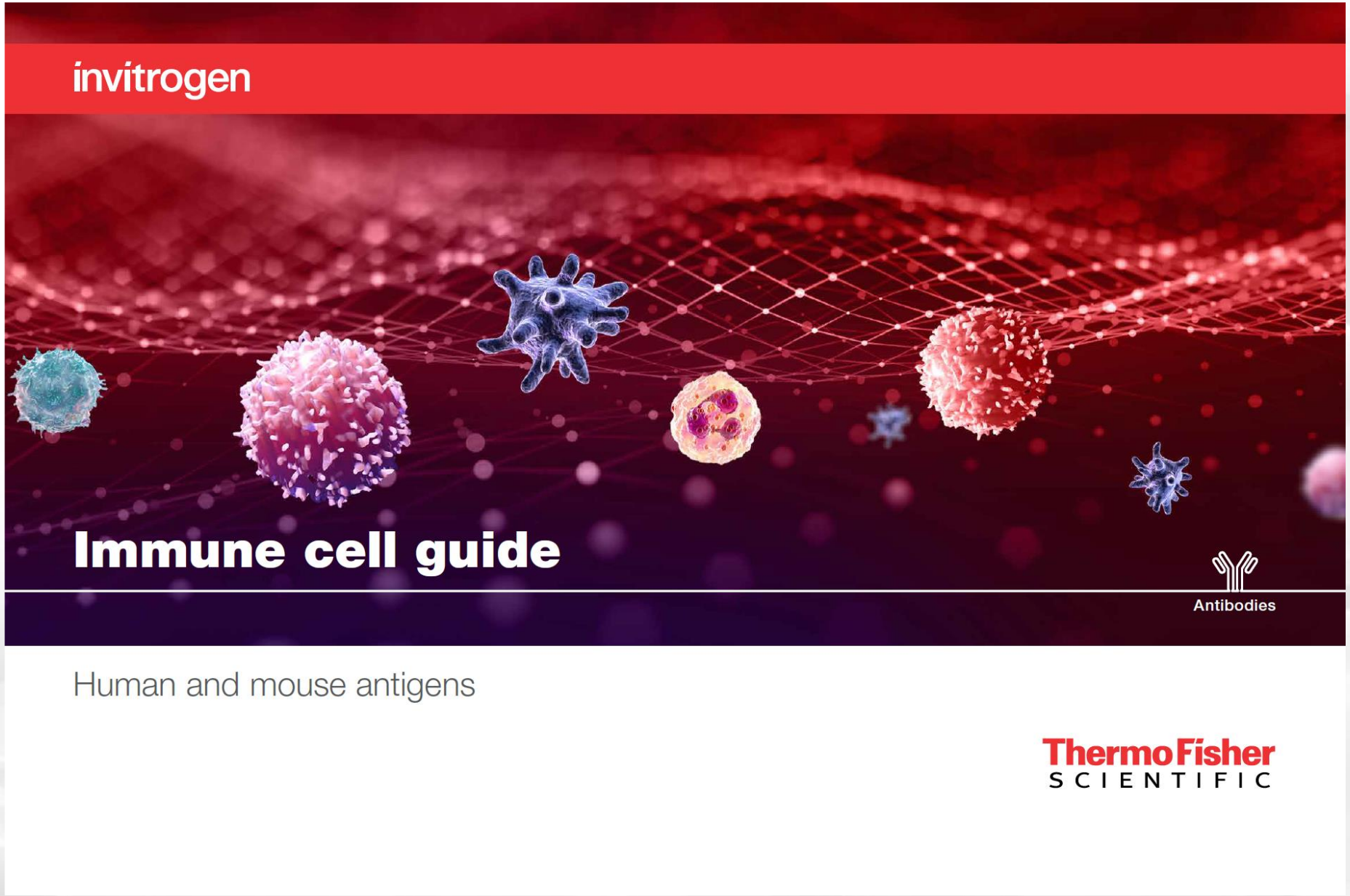
- CD4 Monoclonal Antibody (RM4-5), FITC
- Expression System
  Rat IgG2a kappa
- Recommended Isotype Control
  Rat IgG2a kappa Isotype Control (eBR2a), FITC

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





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

Thank you

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