Intracellular Cytokine Staining using PerFix-NC on the CytoFLEX Flow Cytometer

TECHNICAL INFORMATION BULLETIN

Introduction

Cytokines are important in mediating responses of the adaptive and innate immune systems. TNF-α is a critical inflammatory cytokine produced by both the myeloid and lymphoid lineage and plays an important role in chronic inflammatory diseases such as inflammatory bowel disease and rheumatoid arthritis (1). IFN-γ is another important cytokine that is vital for host defense against viral, bacterial and protozoan infections. IFN-γ is primarily produced by NK(T) cells and both CD4+ and CD8+ T cells (2).

Intracellular cytokine staining is a powerful tool to determine both levels of cytokine production and the cell subset producing the cytokine. However, each cytokine has different production/secretion kinetics, making dual visualization of cytokines difficult. Using a modified procedure with the PerFix-nc kit, we were able to visualize production of both TNF-α and IFN-γ after only four hours of stimulation, allowing for dual visualization of both these proteins.

Methods

Peripheral Blood Mononuclear Cells (PBMC) Isolation

Dispense the blood into an appropriately labeled 50 mL conical tube. Dilute with equal amount of DPBS. Cap and mix by inverting. In a new 50 mL dispense 17 mL of Lymphocyte Separation Medium (LSM). Carefully tilt the tube containing the LSM at an approximate 60 degree angle. Take the blood mixture tube and carefully touch the lip to the LSM tube and slowly decant to overlay blood onto LSM. As the tube fills slowly decrease the angle back to a vertical position. Pay close attention to the interface. Cap the blood overlay tube and centrifuge the tube for 25 minutes at 400g. Set centrifuge to stop without breaking. When centrifuge is stopped, carefully remove the interphase layer containing the PBMCs. Dispense into 15 mL conical tube. Add an equal volume of DPBS. Cap and mix by inverting. Centrifuge for 10 minutes at 400g. Aspirate all supernatant and resuspend to 5 mL with RPMI 1640 complete media. Make a 1:10 dilution of cell suspension with DPBS and perform a cell count.

PMA/Ionomycin Stimulation & PerFix-NC Procedure

PBMCs were stimulated for 4 hours in the presence of Brefeldin A (1 ng/mL) with PMA (80 nM) and Ionomycin (1 ug/mL) at 37° Celsius in a 24-well tissue culture plate. Cells were then washed in a 10x volume of PBS, centrifuged at 400g for 4 minutes. PBMC pellet was resuspended in 50 µL of fetal calf serum. Next, 50 µL of PBMCs, plus or minus PMA/Ionomycin stimulation, were then incubated with 5 µL (low fixation) or 12.5 µL (high fixation) of R1 buffer from the PerFix-NC kit. After incubation for 15 minutes at room temperature, 150 µL of PerFix-NC kit R2 buffer was added to each PBMC sample. Samples were vortexed and all antibodies, both for intracellular and extracellular staining, were added to each sample. Samples were vortexed and incubated for 60 minutes at room temperature in the dark. Next, 3 mL of PBS was added to each sample for 5 minutes. PBMCs were then centrifuged at 400g for 4 minutes. Supernatant was decanted and cell pellets were resuspended in 3 mL, 1x R3 buffer from the PerFix-NC kit. PBMCs were centrifuged at 400g for 4 minutes. Supernatant was decanted and cell pellets were resuspended in 500 µL of 1x R3 from the PerFix-NC kit. Samples were then acquired on a CytoFLEX flow cytometer.

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Laser	405 nm					488 nm						633 nm		
Fluore	Krome Orange	Pacific Blue*	V610	V660	BV785	FITC	PE	ECD	PC5	PC5.5	PC7	APC	APC-A700 (1)	APC-A750 (2)
Marker	CD3					IFN-γ	TNF-α	CD4						CD8
Clone	UCHT1					45.15	IMP2(188)	SFCI12T4D11						B9.11

Results

Intracellular Cytokine Staining with PerFix-NC Allows for Quick Assessment of TNF-α and IFN-γ

PBMCs were stimulated with or without PMA/Ionomycin for 4 hours in the presence of Brefeldin A. The PerFix-NC kit is designed to work with whole blood; therefore, a modified procedure is required to interrogate cytokine expression in purified PBMC preparations lacking protein found in whole blood.

As seen in Figure 1, 12.5 µL of the R1 fixative from the PerFix-NC buffer kit was utilized in the intracellular cytokine procedure. Figure 2 demonstrates data using 5 µL of the R1 fixative from the PerFix-NC buffer kit. The subsequent processing steps remained the same. Either high or low fixation allows for clear staining of TNF-α. However, IFN-γ staining was best observed with the higher fixation method (Table 1). This suggests that a modified PerFix-NC procedure can be utilized for intracellular cytokine staining. Additional effort may be required to optimize staining of a cytokine of interest.

References

(1) Bradley JR. TNF-alpha mediated inflammatory diseases. J. Pathol. 214(2): 149-160, 2008.

(2) Schroder K, Hertzog PJ, Ravasi T, Hume DA. Interferon-gamma: an overview of signals, mechanisms and functions. J. Leukoc. Biol. (75(2): 163-189, 2004.

Authors

"Intracellular Cytokine Staining using PerFix-NC on the CytoFLEX Flow Cytometer "

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Notes

The results demonstrated in this application sheet represent those generated on the Beckman Coulter CytoFLEX Flow Cytometer. As differences exist in the performance between analyzers, the author cannot guarantee a similar appearance with the use of other flow Cytometers.

Reagent Details

Reagent	Supplier	Item number
IFN-γ FITC	Beckman Coulter	IM2716U
TNF-α PE	Beckman Coulter	IM3279U
CD4 ECD	Beckman Coulter	6604727
CD56 PC5.5	Beckman Coulter	A79388
CD25 PC7	Beckman Coulter	A52882
CD8 APC-AF750	Beckman Coulter	A94686
CD3 Krome Orange	Beckman Coulter	B00068
CD44 BV 785	BioLegend	103041
Perfix-NC	Beckman Coulter	B31168
Phorbol 12-myristate 13-acetate (PMA)	Sigma	1585
Ionomycin	Sigma	10634
Brefeldin A	BioLegend	420601
Lymphocyte Separation Medium	Corning	25-072-C1
Dulbecco's Phosphate-Buffered Saline (1X DPBS)	Corning	21-031-CV

(1) APC-Alexa Fluor* 700
(2) APC-Alexa Fluor* 750

Figure 1

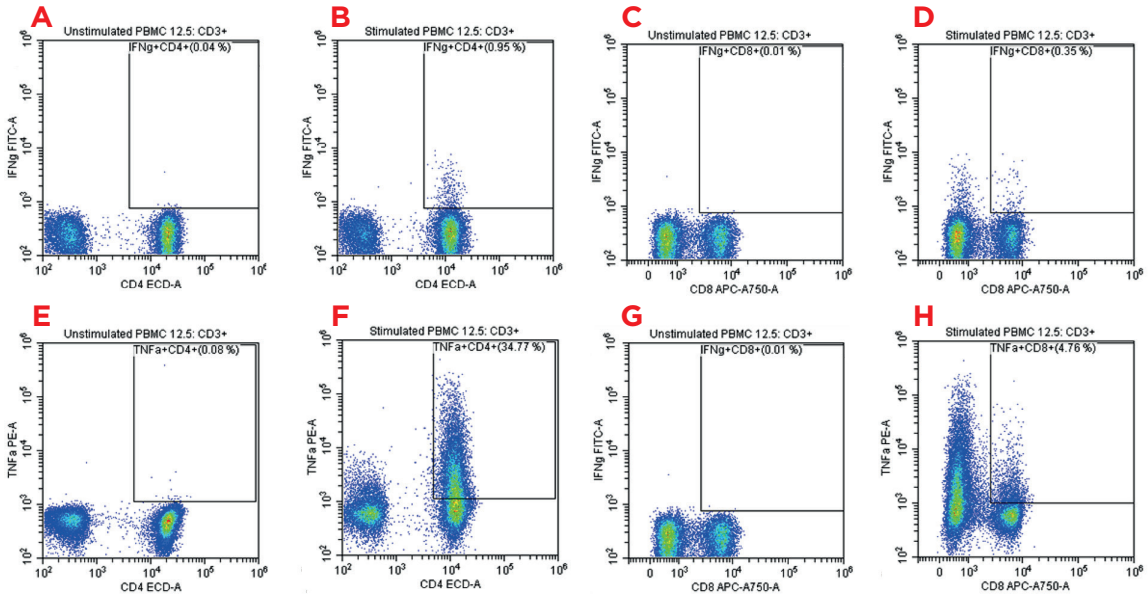

Figure 1. Intracellular Cytokine Staining with PerFix-nc Allows for Quick Assessment of TNF-a and IFN-g PBMCs were unstimulated (Figures 1A, C, E, G) or stimulated with PMA/Ionomycin (Figures B, D, F, H) after a PerFix-nc procedure that utilized 12.5 µL of the R1 fixative.

Figure 2

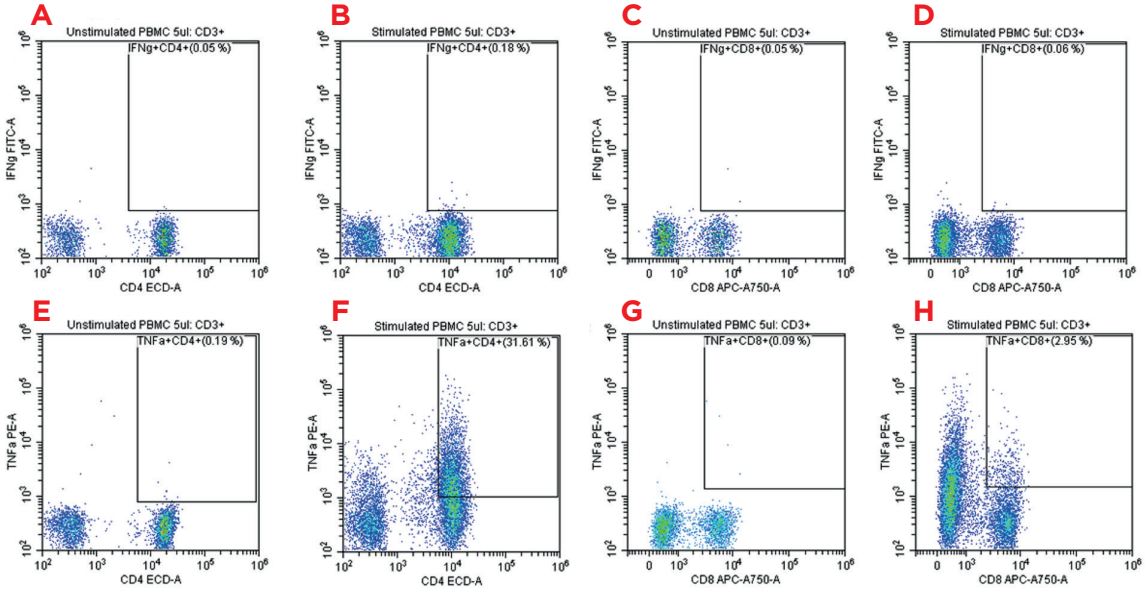

Figure 2. Intracellular Cytokine Staining with PerFix-nc Allows for Quick Assessment of TNF-a and IFN-g PBMCs were unstimulated (Figures 1A, C, E, G) or stimulated with PMA/Ionomycin (Figures B, D, F, H) after a PerFix-nc procedure that utilized 5 µL of the R1 fixative.

	% IFNγ+		% TNFα+	
	CD3+ CD4+	CD3+ CD8+	CD3+ CD4+	CD3+ CD8+
Unstimulated 12.5 µL	0.04	0.01	0.08	0.05
Stimulated 12.5 µL	0.95	0.35	34.77	4.76
Unstimulated 5 µL	0.05	0.05	0.09	0.09
Stimulated 5 µL	0.18	0.06	31.61	2.89

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