Analysis and sorting of cytokine polyfunctionality of human T cells using the MA900 Multi-Application Cell Sorter

Background

Immune-profiling of T cells involves the analysis of cell surface antigen and cytokine profiles. In previous studies, intracellular cytokine staining has been used to assess immunological properties. However, this method cannot detect secreted cytokines. Hydrogel-based particles, called Nanovials (Partillion Bioscience Corp), have been designed to address the need to detect secreted cytokines. These particles are precision engineered, include a cavity, and can be chemically modified to capture cells of interest and secreted cytokines. Nanovials enable researchers to analyze multiple phenotypes of cells, for example, human peripheral blood mononuclear cells (PBMCs), and to collect cells of interest according to their specific characteristics.

Multicolor Panel for the MA900 Multi-Application Cell Sorter

The Sony MA900 Multi-Application Cell Sorter (Figure 1) can be equipped with up to 4 lasers and can detect up to 14 parameters with 12 fluorescence and 2 scatter parameters. We designed the 12-color panel for the MA900 Cell Sorter by referring to the optimized multicolor immunofluorescence panel (OMIP-009) (Table 1). OMIP-009 is a multicolor panel with a total of ten colors: three cytokines, six cell surface antigens, and one viability dye. In our panel, we added two more cell surface antigens.

Figure 1. MA900 Multi-Application Cell Sorter

Laser	Filter	Fluorochrome	Marker
488 nm	525/50	AF488	CD45RA
	585/30	PE	TNF-α
	617/30	PI	PI
	695/50	PerCP-Cy™5.5	CD28
	785/60	PE-Cy™7	CD19
405 nm	450/50	BV421	IFN-γ
	525/50	BV510	CD197 (CCR7)
	585/30	BV570	CD14
	617/30	BV605	CD8
638 nm	665/30	APC	IL-2
	720/60	AF700	CD3
	785/60	APC-Cy7	CD4

APC: Allophycocyanin, AF: Alexa Fluor®, BV: Brilliant Violet, Cy: Cyanine, PE: Phycoerythrin, PI: Propidium Iodide

Table 1. 12-color panel for the MA900 Cell Sorter

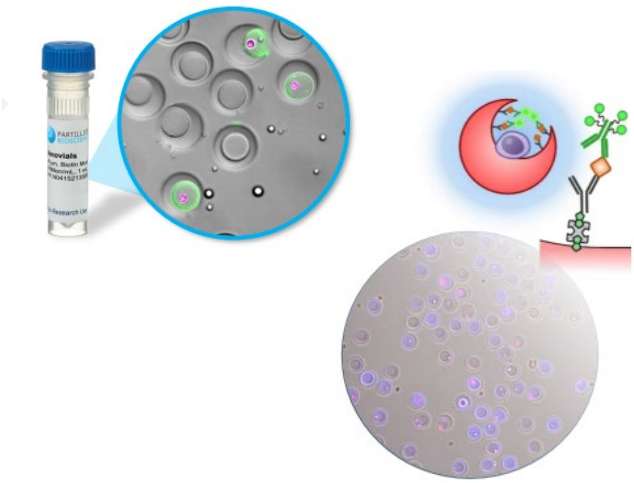
Methods

Nanovial preparation

Biotinylated Nanovials (Product Number NV1-35-BT-01, Partillion Bioscience Corp) were coated with streptavidin, followed by coating with biotinylated antibodies. To capture both cells and cytokines, Nanovials were coated with both biotinylated anti-CD45 and anti-cytokine antibodies.

Dynamic range measurement

Dilution series of recombinant cytokine protein were prepared, mixed with the prepared Nanovials, and incubated at room temperature for 30 minutes. After washing, Nanovials were mixed with fluorochrome-conjugated anti-cytokine antibodies (Table 1) and analyzed using the MA900 Cell Sorter with a 130-µm sorting chip (Part No. LE-C3213, Sony Corporation).



35-µm Nanovials
www.partillion.com

PBMC preparation

Human PBMCs (iQ Biosciences, Inc.) were cultured in KBM 501 (Kohjin Bio) supplemented with 10% fetal bovine serum (FBS), penicillin/streptomycin, anti-CD3, and anti-CD28 antibody at 37°C/5% CO_2. After 3 days, the media was changed to RPMI-1640 with 10% FBS, penicillin/streptomycin, and 5 ng/mL of IL-2. After an additional 3 days, we used these PBMCs to detect cytokine secretion.

Cell experiment with Nanovials

The antibody-coated Nanovials were gently mixed with the prepared PBMCs and incubated for 1 hour at 4°C. The cell-loaded Nanovial suspension was separated from unloaded cells by using a 20-µm cell strainer. We retrieved the sample remaining on top of the cell strainer. Then we stimulated this fraction with 25 ng/mL of phorbol myristate acetate (PMA) and 1 µg/mL of ionomycin for 4 hours at 37°C to induce cytokine secretion. After stimulating the sample, we stained the sample with fluorescent antibody conjugates as shown in Table 1 and analyzed it using the MA900 Cell Sorter with the 130-µm sorting chip.

Intracellular cytokine staining

The PBMCs were incubated in presence of Brefeldin A (BFA) to block cytokine secretion and stimulated with PMA/ionomycin for 4 hours at 37°C to induce cytokine production. After stimulating the sample, we stained the sample with fluorescent antibody conjugates as shown in Table, 1 followed by analysis on the MA900 Cell Sorter with the 130-µm sorting chip.

Results

Cytokine detection on Nanovials in a dynamic range of 10^5 to 10^7 molecules per Nanovial

Our panel was designed to detect three kinds of cytokines. For these cytokines, we explored the detection limit using recombinant proteins. Dilution series were prepared at a concentration in the orders of 0 (negative control), 10^5, 10^6, and 10^7 recombinant proteins per Nanovial particle, mixed with antibody-conjugated Nanovials, and the fluorescence was measured. The results are shown in Figure 2.

Measuring single-cell secretion of cytokines on Nanovials using the Sony MA900 Cell Sorter

Figure 3 shows the forward and back scattering (FSC-BSC scatter) plot of Nanovials. Indicated gates were sorted, and the sorted cells were observed under the microscope, where we found that gate B contained Nanovials with captured cells (see Figure 3-B).

Figure 4 shows the analysis of Nanovials loaded with stimulated or unstimulated PBMCs that were labeled with the 12-color panel. For comparison, four conditions were examined: Nanovials coated only with anti-CD45 antibody that can capture cells, but not secreted cytokines, or Nanovials coated with both anti-CD45 antibody and three cytokine-specific antibodies; for both conditions, stimulated and unstimulated PBMCs also were examined.

In Figure 5, intracellular cytokine staining confirmed cytokine accumulation in stimulated, BFA-treated PBMCs. In this study, cytokines did not accumulate intracellularly in unstimulated cells. However, smaller amounts of cytokines were also seen in stimulated cells without BFA treatment.

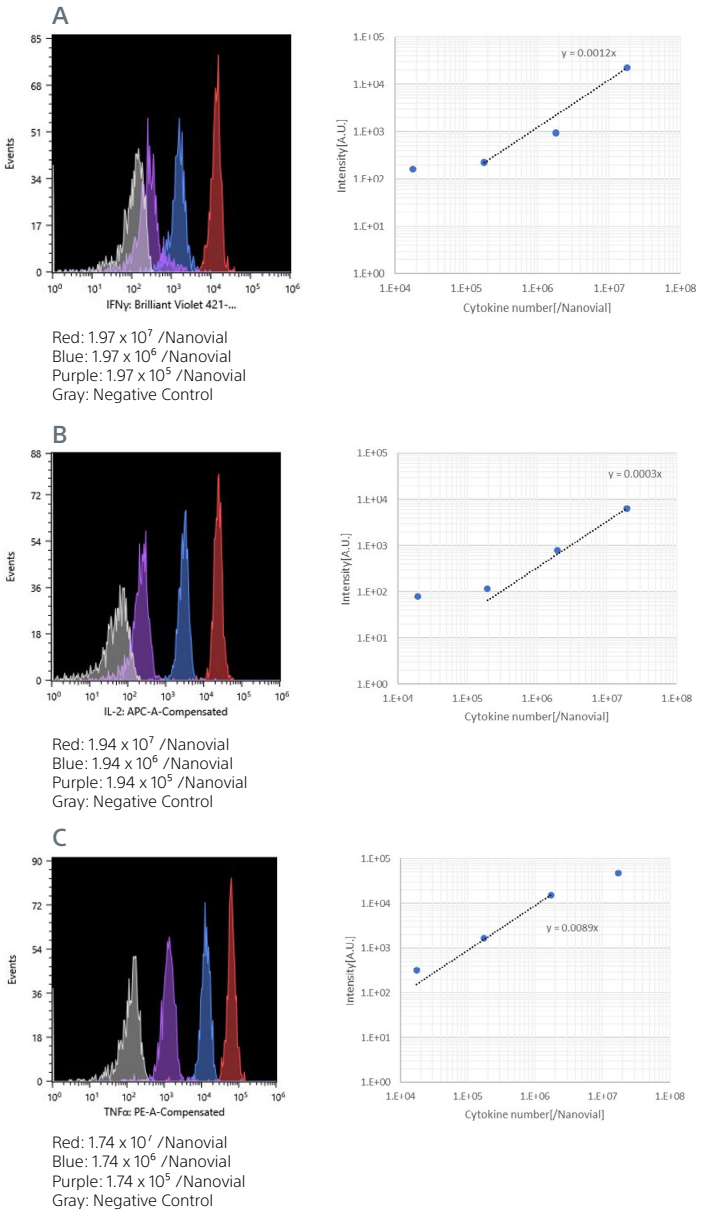


Figure 2. Limit of detection (LOD) and dynamic range determination for Nanovials using flow cytometry
Nanovials were coated with anti-cytokine (IFN-γ, IL-2, and TNF-α) and anti-CD45 antibodies, mixed with 10^5 to 10^7 molecules of (A) recombinant IFN-γ, (B) recombinant IL-2, or (C) recombinant TNF-α. After that, they were stained with fluorescent antibodies. Shown are the measurement results (histograms) and calibration curves.

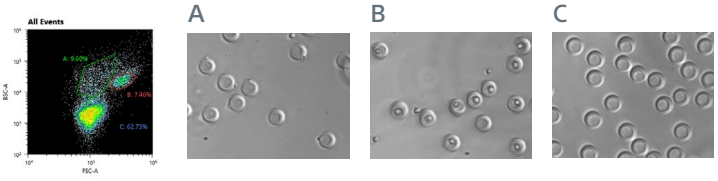


Figure 3. Example of flow cytometry scatter plot of Nanovials incubated with PBMCs
After sorting, in gate A, we found Nanovials bound to debris. In gate B, we confirmed Nanovials capturing single cells. Gate C was found to contain empty Nanovials.

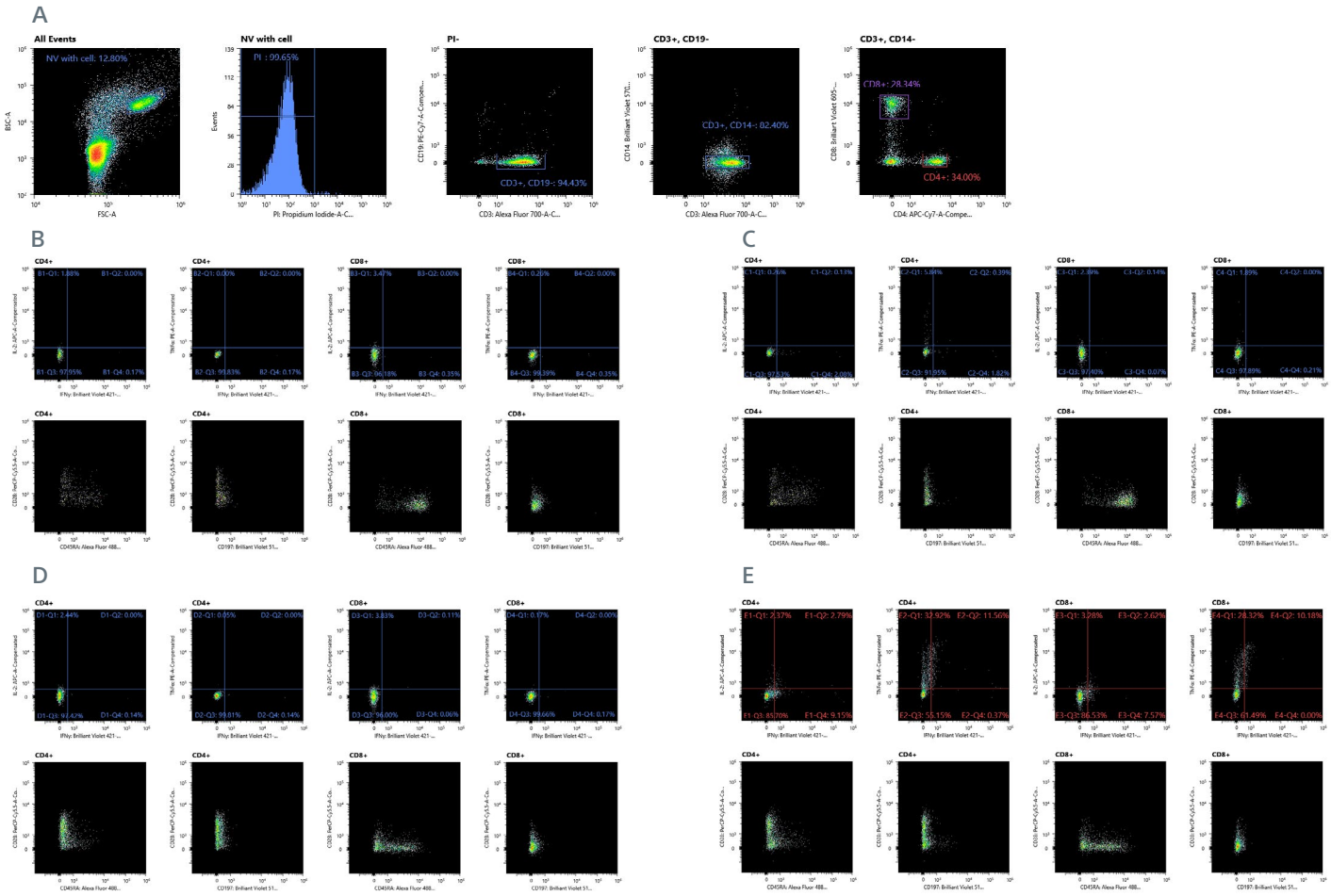


Figure 4. Multiplexed secretion profiling combined with cell surface labeling

A *All Events were gated by FSC-BSC scatter, and singlets were gated on Nanovials containing cells, viability (propidium iodide), and cell surface markers (CD3, CD14, CD19, CD4, and CD8).*

B *Nanovials coated with only anti-CD45 antibody were mixed with unstimulated PBMCs.*

C *Nanovials coated with only anti-CD45 antibody were mixed with PBMCs and incubated with stimulation.*

D *Nanovials coated with anti-CD45 antibody and anti-cytokine antibodies were mixed with unstimulated PBMCs.*

E *Nanovials coated with anti-CD45 antibody and anti-cytokine antibodies were mixed with stimulated PBMCs.*

Summary

The Sony MA900 Cell Sorter was able to analyze and sort Nanovials that were stained with a 12-color multicolor panel, consisting of 8 cell surface antigens, 3 types of cytokines, and 1 viability dye, and was able to sort them in a feature-dependent manner.

We found that the cell-bound Nanovials can be discriminated from empty or debris-bound Nanovials based on the scattering information, and can be sorted by the 130-µm sorting chip. Experiments with recombinant cytokines showed that sandwich-immunostaining on the Nanovial surface was possible when cytokines were present. Cell experiments using Nanovials showed that the cytokines secreted by stimulated PBMCs could be successfully captured by Nanovials. By intracellular cytokine staining, it was confirmed that PBMCs produced cytokines upon PMA/ionomycin stimulation. Intracellular cytokine staining experiments indicated that a certain amount of cytokine accumulates in stimulated cells even if BFA is not added. Our results show that Nanovials can be used to capture living cells distinguishable by multiple cell surface antigens and multiple secreted cytokines.

References

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2. Koo D, Dimatteo R, Lee S, de Rutte J, Di Carlo D. Sorting single T cells based on secreted cytokines and surface markers using hydrogel nanovials. bioRxiv. doi: https://doi.org/10.1101/2022.04.28.489940.

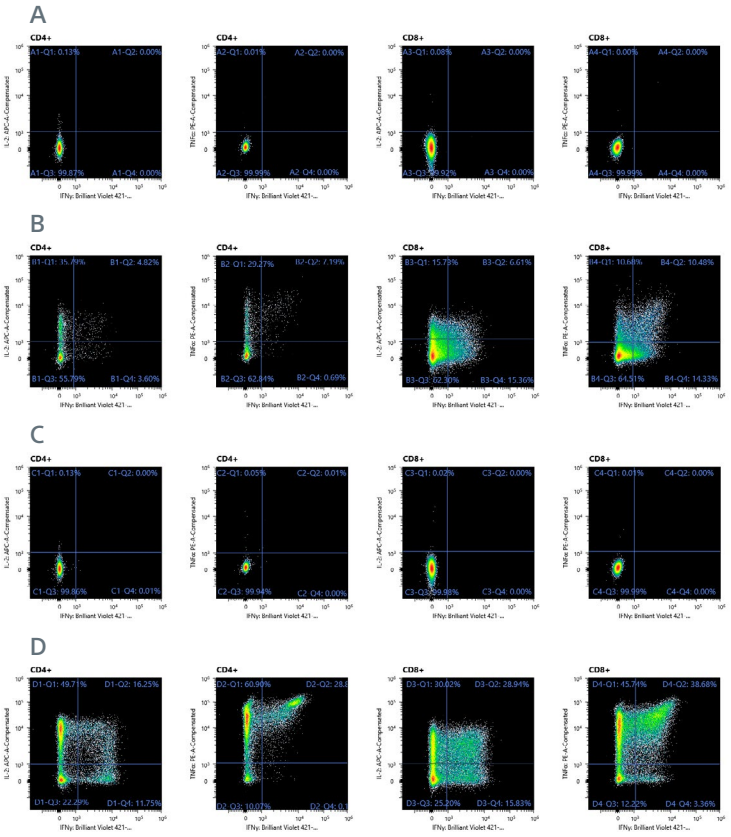

Figure 5. Multiplexed intracellular cytokine staining
A Unstimulated PBMCs, without BFA.
B Stimulated PBMCs, without BFA.
C Unstimulated PBMCs, treated with BFA.
D Stimulated PBMCs, treated with BFA.

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