Designing a T cell backbone panel using the Attune NxT Flow Cytometer

Paul Hutchinson and Myo Thanda Oo, Flow Cytometry Laboratory, Life Sciences Institute, National University of Singapore, Singapore

Introduction

T lymphocytes, a subset of white blood cells, are a major group of cells in the immune system and are essential for protection against infection and disease. Circulating T helper cells identified by the antigen CD4 have been used to study the efficacy of vaccines [1,2], diagnose and monitor infectious diseases [3], and monitor the effects of immunosuppressant drugs [4]. Each CD4 T cell has high specificity for a particular antigen, and can generate an immune response to eradicate organisms that express this antigen. After this, they will remain as memory antigen-specific CD4 T cells. These antigen-specific cells are quite rare in the circulating blood, with frequency ranging from 1 in 100 to less than 1 in 100,000 depending on the antigen and normal range variation [5]. Flow cytometry is an effective technology to monitor and identify rare cells among a mixed population of different cells types. Not only is it capable of rapidly identifying unique cell types, but it can also be used to analyze many other phenotypic features at the single-cell level, making it a valuable tool for understanding the immune system.

In order to accurately identify CD4 T cells, the T cells must first be separated from non–T cells through the use of unique markers (i.e., CD45, CD3, CD4, and CD8 to identify T cell subsets, and CD14, CD16, and CD19 to eliminate non–T cells from the analysis). Then, CD4 T cells can be isolated from the T cell subset. CD4 T cell identification is the basis of many studies and a backbone panel for the initial identification of leukocyte subsets gives the flexibility to add other markers used for deeper characterization of the target cells. For instance, one may want to further characterize the antigen-specific T cells with additional markers of activation and function. In this application note, a viability dye and 7 antibodies, including CD137 and CD69, were used as a backbone panel to identify antigen-specific CD4 T cells [6-9] using the Invitrogen™ Attune™ NxT Flow Cytometer, 4-laser configuration. This panel leaves room for additional markers to further identify CD4 T cell subsets and other changes to CD4 T cell homeostasis such as proliferation, cytokine production, and expression of activation markers on the cell surface.

The Attune NxT Flow Cytometer

Backbone panel design strategy

A 5-color, 8-marker panel was used to detect antigen-specific circulating CD4 T cells in human lymphocytes (Table 1). The antibodies for the backbone panel include CD3 and CD4 to identify the CD4 T cells, and CD137 and CD69 to identify the antigen-specific cells. One channel was chosen for the exclusion of non–T cells and all dead cells, which incorporated the use of additional antibodies to detect CD14 to identify monocytes, CD16 to identify most natural killer (NK) cells and neutrophils, CD19 to identify B cells, and a viability dye (Invitrogen™ LIVE/DEAD™ Fixable Near-IR Dead Cell Stain).

Basic principles of panel design were applied to maximize panel performance and to allow for the incorporation of additional antibodies of interest [10]. Importantly, bright fluorochromes were assigned to identify the cells that were expected to have the least number of antigen molecules available for antibody binding. Thus, Invitrogen™ PE and PE-Cy®7 conjugates were assigned to CD69 and CD137, respectively, to ensure the best resolution possible for these targets. Markers that are used to exclude cells in a single channel are often referred to as a "dump gate" or "dump channel". The 780/60 nm detector with the 637 nm red laser is used in this example as a dump channel, combining the Invitrogen™ eBioscience™ APC–eFluor™ 780 conjugate to detect 3 targets (CD14, CD16, and CD19) and the LIVE/DEAD Fixable Near-IR stain to identify dead cells. CD14, CD16, CD19 and dead cells can easily be excluded from the analysis by combining their detection in one channel. The APC detector (RL1 using 670/14 nm detection with the red 637 nm laser) and the FITC detector (BL1 using 530/30 nm detection with the blue 488 nm laser) are both left open, for drop-in of antibodies needed for each experiment.

Stimulation of antigen-specific T cells

Circulating T cells do not constitutively express CD69 or CD137; stimulants are used to activate T cells and upregulate the expression of these markers to help identify antigen-specific circulating CD4 T cells. In this protocol, we used tuberculin-purified protein derivative (PPD) [6,11] from *Mycobacterium tuberculosis* or a cytomegalovirus (CMV) cell lysate antigen preparation to stimulate whole blood or density gradient–separated peripheral blood mononuclear cells (PBMCs) for 24 hours. Samples cultured without antigen for the same time period were used as the negative control.

Table 1. Five-color human CD4 T cell backbone panel designed using the Attune NxT Flow Cytometer.

Laser/power	Channel	Filter	Marker	Fluor	Clone	Cat. No.	Dilution ratio	Purpose
405 nm (50mW)	VL1	440/50	CD4	Alexa Fluor 405	S3.5	MHCD0426	1:40	Lineage marker
	VL2	512/25	Open					
	VL3	603/48	Open					
	VL4	710/50	Open					
488 nm (50mW)	BL1	530/30	Open					
	BL2	590/40	Open					
	BL3	695/40	CD3	PerCP-eFluor 710	SK7	46-0036-41	1:20	Lineage marker
561 nm (50mW)	YL1	585/16	CD69	PE	CH/4	MHCD6904	1:20	Activation marker
	YL2	620/15	Open					
	YL3	695/40	Open					
	YL4	780/60	CD137	PE-Cy7	4B4-1	A14943	1:40	Antigen-induced activation
637 nm (100mW)	RL1	670/14	Open					
	RL2	723/30	Open					
	RL3	780/60	CD14	APC-eFluor 780	61D3	47-0149-42	1:40	Dump channel
			CD16		eBioCB16	47-0168-41	1:40	
			CD19		SJ25C1	47-0198-42	1:40	
			Viability	LIVE/DEAD Fixable Near-IR	N/A	L10119	1:100	

Figure 1 demonstrates the gating strategy used to identify the CD4 T cells. As shown in Figure 1A–C, the expression of CD69 and CD37 are detected only after cells were treated with the listed antigen preparations. After first identifying the lymphocytes and single cells (Figure 1D, E), the $CD3^+$ T cells are gated to exclude dead and non–T cells (Figure 1F), and then the $CD3^+$ $CD4^+$ T cells are gated on (Figure 1G).

Adding additional markers to the backbone panel

As discussed earlier, backbone panels can be designed such that additional markers can be easily incorporated (Table 2). In this panel, the FITC and APC channels were available for the addition of two markers, CD45RA and CD45RO, that are used to distinguish naive and memory T cells, respectively (option 1, Table 2). In this experiment, whole blood was stimulated with PPD and CMV as described in Figure 1, as well as the inactivated PR8 strain of influenza A virus. Whole blood cultured without antigen was used as the negative control. After gating on live, $CD3^+$ $CD4^+$ lymphocytes as in Figure 1, the expression of CD69 and CD137 was used again to identify the antigen-specific $CD4^+$ T cells that were found only in the samples stimulated with antigen (Figure 2A–D). Figure 2E–H shows the expression of CD45RA and CD45RO on the total $CD4^+$ cells, shown in red, and the $CD69^+$ $CD137^+$ antigen-specific $CD4^+$ T cells, shown in blue. As expected, the cells that are responding to antigen have a memory phenotype and express CD45RO but not CD45RA.

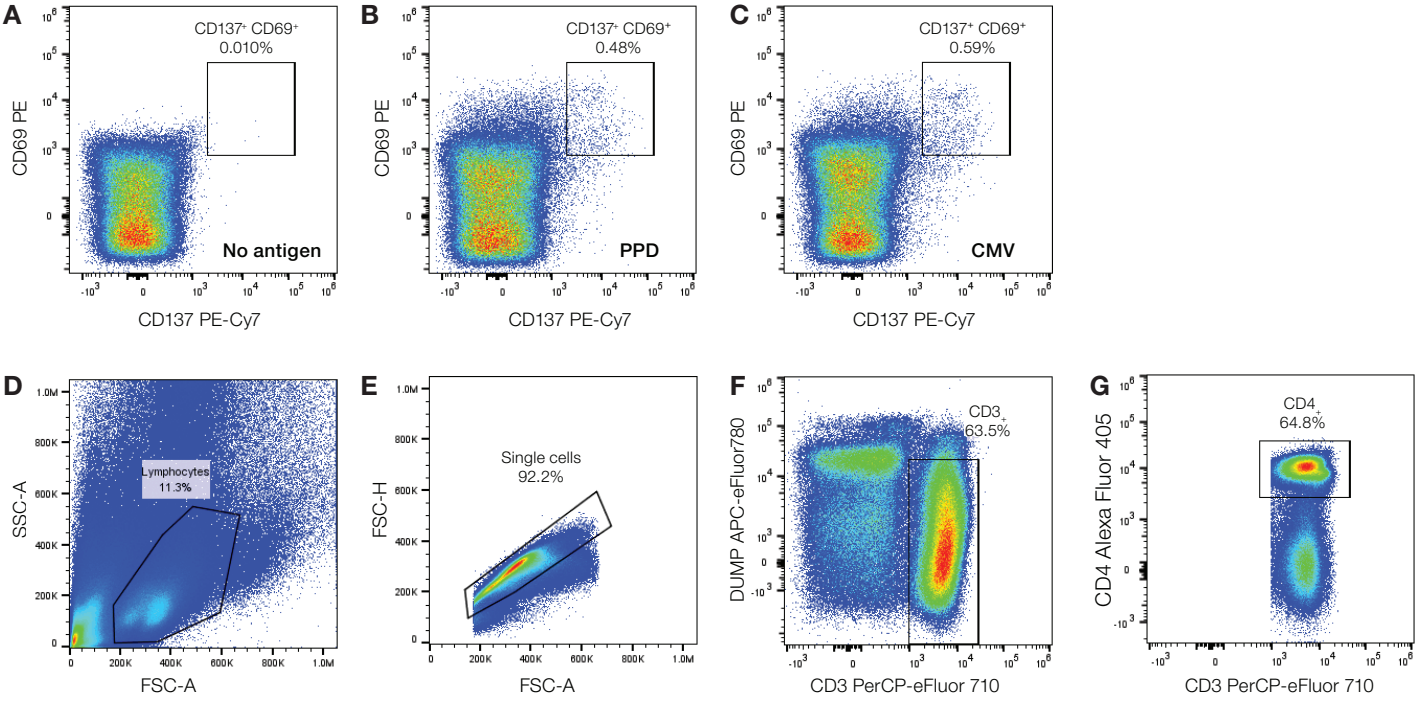

Figure 1. Five-color backbone panel for antigen-specific circulating CD4 T cells. (A) Two-parameter plots showing expression of CD69 and CD137 are shown for undiluted whole blood from a healthy donor that was cultured for 24 hours without antigen **(B)** or with PPD of Mycobacterium tuberculosis **(C)** or a CMV cell lysate antigen preparation. The cells were then harvested and stained with backbone panel antibodies including CD137, CD69, CD3, CD4, CD19, CD16, and CD14 with LIVE/DEAD Fixable Near-IR Dead Cell Stain for viability and analyzed on the Attune NxT Flow Cytometer. **(D)** Lymphocytes were identified using light scatter gates, **(E)** followed by gating on single cells, **(F, G)** and then dump channel$^-$ $CD3^+$ $CD4^+$ cells. Data courtesy of Paul Hutchinson and Myo Thanda Oo, Flow Cytometry Laboratory, Life Sciences Institute, National University of Singapore, Singapore.

Table 2. Options for adding markers to the backbone panel.

Laser/power	Channel	Filter	Marker	Fluor	Clone	Cat. No.	Dilution ratio	Purpose	Description
405 nm (50 mW)	VL1	440/50	CD4	Alexa Fluor 405	S3.5	MHCD0426	1:40	Lineage marker	Backbone, Figure 1
	VL2	512/25	Open						
	VL3	603/48	Open						
	VL4	710/50	Open						
488 nm (50 mW)	BL1	530/30	CD45RA	FITC	MEM-56	MHCD45RA01	1:20	Naive T cells	Option 1, Figure 2
			HLA-DR	FITC	L243	11-9952-41	1:20	Activation marker	Option 2, Figure 3
	BL2	590/40	Open						
	BL3	695/40	CD3	PerCP-eFluor 710	SK7	46-0036-41	1:20	Lineage marker	Backbone, Figure 1
561 nm (50 mW)	YL1	585/16	CD69	PE	CH/4	MHCD6904	1:20	Activation marker	Backbone, Figure 1
	YL2	620/15	Open						
	YL3	695/40	Open						
	YL4	780/60	CD137	PE-Cy7	4B4-1	A14943	1:40	Antigen-induced activation	Backbone, Figure 1
637 nm (100mW)	RL1	670/14	CD45RO	APC	UCHL1	MHCD45RO05	1:20	Memory T cells	Option 1, Figure 2
	RL2	723/30	Open						
	RL3	780/60	CD14	APC-eFluor 780	61D3	47-0149-42	1:40	Dump channel	Backbone, Figure 1
			CD16		eBioCB16	47-0168-41	1:40		
			CD19		SJ25C1	47-0198-42	1:40		
			Viability	LIVE/DEAD Fixable Near-IR	N/A	L10119	1:100		

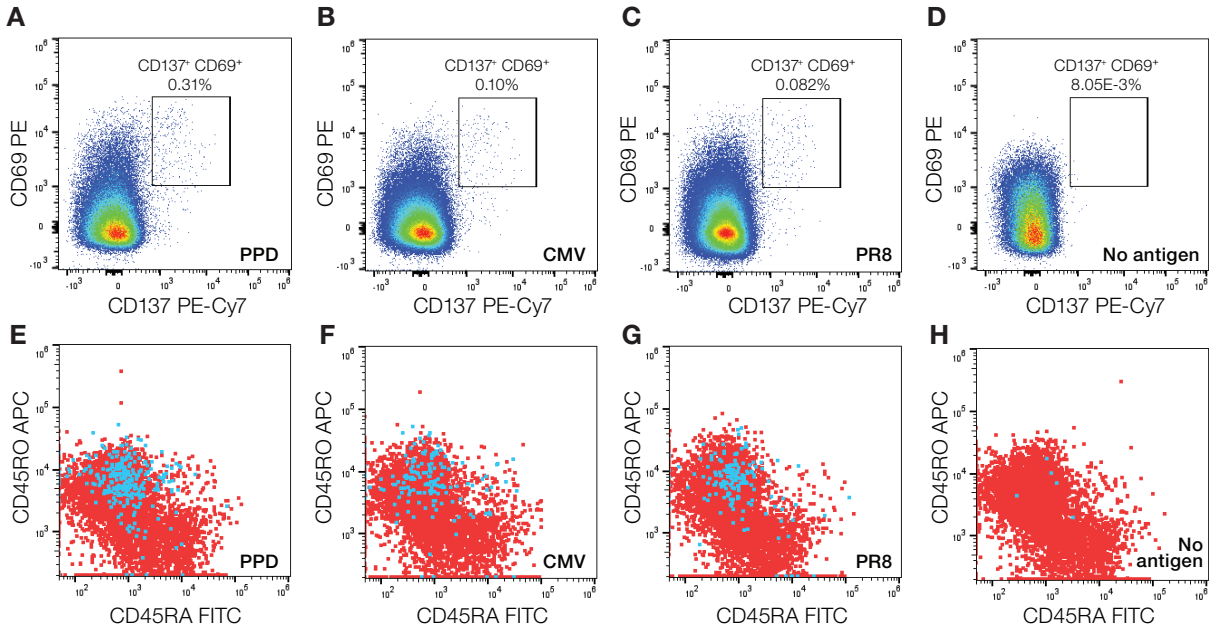

Figure 2. Seven-color panel for detecting CD45RA and CD45RO expression on antigen-specific CD4 T cells. Undiluted whole blood from a healthy donor was cultured for 24 hours with the different antigens listed. The cells were then harvested and stained as per the whole blood assay protocol. In this experiment, the backbone panel was used and anti-CD45RA FITC and anti-CD45RO APC were added (Option 1). The $CD3^+$ $CD4^+$ cells were gated as described in Figure 1 and then **(A–D)** analyzed for CD69 and CD137. **(E–H)** Total $CD3^+$ $CD4^+$ cells (red dots) and the $CD69^+$ $CD137^+$ antigen-specific $CD4^+$ T cells (blue dots) were further analyzed for expression of CD45RO and CD45RA. Data courtesy of Paul Hutchinson and Myo Thanda Oo, Flow Cytometry Laboratory, Life Sciences Institute, National University of Singapore, Singapore.

Using fluorescence minus one samples as control

In multicolor flow cytometry panels, it is essential to have controls to help define populations of interest. One type of control is the fluorescence minus one (FMO) sample that contains all of the fluorochrome-conjugated antibodies used in the panel, except for one. This means that for a 7-color panel, there would be 7 FMO controls: one for each color that is not included (Table 3). This sample is used as a negative control for gating, but also accounts for the spreading error that is introduced into the channel of interest from all other fluorochromes in the panel. The FMO controls are essential to any panel for interpreting the data correctly and have become a standard of practice.

In Figure 3, PBMCs were stimulated with PPD or cultured without antigen and then analyzed for the antigen-specific $CD4^+$ T cells (Figure 3A–C). As before, only when the cells have been stimulated with antigen are the $CD69^+$ $CD137^+$ cells present. In this experiment, the cells were stained with antibodies to human leukocyte antigen (HLA-DR) conjugated to FITC and CD45RO conjugated to APC. Since HLA-DR has a broad range of expression, it can be difficult to determine the positive signal. Figure 3E is the FITC FMO control sample—this is a sample that contains everything in the panel except for the HLA-DR antibody conjugated to FITC. The antigen-specific $CD4^+$ T cells are all expressing both HLA-DR and CD45RO (blue dots), while the total $CD4^+$ cells are heterogeneous for both markers (red dots).

Table 3. The FMO matrix.

FMO control	FITC	PerCP-eFluor 710	PE	PE-Cy7	APC	APC-eFluor 780	APC-eFluor 780	APC-eFluor 780	Alexa Fluor 405
Unstained	–	–	–	–	–	–	–	–	–
CD45RA FITC	–	CD3	CD69	CD137	CD45RO	CD14	CD16	CD19	CD4
CD3 PerCP-eFluor 710	CD45	–	CD69	CD137	CD45RO	CD14	CD16	CD19	CD4
CD69 PE	CD45	CD3	–	CD137	CD45RO	CD14	CD16	CD19	CD4
CD137 PE-Cy7	CD45	CD3	CD69	–	CD45RO	CD14	CD16	CD19	CD4
CD45RO APC	CD45	CD3	CD69	CD137	–	CD14	CD16	CD19	CD4
CD14 APC-eFluor 780	CD45	CD3	CD69	CD137	CD45RO	–	CD16	CD19	CD4
CD16 APC-eFluor 780	CD45	CD3	CD69	CD137	CD45RO	CD14	–	CD19	CD4
CD19 APC-eFluor 780	CD45	CD3	CD69	CD137	CD45RO	CD14	CD16	–	CD4
CD4 Alexa Fluor 405—FMO	CD45	CD3	CD69	CD137	CD45RO	CD14	CD16	CD19	–

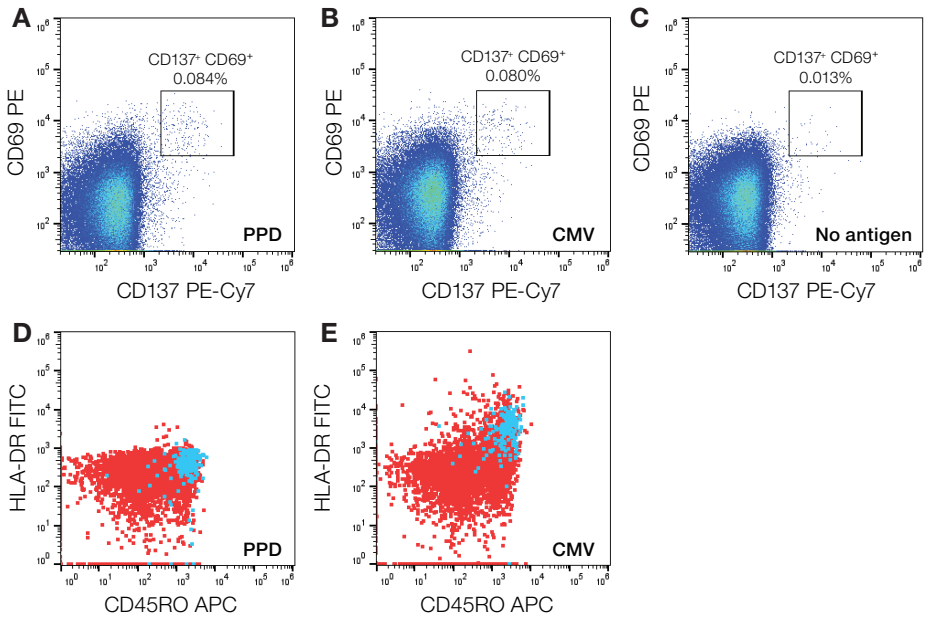

Figure 3. A FITC FMO control was used to define the HLA-DR–positive cells. PBMCs that were isolated from a healthy donor were cultured for 24 hours with or without PPD. The cells were then harvested and stained as per the whole blood assay protocol. In this experiment, the developed backbone panel was used and FITC-conjugated anti–HLA-DR and APC-conjugated anti-CD45RO antibodies were added (option 2 in Table 2). **(A–C)** The $CD3^+$ $CD4^+$ cells were gated as described in Figure 1 and then analyzed for CD69 and CD137. **(D, E)** Total $CD3^+$ $CD4^+$ cells (red dots) and the $CD69^+$ $CD137^+$ antigen-specific $CD4^+$ T cells (blue dots) were further analyzed for expression of HLA-DR and CD45RO, **(D)** and a FITC FMO control was used to determine the gate to define **(E)** HLA-DR–positive cells in the full panel. The samples were run on the Attune NxT Flow Cytometer. Data courtesy of Paul Hutchinson and Myo Thanda Oo, Flow Cytometry Laboratory, Life Sciences Institute, National University of Singapore, Singapore.

Staining protocols and materials

Materials used:

- Gibco™ PBS, pH 7.4 (Thermo Fisher Scientific, Cat. No. 10010031)
- Gibco™ RPMI 1640 Medium (Thermo Fisher Scientific, Cat. No. 11875101)
- Gibco™ Penicillin-Streptomycin (5,000 U/mL) (Thermo Fisher Scientific, Cat. No. 15070063)
- Gibco™ Streptomycin Sulfate, qualified, United States (Thermo Fisher Scientific, Cat. No. 11860038)
- Gibco™ Fetal Bovine Serum, qualified, United States (Thermo Fisher Scientific, Cat. No. 26140079)
- Invitrogen™ eBioscience™ Flow Cytometry Staining Buffer (Thermo Fisher Scientific, Cat. No. 00-4222-26)
- Invitrogen™ AbC™ Anti-Mouse Bead Kit (Thermo Fisher Scientific, Cat. No. A10344)
- Purified Protein Derivative (PPD) from *Mycobacterium tuberculosis* (Statens Serum Insitut, Denmark)
- Cytomegalovirus Cell Lysate Antigen (The Native Antigen Company, Cat. No. CMV-CL-100)
- PR8 virus (lab-made product, see below for details)
- Ficoll-Paque™ PLUS Medium (GE Healthcare, Cat. No. 17144002)
- SepMate™-15 tubes (STEMCELL Technologies, Cat. No. 86415)
- 24-well plate
- 50 mL conical tube
- 12 x 75 mm tubes

Red blood cell (RBC) lysis solution (10X)

- Components
  - 80.2 g NH_4Cl
  - 8.4 g $KHCO_3$
  - 3.7 g (0.01M) EDTA (disodium)
- Usage and storage
  - Dissolve in 1 L distilled water
  - Can be kept for up to 6 months at 4°C
  - Dilute to 1X in distilled water and use fresh

PR8 virus

The influenza A virus (A/PR/8/34, H1N1) was sourced from American Type Culture Collection (ATCC). It was heat-inactivated by keeping it at 80°C for 30 minutes.

Culture of PR8 virus in embryonated chicken eggs

- Components
  - Embryonated chicken eggs (8–10 days of embryonation)
  - Virus stock
  - Sterile PBS
  - Paraffin wax
- Protocol
  - Place embryonated chicken eggs in front of a light source and mark out (with a pencil) the location of the air sac and large blood vessels in the area of the allantoic cavity
  - Disinfect the egg shells with 70% ethanol
  - Use a 21-gauge needle to puncture the eggshell just below the air sac in a non-veined area (take care not to puncture the shell membrane)
  - Inject influenza virus stock diluted ~100 times in sterile PBS (<2 HAU/mL) into the allantoic cavity through the hole
  - Seal the hole with melted wax to prevent contamination
  - Incubate eggs in a 37°C incubator for 2.5 days
  - Refrigerate eggs at 4°C for 4 hours to kill the embryo and induce clotting of the blood
  - Cut the shell open along the air sac and collect the allantoic fluid, taking care not to puncture the egg yolk
  - Clarify the allantoic fluid by centrifugation at 3,000 x *g* at 4°C for 20 min and sterile filter with 0.22 µm filters
  - Aliquot and store at –80°C

Whole blood assay

1. Add 1 mL of undiluted blood (Li-Heparin) to two wells of a 24-well plate. Add desired antigen to one well (PPD–10 µg/mL, CMV lysate–1 µg/mL, or PR8–1:160). The second well will not have antigen.
2. Incubate at 37°C, 5% CO_2 for 24 hours.
3. Harvest cells into a 50 mL conical tube.
4. To lyse RBC, add 25 mL of 1X RBC lysis solution and incubate for 10 min at room temperature.
5. Centrifuge for 8 min at 700 x *g*.
6. Discard the supernatant and resuspend the pellet in 1 mL of 1X PBS.
7. Centrifuge for 4 min at 625 x *g*.
8. Discard the supernatant and resuspend the pellet in 100 µL of 1X PBS.
9. Add fluorochrome-conjugated antibodies and incubate for 20 min on ice in the dark.
10. Wash cells by adding 1 mL of staining buffer; then centrifuge for 4 min at 625 x *g*.
11. Discard supernatant and resuspend pellet in 500 µL of staining buffer.
12. Acquire cells on flow cytometer.

Isolated PBMC assay

1. Add 15 mL of Ficoll-Paque PLUS Medium to a SepMate tube through central hole of the insert. Dilute 15 mL of blood 1:1 with 1X PBS, and then layer on top of Ficoll-Paque PLUS Medium.
2. Centrifuge for 15 min at 1200 x *g* (with the brake on).
3. Pour top layer into 50 mL tube. Add 1X PBS to bring the final volume to 50 mL.
4. Centrifuge for 8 min at 700 x *g*.
5. Pour off supernatant. Resuspend pellet in 10 mL of 1X PBS and count cells.
6. Centrifuge for 8 min at 700 x *g*.
7. Discard supernatant and resuspend cells at 1 x 10^7 cells per mL in RPMI 1640 Medium with 10% FBS.
8. Add 200 µL of PBMCs to two wells of a 96-well round-bottom plate.
9. Add antigen to one well (same concentration as used for the whole blood assay), and do not add antigen to the second well.
10. Resuspend to mix well. Incubate at 37°C, 5% CO_2 for 24 hours.
11. Harvest cells from the wells into a 12 x 75 mm tube, and add 3 mL of staining buffer.
12. Centrifuge for 4 min at 625 x *g*.
13. Discard supernatant and resuspend pellet in 100 µL of staining buffer.
14. Add fluorochrome-conjugated antibodies and incubate for 20 min on ice in dark.
15. Add 1 mL of staining buffer.
16. Centrifuge for 4 min at 625 x *g*.
17. Discard supernatant and resuspend cells in 500 µL of staining buffer.
18. Acquire cells on flow cytometer.

Summary and conclusions

The aim of this study was to identify antigen-specific $CD4^+$ T cells by their upregulation of CD137 and CD69 expression using a core panel of antibodies. Several channels were left open, such as FITC and APC, so that this panel could serve as a backbone to which other markers of interest could be added in order to profile the phenotype and/or analyze the function of the antigen-specific $CD4^+$ T cells. The performance of this panel was demonstrated on whole blood samples and PBMCs from normal healthy human donors, stimulated with various antigens. This study further demonstrated the utility of FMO controls in determining HLA-DR–positive cells.

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