NCL Method ITA-37.2

Immunophenotyping: Analysis of Nanoparticle Effects on the Composition and Activation Status of Human Peripheral Blood Mononuclear Cells

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This protocol assumes an intermediate level of scientific competency with regard to techniques, instrumentation, and safety procedures. Rudimentary assay details have been omitted for the sake of brevity.

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1. Introduction

Immunophenotyping is the use of antigen expression for the identification of distinct immune cell subsets (and their activation statuses) [1-4]. This technique can detect minute changes in cell populations and thus is used to characterize the cell makeup in many diseases as well as determine effects of treatments, such as nanoparticles [5]. It is important to develop a method that allows for immunological evaluation of nanoparticles because some nanoparticles are designed to modify the immune system while others cause immunotoxicity [4, 5]. Currently, the most common technique used to perform immunophenotyping is multicolor flow cytometry [2, 3].

NCL protocol ITA-37 covers two separate immunophenotyping panels (with 11-12 antibody-fluorophore conjugates):

Immunophenotyping Panel #1 (or Lymphocyte Panel):

This panel includes antibody-fluorophore conjugates that allow for analysis of different lymphocyte populations including B cells and T cells (CD8+ T cells, CD4+ T cells, regulatory T (Treg) cells, naïve T cells, and γδ TCR T cells). This panel also determines cellular CD25 and CD154 expression which are markers of proliferation and co-stimulation/presentation, respectively.

Immunophenotyping Panel #2 (or Monocyte, Dendritic cell (DC), Natural Killer (NK) Cell Panel):

This panel includes antibody-fluorophore conjugates that allow for analysis of different cell populations including CD14+ monocytes, DCs (plasmocytoid (p) and myeloid (m) DCs), and NK cells along with NK T cells. This panel also examines cellular CD69 and CD54 expression which are markers of early activation and adhesion, respectively.

When used in conjunction with other immunoassays, the protocol aids in establishing efficacy and safety profiles of engineered nanoparticles used for vaccine or drug delivery. The protocol has two parts, ITA-37.1 described in a separate document and intended for instrument calibration, and ITA-37.2, described herein and intended for the analysis of nanoparticles-treated cells.

2. Principle

This protocol (ITA-37.2) describes the use of peripheral blood mononuclear cells (PBMC) derived from healthy donor volunteers and culture of these specimens in the presence of controls and nanoparticles to assess the effect of nanoparticles on various immune cell populations and their activation statuses. The changes in cell populations and activation status between the untreated negative control samples, the positive control samples, and the treatment samples will allow one to determine the effects of nanoparticles on immune cell status. A separate protocol (ITA-37.1) describes the procedure for the instrument calibration and qualification that is needed before proceeding with ITA-37.2.

The variety and complexity of immune cell phenotypes that can be assessed during immunophenotyping depends on the available flow cytometry instrumentation. This protocol is optimized for the flow cytometer NovoCyte 3005 by Acea Biosciences Inc. (part of Agilent Technologies), which has the capacity to acquire data from 14 distinct fluorophores simultaneously. NovoCyte 3005 is equipped with 3 lasers (405 nm, 488 nm, and 640 nm) and 6 detectors (445/45 nm, 530/30 nm, 572/28 nm, 660/20 nm, 725/40 nm and 780/60 nm) [6].

If another cytometer is used, the procedure described herein would require an optimization according to the technical specifications of that instrument.

3. Reagents, Materials, and Equipment

Note: The NCL does not endorse any of the suppliers listed below; these reagents were used in the development of the protocol and their inclusion is for informational purposes only. Equivalent supplies from alternate vendors can be substituted. Please note that suppliers may undergo a name change due to a variety of factors. Brands and part numbers typically remain consistent but may also change over time.

3.1 Reagents for PBMC cultures

3.1.1 Human blood anti-coagulated with Li-heparin and obtained from healthy donors

3.1.2 Phosphate buffered saline (PBS) (GE Life Sciences, SH30256.01)

3.1.3 RPMI-1640 (GE Life Sciences, HyClone, SH30096.01)

3.1.4 Fetal bovine serum (GE Life Sciences, HyClone, SH30070.03)

3.1.5 Penicillin streptomycin solution (GE Life Sciences, Hyclone, SV30010)

3.1.6 L-glutamine (GE Life Sciences, Hyclone, SH30034.01)

3.1.7 Ficoll Paque Premium (GE Healthcare, 17-5442-03)

3.1.8 Hank's balanced salt solution (HBSS) (Gibco, 14175-095)

3.1.9 ViaStain AOPI Staining solution (Nexcelom Biosciences, CS2-0106-5mL)

3.2 Controls

3.2.1 Ultrapure LPS from K12 E.coli (Invivogen, tlrl-peklps)

3.2.2 Phytohemagglutinin (PHA-M) (Sigma, L8902)

3.2.3 Phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich, P1585)

3.2.4 Ionomycin (STEMCELL Technologies, 73722)

3.2.5 Oligodeoxyribonucleotide, Human TLR9 ligand (ODN2216) (InvivoGen, tlrl-2216-1)

Table 1. Guidance on Concentrations and Purpose of Positive Controls

Description/ Control	LPS	PHA-M	PMA	Ionomycin	ODN2216
Primary purpose	Positive control for lymphocyte activation (CD25)	Positive control for lymphocyte activation (CD25; CD69; CD54)	Positive control for lymphocyte activation (CD25; CD154)	Positive control for lymphocyte activation (CD25; CD154)	Positive control for monocyte and dendritic cell activation (CD69; CD54)
Final concentration in assay	20 ng/mL	10 µg/mL	50 ng/mL	1 µg/mL	5 µg/mL

Note: Other agents can be used as the assay positive control.

3.3 Reagents for Flow Cytometry

3.3.1 eBioscience Flow Cytometry Staining Buffer (Invitrogen, 00-4222-26)

3.3.2 Paraformaldehyde (PFA) 20% Solution (Electron Microscopy Science, 15713)

3.3.3 NovoRinse (Agilent Technologies, 872B603)

3.3.4 NovoClean (Agilent Technologies, 872B602)

3.3.5 NovoFlow (Agilent Technologies, 871B607)

3.3.6 Antibodies, Dyes, and Isotype controls (Tables 2-5)

3.4 Equipment and Materials

3.4.1 Pipettes covering a range of 0.05 to 10 mL

3.4.2 24-well round bottom plates

3.4.3 Polypropylene tubes, 15 and 50 mL

3.4.4 Microcentrifuge tubes

3.4.5 5 mL Polystyrene Round bottom tube; 12x75 mm

3.4.6 Centrifuge

3.4.7 Refrigerator, 2-8 ºC

3.4.8 Freezer, -20 ºC

3.4.9 Freezer, -80 ºC

3.4.10 Cell culture incubator with 5% CO_2 and 95% humidity.

3.4.11 Biohazard safety cabinet approved for level II handling of biological material

3.4.12 Water Bath

3.4.13 Vortex

3.4.14 Cellometer Auto2000

3.4.15 Acea Novocyte 3005

Table 2. Anti-human Labeling Antibodies used in the Immunophenotyping Panel 1 (Lymphocyte Panel)

Fluorochrome	Marker	Cell Type	Purpose	Company	Concentration (µg/mL)	Catalog #
FITC	CD8a	T cells	Cytotoxic T cells	BioLegend	200	300905 or 300906
PE	CD4	T cells	T-helper	BioLegend	100	317409 or 317409
PE-Cy7	CD19	B cells	LPS co-receptor	BioLegend	100	302215 or 302216
APC	CCR4	T-reg	CD194	BioLegend	50	359407 or 359408
AF700	CD45RA	Naïve cells	Memory status	Invitrogen	50	56-0458-42
APC-Fire 750	TCR-γδ	TCR- γδ T cells	TCR- γδ receptor	BioLegend	400	331227 or 331228
PacBlue	CD45	All leukocytes	Pan-leukocyte	BioLegend	100	368539 or 368540
Zombie Aqua	Live/dead	All cells	Excludes dead cells	BioLegend	Not Available	423101 or 423102
BV570	CD3	T cells	T cells	BioLegend	80	300435 or 300436
BV650	CD25	T-reg and activation status of cells	IL-1 receptor α	BioLegend	100	302633 or 302634
BV711	CD154	Lymphocytes	CD40L	BioLegend	100	310837 or 310838
BV785	CD127	T-reg	IL-7 receptor α	BioLegend	50	351329 or 351330

Table 3. Isotype Antibodies used in the Immunophenotyping Panel 1 (Lymphocyte Panel)

Fluorochrome	Isotype	Clone	Company	Concentration (µg/mL)	Catalog #
FITC	Mouse IgG1, K	MOPC-21	BioLegend	200	400109 or 400110
PE	Mouse IgG2b, K	MPC-11	BioLegend	200	400313 or 400314
PE-Cy7	Mouse IgG1, K	MOPC-21	BioLegend	200	400125 or 400126
APC	Mouse IgG1, K	MOPC-21	BioLegend	200	400121 or 400122
AF700	Mouse IgG2b kappa	eBMG2b	Invitrogen	200	56-4732-80
APC-Fire 750	Mouse IgG1, K	MOPC-21	BioLegend	200	400195 or 400196
PacBlue	Mouse IgG1, K	MOPC-21	BioLegend	500	400131
BV570	Mouse IgG1, K	MOPC-21	BioLegend	100	400159 or 400160
BV650	Mouse IgG1, K	MOPC-21	BioLegend	100	400163 or 400164
BV711	Mouse IgG1, K	MOPC-21	BioLegend	100	400167 or 400168
BV785	Mouse IgG1, K	MOPC-21	BioLegend	100	400169 or 400170

Table 4. Anti-human Labeling Antibodies for Immunophenotyping Panel 2 (Monocyte, DC, NK Cell Panel)

Fluorochrome	Marker	Cell Type	Purpose	Company	Concentration (µg/mL)	Catalog #
FITC	CD56	NK cells	Neural cell adhesion molecule	BioLegend	200	362545 or 362546
PE	CD14	Monocytes	LPS co-receptor	BioLegend	200	301805 or 301806
PE-Cy7	CD19	B cells	LPS co-receptor	BioLegend	100	302215 or 302216
APC	CD123	pDCs	Interleukin-3 receptor	BioLegend	100	306011 or 306012
AF700	CD54	Lymphocytes and monocytes	Adhesion	BioLegend	400	353125 or 353126
APC-Fire 750	CD20	B cells	B cells	BioLegend	200	302357 or 302358
PacBlue	CD45	All leukocytes	Pan-leukocyte	BioLegend	100	368539 or 368540
Zombie Aqua	Live/dead	All cells	Excludes dead cells	BioLegend	Not Available	423101 or 423102
BV570	CD3	T cells	T cells	BioLegend	80	300435 or 300436
BV650	CD69	Neutrophils/ monocytes	Early activation	BioLegend	50	310933 or 310934
BV785	CD11c	mDCs	Integrin α x	BioLegend	160	301643 or 301644

Table 5. Isotype Antibodies for Immunophenotyping Panel 2 (Monocyte, DC, NK Cell Panel)

Fluorochrome	Isotype	Clone	Company	Concentration (µg/mL)	Catalog #
FITC	Mouse IgG1, K	MOPC-21	BioLegend	200	400109 or 400110
PE	Mouse IgG2a, K	MOPC-173	BioLegend	200	400211
PE-Cy7	Mouse IgG1, K	MOPC-21	BioLegend	200	400125 or 400126
APC	Mouse IgG1, K	MOPC-21	BioLegend	200	400121 or 400122
AF700	Mouse IgG1, K	MOPC-21	BioLegend	500	400143
APC-Fire 750	Mouse IgG2b, K	MPC-11	BioLegend	200	400371 or 400372
PacBlue	Mouse IgG1, K	MOPC-21	BioLegend	500	400131
BV570	Mouse IgG1, K	MOPC-21	BioLegend	100	400159 or 400160
BV650	Mouse IgG1, K	MOPC-21	BioLegend	100	400163 or 400164
BV785	Mouse IgG1, K	MOPC-21	BioLegend	100	400169 or 400170

4. Preparation of Reagents and Controls

4.1 Complete RPMI-1640 medium

The complete RPMI medium should contain the following reagents:

10% FBS (heat inactivated)

2 mM L-glutamine

100 U/mL penicillin

100 µg/mL streptomycin

Store at 2-8 °C protected from light for no longer than 1 month. Before use, warm the media in a water bath.

4.2 Heat inactivated fetal bovine serum

Thaw a 50 mL aliquot of fetal bovine serum and equilibrate to room temperature. Place the tube in a water bath set up to 56 °C and incubate with mixing for 35 min. The heat inactivation takes 30 min and the initial 5 min is used to bring the entire content of the vial to 56 °C. Chill the serum and use to prepare complete culture media.

4.3 Lipopolysaccharide (LPS, 1 mg/mL stock)

E.coli K12 LPS is supplied as lyophilized powder. Reconstitute by adding 1 mL of sterile water to 1 mg of LPS in the vial and vortex to mix. Stocks with higher concentration (5-10 mg/mL) can also be prepared. Aliquot 20 µL and store at a nominal temperature of -20 °C. Avoid repeated freeze- thaw cycles. On the day of experiment thaw one aliquot and use such as its final concentration in PBMC or WB culture is 20 ng/mL.

4.4 Phytohemagglutinin (PHA-M, 1mg/mL stock)

Add 1 mL of sterile PBS or cell culture medium per 1 mg of PHA-M to the vial and gently rotate to mix. Store daily use aliquots at a nominal temperature of -20ºC. Avoid repeated freezing/thawing. On the day of experiment dilute stock PHA-M solution in cell culture medium so the final concentration in the positive control sample is 10 µg/mL.

4.5 PMA (1 mg/mL stock)

Reconstitute in DMSO to a final concentration of 1 mg/mL. Prepare single use 20 µL aliquots and store at -20 °C. On the day of experiment thaw an aliquot at room temperature and dilute in culture media so the final concentration in the test sample is 50 ng/mL.

4.6 Ionomycin (10 mg/mL stock)

Ionomycin is supplied in ionomycin free acid 1%, ethyl alcohol 99%. Prepare single use 5 µL aliquots and store at -20 °C. On the day of experiment thaw an aliquot at room temperature and dilute in culture media so the final concentration in the test sample is 1 µg/mL.

4.7 ODN 2216 (1mg/mL stock)

This oligonucleotide is supplied as lyophilized powder. Reconstitute in pyrogen-free, nuclease-free water to a final concentration of 1 mg/mL. Prepare single use 5 µL aliquots and store at -20 °C. On the day of experiment thaw an aliquot at room temperature and dilute in culture media so the final concentration in the test sample is 5 µg/mL.

4.8 Vehicle Control

Vehicle control is the buffer or media used to formulate test nanomaterials. Common excipients used in nanoformulations are trehalose, sucrose, and albumin. However, other reagents and materials are also used alone or in combination. Vehicle control should match formulation buffer of the test-nanomaterial by both composition and concentration.

5. Preparation of Nanoparticles

The total amount of nanoparticle sample needed for testing these two immunophenotyping panels is at least 1.0 mL per donor at testing concentration (when minimal required dilution (MRD) is 10). The nanoparticle concentration is selected based on the plasma concentration of the nanoparticle at the intended therapeutic dose. For the purpose of this protocol this concentration is called "theoretical plasma concentration". Considerations for estimating theoretical plasma concentration were reviewed elsewhere [7] and are summarized in Box 1 below.

Box 1. Example Calculation to Determine Nanoparticle Concentration for In Vitro Tests

In this example, we assume a mouse dose of 123 mg/kg. Therefore, the scaled equivalent human dose would be:

$$Human\ dose = \frac{mouse\ dose}{12.3} = \frac{123\ mg/kg}{12.3} = 10\ mg/kg$$

Blood volume constitutes approximately 8% of the body weight. Therefore, an average human of 70 kg body weight has approximately 5.6 L of blood. Assuming all the nanoparticle injected goes into the systemic circulation, this provides a rough approximation of the potential maximum nanoparticle concentration in blood, which is used as the in vitro test concentration.

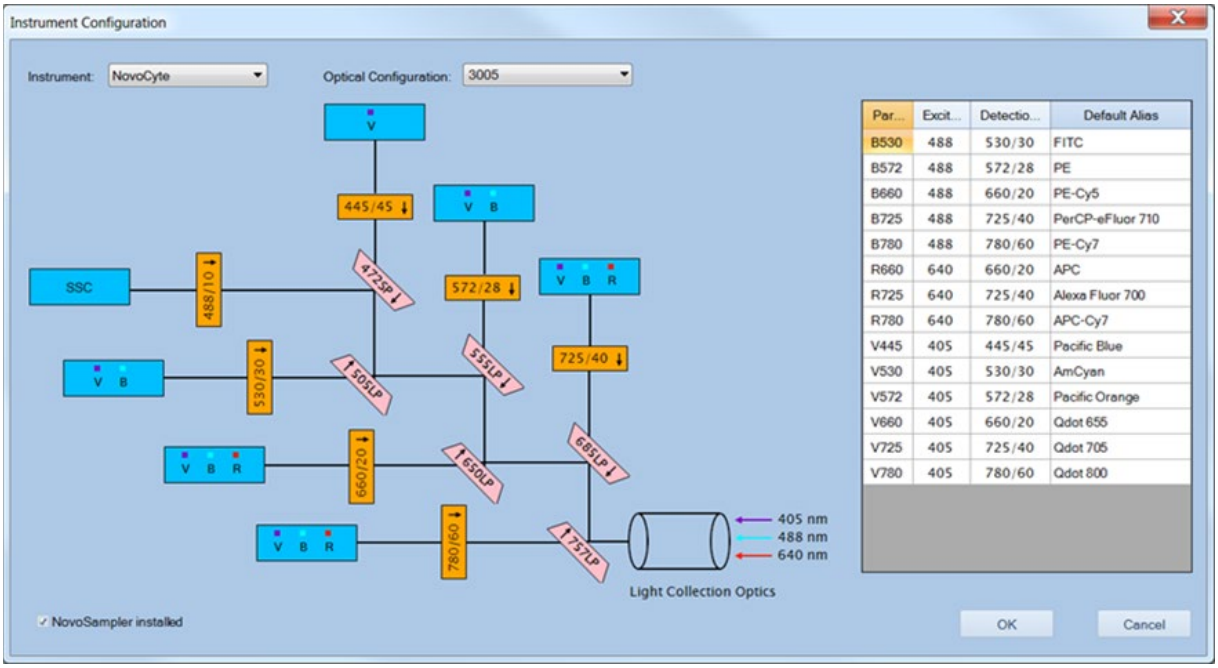
$$in\ vitro\ concentration_{human\ matrix} = \frac{human\ dose}{human\ blood\ volume} = \frac{70\ kg * 10\ mg/kg}{5.6\ L} = 0.125\ mg/mL$$

The assay can evaluate 1-3 concentrations of one nanoparticle at a time: 10X (or when feasible 100X, 30X or 5X) of the theoretical plasma concentration, theoretical plasma concentration, and one 1:5 serial dilution of the theoretical plasma concentration. When the intended therapeutic concentration is unknown, the highest final concentration is 1 mg/mL or the highest reasonably achievable concentration using a MRD of 10.

For example, if the final theoretical plasma concentration to be tested is 0.2 mg/mL, then a stock of 10 mg/mL will be prepared and diluted 5-fold (2 mg/mL), followed by a dil 5 serial dilution (0.4 mg/mL). When 100 µL of each of these samples are combined in a culture plate well with 800 µL of cells + 100 µL complete media (1 mL total volume), the final concentrations of nanoparticles are 0.04, 0.2, and 1 mg/mL.

6. NovoCyte 3005 Instrument Settings

Important Note: The following experiments were optimized on the NovoCyte 3005 with the settings shown in Figure 1. If a different cytometer is used, then instrument calibration or fluorescent labels may need to be changed.



Par...	Excit...	Detectio...	Default Alias
B530	488	530/30	FITC
B572	488	572/28	PE
B660	488	660/20	PE-Cy5
B725	488	725/40	PerCP-eFluor 710
B780	488	780/60	PE-Cy7
R660	640	660/20	APC
R725	640	725/40	Alexa Fluor 700
R780	640	780/60	APC-Cy7
V445	405	445/45	Pacific Blue
V530	405	530/30	AmCyan
V572	405	572/28	Pacific Orange
V660	405	660/20	Qdot 655
V725	405	725/40	Qdot 705
V780	405	780/60	Qdot 800

Figure 1. Settings on Novocyte 3005 [8].

7. Experimental Conditions Necessary for Preparation of Immunophenotyping Panels #1 and #2

Important Notes: Please see Tables 6-7 for the conditions/samples needed for Immunophenotyping Panels #1 and #2 (two replicates per condition). If there more than 65 samples total (between the two immunophenotyping panels), it is suggested to perform the two immunophenotyping panels separately. Please see Tables 2-5 for antibodies/dyes included in isotype control samples and fully stained labeling antibody samples.

Table 6. Conditions for Immunophenotyping Panel #1

Unstained Samples	Isotype Control Samples	Fully Stained Labeling Antibody Samples
Negative Control: Unstimulated	Negative Control: Unstimulated (x2)	Negative Control: Unstimulated (x2)
Positive Control: LPS/PHA-M	Positive Control: LPS/PHA-M (x2)	Positive Control: LPS/PHA-M (x2)
Positive Control: PMA/Ionomycin	Positive Control: PMA/Ionomycin (x2)	Positive Control: PMA/Ionomycin (x2)
Vehicle Controls	Vehicle Controls (x2)	Vehicle Controls (x2)
Test samples (MRD 10)	Test samples (MRD 10) (x2)	Test samples (MRD 10) (x2)

Table 7. Conditions for Immunophenotyping Panel #2

Unstained Samples	Isotype Control Samples	Fully Stained Labeling Antibody Samples
Negative Control: Unstimulated	Negative Control: Unstimulated (x2)	Negative Control: Unstimulated (x2)
Positive Control: ODN2216/PHA-M	Positive Control: ODN2216/PHA-M (x2)	Positive Control: ODN2216/PHA-M (x2)
Vehicle Controls	Vehicle Controls (x2)	Vehicle Controls (x2)
Test samples (MRD 10)	Test samples (MRD 10) (x2)	Test samples (MRD 10) (x2)

Note: Experimental procedure takes at least 2 days but realistically takes 3 days per donor as each donor needs its own unstained and isotype control samples. Day 1 consists of isolation and treatment of PBMC. Day 2 involves staining of PBMC for flow cytometry. Flow cytometry samples can be run on Day 2 or 3.

8. Collection and Handling of Whole Blood (Day 1)

This step requires approval by an Institutional Review Board (IRB) or another relevant board; please consult your organization for details on appropriate regulations within your research organization. Collect whole blood from healthy donor volunteers who have not been on medication and clear from infection for at least 2 weeks prior to blood donation. Use Li-heparin tubes and discard first 10 cc. For the best results whole blood should be used within 1 hour after collection. Prolonged storage (> 2 hr) of whole blood will lead to a decrease in cell function.

9. Preparation of PBMC

9.1 Place freshly drawn blood into 15- or 50-mL conical centrifuge tubes, add an equal volume of room-temperature PBS, and mix well.

9.2 Slowly layer the Ficoll-Paque solution underneath the blood/PBS mixture by placing the tip of the pipet containing Ficoll-Paque at the bottom of the blood sample tube. Alternatively, the blood/PBS mixture may be slowly layered over the Ficoll-Paque solution. Use 3 mL of Ficoll-Paque solution per 4 mL of blood/PBS mixture. For example, 15 mL Ficoll-Paque per 20 mL of diluted blood in a 50 mL tube.

Note: To maintain Ficoll-blood interface it is helpful to hold the tube at a 45º angle.

9.3 Centrifuge 30 min at 900 x g, 18-20ºC, without brake.

Note: Depending on the type of centrifuge, one may also need to set acceleration speed to minimum.

9.4 Using a sterile pipet, remove the upper layer containing plasma and platelets and discard it.

9.5 Using a fresh sterile pipet, transfer the mononuclear cell layer into another centrifuge tube.

9.6 Wash cells by adding an excess of HBSS and centrifuging for 10 min at 400xg, 18-20 ºC. The HBSS volume should be ~3 times the volume of mononuclear layer.

Note: Usually 4 mL of blood/PBS mixture results in ~ 2 mL of mononuclear layer and requires at least 6 mL of HBSS for the wash step. We use 10 mL of HBSS per each 2 mL of cells.

9.7 Discard supernatant and repeat wash step one more time.

9.8 Re-suspend cells in complete RPMI-1640 medium. Dilute cells 1:2 with AOPI, count cells and determine viability using AOPI exclusion. If viability is at least 90%, proceed to next step.

10. Experimental Procedure for Nanomaterial Treatment of PBMC (24-well)

10.1 Adjust the concentration of PBMC from step 9.8 to 1.25 x 10^6 viable cells/mL using complete RPMI medium.

10.2 Dispense 800 µL of PBMC from step 10.1 per well in 24-well plate. Gently shake plates to allow all components to mix.

10.3 Dispense 200 µL of blank complete media (unstimulated negative control) or positive control into corresponding wells of 24-well plate containing 800 µL of PBMCs for a total of 1 mL per well.

10.4 Dispense 100 µL of vehicle control or test sample into corresponding wells of 24-well plate containing 800 µL of PBMCs. Add 100 µL of fresh complete media for a total of 1 mL per well. (Vehicle control or test sample = MRD 10)
Note: If MRD of 5 is to be used, 200 µL of vehicle control or test sample should be added to the 800 µL of PBMC. Also, if one wants to understand whether nanoparticle may potentiate or inhibit cellular response to the assay positive control (LPS, PHA-M, PMA, Ionomycin, and/or ODN2216), the positive control should be co-cultured with nanoparticles in the presence of cells.

10.5 Incubate for about 24 hours in a humidified 37˚C, 5% CO_2 incubator.

11. Experimental PBMC Staining Procedure (Day 2)

11.1 Master Mix (MM) Preparation

11.1.1 Prepare MMs for the isotype controls and the antibody-stained samples in labeled microcentrifuge tubes based on the previously determined antibody concentrations.

Note: Prepare amount of MM for sample number (n) + 2. Concentrations to be used for the isotype control antibodies are the same as the concentration of corresponding fluorescent labeling antibody. See Tables 8-9 for Immunophenotyping Panel #1 and Tables 10-11 for Immunophenotyping Panel #2.

a. Human Immunophenotyping Panel #1 Antibodies
Antibody Titration Concentration Determined: Dil 25: CD8-FITC, CD45RA-AF700, CD45-PacBlue, CD3-BV570, CD127-BV785; Dil 50: TCR-gamma/delta APC-Fire750, CD154-BV711; Dil 100: CD25-BV650; Dil 125: CD4-PE, CD19-PE-Cy7, CCR4-APC

b. Human Immunophenotyping Panel #2 Antibodies
Antibody Titration Concentration Determined: Dil 25: CD123-APC, CD20-APC-Fire750, CD45-PacBlue, CD3-BV570, CD69-BV650, CD11c-BV785; Dil 125: CD56-FITC, CD14-PE, CD19-PE-Cy7, CD54-AF700

Table 8. Immunophenotyping Human Panel #1 Labeling Antibody MM

Optimal Antibody (Ab) dilution (ITA-37.1 Step 6.1)	Final Antibody Dilution	Volume of stock Ab needed (µL) per 100 µL samples	Number of samples (n +2)	Total volume of stock Ab needed (µL)	Number of Abs in panel	Total MM volume (µL)
Dil 25	Dil ~250	1.6	n+2	(n+2)(1.6)	5	5(n+2)(1.6)
Dil 50	Dil ~500	0.8	n+2	(n+2)(0.8)	2	2(n+2)(0.8)
Dil 100	Dil ~1000	0.4	n+2	(n+2)(0.4)	1	1(n+2)(0.4)
Dil 125	Dil ~1250	0.32	n+2	(n+2)(0.32)	3	3(n+2)(0.32)
Total MM Volume (µL)						**Sum of volumes in above column**
Total MM volume added to each sample = (Total MM Volume)/ (n+ 2)						**10.96 µL**

Table 9. Immunophenotyping Human Panel #1 Isotype Control MM

Isotype control Ab	Conc. of isotype control Ab (µg/mL)	Conc. of corresponding labeling Ab (µg/mL)	Ratio of isotype Ab conc: labeling Ab conc	Quantity of stock labeling Ab added labeling Ab MM (µL)	Quantity of stock isotype Ab to add to isotype control MM (µL)
FITC Mouse IgG1, K	200	200	1:1	(n+2)(1.6)	(n+2)(1.6)/1
PE Mouse IgG2b, K	200	100	2:1	(n+2)(0.32)	(n+2)(0.32)/2
PE-Cy7 Mouse IgG1, K	200	100	2:1	(n+2)(0.32)	(n+2)(0.32)/2
APC Mouse IgG1, K	200	50	4:1	(n+2)(0.32)	(n+2)(0.32)/4
AF700 Mouse IgG2b, K	200	50	4:1	(n+2)(1.6)	(n+2)(1.6)/4
APC-Fire750 Mouse IgG1, K	200	400	1:2	(n+2)(0.8)	(n+2)(0.8)/(1/2)
PacBlue Mouse IgG1, K	500	100	5:1	(n+2)(1.6)	(n+2)(1.6)/5
BV570 Mouse IgG1, K	100	80	1:0.8	(n+2)(1.6)	(n+2)(1.6)/(1/0.8)
BV650 Mouse IgG1, K	100	100	1:1	(n+2)(0.4)	(n+2)(0.4)/1
BV711 Mouse IgG1, K	100	100	1:1	(n+2)(0.8)	(n+2)(0.8)/1
BV785 Mouse IgG1, K	100	50	2:1	(n+2)(1.6)	(n+2)(1.6)/2
Total MM volume (µL)					**Sum of volumes in above column**
Total MM volume added to each sample = (Total MM Volume)/ (n+ 2)					**7.6 µL**

Table 10. Immunophenotyping Human Panel #2 Labeling Antibody MM

Optimal Antibody (Ab) dilution (ITA-37.1 Step 5.1)	Final Antibody Dilution	Volume of stock Ab needed (µL) per 100 µL	Number of samples (n +2)	Total volume of stock Ab needed (µL)	Number of Abs in panel	Total Master mix volume (µL)
Dil 25	Dil ~250	1.6	n+2	(n+2)(1.6)	6	6(n+2)(1.6)
Dil 125	Dil ~1250	0.32	n+2	(n+2)(0.32)	4	4(n+2)(0.32)
Total MM Volume (µL)						**Sum of volumes in above column**
Total MM volume added to each sample = (Total MM Volume)/ (n+ 2)						**10.88 µL**

Table 11. Immunophenotyping Human Panel #2 Isotype Control MM

Isotype control Ab	Conc. of isotype control Ab (µg/mL)	Conc. of corresponding labeling Ab (µg/mL)	Ratio of isotype Ab conc: labeling Ab conc	Quantity of stock labeling Ab added to labeling Ab MM (µL)	Quantity of stock isotype Ab to add to isotype control MM (µL)
FITC Mouse IgG1, K	200	200	1:1	(n+2)(0.32)	(n+2)(0.32)/1
PE Mouse IgG2a, K	200	200	1:1	(n+2)(0.32)	(n+2)(0.32)/1
PE-Cy7 Mouse IgG1, K	200	100	2:1	(n+2)(0.32)	(n+2)(0.32)/2
APC Mouse IgG1, K	200	100	2:1	(n+2)(1.6)	(n+2)(1.6)/2
AF700 Mouse IgG1, K	500	400	1.25:1	(n+2)(0.32)	(n+2)(0.32)/1.25
APC-Fire750 Mouse IgG2b, K	200	200	1:1	(n+2)(1.6)	(n+2)(1.6)/1
PacBlue Mouse IgG1, K	500	100	5:1	(n+2)(1.6)	(n+2)(1.6)/5
BV570 Mouse IgG1, K	100	80	1.25:1	(n+2)(1.6)	(n+2)(1.6)/1.25
BV650 Mouse IgG1, K	100	50	2:1	(n+2)(1.6)	(n+2)(1.6)/2
BV785 Mouse IgG1, K	100	160	1:1.6	(n+2)(1.6)	(n+2)(1.6)/(1/1.6)
Total MM Volume (µL)					**Sum of volumes in above column**
Total MM volume added to each sample = (Total MM Volume)/ (n+ 2)					**8.42 µL**

11.2 Harvesting of treated PBMC for staining

11.2.1 Collect cultured PBMC in properly labeled 1.5 mL microcentrifuge tubes and spin in a centrifuge at 400xg for 5 minutes. Transfer supernatants into fresh tubes store at -80 °C. Supernatants can be stored for ELISAs to be performed at later time point, if desired.

11.2.2 Wash each cell sample 2x with 1X PBS.

a. Add 1 mL 1X PBS to each tube.

b. Centrifuge sample for 7 min at 400xg.

c. Aspirate off each supernatant.

11.3 Staining Procedure

11.3.1 Dilute Zombie Aqua Dye 5-fold using 1X PBS as the diluent.
Note: Need 0.8 µL of dil 5 Zombie Aqua dilution x n conditions = 0.8(n+2) µL of dil 5 dilution. N = the number of fully stained labeling antibody samples in both Panel #1 and Panel #2.

11.3.2 Add 0.8 µL of the Dil 5 Zombie Aqua dye from step 11.3.1 to all fully stained labeling Ab samples in both Immunophenotyping Panels #1 and Panel #2 (see Tables 6-7).

11.3.3 Incubate samples for 30 minutes at room temperature in the dark.

11.3.4 Wash each sample twice with 500 µL staining buffer.

11.3.5 Resuspend each sample in 40 µL staining buffer.

11.3.6 Resuspend samples in staining buffer.

a. Panel #1: Unstained samples: 100 µL staining buffer; Isotype Control samples: 92.4 µL staining buffer; Fully stained labeling antibody samples: 89.04 µL staining buffer

b. Panel #2: Unstained samples: 100 µL staining buffer; Isotype Control samples: 91.58 µL staining buffer; Fully stained labeling antibody samples: 89.12 µL staining buffer

11.3.7 Add appropriate MM volume and staining buffer to appropriate cell sample (Final volume 100 µL).

a. Panel #1:

i. Isotype Control samples: 7.6 µL MM

ii. Fully stained labeling antibody samples: 10.96 µL MM

b. Panel #2:

i. Isotype Control samples: 8.42 µL MM

ii. Fully stained labeling antibody samples: 10.88 µL MM

11.3.8 Incubate samples for 30 minutes at room temperature in the dark.

11.3.9 Wash each sample with 500 µL staining buffer.

11.3.10 Fix cells by resuspending each cell sample in 100 µL 2% PFA and incubating for 15 minutes at room temperature in dark.

11.3.11 Wash each sample 2x with 500 µL staining buffer.

11.3.12 Resuspend each sample in 500 µL staining buffer.

11.3.13 Save samples in 4˚C fridge. Samples can be run the following day.

12 Data Acquisition on Novocyte 3005 (Day 3)

12.1 Preparation of Panel #1 Samples

12.1.1 Remove the Panel #1 samples from the 4˚C fridge and let samples come to room temperature.

12.1.2 Obtain flow tubes and label them appropriately.

12.1.3 Transfer 500 µL of each sample to the appropriately labeled flow tube. Briefly vortex samples.

12.1.4 Samples are ready for flow cytometry.

12.2 Data Acquisition with NovoCyte 3005 (Panel #1)

12.2.1 Turn on computer > NovoExpress > username and password

12.2.2 Turn on flow cytometer

12.2.3 Make sure all parameters (channels and laser intensity) are the corresponding FMO experiment. *Note: The FMO control experiment can be saved as a template from which the new experiment can be made.*

12.2.4 Save file.

12.2.5 Check the experimental parameters

a. Plate Manager

i. Mode: custom; 24 tube rack
ii. Indicate samples to run horizontal.
iii. Click on proper squares to create sample. Rename each sample accordingly in work list.
iv. Mix: 1 cycle every, 2 wells (1500 rpm; 10 sec); Rinse: 1 cycle, every 3 wells

b. Experimental Run. Stop conditions: Events >500,000; 300 µL. Fast (Apply changes to all samples)
c. Cytometer Settings: Parameters, please see ITA-37.1 Table 4 in Section 6.1.4.4.

12.2.6 Insert samples in tube rack.
12.2.7 Click run plate. Highlight all boxes that are to be run (all). → run → okay
12.2.8 Save file.
12.2.9 Export FCS files.

12.3 Preparation of Panel #2 Samples

12.3.1 Remove the Panel #2 samples from the 4˚C fridge and let samples come to room temperature.
12.3.2 Obtain flow tubes and label them appropriately.
12.3.3 Transfer 500 µL of each sample to the appropriately labeled flow tube. Briefly vortex samples.
12.3.4 Samples are ready for flow cytometry.

12.4 Data Acquisition with NovoCyte 3005 (Panel #2)

12.4.1 NovoExpress > username and password > New NovoExpress file
12.4.2 Make sure all parameters (channels and laser intensity) are the corresponding FMO experiment. *Note: The FMO control experiment can saved as a template from which the new experiment can be made.*
12.4.3 Save file.
12.4.4 Check the experimental parameters

a. Plate Manager
    i. Mode: custom; 24 tube rack
    ii. Indicate samples to run horizontal.
    iii. Click on proper squares to create sample. Rename each sample accordingly in work list.
    iv. Mix: 1 cycle every, 2 wells (1500 rpm; 10 sec); Rinse: 1 cycle, every 3 wells
b. Experimental Run. Stop conditions: Events >500,000; 300 µL. Fast (Apply changes to all samples)
c. Cytometer Settings: Parameters, please see ITA-37.1 Table 4 in Section 6.1.4.4.

12.4.5 Insert samples in tube rack

12.4.6 Click run plate. Highlight all boxes that are to be run (all). → run → okay

12.4.7 Save file.

12.4.8 Export FCS files.

13 Data Analysis of Immunophenotyping Panels with FCS Express

13.1 Application of Compensation Matrix to Immunophenotyping Panels

Important Notes: There are multiple ways to apply the compensation matrix of the FMO controls to the immunophenotyping panels. Below is one method. Steps 13.1 -13.4 should be applied to both Immunophenotyping Panel #1 and #2 using the appropriate FMO controls for the applied compensation.

13.1.1 Make a copy of the compensated FMO control file (.ncf) that correlates to the immunophenotyping file.

13.1.2 Upload the FCS files from the appropriate immunophenotyping experiment into the FMO control file (.ncf).
a. Right click on file name in Experimental Manager.
b. Click on Import FCS files.

c. Browse for the folder with appropriate FCS files.

13.1.3 Apply the compensation matrix from the FMO control sample to the immunophenotyping samples.

a. Under "Compensation Specimen" click on the "+" for any sample.
b. Right click on Compensation and select Copy.
c. Right click on overarching immunophenotyping sample and click on Paste.
d. "Are you sure you want to paste Compensation to all samples in *experimental sample name*" Yes
e. Can then delete the FMO control sample.

13.1.4 Rename the file with the compensated immunophenotyping samples and save.

13.2 Analysis of Immunophenotyping Panel #1 using FCS Express

13.2.1 Make sure that FCS Express is set up to use the compensation of the FCS files: File > Options > Choose default compensation options > FCS file

13.2.2 Import the compensated FCS files to appropriate FCS Express file. *Important Note: Negative control, positive controls, vehicle controls, and test sample files should be imported into separate FCS Express files and analyzed separately.*

13.2.3 Set up histograms and dot plots as per cell definition for fully stained sample as indicated Figure 2.
Note: Corresponding isotype controls should be overlayed onto each plot and used to set the gates which are then applied to the fully stained labeling antibody samples.

13.2.4 Save all files.

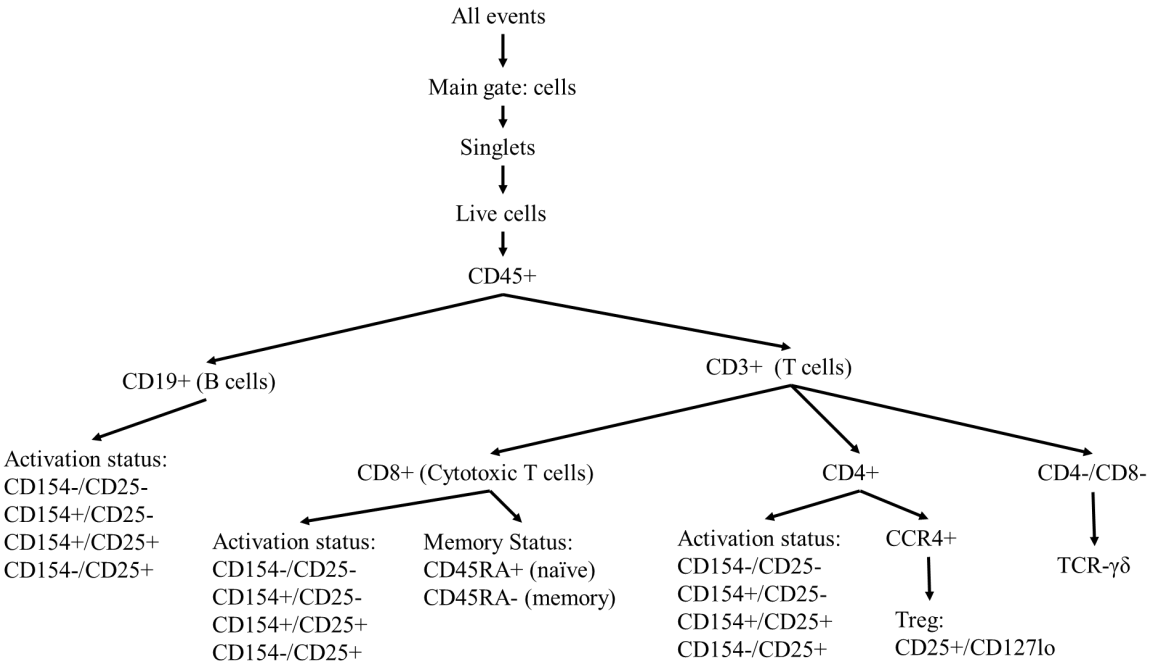


Figure 2. Gating Strategy for Immunophenotyping Panel #1.

Gates—

- **<u>Live cells:</u> Cells**: SSC-H v FSC-H → **Singlets**: FSC-H v FSC-A → **Live cells:** FSC-H v L/D-Zombie Aqua: Gate Dead cells and exclude gate

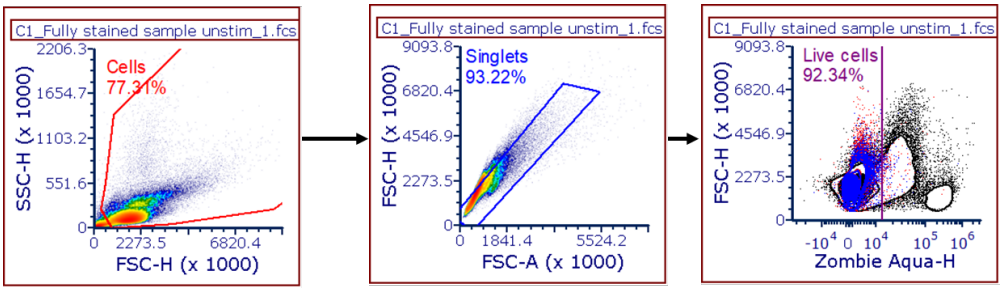


Figure 3. Live Cell Gating. Sequential gates to determine live cell population.

- **<u>B cells (CD19+):</u> Cells → Singlets → Live cells → CD45+:** FSC-H v CD45-PacBlue-H → **CD19+ (B cells):** FSC-H v CD19-PE-Cy7-H
  - **Activation** (CD154 v CD25): CD154-BV711-H v CD25-BV650-H: CD25-/CD154-, CD25-/CD154+, CD25+/CD154+, CD25+/CD154-

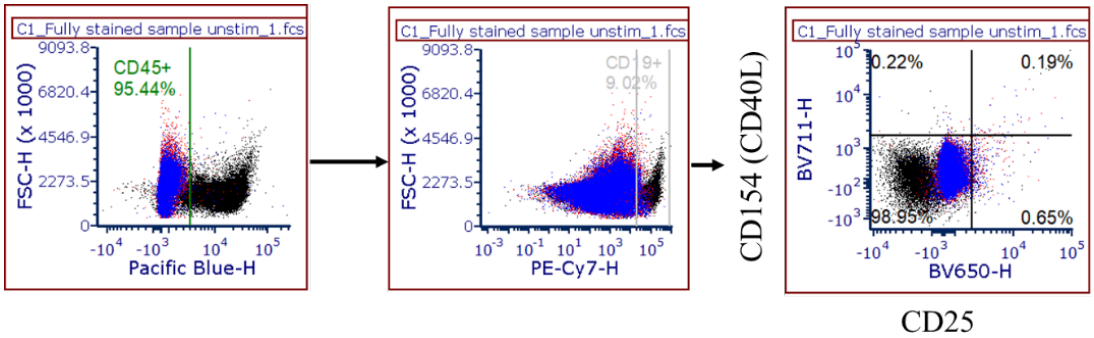


Figure 4. B Cell Gating. Sequential gates to determine CD19+ B cell population and activation status.

- **<u>T cells (CD3+):</u> Cells → Singlets → Live cells → CD45+ → CD3+:** FSC-H v CD3-BV570-H
  - **<u>CD8+ vs CD4+ T cells:</u> Cells → Singlets → Live cells → CD45+ → CD3+ → CD8 v CD4:** CD8-FITC-H v CD4-PE-H: CD4-/CD8-, CD4-/CD8+, CD4+/CD8+, CD4+/CD8-
    - **CD4-/CD8+ → Activation** (CD154 v CD25): CD154-BV711-H v CD25-BV650-H: CD25-/CD154-, CD25-/CD154+, CD25+/CD154+, CD25+/CD154-
    - **CD4+/CD8- → Activation** (CD154 v CD25): CD154-BV711-H v CD25-BV650-H: CD25-/CD154-, CD25-/CD154+, CD25+/CD154+, CD25+/CD154-
  - **<u>CD8+ Naïve T cells:</u> Cells → Singlets → Live cells → CD45+ → CD3+ → CD8+/CD4- → CD45RA+:** FSC-H v CD45RA-AF700-H
  - **<u>Regulatory T cells (Treg):</u> Cells → Singlets → Live cells → CD45+ → CD3+ → CD4+/CD8- → CCR4+:** Count v CCR4-APC-H → **Treg (CD25+, CD127low)**: CD25-BV650-H v CD127-BV785-H
  - **<u>TCR γ/δ cells:</u> Cells → Singlets → Live cells → CD45+ → CD3+ → CD4-/CD8- → TCR γ/δ:** Count v TCRγ/δ-APC-Fire750-H

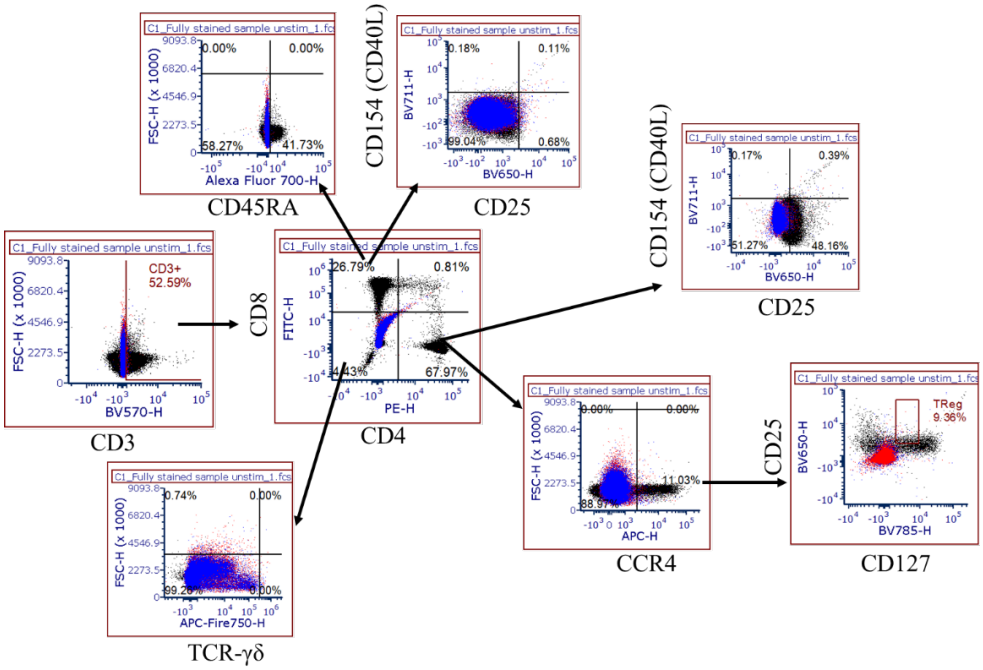


Figure 5. T Cell Gating. Gating to determine the different T cell populations and activation statuses.

13.3 Analysis of Immunophenotyping Panel #2 using FCS Express

13.3.1 Make sure that FCS Express is set up to use the compensation of the FCS files: File > Options > Choose default compensation options > FCS file

13.3.2 Import the compensated FCS files to appropriate FCS Express file.
Important Note: Negative control, positive controls, vehicle controls, and test sample files should be imported into separate FCS Express files and analyzed separately.

13.3.3 Set up histograms and dot plots as per cell definition for fully stained sample as indicated in Figure 6.
Note: Corresponding isotype controls should be overlayed onto each plot and used to set the gates which are then applied to the fully stained labeling antibody samples. Define initial cell population into lymphocytes and monocytes by SSC v FSC and then by markers.

13.3.4 Save all files.

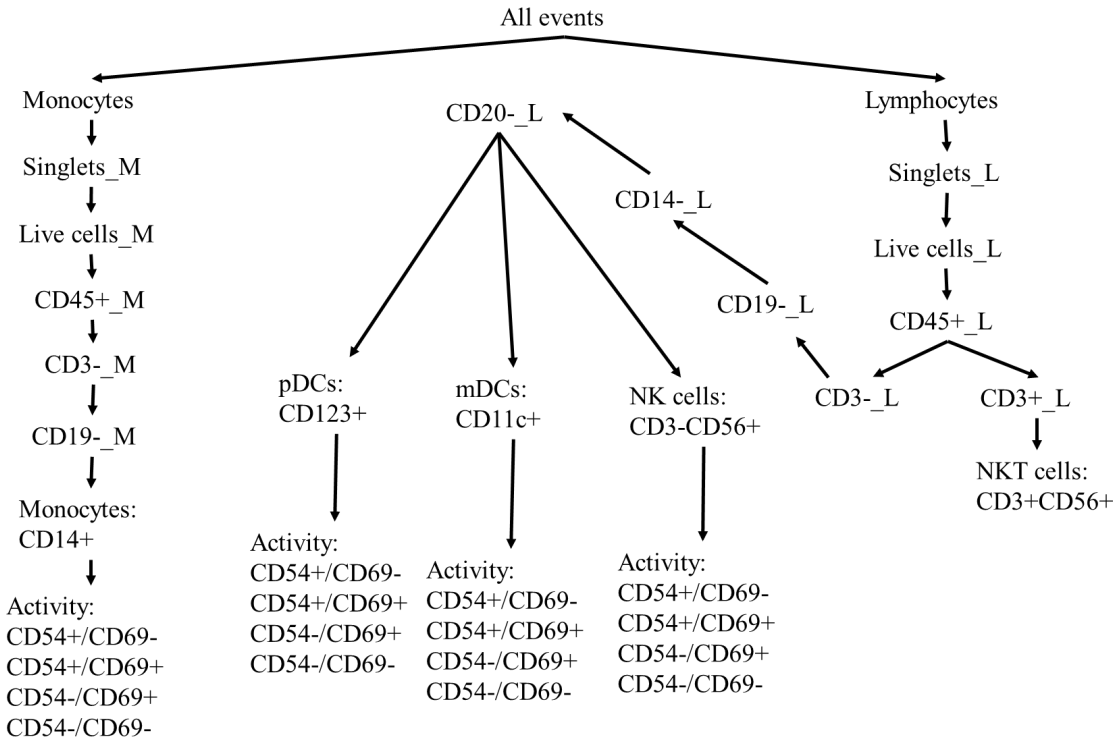


Figure 6. Gating Strategy for Immunophenotyping Panel #2.

Gates—

- **<u>Monocytes (CD14+)</u>: Monocyte population via scatter:** SSC-H v FSC-H → **Singlets_M**: FSC-H v FSC-A → **Live_M:** FSC-H v L/D-Zombie Aqua: Gate dead cells and exclude gate → **CD45+_M:** FSC-H v CD45-PacBlue-H → **CD3-_M:** FSC-H v CD3-BV570-H → **CD19-_M:** FSC-H v CD19-PE-Cy7 → **Monocytes (CD14+):** FSC-H v CD14-PE-H
    - **Activation:** (CD54 v CD69): CD54-AF700-H v CD69-BV650-H: CD54-/CD69-, CD54-/CD69+, CD54+/CD69+, CD54+/CD69-

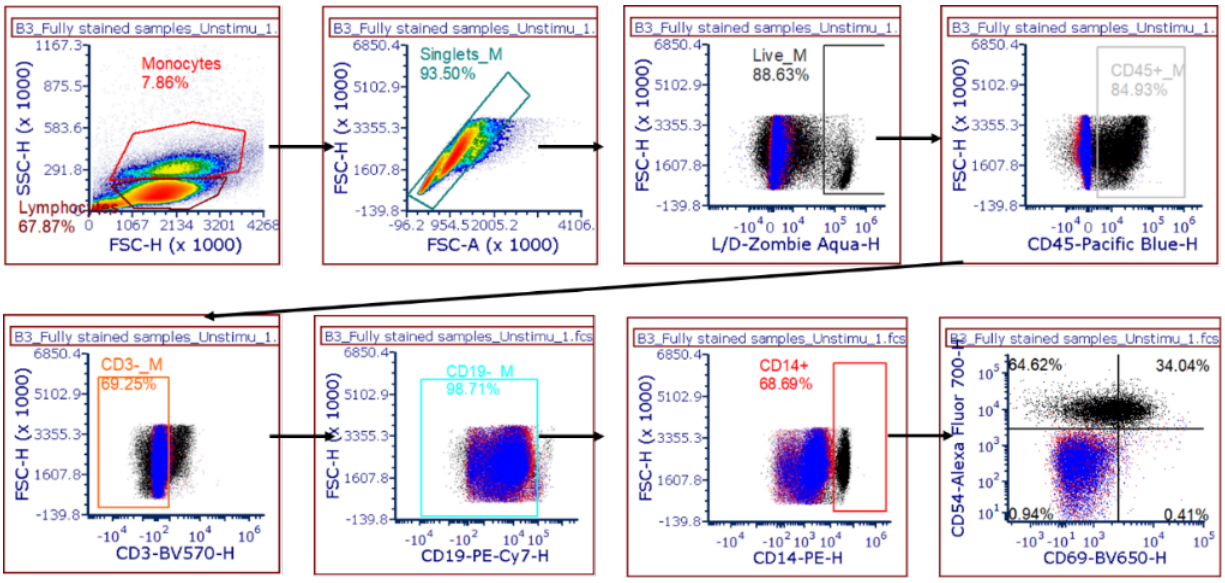


Figure 7. Monocyte Gating. Sequential gating to determine monocyte population and activation status.

- **<u>CD45+ Lymphocytes</u>: Lymphocyte population via scatter:** SSC-H v FSC-H → **Singlets_L**: FSC-H v FSC-A → **Live_L:** FSC-H v L/D-Zombie Aqua: Gate Dead cells and exclude gate → **CD45+_L:** FSC-H v CD45-PacBlue-H

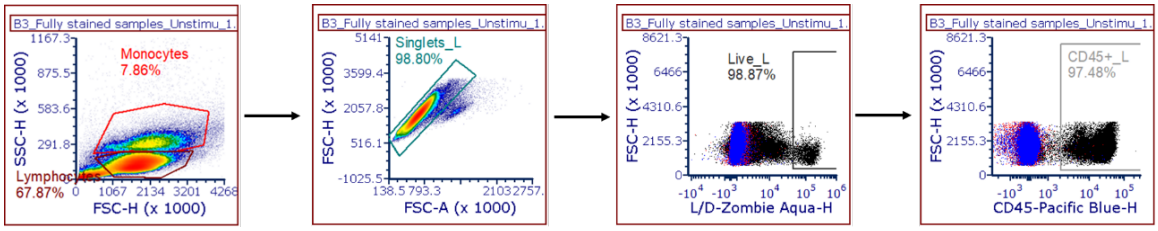


Figure 8. CD45+ Lymphocyte Gating. Sequential gating to CD45+ Lymphocyte population.

- **<u>NK T cells:</u> Lymphocyte population via scatter → Singlets_L → Live_L → CD45+_L → CD3+_L:** FSC-H v CD3-BV570-H → **CD56+:** FSC-H v. CD56-FITC-H

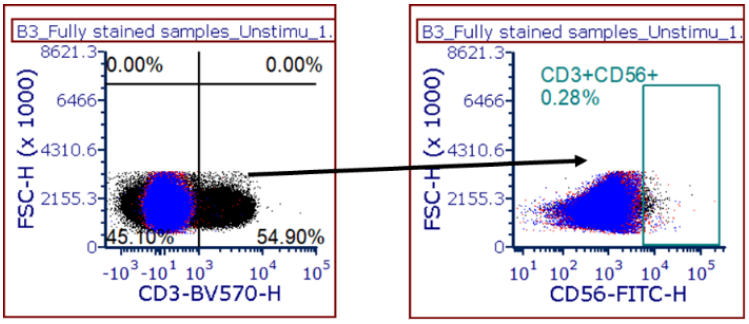


Figure 9. NK T Cell Gating. Gating to determine the CD3+ CD56+ T cell population.

- **<u>CD45+ CD3- CD14- CD20- cells</u>: Lymphocyte population via scatter → Singlets_L → Live_L → CD45+_L → CD3-_L:** FSC-H v CD3-BV570-H → **CD19-_L:** FSC-H v CD19-PE-Cy7 → **CD14-_L:** FSC-H v CD14-PE-H → **CD20-_L:** FSC-H v CD20-APC-Fire750-H

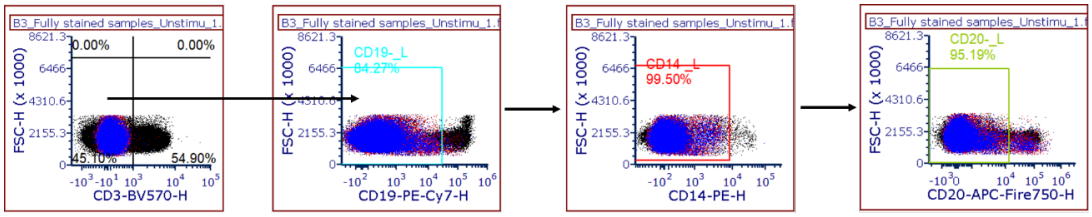


Figure 10. CD45+ CD3- CD14- CD20- Cell Population.

- **<u>pDCs</u>: Lymphocyte population via scatter → Singlets_L → Live_L → CD45+_L → CD3-_L→ CD19-_L → CD14-_L → CD20-_L: → CD123+_L:** FSC-H v CD123-APC-H
  - **Activation:** (CD54 v CD69): CD54-AF700-H v CD69-BV650-H: CD54-/CD69-, CD54-/CD69+, CD54+/CD69+, CD54+/CD69-
- **<u>mDCs</u>: Lymphocyte population via scatter → Singlets_L → Live_L → CD45+_L → CD3-_L→ CD19-_L → CD14-_L→ CD20-_L→ CD11c+_L:** FSC-H v CD11c-BV785-H
  - **Activation:** (CD54 v CD69): CD54-AF700-H v CD69-BV650-H: CD54-/CD69-, CD54-/CD69+, CD54+/CD69+, CD54+/CD69-
- **<u>NK cells</u>: Lymphocyte population via scatter → Singlets_L → Live_L → CD45+_L → CD3-_L→ CD19-_L → CD14-_L→ CD20-_L→ CD56+:** FSC-H v CD56-FITC-H
  - **Activation:** (CD54 v CD69): CD54-AF700-H v CD69-BV650-H: CD54-/CD69-, CD54-/CD69+, CD54+/CD69+, CD54+/CD69-

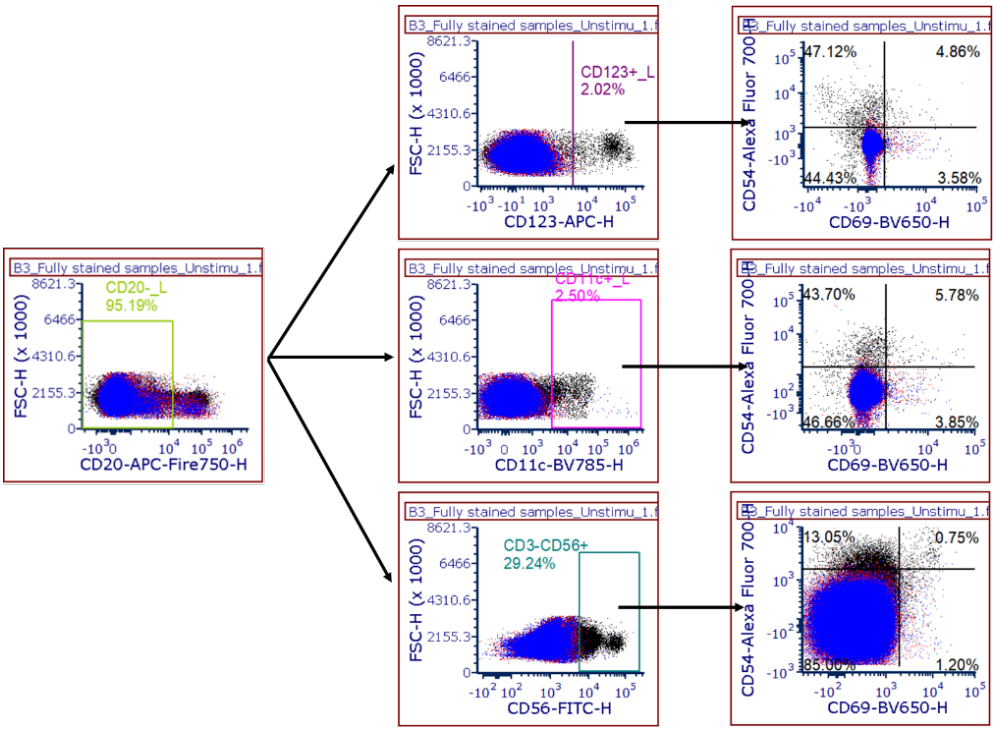


Figure 11. Gating for pDCs, mDCS, NK cells. Gating strategy for different lymphocyte populations.

14 References

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15 Abbreviations

Ab	antibody
PBMC	peripheral blood mononuclear cells
Treg	regulatory T cells
DCs	dendritic cells
NK	natural killer
pDC	plasmacytoid DC
mDC	myeloid DC
FMO	fluorescence minus one
FBS	fetal bovine serum
PBS	phosphate buffered saline
LPS	lipopolysaccharide
PHA-M	phytohemagglutinin
ODN	oligodeoxyribonucleotide
PFA	paraformaldehyde

RT	room temperature
MRD	minimal required dilution
MM	Master Mix
FSC	forward scatter
SSC	side scatter