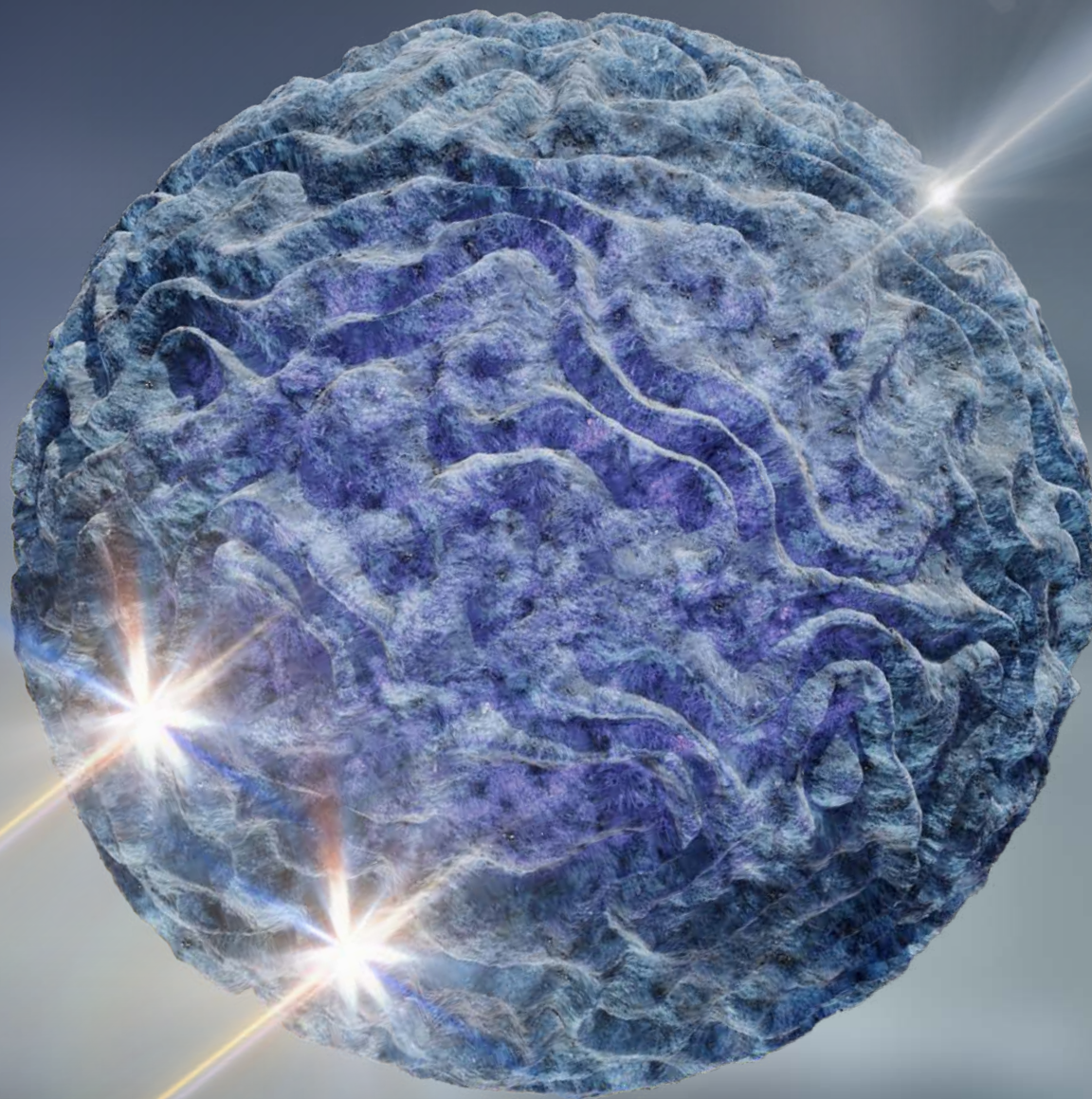# FLOW CYTOMETRY
HANDBOOK

bio-techne®

AVAILABLE FLUOROCHROME OPTIONS BY LASER

CONJUGATE	LASER & EXCITATION PEAK		EMISSION PEAK		BRIGHTNESS
DyLight 350	UV	354 nm	Blue	432 nm	Moderate
Alexa Fluor® 350	UV	346 nm	Blue	442 nm	Moderate
DyLight 405	Violet	400 nm	Blue	420 nm	Moderate
Alexa Fluor® 405	Violet	401 nm	Blue	421 nm	Moderate
DyLight 488	Blue	493 nm	Green	518 nm	Moderate
FITC	Blue	494 nm	Green	520 nm	Moderate
Alexa Fluor® 488	Blue	490 nm	Green	525 nm	Moderate
PE	Blue	496 nm, 565 nm	Yellow	578 nm	Bright
PE-Atto 594	Blue	496 nm, 565 nm	Red	632 nm	Bright
PerCP	Blue	482 nm	Red	678 nm	Dim
PE-Cy5.5	Blue	496 nm, 565 nm	Red	690 nm	Moderate
PE-Cy7	Blue	496 nm, 565 nm	Near Infrared (NIR)	782 nm	Moderate
Alexa Fluor® 532	Yellow-Green	532 nm	Yellow	554 nm	Moderate
Janelia Fluor® 549	Yellow-Green	549 nm	Yellow	571 nm	Bright
DyLight 550	Yellow-Green	562 nm	Yellow	576 nm	Bright
PE	Yellow-Green	496 nm, 565 nm	Yellow	578 nm	Bright
Alexa Fluor® 594	Yellow-Green	590 nm	Red	617 nm	Moderate
DyLight 594	Yellow-Green	593 nm	Red	618 nm	Moderate
PE-Atto 594	Yellow-Green	496 nm, 565 nm	Red	632 nm	Bright
PE-Cy5.5	Yellow-Green	496 nm, 565 nm	Red	690 nm	Moderate
PE-Cy7	Yellow-Green	496 nm, 565 nm	Near Infrared (NIR)	782 nm	Moderate
APC	Red	650 nm	Red	660 nm	Bright
Janelia Fluor® 646	Red	646 nm	Red	664 nm	Moderate
Alexa Fluor® 647	Red	650 nm	Red	665 nm	Moderate
DyLight 650	Red	652 nm	Red	672 nm	Bright
DyLight 680	Red	692 nm	Near Infrared (NIR)	712 nm	Bright
Alexa Fluor® 700	Red	695 nm	Near Infrared (NIR)	720 nm	Dim
APC-Cy7	Red	650 nm	Near Infrared (NIR)	767 nm	Moderate
Alexa Fluor® 750	Red	749 nm	Near Infrared (NIR)	775 nm	Moderate
DyLight 755	Red	754 nm	Near Infrared (NIR)	776 nm	Moderate

TABLE OF CONTENTS

INTRODUCTION

Flow cytometry is a powerful, high-throughput technology that is used to characterize suspensions of single cells or particles. Traditional applications involve immunophenotyping of cells prepared from culture or tissue using fluorescent-dye conjugated antibodies that recognize specific cell surface or intracellular antigens (**FIGURE 1**). By combining different fluorescent probes in a multi-color panel, several parameters are measured simultaneously on thousands to millions of individual cells. The relative abundance of antigens/probes in a population of cells is determined by calculating the fluorescence intensity for each parameter and analyzed to compare gene expression patterns between samples (**FIGURE 2, 3, AND 4**).
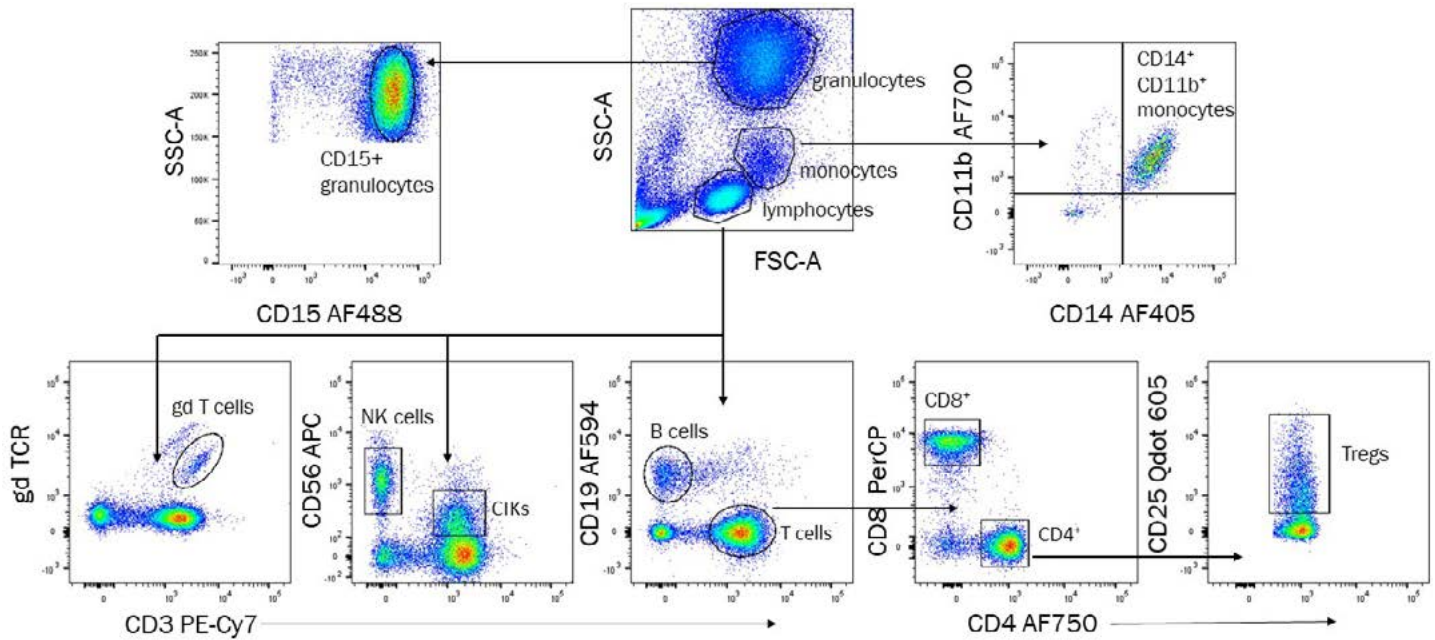

FIGURE 1. A 10-parameter flow cytometry in human whole blood. Human whole blood was stained with the following panel of surface markers: hCD11b AF700 (R&D Systems Catalog # FAB1699N), hCD14 AF405 (R&D Systems Catalog # FAB3832V), hCD15 AF488 (R&D Systems Catalog # FAB7368G), hCD3 PE-Cy7 (R&D Systems Catalog # FAB100), hCD4 AF750 (R&D Systems Catalog # FAB3791S), hCD8 PerCP (R&D Systems Catalog # FAB1509C), hCD19 AF594 (R&D Systems Catalog # FAB4867T), hγδTCR AmCyan, hCD25 Qdot 605 (R&D Systems Catalog # FAB1020), and hCD56 APC (R&D Systems Catalog # FAB2408A).
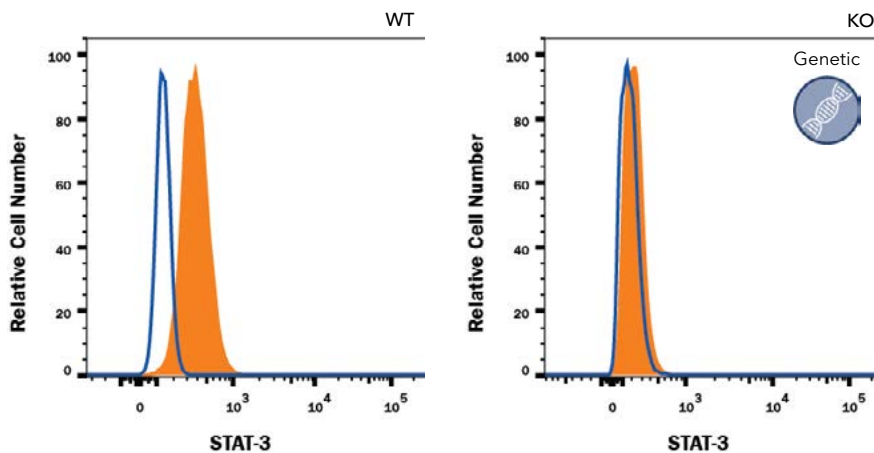

FIGURE 2. Knockout validation of STAT3 staining. STAT3 knockout and parental HeLa cell lines were stained with Mouse Anti-Human/Mouse/Rat STAT3 Monoclonal Antibody (R&D Systems Catalog # MAB1799, filled histogram) or isotype control antibody (R&D Systems Catalog # MAB0041, open histogram) followed by anti-Mouse IgG PE-conjugated secondary antibody (R&D Systems Catalog # F0102B). To facilitate intracellular staining, cells were fixed with Flow Cytometry Fixation Buffer (R&D Systems Catalog # FC004) and permeabilized with Flow Cytometry Permeabilization/Wash Buffer I (R&D Systems Catalog # FC005). No staining in the STAT3 knockout HeLa cell line was observed.

INTRODUCTION

FIGURE 3. Knockout validation of MICA staining. MICA knockout and parental K562 human chronic myelogenous leukemia cell lines were stained with Mouse Anti-Human MICA Monoclonal Antibody (R&D Systems Catalog # MAB1300, filled histogram) or isotype control antibody (R&D Systems Catalog # MAB0041, open histogram) followed by anti-Mouse IgG PE-conjugated secondary antibody (R&D Systems Catalog # F0102B). No staining in the MICA knockout K562 cell line was observed.
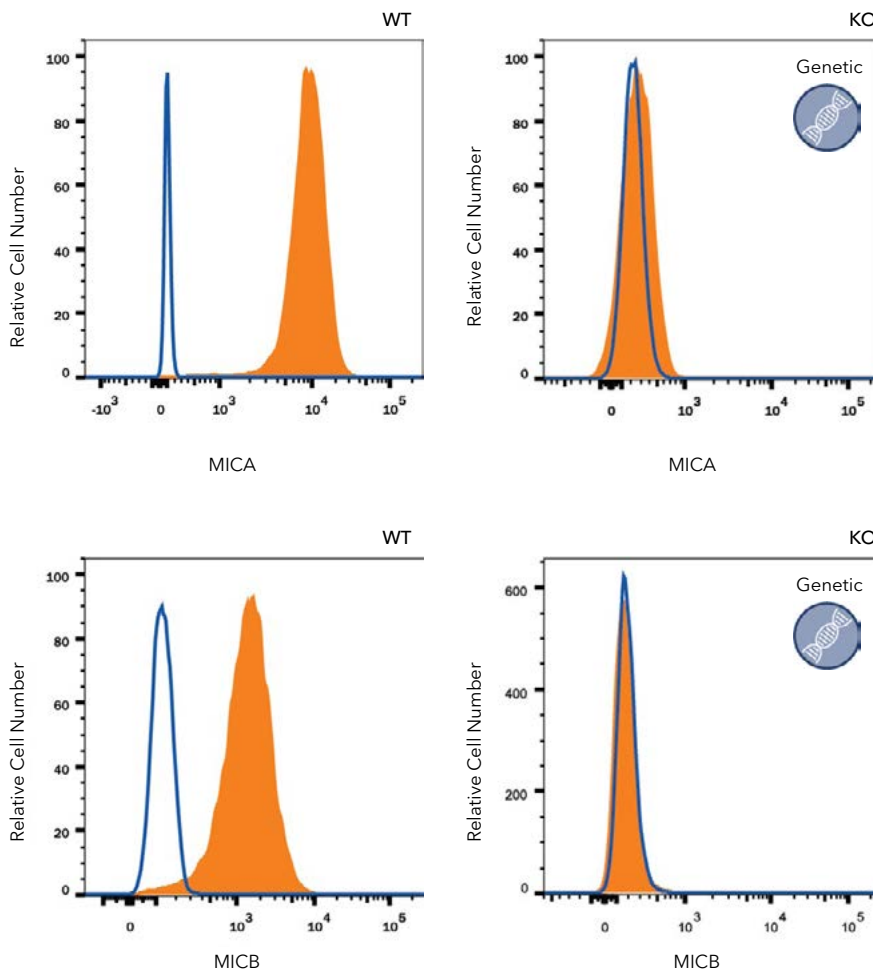

FIGURE 4. Knockout validation of MICB staining. MICB knockout and parental K562 human chronic myelogenous leukemia cell lines were stained with Mouse Anti-Human MICB Monoclonal Antibody (R&D Systems Catalog # MAB1599, filled histogram) or isotype control antibody (R&D Systems Catalog # MAB0041, open histogram) followed by anti-Mouse IgG PE-conjugated secondary antibody (R&D Systems Catalog # F0102B). To facilitate intracellular staining, cells were fixed with Flow Cytometry Fixation Buffer (R&D Systems Catalog # FC004) and permeabilized with Flow Cytometry Permeabilization/Wash Buffer I (R&D Systems Catalog #FC005). No staining in the MICB knockout K562 cell line was observed.

Flow cytometry is used in a variety of research applications and for clinical diagnostics, such as in the diagnosis of hematological disorders, and continues to advance as a leading biomedical technology. Applications include:

- Evaluation of gene expression through fluorescent protein detection

- Analysis of DNA content using nucleic acid binding fluorescent dyes

- Assessing cytokine/chemokine production via capture beads

- Evaluation of cell viability and vitality

- Monitoring cellular proliferation or cell activation

- Detection of RNA transcripts

Recently, the number of parameters cytometers are capable of measuring has increased to more than 20, made possible by the development of new fluorescent probes and advances in instrument design, fluorescence detection, and fluorochrome excitation. This guide will give an overview of common considerations and general guidelines for performing flow cytometry assays. For more in-depth knowledge of flow cytometry techniques, review our Springer publication.[1]
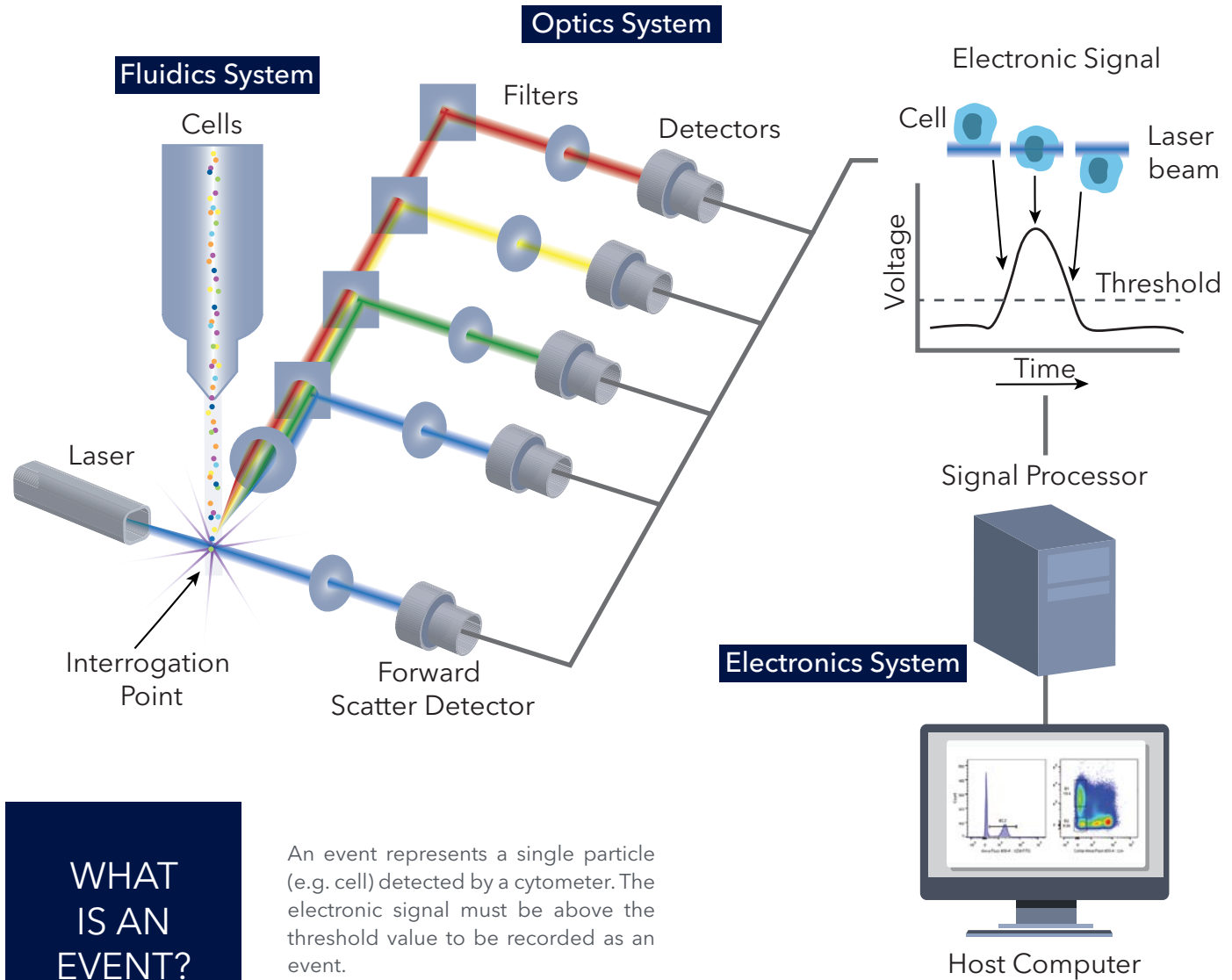
BASIC COMPONENTS OF A FLOW CYTOMETER

The universal components of a flow cytometer are fluidics, optics, and electronics.

The **fluidics system** uses pressure, vacuum, or pumps to move cells from the sample tube to the flow cell where the sample stream is focused, to line up the cells for analysis one cell at a time.

In the **optics system**, lasers are directed to the analysis point (interrogation point) as a source of excitation for fluorescent probes. Fluorescence emission (photons) and light scatter are directed to detectors via fiber optics or lenses/mirrors.

The **electronics system** converts emitted photons into a voltage pulse, an electronic signal over time. Each event is processed and visualized using one and two dimensional plots.

Optics System

Fluidics System

Cells

Filters

Detectors

Electronic Signal

Cell

Laser beam

Threshold

Voltage

Time

Signal Processor

Laser

Interrogation Point

Forward Scatter Detector

**Electronics System**

Host Computer

WHAT IS AN EVENT?

An event represents a single particle (e.g. cell) detected by a cytometer. The electronic signal must be above the threshold value to be recorded as an event.
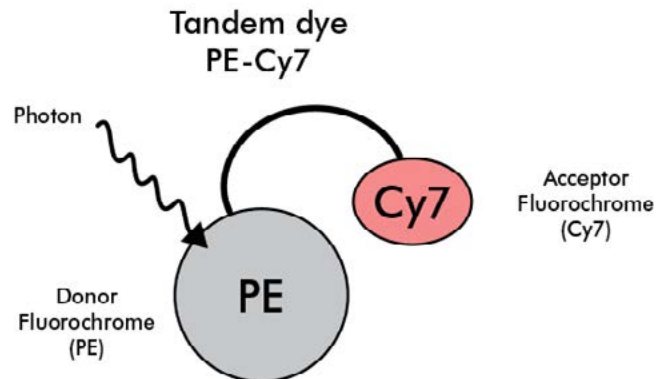
BASIC COMPONENTS OF A FLOW CYTOMETER

WHAT IS FLUORESCENCE?

Fluorescence is defined as the emission of light by a molecule that has absorbed light at a shorter wavelength. Fluorochromes are fluorescent molecules, including proteins or peptides (e.g. GFP), small organic compounds (e.g. FITC), synthetic polymers, and nanoparticles (e.g. quantum dots).[2] Each fluorochrome has a distinct excitation and emission spectra as illustrated below for Alexa Fluor® 488 (AF488). The excitation spectrum (dashed green histogram) is the range of wavelengths of light that are absorbed by the fluorochrome, roughly from 450 nm to 530 nm with an excitation maximum at 490 nm for AF488. The emission spectrum (solid green histogram) depicts the normalized fluorescence intensity of a fluorochrome across a range of wavelengths, which is around 490 nm to over 600 nm for AF488 with an emission maximum at 525 nm.
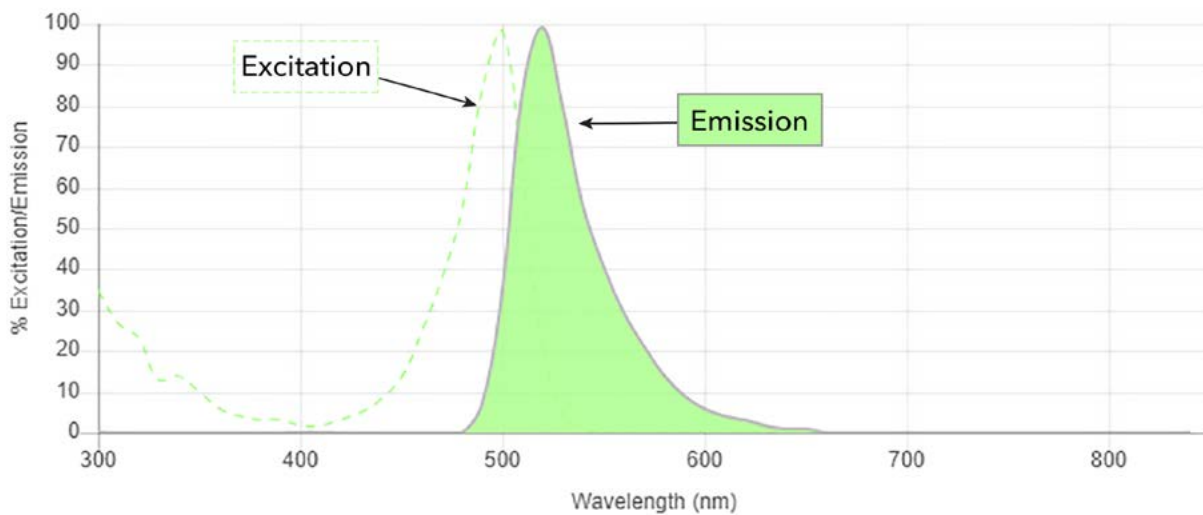
WHAT ARE TANDEM DYES?

Tandem dyes are also referred to as FRET (fluorescence resonance energy transfer) dyes because the emission of the donor fluorochrome (e.g. PE) excites the acceptor fluorochrome (e.g. Cy7). The energy transfer between these covalently bonded fluorochromes (e.g. PE-Cy7) expands the range of detectable wavelengths excited by a single laser. However, the potential instability of these dyes and their sensitivity to light and certain reagents means tandem dyes should be used with caution.

Spectra viewers are a great resource for determining the excitation and emission of various fluorochromes. Explore the Novus Spectra Viewer www.novusbio.com/spectraviewer

BASIC COMPONENTS OF A FLOW CYTOMETER

OPTICS SYSTEM

LASERS

Flow cytometers use lasers as excitation sources for fluorochromes. Laser wavelengths vary and determine what fluorochromes can be measured on any particular instrument. Generally, the blue laser is also used to measure light scatter.

| DIFFERENT LASER OPTIONS	
LASER	COLOR
355 nm or 377 nm	UV or Near UV
405 nm	Violet
488 nm	Blue
561 nm	Yellow-Green
638 nm or 640 nm	Red
808 nm	Infrared (IR)

WHAT IS SCATTERED LIGHT?

Forward scatter (FSC) and side scatter (SSC) are signals generated from the scattering or refraction of the laser light by the cell or particle being measured (**A**). FSC is measured in the forward direction as the laser passes through the cell and is refracted, whereas SSC is measured at a 90° angle. Both of these parameters provide important details about cell appearance (**B**). FSC and SSC parameters are key to discriminate cells from debris and noise.

Scatter plots are used to eliminate debris from analysis and serve as a good starting point to define specific cellular populations such as lymphocytes, monocytes, and granulocytes (**C**).

A — Laser Beam, Side-scatter, Forward-scatter

C — SSC vs FSC: granulocytes, monocytes, lymphocytes
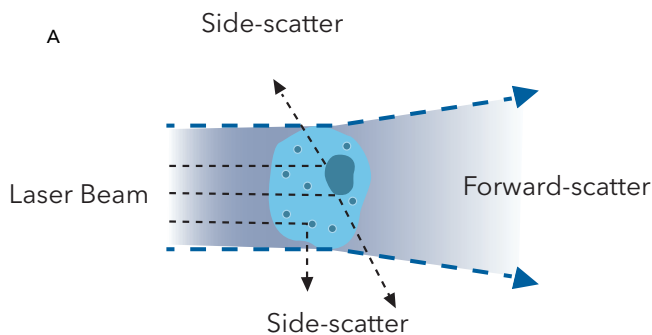
PARAMETER	CORRELATES WITH
FSC	Cell size
SSC	Granularity/complexity of cells

B
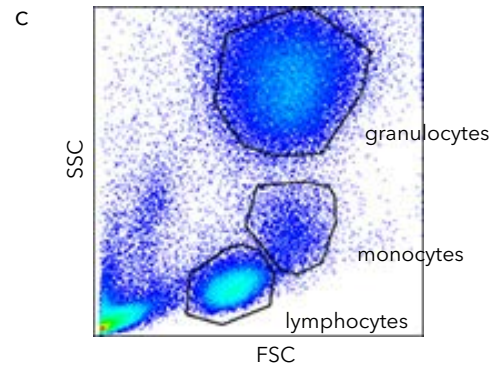
OPTICAL FILTERS

The fluorescence emission of each fluorochrome is captured using optical filters, pieces of glass coated with specialized materials, which determine how light is reflected and transmitted. While laser wavelength is necessary to determine if a fluorochrome can be excited, the optical filters determine if a fluorochrome can be detected. There are generally three types of optical filters found in flow cytometers:

BASIC COMPONENTS OF A FLOW CYTOMETER

1. **Longpass (LP) filters:** allow any longer wavelength through (in this example, >500 nm)

2. **Shortpass (SP) filters:** allow any shorter wavelength through (in this example <600 nm)

3. **Bandpass (BP) filters:** allow a specific range of wavelength through. On a bandpass filter, the numbers indicate the median wavelength and the range of the pass. For example with BP 550/50, 550 nm indicates the median wavelength and light with wavelengths 525-575 nm will pass through (This filter spans 50 nm, 25 nm on either side of the median bandpass).

4. Within a detection system, light often bounces between various detectors to separate out distinct fluorescence signals. Some filters, such as LP and SP filters, are set up as dichroic mirrors. Positioned at a 45° angle from the incoming light, dichroic mirrors allow any blocked light to deflect to the next filter.

Filters

Longpass   Shortpass   Bandpass

LP 500   SP 600   550/50
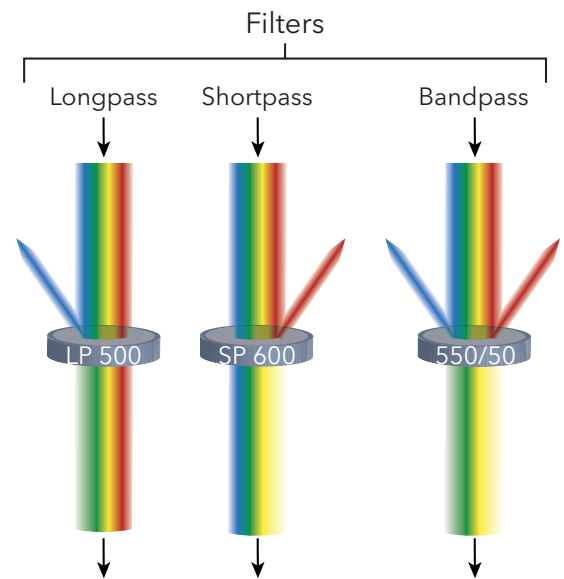
PMT
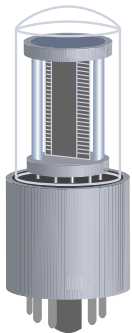
DETECTORS

Detectors (e.g. photomultiplier tubes, PMTs) are placed behind the optical filters to convert the passing photons into an electronic signal. The signal is amplified by controlling voltage or gain settings on each detector (see section on setting instrument voltages, pg 20).

HOW TO DETERMINE THE OPTIMAL LASER AND FILTER FOR A FLUOROCHROME

The wavelength of the laser beam must fall within the excitation spectrum of a fluorochrome in order to efficiently excite the molecule of interest. For example, AF488 would be optimally excited by a laser with a wavelength of 488 nm. Following excitation, AF488 emits light above 475 nm and the fluorescence signal between 515 nm and 545 nm is captured by the 530/30 bandpass filter.

488 Laser   Filter 530/30

Emission signal

% Excitation/Emission

Wavelength (nm)
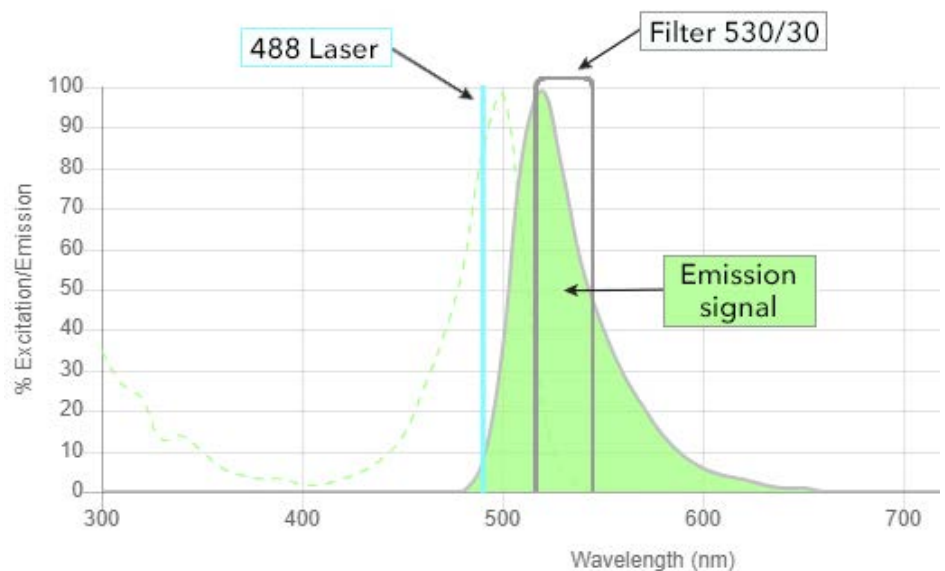
BASIC COMPONENTS OF A FLOW CYTOMETER

Most instruments are configured to detect multiple fluorochromes from each laser line. This is accomplished by "bouncing" light through a series of filters. As illustrated in **FIGURE 5**, light excited by the 405 nm laser would enter the light collection optics and hit a 600 nm dichroic LP mirror. With this filter, any wavelength longer than 600 nm would be sent to the 610/20 BP filter (range of 600-620 nm), and any light in that range would pass through to the Qdot 605™ detector. Any light with a wavelength shorter than 600 nm would be directed to the 505 LP mirror. From here, anything longer than 505 nm would pass through to the 525/50 BP filter (AmCyan), and anything shorter would be directed to the 450/40 BP filter (Alexa Fluor™ 405) and so on. This same process occurs for each laser on the instrument.
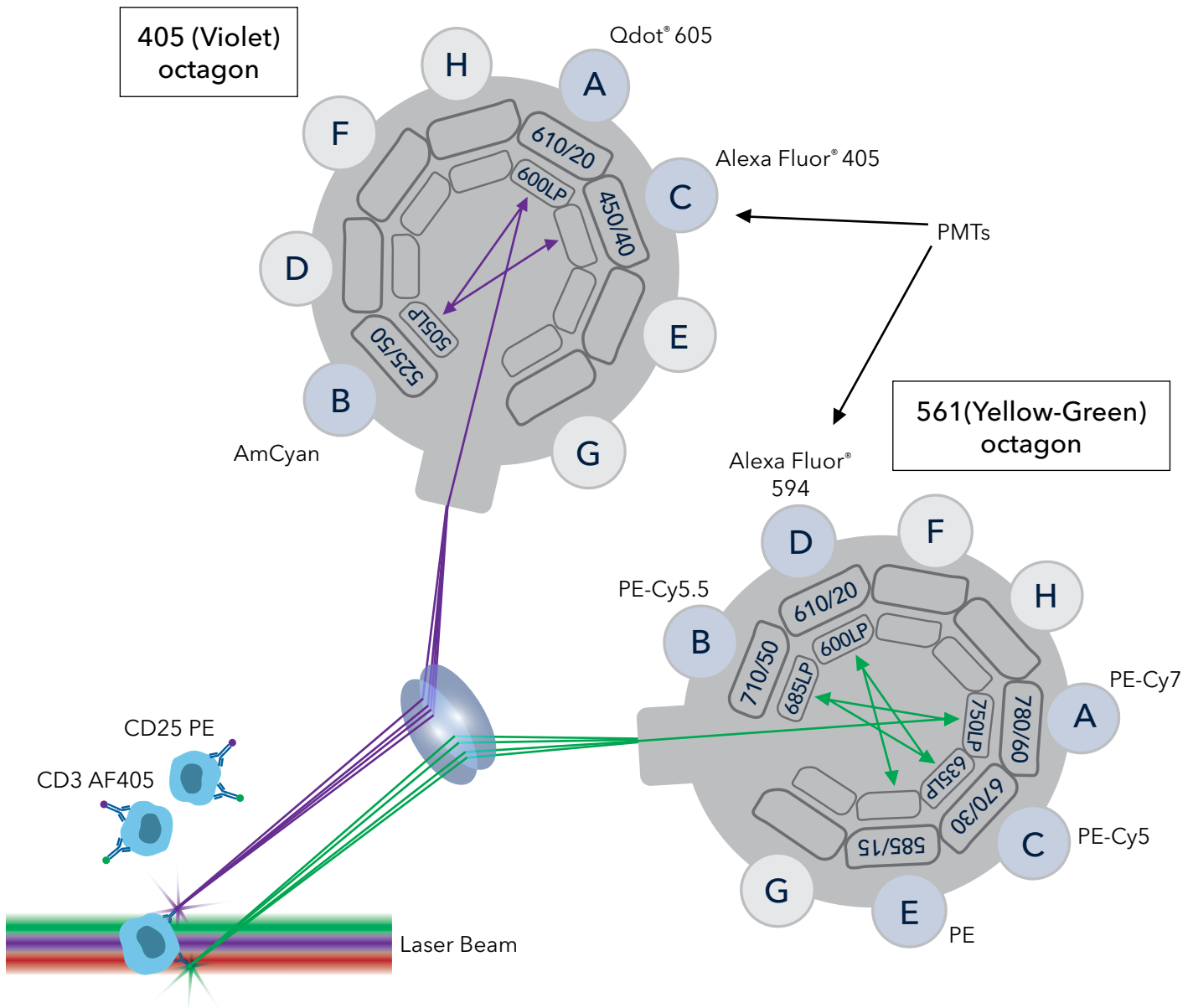
FIGURE 5. Schematic of 405 and 561 nm laser detector modules on the BD LSRFortessa. The various detectors (letters A-G) are represented on the BD LSRFortessa for the 405 and 561 octagons (adapted from the cytometer configuration). The bandpass filters are shown in rectangular boxes in the outer circle and the longpass mirrors are directly below the bandpass filters in the inner circle. Fluorochrome conjugated antibodies on cells are excited by lasers in the flow cell. The emitted light then passes through a series of mirrors/filters within the optics system and direct the light to the appropriate detector. The emitted light is directed from high to low wavelengths in the octagon, or in order from detector A to B to C as shown in the 405 octagon.

TYPES OF CYTOMETERS

While each cytometer contains components to process and analyze cells, there is flexibility in how these components are engineered. In the last decade, adaptations to flow cytometry have resulted in technological advances and unique applications.

SPECTRAL CYTOMETRY

A recent commercial development of flow cytometry is spectral cytometry. Spectral cytometers differ from conventional flow cytometers in the way that light is collected. Spectral cytometers disperse fluorescence signals across a detector array to analyze the full emission wavelengths of each fluorochrome (**FIGURE 6**) rather than using a specific bandpass filter. The emission pattern of each fluorochrome across the detector array generates a reference signature that is used to mathematically unmix the proportion of the signal associated with each fluorochrome from the fully stained sample. Once unmixed, the data can be analyzed as in conventional flow cytometry.

Since spectral cytometry eliminates the need for dedicated optical filters for each fluorochrome, there is more flexibility in the types and combinations of fluorescent probes that can be used. For example, highly overlapping fluorochromes can be separated if there are enough differences in their spectral signatures across the detector array. In addition, the spectral signature of cellular autofluorescence can be extracted from the data to minimize background noise.
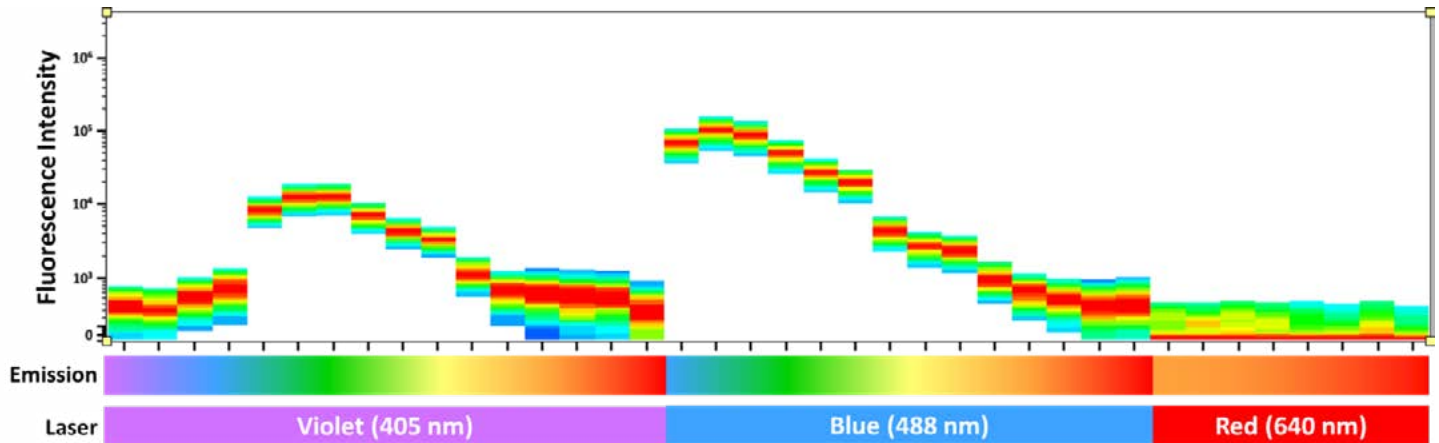

FIGURE 6. Spectral Signature of FITC on Cytek Aurora. This spectral plot displays the signature of CD4 FITC stained antibody capture beads across the detector arrays from the violet, blue and red lasers on the Aurora (three laser, 38-channel) spectral cytometer (Cytek Biosciences).

IMAGING FLOW CYTOMETRY

Combining concepts of microscopy with flow cytometry led to the development of a class of instruments called imaging flow cytometers. Imaging flow cytometry uses objective lenses to capture images of the cells at the interrogation point and relies on charge-coupled device (CCD) cameras to capture fluorescence at multiple wavelengths. The composite images allow the researcher to understand the spatial distribution of fluorescent probes throughout the cell. This is useful for applications such as localization of a specific probe to parts of the cell, evaluation of cell-cell interaction, morphological analysis, and spot counting.

TYPES OF CYTOMETERS

MASS CYTOMETRY

Mass cytometry utilizes techniques of mass spectrometry to achieve high parameter analysis of single cells. Mass cytometry uses antibodies conjugated to stable isotopes of a specific mass which are then measured using time-of-flight (TOF) mass spectrometry (**FIGURE 7**). The advantage of this technology lies in the wide range of stable isotopes that can be used, avoiding the constraints of spectral overlap associated with fluorochromes. Mass cytometry has resulted in cell phenotyping panels upwards of 30 parameters and surpassing many high-end flow cytometers.
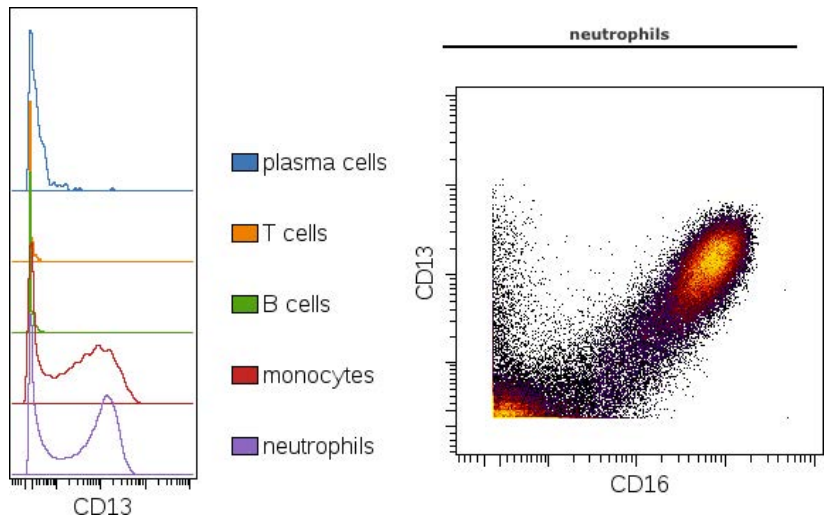

FIGURE 7. CyTOF data. Human bone marrow underwent RBC lysis and surface staining with Gd-160 conjugate of CD13 (22A5) antibody (Novus Biologicals Catalog # NBP1-28429) at 2 ug/mL, followed by gating on standard surface markers. Neutrophils demonstrate characteristic CD13 loss and reacquisition, with partial reactivity on monocytes. Image from customer review.

CELL SORTING

Cell sorters contain the basic components of a flow cytometer with the addition of a module to enable isolation of single cells of interest. Most cell sorters use electrostatic methods, also known as jet-in-air, whereby the stream exiting the flow cell is vibrated to generate droplets. The objective is to isolate single cells inside individual droplets and when a target cell is identified, apply an electrostatic charge, either positive or negative, to the drop. The target drop is then deflected by charged plates (+/-) and deposited in a collection tube. Uncharged drops are directed to a waste receptacle. The figure on the right depicts two-way sorting plus a waste stream. Droplet cell sorting using fluorescence was developed at Stanford University in the 1970's by the Herzenberg lab who coined the term Fluorescence Activated Cell Sorting or FACS.

Electrostatic cell sorting has remained the technology of choice for cell isolation due to its speed and ability to be applied to high-end flow cytometers to identify and purify specific cell populations using complex staining panels. Most high-end cell sorters are able to simultaneously isolate up to four and sometimes six populations with high purity at speeds of 5,000 to 20,000 cells per second. In addition, many cell sorters are able to sort single cells into multi-well plates enabling downstream assays such as single cell genomics.

Other types of cell sorters use different methods of cell isolation such as air diversion for large particles and microfluidic actuators to divert cells of interest inside a contained apparatus.

Principle of FACS
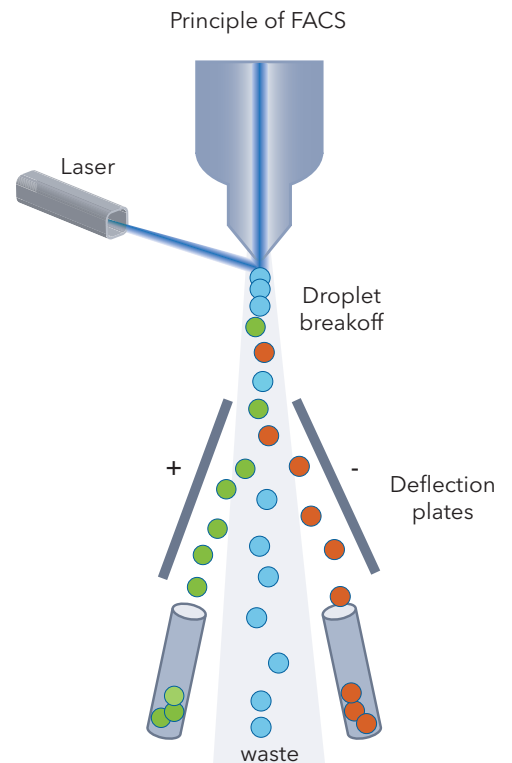
TYPES OF CYTOMETERS: OVERVIEW

CATEGORY	TYPES	SAMPLE TYPES	PURIFY CELLS OR PARTICLES	DETECTION METHOD	SPILLOVER CORRECTION	RESOLVES SUB-CELLULAR LOCALIZATION	DATA FILE TYPE
Analytical Cytometers	Conventional	Single Cell Suspension	N	Peak fluorescence is measured in dedicated channel	Compensation	N	FCS
	Spectral	Single Cell Suspension	N	Full fluorescence emission is captured across detector arrays	Spectral Unmixing	N	FCS
	Imaging Flow	Single Cell Suspension	N	Peak fluorescence is measured in dedicated channel	Compensation	Y – Images are generated	Proprietary file types
	Mass	Single Cell Suspension	N	Time-of-Flight (TOF) of metal tag is measured		N	IMD (integrated mass data)
	Imaging Mass	Tissue sections or cell smears on glass slides	N	Time-of-Flight (TOF) of metal tag is measured		Y – Images are reconstructed following laser ablation	MCD (mass cytometry data)
Cell Sorters	Electrostatic/ Droplet	Single Cell Suspension	Y	Peak fluorescence is measured in dedicated channel	Compensation	N	FCS
	Microfluidic	Single Cell Suspension	Y	Peak fluorescence is measured in dedicated channel	Compensation	N	FCS

WHAT'S AN ALTERNATIVE METHOD FOR QUANTIFYING DIFFICULT TARGETS IN SINGLE CELLS?

Single-Cell Westerns make it possible to run Westerns on individual cells in order to measure variation of protein expression across heterogeneous cells in a sample. Milo™ is the world's first Single-Cell Western platform. Milo chemically lyses cells before analysis for easy access to protein epitopes that can be challenging to detect with intact cells using flow cytometry. This semi-automated technology generates Western-based protein expression data on a thousand single cells per run and simultaneously detects up to 12 targets per cell.

Benefits of Single-Cell Westerns:

- Eliminate fixation & permeabilization steps

- Use Western-validated antibodies

- Resolve and detect intranuclear proteins, protein isoforms, post-translational modifications, and more..

- Analyze heterogeneity in highly enriched flow-sorted samples

Learn more about Milo at www.proteinsimple.com/milo

FLOW CYTOMETRY WORKFLOW: FOUR MAIN STEPS

Experimental Setup	→	Sample Preparation	→	Sample Staining	→	Sample Acquisition/Analysis

This workflow, covering the four main steps of a multicolor flow cytometry experiment, will explain how to take cells and generate the data shown in dot plots and histograms.

I. EXPERIMENTAL SETUP

For any panel design, there are two critical steps:

1. Know the instrument configuration -> understand which fluorochromes can and cannot be used.

2. Understand the biological markers -> list the markers of interest and have a general idea of their cellular expression and distribution.

Flow cytometers are generally equipped with multiple lasers that produce light at a specific wavelength. Since light emitted by each fluorochrome is filtered, generally through a bandpass filter in combination with short or long pass filters, the cytometer will only detect fluorescence within a specific range of wavelengths. When selecting a fluorochrome, verify that the instrument has both a laser capable of exciting the fluorescent probe of interest and a filter set capable of detecting the probe's emission.

ANTIBODY SELECTION / PANEL DESIGN

Generally, the more fluorescent probes that are applied to a sample, the more information can be collected from that sample. When combining multiple fluorochromes within a single sample, there are a number of basic recommendations to follow for panel design:

<10 COLORS

1. Match antigen density* with fluorochrome brightness (see the table of fluorochromes on the inside cover) a. Most importantly, low density or difficult to detect markers are paired with bright fluorochromes. b. High density markers are matched with moderate or dim fluorochromes.

2. Minimize spillover by selecting fluorescent probes that span the color spectrum.

3. Test!

>10 COLORS

1. Match markers with a low level of expression with bright fluorochromes.

2. Minimize spillover especially for low expressing antigens.

3. Use a spillover spread matrix** to determine channels where resolution may be impacted.

4. Reserve suboptimal fluorochrome combinations for mutually exclusive antigens (antigens that are never expressed on the same cell type).

5. Test!

The final step before running an experiment is to test the panel to demonstrate that staining is biologically relevant and to verify that all stains are compatible.

FLOW CYTOMETRY WORKFLOW: FOUR MAIN STEPS

Antigen density refers to the level of a protein expressed in or on a cell (see the example below) and is an important consideration in panel design. Antigens that are expressed at a low level, are intracellular, or have a continuous expression pattern (not induced) should be paired with the brightest fluorochromes to ensure detection. Antigen density and expected expression patterns can be found online or in the literature.

MARKER	DENSITY	FLUOROCHROME BRIGHTNESS
CD3	High	Low to Moderate
CD25	Low	Bright
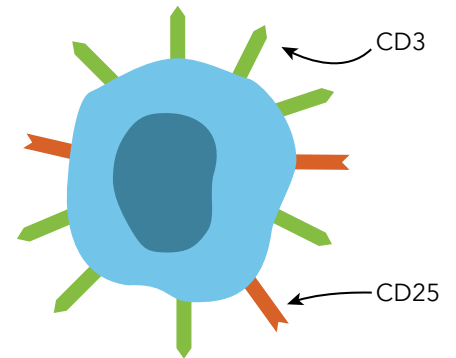
Spillover indicates that fluorochrome signals are "spilling" over into other detectors. This is a product of the fluorochrome emission and the optical setup of the instrument. As shown below the green signal (AF488) is detected in the FITC filter (515-545 nm), as well as in the adjacent PE filter (564-606 nm). This spillover is corrected by compensation, which is covered in the next section.
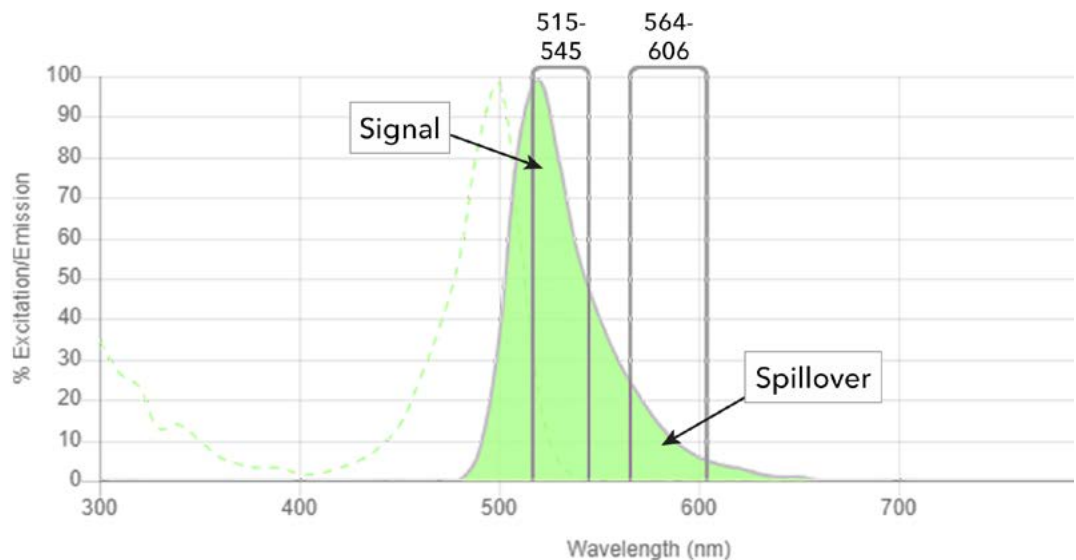
Spillover spreading is due to a measurement error from multiple fluorochromes spilling into each detector and leads to loss of resolution in some channels.[3] This becomes more important as the number of fluorochromes in an experiment increases and the spread is higher as fluorescence gets brighter. Channels that may have spillover spreading issues are identified by creating a spillover spread matrix (SSM). In general, dim signals should be protected from this phenomenon.

In the example on the right, fluorescence from detector 2 spreads into detector 1 and this spread increases as the signal in detector 2 increases. Therefore, if a low expressed antigen with a fluorochrome in detector 1 (light grey circle) and a highly expressed antigen in detector 2 (orange) are analyzed, detecting the dim antigen would not be possible.
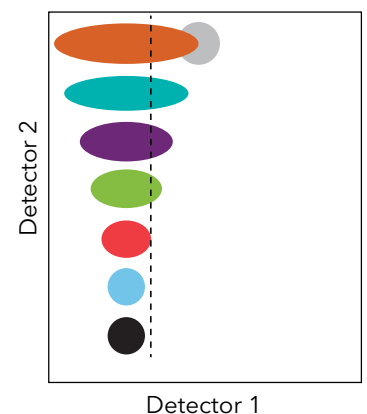
FLOW CYTOMETRY WORKFLOW: FOUR MAIN STEPS

UNDERSTANDING CONTROLS

Flow cytometry controls can be split into five categories[5]:

1. INSTRUMENT CONTROLS

Instrument controls confirm that the instrument is performing properly by tracking laser, detectors, and fluidic performance.[6] In general, these controls involve calibration particles supplied or sold by the instrument's manufacturer or fluorescent beads. These quality control particles should be run every day the instrument is in use.

WHAT IS COMPENSATION?

2. COMPENSATION CONTROLS

As mentioned in the previous section, when the emission spectra between fluorochromes overlap, these signals can spread into multiple channels. The process of correcting for spillover is known as compensation.[7] Compensation ensures that the detector only calculates fluorescence emission specific to that channel (**FIGURE 8**).

For compensation, a single stained sample (only one color per sample) is needed from each fluorochrome in the experiment. These can be set up using cells, compensation beads, or antibody capture beads*. When creating compensation controls, there are four essential rules:

1. The compensation fluorochrome **MUST** be an exact match to the experimental fluorochrome.

    a. Fluorochromes in the same channel will still have different emission spectra and are not interchangeable. For instance, FITC cannot be used as a compensation control for GFP.

    b. Due to differences in tandem chemistry, the tandem conjugated antibody in the experiment should be the same one (from the same tube) used for compensation calculations. Using the same tandem fluorochrome but conjugated to a different antibody, or even using a different lot, can lead to inaccurate calculations.

2. Controls must be as bright or brighter than experimental samples.

3. Autofluorescence for negative and positive populations must be the same for any given control. Best practices are to individually gate positive and negative populations for each channel and to avoid using a universal negative.

4. Collect enough events! Compensation relies on an accurate calculation of median fluorescence intensity and if there are too few events, this will not be calculated accurately. The minimum number of events collected should be above 5,000.
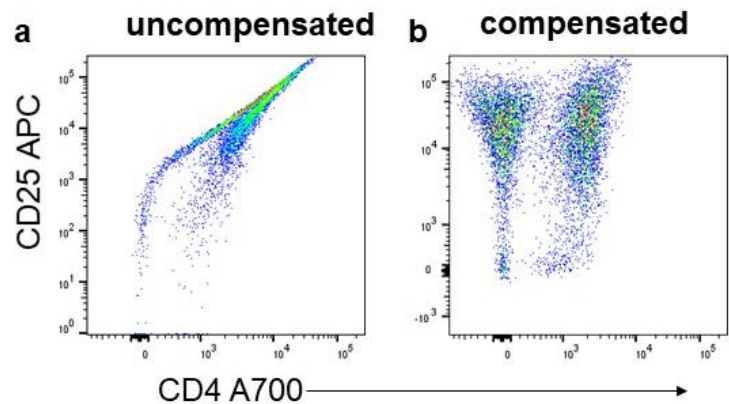

FIGURE 8. Uncompensated versus compensated data. Human CD3+ T cells were activated with anti-CD3/anti-CD28 for 7 days. The cells were stained with antibodies against hCD4 A700 (R&D Systems Catalog # FAB3791N) and hCD25 APC (R&D Systems Catalog # FAB1020A). Dot plot of hCD4 and hCD25 without compensation (**a**) and with compensation applied (**b**).

FLOW CYTOMETRY WORKFLOW: FOUR MAIN STEPS

*Antibody capture beads bind the antibody heavy chain and, unlike the biological sample, do not require specific antigen recognition. These beads serve as useful compensation reagents that can improve the consistency of compensation and are particularly suited when:

- The positive population is dim or rare. Compensation beads have an intense fluorescence signal, contributing to compensation controls being as bright or brighter than experimental samples.

- Working with multiple fluorochromes.

- Cells, used for making controls, are in limited supply.

3. GATING CONTROLS

One of the most critical aspects of analyzing flow cytometry data is properly setting gates. When cell populations aren't clearly defined or are rare, the Fluorescence Minus One (FMO) control is necessary for interpreting flow data and for setting gates. Since FMO controls provide a reliable assessment of background in a multicolor experiment, they can accurately discriminate between negative and positive cell populations.

Preparing a FMO control involves staining cells with all reagents except one. For example, if running a four-color experiment with AF488, PE, APC, and DAPI, FMO controls would be stained as follows:

SETTING UP FMO CONTROLS				
CONTROL	FITC	PE	DAPI	APC
AF488 FMO	X	✓	✓	✓
PE FMO	✓	X	✓	✓
DAPI FMO	✓	✓	X	✓
APC FMO	✓	✓	✓	X

4. PROTOCOL CONTROLS

Antibodies bind to cells in three fundamental ways:

1. Fab region binds the antigen (ideal scenario).

2. Fc region binds to the Fc-receptor (generally unwanted).

3. Non-specifically (off-target antigens, to "sticky" membranes, etc).

In any flow cytometry assay, the best way to minimize non-specific binding includes:

- Proper titration of all reagents

- Fc-receptor blocking of cells

- Addition of a viability dye to exclude dead cells

TYPES OF ANTIBODY BINDING

FAB Binding
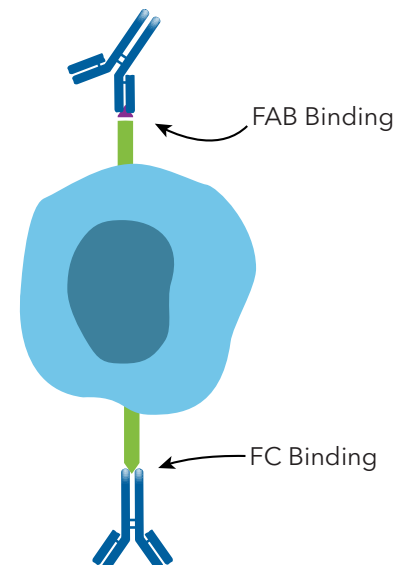
FC Binding

FLOW CYTOMETRY WORKFLOW: FOUR MAIN STEPS

<div style="float:left">

WHAT ARE ISOTYPE CONTROLS?

</div>

Isotype controls are used to assess non-specific binding due to antibody class. An isotype control is an antibody that lacks specificity to the target of interest, but matches the class, type, and fluorescent conjugate of the primary antibody. By measuring background staining, isotype controls will indicate if a protocol requires further optimization. Isotype controls are useful to determine if:

- **Cells have been adequately blocked.** If not fully blocked, isotype controls will bind Fc receptors. If the cause of non-specific binding is due to cell "stickiness", especially from the presence of dead cells, add DNase to the staining buffer.

- **Cells have been adequately washed during an intracellular stain.** It is common for antibodies to intracellular targets to get trapped inside the cell, even when not bound to antigens.

Historically, isotype controls have been one of the most frequently used negative controls in flow cytometry. However, a perfectly matched isotype control must have the same heavy and light chain class of immunoglobulin as well as the same ratio of fluorochrome to antibody as the test antibody. In conditions where isotype controls do not meet these requirements, the user must be cautious in overinterpretation of background staining.[5,8] In multicolor flow cytometry panels, isotype controls do not account for background fluorescence attributed to reagents in other channels. The FMO control is the appropriate control for this assessment (see section on gating controls, pg 14).

<div style="float:left">

WHAT ARE ISOCLONIC CONTROLS?

</div>

Isoclonic controls involve the addition of excess unlabeled antibody to detect non-specific binding. If antibody binding is specific, adding an excess of unlabeled antibody will result in a decrease in fluorescence intensity. No reduction in fluorescence indicates non-specific binding.

5. EXPERIMENTAL CONTROLS

Experimental controls fall into a few principal categories:

- **Biological Negative Controls** provide background expression for all tested markers. This control is particularly important for comparing the differences in expression of a protein on chemically or biologically treated cells (**FIGURE 9**).

- **Biological Positive Controls** demonstrate positive staining by an antibody in pertinent cells and indicate that the antibody is working in the experiment.

- **Longitudinal Controls / Reference** controls are quality controls for long term studies to ensure consistent staining across experiments.
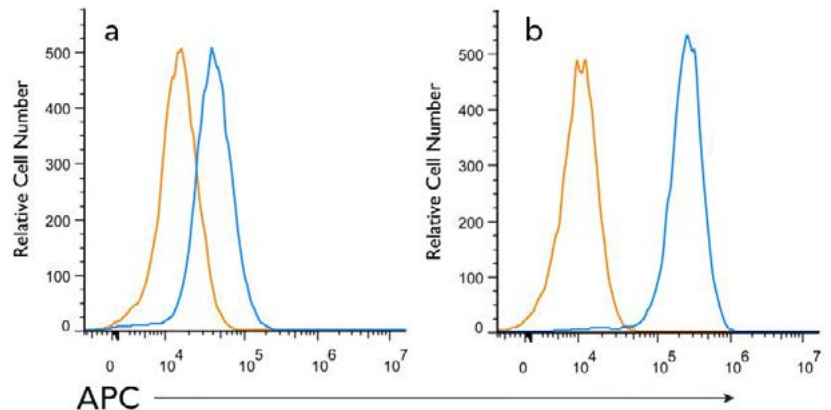
FIGURE 9. Negative controls for induced markers. Jurkat cells were either untreated (**a**) or treated with 50uM Chloroquine (Tocris Bioscience Catalog# 4109) for 24 hours (**b**). An intracellular stain was performed with LC3B (1251A) antibody (Novus Biologicals Catalog # NBP2-46892, blue) and a matched isotype control (R&D Systems Catalog # MAB1050, orange). Cells were fixed with 4% formaldehyde, following fixation, cells were permeabilized with 0.1% saponin. Cells were incubated in an antibody dilution of 1 ug/mL for 30 minutes at room temperature, followed by rabbit IgG APC-conjugated secondary antibody (R&D Systems Catalog # F0111).

FLOW CYTOMETRY WORKFLOW: FOUR MAIN STEPS

| Experimental Setup | → | Sample Preparation | → | Sample Staining | → | Sample Acquisition/Analysis |

II. SAMPLE PREPARATION

Samples for flow cytometry are prepared as a single particle suspension from a variety of sources to analyze viable or fixed cells, nuclei, microorganisms or small particles depending on the objectives of the assay. Having or developing a good protocol for sample preparation forms the basis for a successful experiment. The main consideration when generating a single cell suspension is to minimize cell death and debris. Dead cells can cause clumping in the sample, tend to stick to free antibody during immunostaining, and exhibit a higher level of autofluorescence, which interferes with accurate measurement of fluorescence. Excess debris from the preparation protocol can increase the level of noise and, if excessive, can interfere with measurement of the cells of interest.

While there is no magic in flow cytometry (Howard Shapiro's Zero-th Law of Flow Cytometry), sample preparation can be optimized by following the below steps:

1. Get and maintain a single cell suspension.

    a. Filters are a great resource for removing clumps from samples. It is recommended to start with a 40 micron filter.

    b. Certain staining buffer additives can reduce cell clumping.

        i. EDTA (~1-5 mM) helps to avoid cation-dependent cell adhesion.

        ii. DNase (~0.1 mg/mL) breaks down DNA from dead cells and reduces cell adhesion.

    c. Liberating a cell population from whole tissue can be challenging. Enzymes such as collagenase and trypsin are commonly used to break up tissue and release cells. Care must be taken to avoid over-digestion which can destroy antigen(s) of interest or compromise cell viability.

2. Know the biological samples and keep cells alive. A standard protocol for cell prep will depend on the cell source.

    a. Keeping cells on ice and using cool buffers helps maintain viability in some cells. (NOTE: this may not be applicable to all cells or applications!)

    b. Minimize the time, vortexing, and excessive centrifuge speeds/times, all of which can affect cell viability. For example, peripheral blood, lymphoid tissues and cultured cells require less rigorous methods to generate a single cell suspension.

    c. Process cells as quickly as possible before fixing to minimize artifacts and cell death.

    d. Consider adding HEPES to increase CO_2 buffering in the staining solution.

    e. Know the cell size and whether they express any fluorescent proteins or have any fluorescent properties.

FLOW CYTOMETRY WORKFLOW: FOUR MAIN STEPS

Experimental Setup → Sample Preparation → Sample Staining → Sample Acquisition/Analysis

III. SAMPLE STAINING

Developing a flow cytometry protocol and workflow will take some troubleshooting as there are many variables that must be optimized. When starting a new flow cytometry experiment, it is always best to do trial runs with various conditions in order to optimize the workflow. Protocols and staining conditions may not be directly transferable between cell types or when analyzing different antigens.

Antibody incubation time and temperature can affect receptor staining and this step may require further development (**FIGURE 10**).
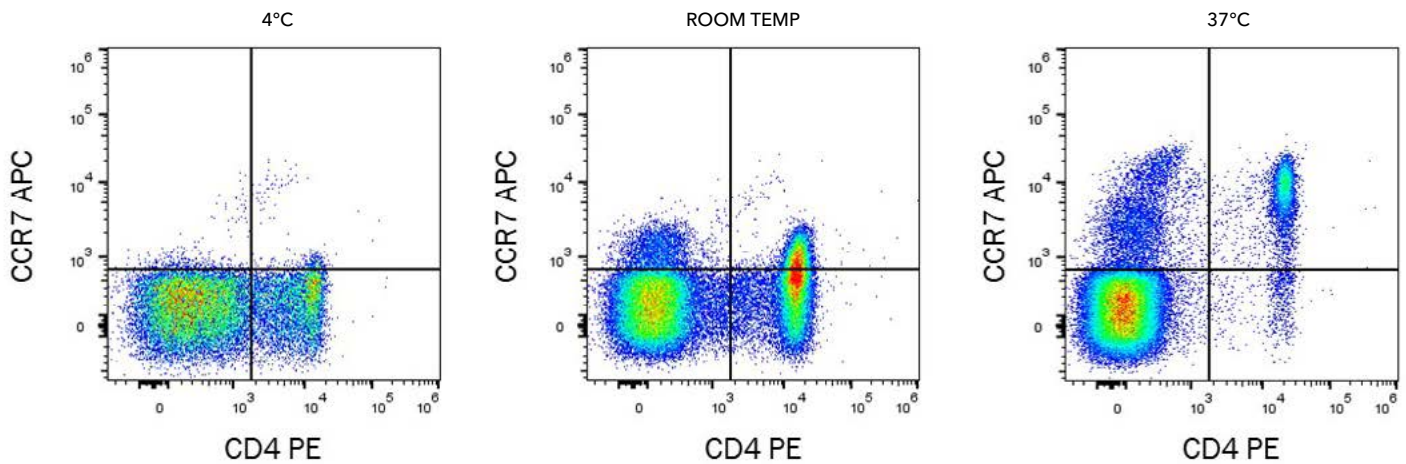

FIGURE 10. **Effect of temperature on staining.** The epitope of CCR7 is not accessible without the optimal staining conditions. Cells stained at 4° C have low CCR7 signal. Detection of CCR7 (R&D Systems Catalog # FAB197A) is improved when the antibody incubation temperature is increased to room temperature and 37° C under the same cell preparation conditions. While CCR7 staining is ideal at 37° C, staining of other receptors may be optimal at 4° C.
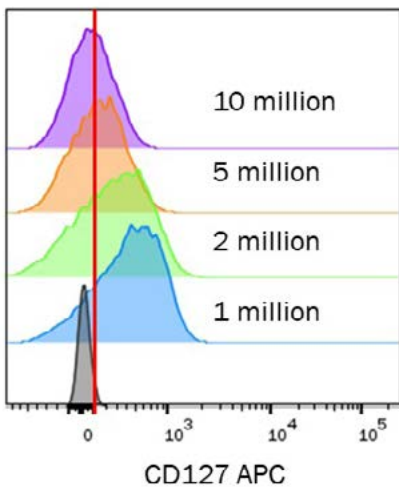
Manufacturers typically provide antibody dilutions using a fixed number of cells, ~one million cells per sample. This is particularly important for low density markers such as CD127, in which high cell number has a considerable impact on staining. (**FIGURE 11**).
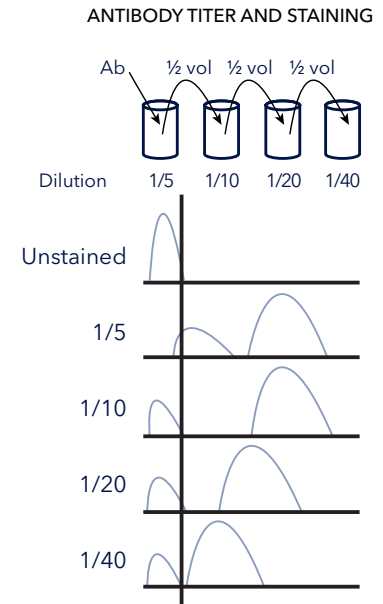
FIGURE 11. Effect of cell density on staining. Varying numbers of CD127+ T-cells have been stained with CD127 APC (R&D Systems Catalog # FAB306A) at a fixed concentration. Staining is optimal at one million cells because staining > two million cells results in a decrease in signal. This loss is due to the antibody being too dilute to bind all of the available antigen binding sites.

FLOW CYTOMETRY WORKFLOW: FOUR MAIN STEPS

Additional tips to optimize sample staining conditions include:

1. Always add a viability dye.

2. Always titrate reagents to achieve optimal signal to noise (primary antibodies, secondary antibodies, viability dyes, etc.). In the example on the right, a 1:10 dilution demonstrates the best signal to noise.

3. Special considerations are needed for intracellular stains.

   i) To stain intracellular molecules, cells need to be fixed in suspension and then permeabilized before the antibody is added. This fixation/permeabilization treatment allows the antibody to pass through the plasma membrane into the cell interior, while maintaining the morphological characteristics used to sort cells.

ANTIBODY TITER AND STAINING

Ab   ½ vol   ½ vol   ½ vol

Dilution   1/5   1/10   1/20   1/40

Unstained

1/5

1/10

1/20

1/40

FIXATION

- Keep fixatives cold-fixation is an exothermic reaction!

- Mix gently while adding the fixative to reduce cell clumping.

- Epitopes and fluorochromes can be fixation-sensitive. Be sure to test each antibody and check the product datasheet.

- Stain surface antigens before fixation and permeabilization.

- Fixation/permeabilization reagents alter the scatter properties as well as cellular autofluorescence, therefore, include an unstained control that has been treated with the same reagents.

- For staining phosphorylated proteins, the cells should be fixed and permeabilized immediately after the stimulation as phosphorylation is a transitory phase.

PERMEABILIZATION

- Commonly used detergents include saponin, Triton® X-100, or Tween® 20.

- Some detergents should be added to buffers throughout the entire protocol, including washes.

ii) For staining secreted proteins such as cytokines, a protein transport inhibitor (Monensin or Brefeldin A) is required to trap the cytokines inside the cell. These compounds prevent the export of newly synthesized proteins by disrupting the ER-Golgi transport machinery. For experimental treatments with stimulation periods of up to 4-6 hours, the secretion inhibitor can be present during the entire incubation period. If the stimulation period is longer than 4-6 hours, the secretion inhibitor should be added for only the last two hours of the incubation.

iii) Avoid tandem dyes for intracellular targets—these conjugates are large and may have difficulty penetrating cellular membranes as well as being washed out of the cell.
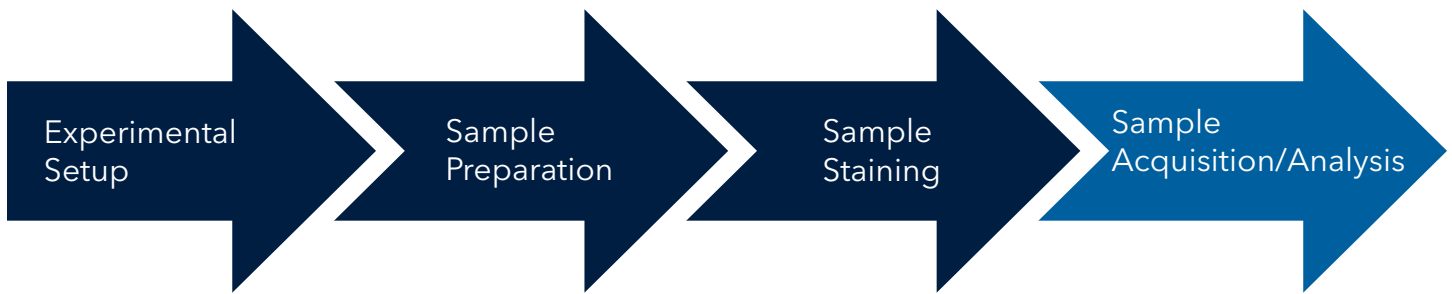
No data analysis technique can make good data out of bad data (Howard Shapiro's Seventh Law).

FLOW CYTOMETRY WORKFLOW: FOUR MAIN STEPS

SAMPLE PREPARATION AND STAINING FLOWCHART

FLOW CYTOMETRY WORKFLOW: FOUR MAIN STEPS
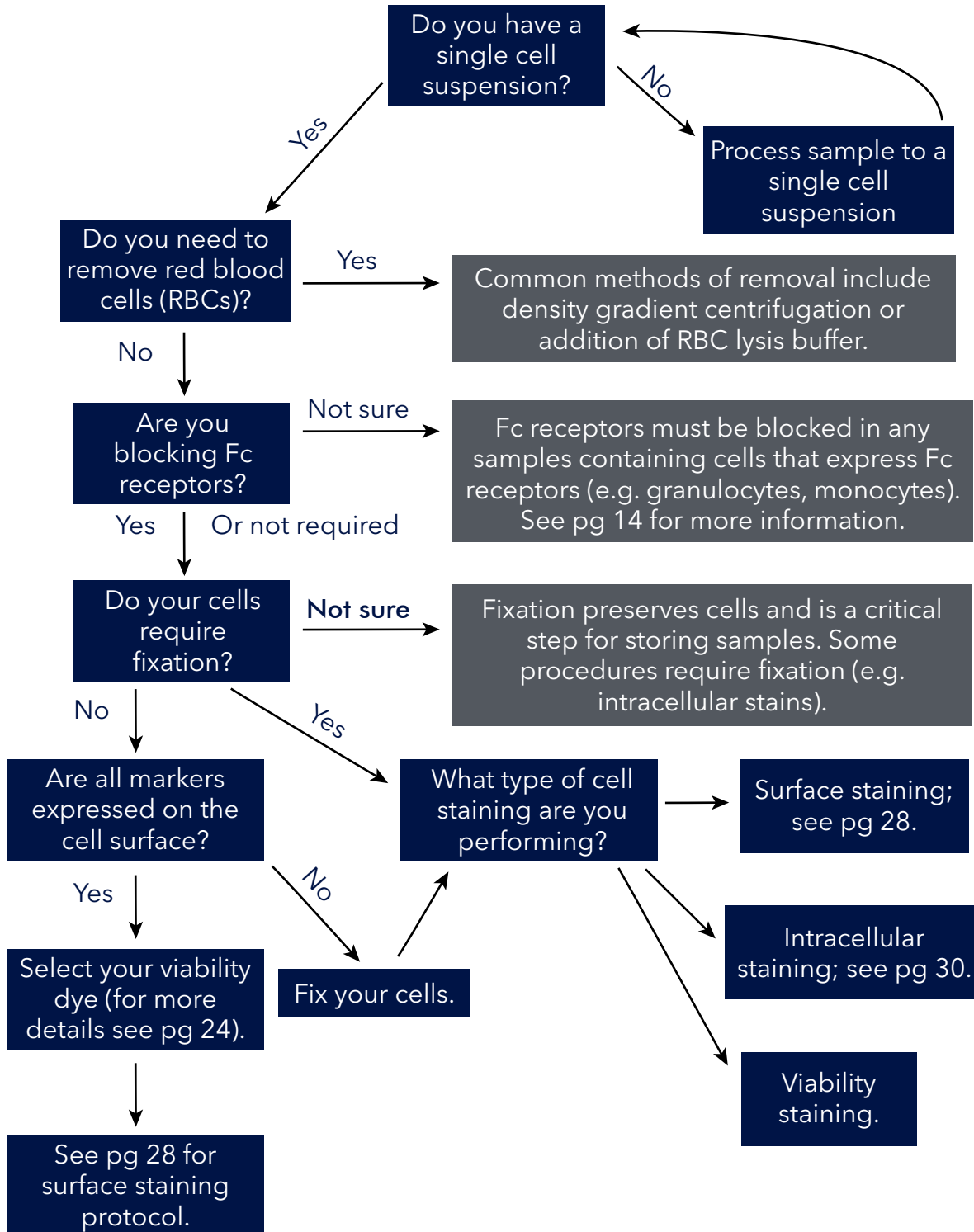
| Experimental Setup | Sample Preparation | Sample Staining | Sample Acquisition/Analysis |

IV. SAMPLE ACQUISITION

When setting instrument voltages, there are two general rules to follow:

1. The background signal should be in a region with minimal contribution from electronic noise.

2. The positive signal must be on-scale and within the linear range of the detector.

Instrument settings following instrument calibration are generally a good place to start when setting voltages. With these settings, check that the positive signal for each channel is on scale (**FIGURE 12**). If the positive signal is too high, reduce the voltage in the channel to ensure the stained population is on scale. This step is necessary for testing cells used in the experiment, not with other reagents such as capture beads.

a off-scale voltage (800 volts)
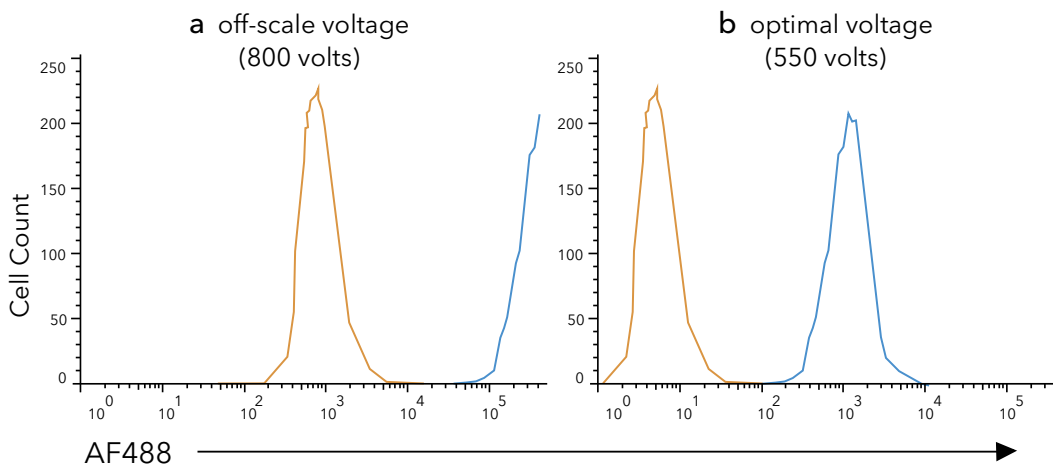
b optimal voltage (550 volts)

FIGURE 12. Off-scale voltage versus optimal voltage. When setting voltages for a flow cytometry experiment, it is important that all positive signals be on-scale (within the limit of fluorescence that the instrument can detect). When voltages are set too high (**a**), positive signals are too bright for the instrument and cannot be resolved. Care should always be taken to ensure that positive signals are on-scale (**b**).

RUN SINGLE STAINED CONTROLS FOR COMPENSATION

Once the optimal voltages have been set for each channel, move on to measuring compensation. Note:

• Due to the high binding capacity of antibody capture beads, it is not recommended to use compensation beads to set (or reset) voltages. *If the signal is saturated, titer down the amount of antibody applied to the capture beads.*

• The voltage in every channel must be the same for each compensation control.

Run each fluorochrome control on the appropriate tube, ensuring adequate positive events have been collected to accurately calculate compensation. Compensation can either be calculated prior to acquisition using an algorithm provided with the instrument software or post acquisition using flow cytometry analysis software.

FLOW CYTOMETRY WORKFLOW: FOUR MAIN STEPS

<table>
<tr><td>

HOW MANY EVENTS SHOULD BE COLLECTED?

</td><td>

One strength of flow cytometry is the ability to analyze a large population of cells in a short amount of time, resulting in statistically robust data. However, in order to ensure data is statistically significant, a sufficient number of events must be collected.[9,10] To determine the number of events needed, have a sense of the expected frequency of the positive population. The frequency is used to calculate the CV (coefficient of variation). A low CV is better and the CV can be lowered by increasing the number of cells collected. See below example for a population with an expected frequency of 1:100.

</td></tr>
</table>

For rare populations, a greater number of events will need to be collected for reproducible results. Finding the one in a million cell will require collecting more than 10,000 events, or even 1,000,000 events. There is no steadfast rule for this and no arbitrary "correct" number. Using the correct controls and understanding the biology of the system under study is the best place to start.

EVENTS COLLECTED	CV	POSITIVE EVENTS
10,000	10%	100
50,000	4.4%	500
100,000	3.2%	1,000

IV. ANALYSIS

Flow cytometry data analysis software traditionally uses worksheets containing a series of plots to visualize the data. Single parameter (histograms) or two parameter plots (dot plots) display the pattern of expression of each marker (**FIGURE 13**). Cell populations of interest are identified and segregated by drawing gates to form data boundaries and to isolate cells with common phenotypic patterns. Additional plots and gates narrow down the population(s) of interest using gating hierarchy or boolean logic. Statistics are calculated from the data points contained within the gates (e.g. frequency).
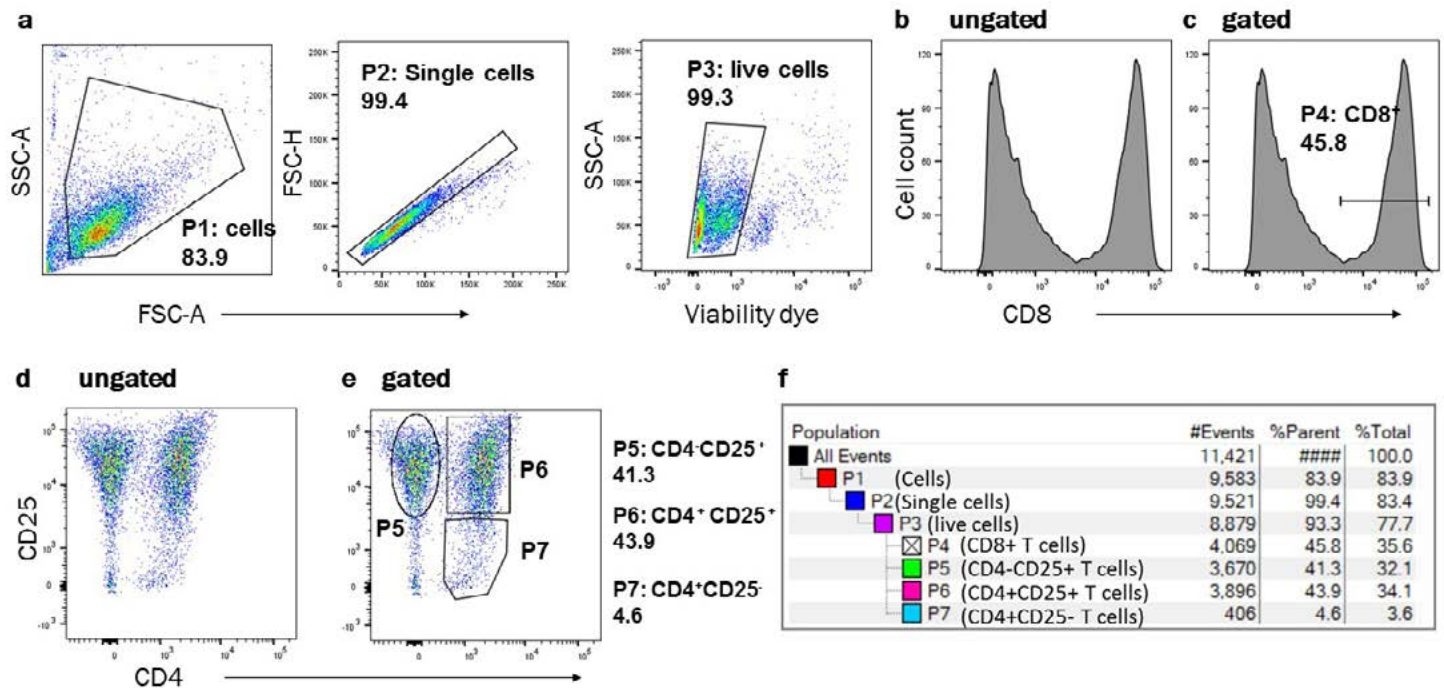

FIGURE 13. Histograms, dot plots, and population hierarchies. Human CD3+ T cells were activated with anti-CD3/anti-CD28 for 7 days. The cells were stained with antibodies against hCD4 (R&D Systems Catalog # FAB3791), hCD8 (R&D Systems Catalog # FAB1509), and hCD25 (R&D Systems Catalog # FAB1020). (**a**) Plots were drawn for cells (P1), single cells (P2), and live cells (P3). Ungated (**b**) and gated (**c**) histograms for hCD8. Dot plot of hCD4 and hCD25 without gates (**d**) and with gates (**e**). Dot plot of hCD4 and hCD25 with various gates drawn. Polygon gate around CD4+CD25- T cells, rectangular gate around CD4+CD25+ T cells, and oval gate around CD4-CD25+ cells (**e**). The gating hierarchy is shown in (**f**) where the CD8+ histogram gate (**c**) is a subgate of P1, P2, and P3; the same is true of the gates (P5-P7) shown on the dot plot for CD4/CD25 (**e**).

FLOW CYTOMETRY WORKFLOW: FOUR MAIN STEPS

FILE FORMAT - THE FCS FILE:

Flow cytometers generate data in the FCS (flow cytometry standard) file format. The FCS file was established and is maintained by the Data Standards taskforce of the International Society for Advancement of Cytometry (ISAC) to provide 'the specifications needed to completely describe flow cytometry data sets' and a uniform file format that can be analyzed in different software platforms. The current version of FCS is 3.1.

FILE ANNOTATION:

FCS files contain many keywords that can be used during analysis. Some of these are required by the file standard and auto-populated by the manufacturer's software. Other keywords require input from the user. Best practices for data annotation are outlined in the Minimum Information about a Flow Cytometry Experiment (MIFlowCyt), which is an accepted ISAC standard.[11] Examples of annotations include, sample name, marker/dye names, treatment, etc. A more thorough list, shown in the right table, is derived from the MIFlowCyt publication. Following best practices, details about experimental variables should be associated with the data file.

DATA RESOLUTION AND DISPLAY:

Data Scaling - Each flow cytometer processes the data into bins of fluorescence. The number of bins determines the resolution of the data scale, expressed in bits. In general, data is acquired in anywhere from 10 to 24 bits. Data can be acquired in linear and logarithmic mode depending on the dynamic range of the fluorescent populations (**FIGURE 14**).
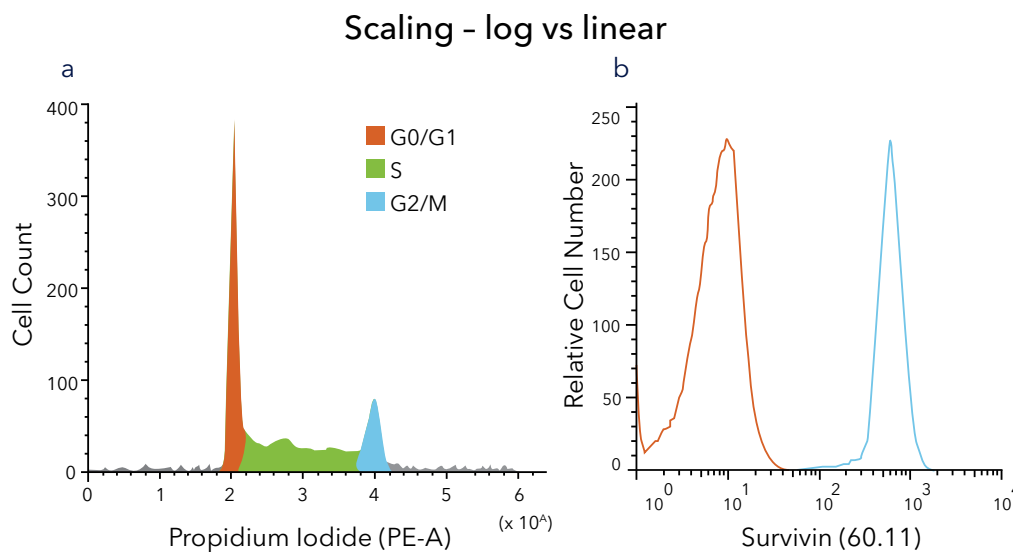
MIFlowCyt DATA ANNOTATION TABLE	
Experiment Overview	Purpose / Goal / Hypothesis
	Experiment Variables
	Conclusions
	Quality Control
Flow Sample (Specimen)	Material
	Source / Organism / Location
	Treatment
	Reagent / Analyte / Detector / Reporter
Data Analysis	List-mode Data
	Compensation
	Gating
	Descriptive Statistics
Instrument Details	Instrument Identification
	Fluidics Configuration
	Optical Configuration
	Electronic Configuration

Scaling – log vs linear

FIGURE 14. **Linear versus logarithmic scaling.** In applications such as DNA content analysis, there is a linear shift in the fluorescence from G1 to G2 with twice the amount of DNA in G2 as in G1 requiring a linear display of the data (**a**). For most other applications (e.g. immunophenotyping), data is acquired in log mode when the level of fluorescence varies over a large dynamic range (**b**).

FLOW CYTOMETRY WORKFLOW: FOUR MAIN STEPS

DATA TRANSFORMATION - Data transformation is another tool for displaying data with a high dynamic range. In flow cytometry, data transformation facilitates the visualization of data points that fall near zero due to background subtraction and spreading error. These data points get assigned to the first log channel resulting in events that pile up on the axis.[12,13] Biexponential and hyperlog are common algorithms used for data transformation (**FIGURE 15**). Data transformation compresses data below and above zero to more accurately visualize the data in this lower range.

FIGURE 15. **Logarithmic versus biexponential scaling.** Human CD3+ T cells were activated with anti-CD3/anti-CD28 for 7 days and were stained with antibodies against hCD8 (R&D Systems Catalog # FAB1509) and hCD25 (R&D Systems Catalog # FAB1020). Logarithmic scaling is shown in (**a**) and Biexponential scaling is shown in (**b**).
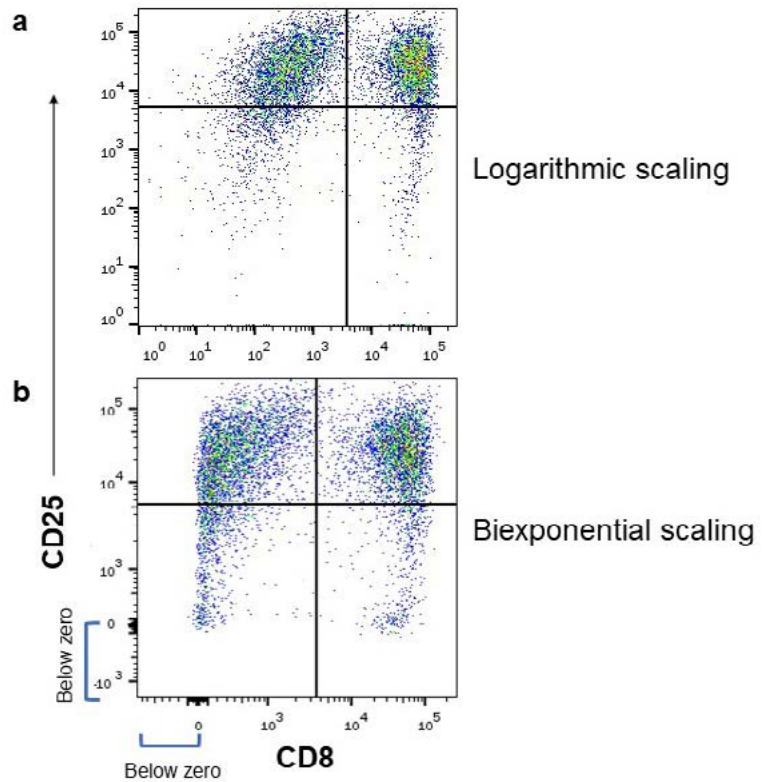
DATA CLEANUP:

As other technologies, flow cytometry is prone to anomalies in the data that arise from instrument problems, sample prep issues, or user error. It is important to QC and, if necessary, clean up the data to avoid misinterpretation. Gating strategies for data cleanup include:
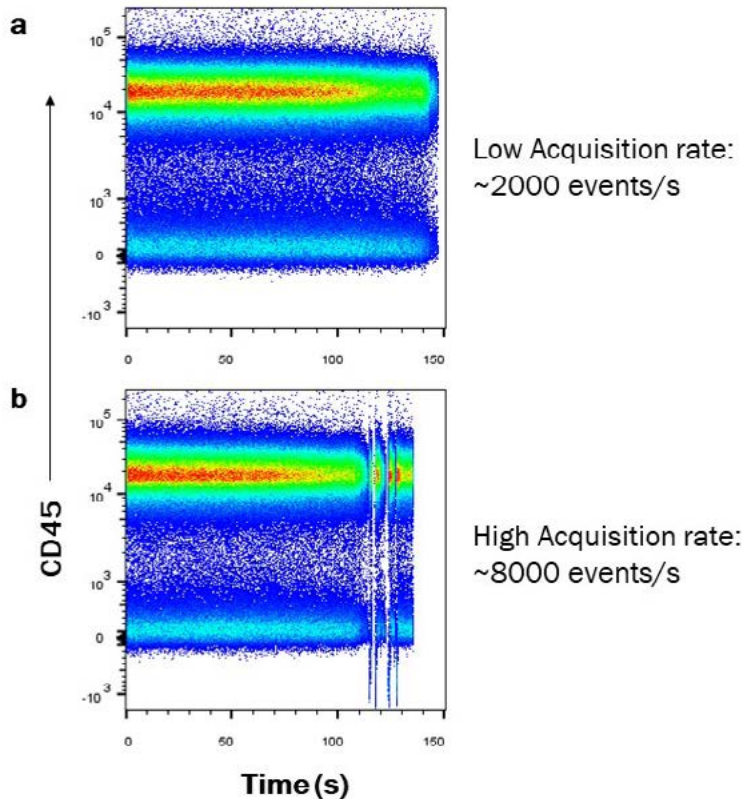
TIME GATING - The time duration of the recorded sample is a parameter that is included in every FCS file. This can be plotted against any other parameter in a dot plot to look for instability in the fluidics. In the case of a clog or bubble, for example, the user can gate on the duration of time outside of the anomaly to ensure the fluorescence being measured is error free (**FIGURE 16**). In some cases, samples may need to be eliminated from the analysis or data re-recorded once the issue is resolved. This time plot can even be monitored during acquisition to immediately identify and resolve any problems.

FIGURE 16: **Low versus high acquisition time plots.** PBMCs were stained with hCD45 (R&D Systems Catalog # FAB1430) and acquired on either low (**a**) or high (**b**) on the Fortessa. (**a**) Low (~2000 events/s) acquisition rates show a steady flow rate on the corresponding time/CD45 plot whereas high (8000 events/s) acquisition rates (**b**) show multiple breaks/disruptions in the flow rate.

FLOW CYTOMETRY WORKFLOW: FOUR MAIN STEPS

SCATTER GATING - The FSC and SSC plot provides a rough visualization of the cells based on morphology and light scatter characteristics (**FIGURE 17**). Noise is excluded from detection by using a threshold set on the FSC parameter. Threshold determines the minimum value required for an electronic signal to be counted as an event. In addition, the scatter plot gives an impression of the level of cell death, amount of debris, and proportion of cell clumps to generate an initial cleanup gate. Alternatively, the scatter gate can be drawn after the time and singlet gates as a final cleanup prior to viability gating.

SINGLET GATING - Flow cytometry is a single cell technology. However, cells can stick together or pass through the analysis point in tandem (coincident events). Without eliminating clumps from analysis, which can contain both cells of interest and negative populations, results will be inaccurate.

Singlet gating distinguishes single cells from clumps by using the different pulse measurements of height (H), width (W) and area (**A**). Combining two of these pulse measurements, enables identification of cell aggregates and gating on the single cell population (**FIGURE 17b**). In some applications, it may be useful to perform singlet gating on a fluorescent marker (e.g. DNA binding dyes). Note: not all of these measurements are automatically included in the data file, therefore it is important to turn them on in the software prior to analysis.
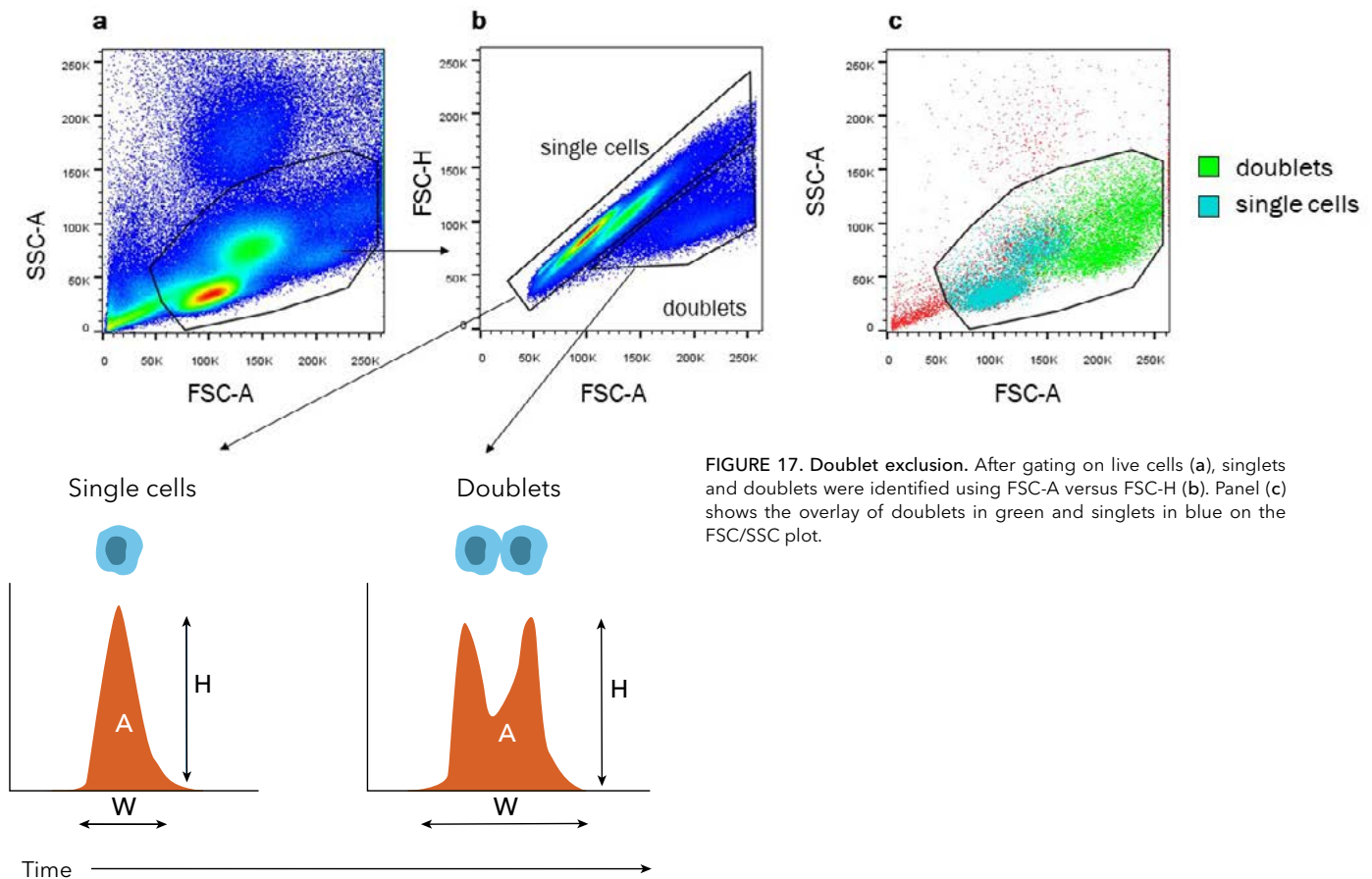

FIGURE 17. **Doublet exclusion.** After gating on live cells (**a**), singlets and doublets were identified using FSC-A versus FSC-H (**b**). Panel (**c**) shows the overlay of doublets in green and singlets in blue on the FSC/SSC plot.

VIABILITY GATING - Dead cells can be problematic when present in a sample. Ideally, the sample preparation will minimize cell death. However, viability dyes facilitate the elimination of dead cells from subsequent analysis. The viability dye can be plotted against a scatter parameter or another fluorescent marker depending on the panel (see **FIGURE 18**).

FLOW CYTOMETRY WORKFLOW: FOUR MAIN STEPS

FIGURE 18. Detection of apoptotic cells by viability dyes. KG.1 cells were either left untreated (a and c) or were treated with 5μM Camptothecin (Tocris Bioscience Catalog # 1100) for 12 hours to induce apoptosis (b and d). Cells were then harvested and labeled with an amine-reactive dye (a and b) or Annexin V (c and d). Dead cells were identified as those cells showing positive fluorescence for the amine-reactive dye or Annexin V. The differences in the percentage of dead cells recognized by the amine-reactive dye and Annexin V are reflected by the ability of Annexin V to recognize both early and late apoptotic cells while amine-reactive dyes label late apoptotic cells after the plasma membrane has become porous.
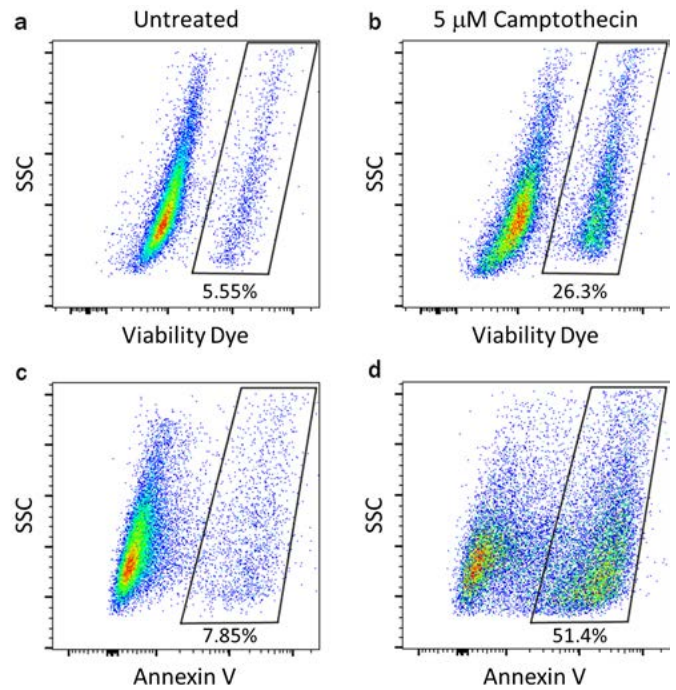
OTHER GATING STRATEGIES:

DUMP CHANNEL - In certain flow cytometry panels, there may be a desire to eliminate multiple cell types from subsequent analysis. In this case a dump channel can be used. Essentially, markers for multiple cell types labeled with the same color are used to stain the sample. Any cell type positive for those markers are identified and eliminated from the analysis by gating on the negative population (FIGURE 19).
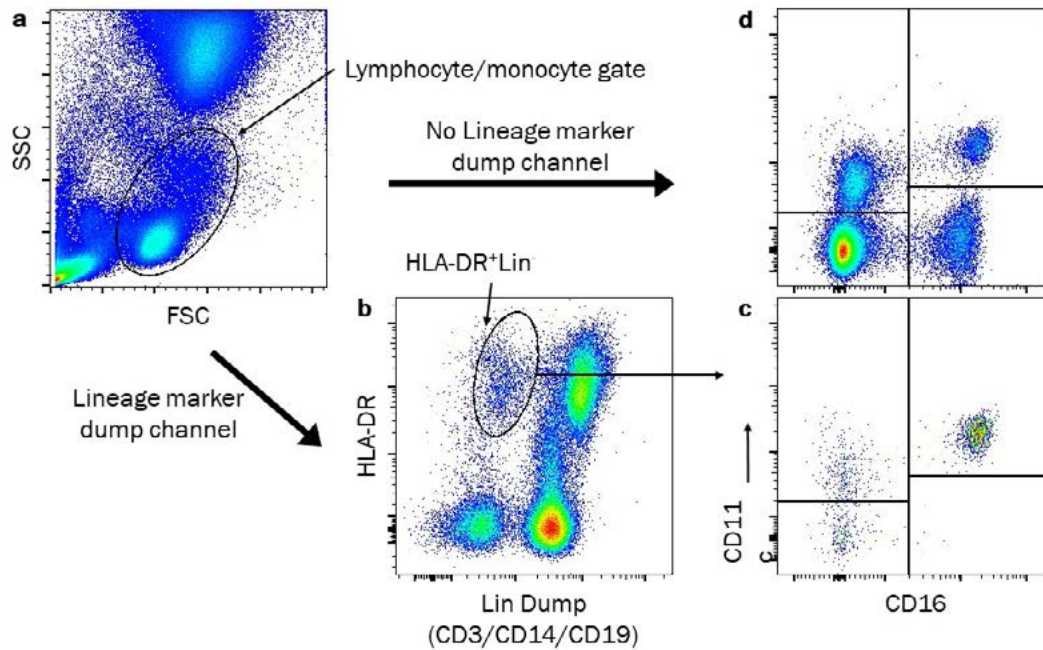

FIGURE 19. Use of a lineage marker dump channel to identify human blood dendritic cells. Blood dendritic cells are a minor cell population in human blood that are found within the lymphocyte population. Lymphocytes/monocytes were gated (a) and then blood dendritic cells were identified as HLA-DR+, lineage "Lin" negative (CD3-CD14-CD19-) cells (b). To facilitate the detection of blood dendritic cells, a lineage "Lin" dump channel was created by using antibodies to the lineage markers CD3 (R&D Systems Catalog # FAB100), CD14 (R&D Systems Catalog # FAB3832) and CD19 (R&D Systems Catalog # FAB4867) all conjugated to AF405. HLA-DR+Lin- cells can then be further separated into discrete populations based on the expression of other cell surface proteins such as CD11c (R&D Systems Catalog # FAB1777) and CD16 (R&D Systems Catalog # FAB2546) (c). Dot plot (d) demonstrates the size and distribution of the CD11c and CD16 populations without using a lineage dump channel.

FLUORESCENCE MINUS ONE - Certain populations can be difficult to identify and thus, a challenge to determine where gate boundaries should be placed. In these cases, the fluorescence minus one control or FMO can provide a guideline for more accurate gate placement. The FMO control is stained in the same way as the sample except eliminating one of the markers of interest. The background fluorescence contributed by all of the reagents except for one is revealed by this control to more accurately dictate where the boundary of the gates should begin (FIGURE 20).
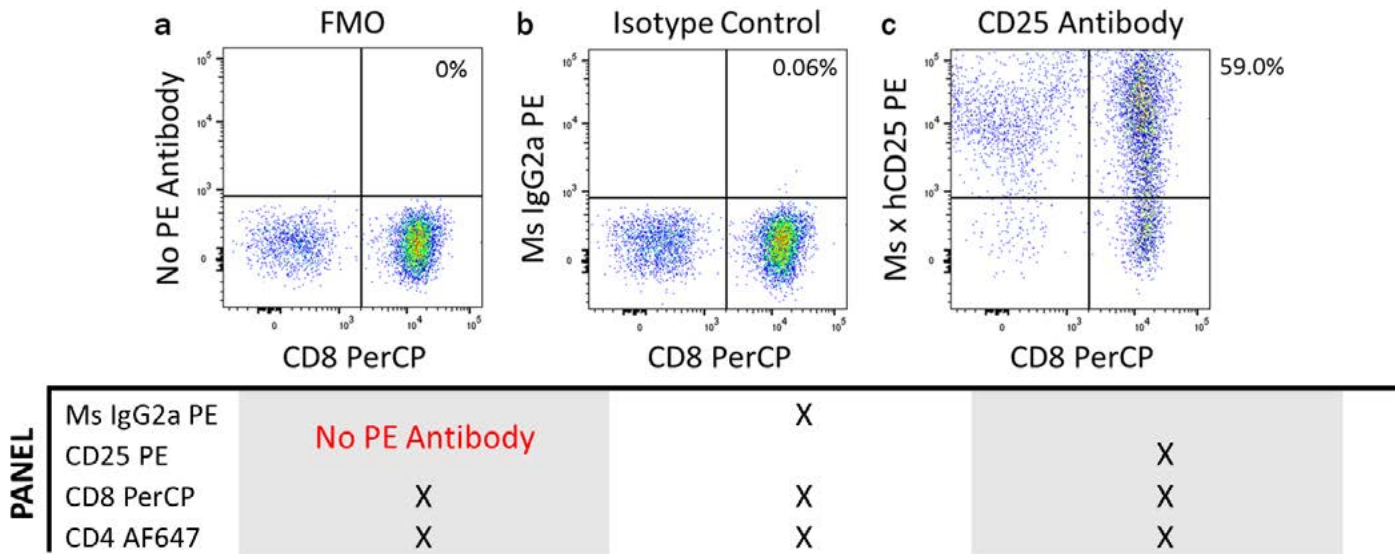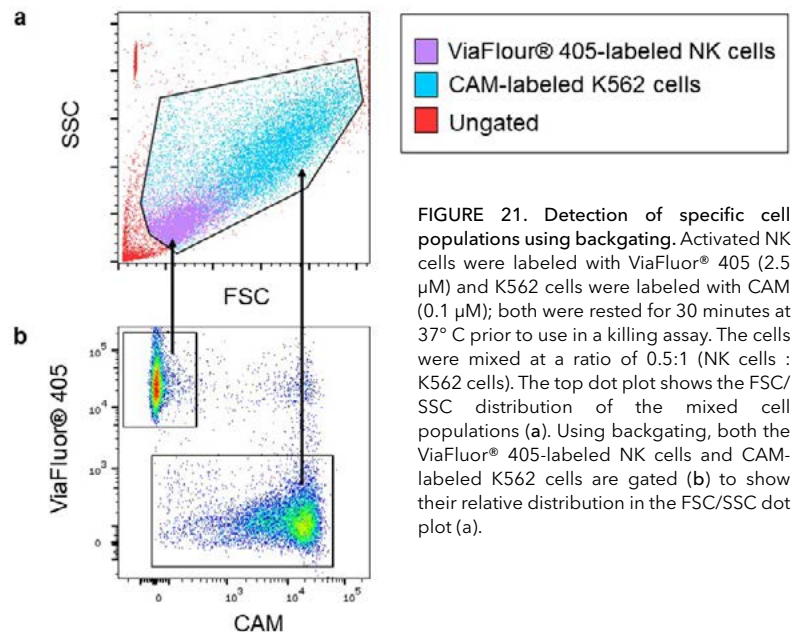
FLOW CYTOMETRY WORKFLOW: FOUR MAIN STEPS

FIGURE 20. Illustrated comparison of FMO and isotype controls. CD3+ T cells were stimulated with plate-bound αCD3/αCD28 antibodies (5µg/mL; 2µg/mL, respectively) for nine days. Cells were then harvested and stained with CD4 AF647 (R&D Systems Catalog # FAB3791R), CD8 PerCP (R&D Systems Catalog # FAB1509C) and CD25 PE (R&D Systems Catalog # FAB1020P) antibodies. (a) To establish the FMO control for CD25, all antibodies in the panel (CD4 AF647 and CD8 PerCP) were included except for one (CD25 PE). To illustrate the use of an isotype control, a Ms IgG2a PE antibody is included along with the CD4 AF647 and CD8 PerCP antibodies in panel (b). Staining of CD3 T cells with all three primary antibodies (CD4 AF647, CD8 PerCP and CD25 PE) is shown in (c). Notice how the relative MFIs of the PE channel on the CD8+ cells are comparable between the FMO control (a; MFI = 143) and the isotype control (b; MFI = 169) when the concentration of the Ms IgG2a PE isotype control antibody, and corresponding CD25 PE antibody (c) have been correctly titrated.

BACKGATING - Backgating is a useful tool to verify that the gating strategy will correctly identify, and not exclude any, events of interest. Populations can be colored in the analysis software to easily distinguish discrete populations in other plots (FIGURE 21). Gates can be adjusted if needed to better encase the cells of interest or exclude any unwanted cells.

STATISTICS:

There are two primary data values used in flow cytometry, frequency and fluorescence intensity (FIGURE 22).

- **Frequency** reveals the proportion of a specific population in the whole sample or a subset of cells.

- **Fluorescence intensity** should be reported as median in logarithmically displayed data (MFI, where M is median). Caution should be taken when comparing MFI across experiments, in particular between instruments because instrument configuration and setup drastically influence this value. For accurate comparisons, it may be necessary to standardize instrument setup using beads by targeting an MFI while adjusting voltages.

FIGURE 21. Detection of specific cell populations using backgating. Activated NK cells were labeled with ViaFluor® 405 (2.5 µM) and K562 cells were labeled with CAM (0.1 µM); both were rested for 30 minutes at 37° C prior to use in a killing assay. The cells were mixed at a ratio of 0.5:1 (NK cells : K562 cells). The top dot plot shows the FSC/SSC distribution of the mixed cell populations (a). Using backgating, both the ViaFluor® 405-labeled NK cells and CAM-labeled K562 cells are gated (b) to show their relative distribution in the FSC/SSC dot plot (a).
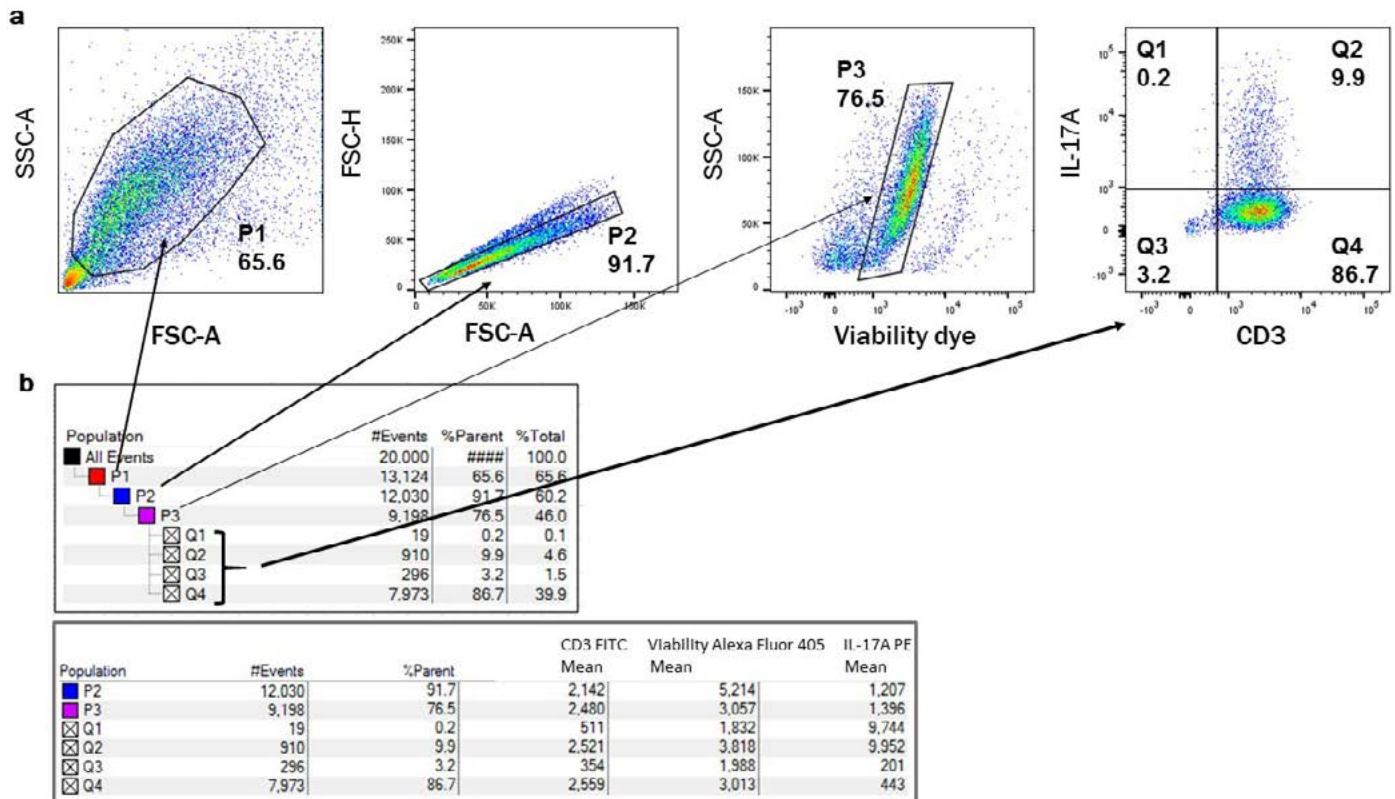
FLOW CYTOMETRY WORKFLOW: FOUR MAIN STEPS

FIGURE 22. **Analysis with statistics.** Purified CD4+ T cells were polarized into hTh17 cells for 5 days. After restimulation with the Tocris activation cocktail (Tocris Bioscience Catalog # 5476), hTh17 cells were stained with a viability dye followed by CD3. Intracellular staining was carried out for IL-17A using the FoxP3 buffer kit (R&D Systems Catalog # FC012). Analysis was carried out on a Fortessa. (**a**) Dot plots showing the lymphocyte gate (P1), single cells (P2), live cells (P3), and CD3+IL-17A+ T cells (Q2) (R&D Systems Catalog # FAB100F and R&D Systems Catalog # IC317P, respectively). (**b**) Population hierarchies of each dot plot are shown and the corresponding gate is shown for each by an arrow (top panel). Statistics for cell number, percent (frequency) of the parent population, and the MFI values of CD3 FITC, Viability, Alexa Fluor®405, and IL-17A PE are shown in the bottom panel.

PROTOCOLS

MATERIALS

- Fc Receptor Blocking Reagents (Fc Receptor Blocking Antibodies or IgG Solutions)

- Flow Cytometry Red Blood Cell Lysis Solution [Flow Cytometry Human Lyse Buffer (10X; R&D Systems Catalog # FC002) or Flow Cytometry Mouse Lyse Buffer (10X; R&D Systems Catalog # FC003) or equivalent]

- Flow Cytometry Staining Buffer (R&D Systems Catalog # FC001, or an equivalent solution containing BSA and sodium azide)

- Fluorochrome-conjugated Antibodies suitable for use in flow cytometry

- Isotype Control Antibodies

- FACS™ Tubes (5 mL round-bottom polystyrene tubes)

- Pipette Tips and Pipettes

- Centrifuge

METHODS

01   Sample preparation:

     a. For staining peripheral blood cells, whole blood should be collected in evacuated tubes containing EDTA or heparin as the anticoagulant. Contaminating serum components should be removed by washing the cells three times in Flow Cytometry Staining Buffer and centrifuge at 500 x g for 5 minutes.

     b. For staining cell lines or cells from activated cell cultures, the cells should be centrifuged at 500 x g for 5 minutes and washed three times in Flow Cytometry Staining Buffer to remove any residual growth factors that may be present in the culture medium.

     c. Cells that require trypsinization to enable removal from their substrates should be further incubated in medium for 1-2 hours on a rocker platform to enable regeneration of the receptors. The use of the rocker platform will prevent reattachment to the substrate.

Note: Titration experiments should be performed to determine optimal reagent amounts.

02   Harvest cells and aliquot up to 1 x 10^6 cells/100 μL into FACS tubes. Block cells with IgG (1 μg IgG/10^6 cells) or Fc block for 15 minutes at room temperature.

Note: Do not wash excess block from this reaction.

PROTOCOLS

03   Add conjugated antibody or isotype control antibody and mix. Incubate cells for 30 minutes at room temperature in the dark.

04   Remove any unbound antibody by washing the cells in Flow Cytometry Staining Buffer. Centrifuge the suspended cells at 300 x g for 5 minutes and decant the supernatant. Resuspend the cells by adding 2 mL of Flow Cytometry Staining Buffer. Repeat the wash two times.

     Note: If using whole blood, samples should go through a red blood cell lysis step at this point using Flow Cytometry Human or Mouse Lysis Buffer.

05   Lysis of Red Blood Cells: Add 2 mL of 1X Human (R&D Systems Catalog # FC002) or Mouse (R&D Systems Catalog # FC003) Lyse Buffer to each tube, mix, and incubate in the dark at room temperature for 10 minutes. Mix cells a second time and incubate samples for an additional 10 minutes. Centrifuge and wash cells in Flow Cytometry Staining Buffer as described in step 4 above.

     Note: If an unconjugated primary antibody was used, incubation with an appropriate secondary antibody should occur now. Incubate for 20-30 minutes in the dark and wash as in step 4.

06   Resuspend the cells in 200-400 µL of Flow Cytometry Staining Buffer for final flow cytometric analysis.

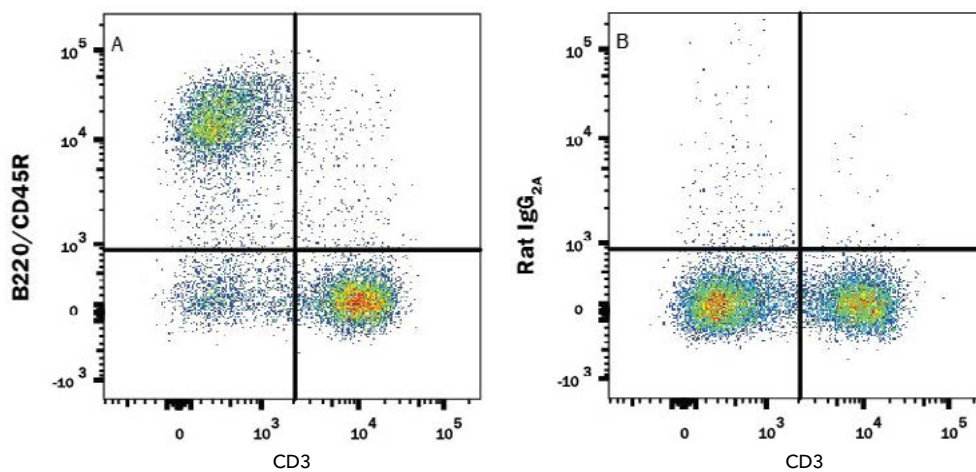FIGURE 23 below shows an example of surface staining mouse splenocytes with B220 and CD3.

FIGURE 23. Detection of B220/CD45R in mouse splenocytes. A) Mouse splenocytes were stained with Rat Anti-Mouse CD3 PE-conjugated Monoclonal Antibody (R&D Systems Catalog # FAB4841P) and Rat Anti-Mouse B220/CD45R APC-conjugated Monoclonal Antibody (R&D Systems Catalog # FAB1217A) or B) RatIgG2A APC-conjugated isotype control antibody (R&D Systems Catalog # IC006A).

PROTOCOLS

MATERIALS

- Fc Receptor Blocking Reagents (Fc Receptor Blocking Antibodies or IgG Solutions)

- PBS (1X): 0.137 M NaCl, 0.05 M NaH_2PO_4, pH 7.4 or Hank's Balanced Salt Solution (HBSS; 1X)

- Flow Cytometry Fixation Buffer (R&D Systems Catalog # FC004, or an equivalent solution containing 1 - 4% formaldehyde)

- Flow Cytometry Permeabilization Buffer/Wash Buffer I (1X; R&D Systems Catalog # FC005, or an equivalent solution containing a permeabilization agent such as saponin or Triton X-100)

- Intracellular Antibodies

- Isotype Control Antibodies

- FACS™ Tubes (5 mL round-bottom polystyrene tubes)

- Pipette Tips and Pipettes

- Centrifuge

METHODS

01 Harvest the cells and wash 2 times by adding 2 mL of 1X PBS (or HBSS), centrifuging at 300 x g for 5 minutes, and then decanting buffer from pelleted cells.

   Note: **Staining of surface antigens** may be done at this point (Fc block prior to carrying out surface staining).

02 Aliquot up to 1 x 10^6 cells/100 µL into FACS tubes. Add 0.5 mL of cold Flow Cytometry Fixation Buffer* and mix. Incubate at room temperature for 10 minutes. Mix the cells intermittently in order to maintain a single cell suspension.

   Note: Fixation buffer percentages need to be optimized based on the intracellular marker being detected. Recommended range is from 1-4% (4% is the stock).

03 Centrifuge cells and decant the Fixation Buffer. Wash the cells 2 times with 1X PBS (or HBSS) as in step 1. Wash the cells in 2 mL of Flow Cytometry Permeabilization/Wash Buffer I. Centrifuge and decant supernatant.

>>>> **PROTOCOL: STAINING INTRACELLULAR MOLECULES USING DETERGENTS TO PERMEABILIZE THE CELL MEMBRANE**

04 Block cells with IgG or Fc block for 10-15 minutes. Add conjugated antibody or isotype control antibody and mix. Incubate cells for 30 minutes at room temperature in the dark.

> *Note:* Because saponin-mediated cell permeabilization is a reversible process, it is important to keep the cells in the presence of Permeabilization Buffer I during intracellular staining.

05 Wash cells 2 times with Flow Cytometry Permeabilization/Wash Buffer I as in step 3.

> *Note:* If an unconjugated primary antibody was used, incubation with an appropriate secondary antibody should occur now. Incubate for 20-30 minutes in the dark and wash as in step 3.

06 Resuspend the cells in 200-400 µL 1X PBS (or HBSS) buffer for flow cytometric analysis.

Shown below is an example of intracellular staining using an IFN-γ antibody on stimulated human PBMCs (**FIGURE 24**).
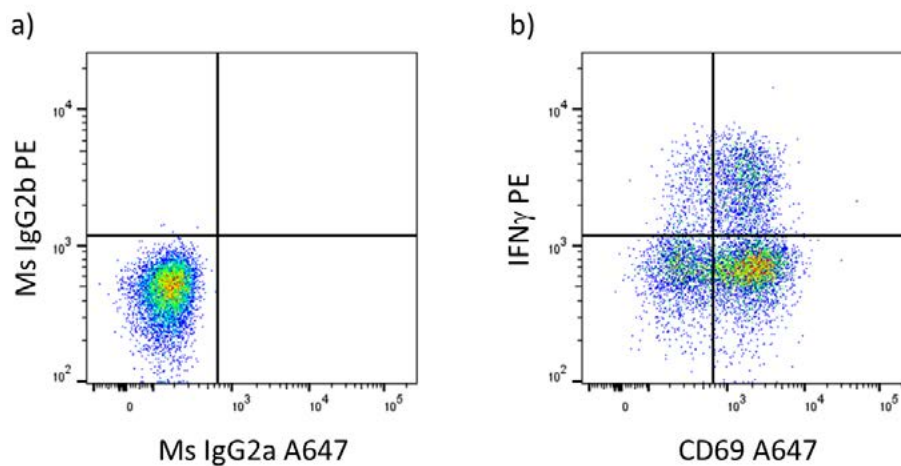

FIGURE 24. Intracellular staining for IFNγ using a saponin-based permeabilization buffer. Human PBMCs were stimulated with a restimulation cocktail (Tocris Bioscience Catalog # 5476/1ML) for three hours per the manufacturer's recommendation. Cells were then harvested and stained with a fixable viability dye prior to surface staining with CD3 AF700 (R&D Systems Catalog # FAB100N), CD45RA AF488 (R&D Systems Catalog # FAB1430G) and Ms IgG$_{2a}$ AF647 isotype control antibody (R&D Systems Catalog # IC003R) (**a**) or isotype matched CD69 AF647 antibody (R&D Systems Catalog # IC0041R) (**b**). Cells were then washed, fixed with 1% paraformaldehyde and permeabilized prior to staining with a Ms IgG$_{2b}$ PE isotype control antibody (**a**) or isotype matched IFNγ PE antibody (**b**).

PROTOCOLS

>>> **PROTOCOL: DETECTION OF TRANSCRIPTION FACTORS/INTRACELLULAR ANTIGENS USING THE FLOWX™ FOXP3/TRANSCRIPTION FACTOR STAINING KIT**

MATERIALS

- Fc Receptor Blocking Reagents (Fc Receptor Blocking Antibodies or IgG Solutions)

- PBS (1X): 0.137 M NaCl, 0.05 M NaH_2PO_4, pH 7.4

- Flow Cytometry Staining Buffer (R&D Systems Catalog # FC001)

- FlowX™ FoxP3/Transcription Factor staining kit (R&D Systems Catalog # FC012)

  - FoxP3 Fixation/Transcription Factor concentrate (4X)

  - FoxP3 Fixation/Transcription Factor Diluent (1X)

  - FoxP3/Transcription Factor Permeabilization and Wash buffer (10X)

- Intracellular Antibodies

- Isotype Control Antibodies

- FACS™ Tubes (5 mL round-bottom polystyrene tubes)

- Pipette Tips and Pipettes

- Centrifuge

METHODS

01 Wash human PBMCs or mouse splenocytes (1 x 10^6 cells per sample) with 2 mL of Flow Cytometry Staining Buffer (R&D Systems Catalog # FC001) or other BSA-containing buffer, by spinning at 300 x g for 5 minutes, using 5 mL flow cytometry tubes.

02 Fc-block cells with blocking IgG (1 µg IgG/10^6 cells) for 15 minutes at 2-8°C.

03 Surface stain with desired antibody for 30 minutes at 2-8°C.

04 Wash cells two times with cold 1x PBS. During washes, make up fresh 1x FoxP3/Transcription Factor Fixation Buffer by diluting FoxP3/Transcription Factor Fixation Concentrate (4X) with FoxP3/Transcription Factor Fixation Diluent (i.e. 100 µL FoxP3/Transcription Factor Fixation Concentrate (4X) + 300 µL FoxP3/Transcription Factor Fixation Diluent).

PROTOCOLS

05 Resuspend cells in fresh 1X FoxP3/Transcription Factor Fixation Buffer using 0.5 mL/tube. Incubate at 2-8°C for 30 minutes. During this incubation, make up 1X FoxP3/Transcription Factor Permeabilization and Wash Buffer by diluting FoxP3/Transcription Factor Permeabilization and Wash Buffer (10X) with distilled water (ie.100 µL FoxP3/Transcription Factor Permeabilization and Wash Buffer (10X) + 900 µL diH$_2$O) and keep at 2-8°C.

06 Wash two times with fresh, cold, 1X FoxP3/Transcription Factor Permeabilization and Wash Buffer.

07 Block cells again with Fc block for 10-15 minutes, and then add FoxP3 or other transcription factor antibody to cells and incubate for 30 minutes at 2-8°C.

08 Wash cells one time with cold 1X FoxP3/Transcription Factor Permeabilization and Wash Buffer.

09 Resuspend cells in Flow Cytometry Staining Buffer and run on a flow cytometer.

Shown below is an example of mouse splenocytes stained with CD4, and CD25, and FoxP3 antibody (**FIGURE 25**).
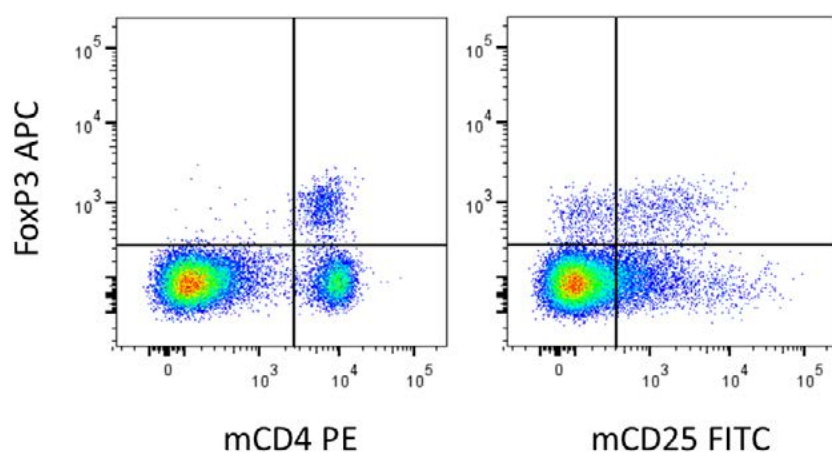

FIGURE 25. Detection of FoxP3 in mouse splenocytes. Mouse splenocytes were surface stained with mCD4 PE and mCD25 FITC (R&D Systems Catalog # FAB554P, FAB9164). To facilitate intracellular staining, cells were first fixed, and then permeabilized using the FoxP3/Transcription factor kit buffers. The cells were then stained intracellularly with FoxP3 APC (R&D Systems Catalog # IC8214A) or a Rabbit IgG APC isotype control (R&D Systems Catalog # IC1051A; not shown).

PROTOCOLS

MATERIALS

- Fc Receptor Blocking Reagents Fc Receptor Blocking Antibodies or IgG Solutions)

- PBS (1X): 0.137 M NaCl, 0.05 M NaH_2PO_4, pH 7.4

- Flow Cytometry Staining Buffer (R&D Systems Catalog # FC001)

- Flow Cytometry Fixation Buffer (R&D Systems Catalog # FC004)

- Methanol (-20°C)

- Phospho-Specific Antibodies

- Isotype Control Antibodies

- FACS™ Tubes (5 mL round-bottom polystyrene tubes)

- Pipette Tips and Pipettes

- Centrifuge

METHODS

Note: Staining of surface antigens may be done at this point (carry out Fc blocking first).

Note: If an unconjugated primary antibody was used, incubation with an appropriate secondary antibody should be carried out for 20-30 minutes in the dark; carry out washes as outlined in step 3.

01 Harvest the cells quickly and wash 2 times by adding 2 mL of **cold** 1x PBS (or HBSS), centrifuging at 300 x g for 5 minutes, and then decanting buffer from pelleted cells.

02 Aliquot up to 1 x 10^6 cells/100 µL into FACS tubes. Add 0.5 mL of **cold** Flow Cytometry Fixation Buffer* and vortex. Incubate at room temperature for 10 minutes. Vortex the cells intermittently in order to maintain a single cell suspension.

*Note: Fixation buffer percentages need to be optimized based on the phospho marker being detected. Recommended range is from 1-4% (4% is the stock).

03 Centrifuge cells at 300 x g for 5 minutes and decant Fixation Buffer. Wash cells 2 times with PBS (or HBSS) as in step 1. Resuspend the cells in 900 µL of -20°C methanol. Incubate for 30 minutes at 4°C.

PROTOCOLS

04 Centrifuge the cells for 5 minutes at 300 x g. Remove and discard supernatant. Wash 2 times with Flow Cytometry Staining Buffer.

05 Block cells for 10-15 minutes with Fc block, and then add the conjugated phosphorylated antibody or isotype control antibody, and vortex. Incubate cells for 30 minutes at room temperature in the dark.

06 Wash cells 2 times with Flow Cytometry Staining Buffer.

07 Resuspend the cell pellet in 200 - 400 µL of Flow Cytometry Staining Buffer for flow cytometric analysis.

Shown below is an example of intracellular staining using phospho-STAT2 on Daudi cells treated with human IFN-α (**FIGURE 26**).
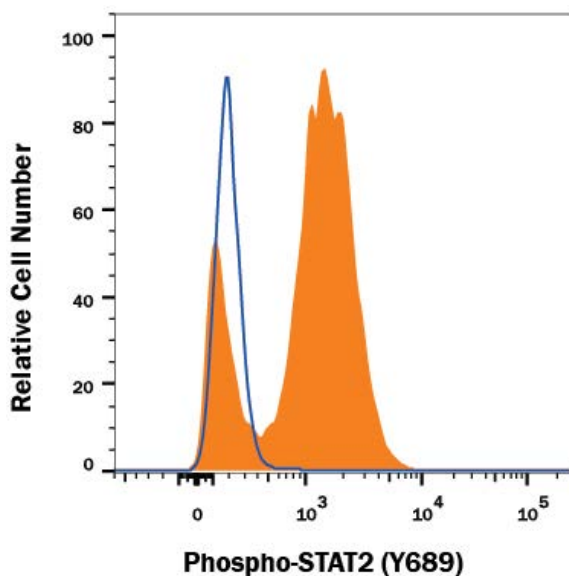

FIGURE 26. Detection of STAT2 in Daudi human cell line. Daudi human Burkitt's lymphoma cell line untreated (open histogram) or treated with 500 U/mL Recombinant Human IFN α A (R&D Systems Catalog # 11100-1, filled histogram) for 20 minutes was stained with Rabbit Anti-Human Phospho-STAT2 (Y689) Monoclonal Antibody (R&D Systems Catalog # MAB2890), followed by Fluorescein-conjugated Anti-Rabbit IgG Secondary Antibody (R&D Systems Catalog # F0112). To facilitate intracellular staining, cells were fixed with Flow Cytometry Fixation Buffer (R&D Systems Catalog # FC004) and permeabilized with 90% methanol.

TROUBLESHOOTING GUIDE

ISSUE	POSSIBLE CAUSE	POTENTIAL SOLUTION
No / Weak Staining	Incorrect antibody concentration	Titrate antibody to find optimal concentration.
	Target is inaccessible	Check expected location of target (surface, intracellular, nuclear). If a membrane protein, determine if the epitope is expressed on the inner leaflet of the membrane.
		Keep cells on ice to prevent internalization of surface antigens. Addition of sodium azide can help with this.
		If trypsin was used to detach cells, this may result in loss of surface antigens. Try a gentler detachment procedure.
		Permeabilization may have been insufficient. If the target of interest is intracellular or nuclear, ensure the appropriate fixation and permeabilization protocol is used. Different fixation and permeabilization buffers may be required, depending on the target and other reagents used. Formaldehyde fixation can be used in conjugation with saponin, Triton X-100 or 90% methanol (ice-cold), for cell permeabilization. 70% ethanol (ice-cold) can sometimes be used as an alternative method of fixation, particularly when performing cell cycle analysis. Adjusting the concentration of the fixative or permeabilization agent may also be required.
	Intracellular staining – fluorochrome is too large	Ensure a low molecular weight fluorochrome is used for intracellular staining. Avoid tandem dyes.
	Protein expression is too low	Ensure cells express the protein of interest.
		Adjust activation protocols to increase protein expression.
		Ensure an inhibitor for Golgi transport is included for any secreted protein.
	Fluorochrome has been inappropriately stored	Try a new lot of conjugated antibody.
	Low density antigen is paired with a dim fluorochrome	Adjust panel to ensure all low expressed proteins are with bright fluorochromes.
	Cytometer is not optically set up to detect the fluorochrome	Ensure the correct laser and filters are in place to excite and detect the fluorochrome.
	Voltage is set too high	Ensure the positive population is on scale and not lost off the high end of the plot.
	If using a secondary antibody, the primary and secondary are not compatible	Ensure the secondary is raised against the same species as the host of the primary antibody (e.g. use an anti-rabbit antibody to detect a rabbit primary antibody).
	Insufficient staining time or incorrect temperature	Optimize staining time and incubation temperature. This may be different for primary and secondary antibodies or for surface and intracellular antigens.
High staining in negative population	Cell autofluorescence	Run unstained cells to check for autofluorescence.
	Non-specific binding	Titrate antibody to find optimal concentration.
		Block Fc receptors with Fc-block reagents or serum.
		Include a viability dye to remove dead cells from analysis.
		Include additional wash steps.
	PMT voltage is set too high	Adjust voltage to keep populations on scale.

TROUBLESHOOTING GUIDE

ISSUE	POSSIBLE CAUSE	POTENTIAL SOLUTION
Unexpected staining patterns	Cell doublets present	Ensure analysis gate that excludes doublets is used.
	Dead cells	Include a viability dye to remove dead cells from analysis.
	Fluidics or laser delay issues	Use a time plot to check fluidic stability, check calibrations for laser delays and ensure the sheath tank is full and pressurized.
	Incorrect instrument settings	Ensure correct settings are used.
	Clogged flow cell	Follow instrument cleaning instructions. Ensure samples are filtered prior to running.
	Reagents	Some reagents such as trypsin, EDTA, and lysing solutions may affect certain markers. Select a method that does not interfere with detection.
Unusual scatter patterns	Incorrect instrument settings	Ensure correct settings are used.
	Activated cells	Cell activation can impact scatter properties of cells. Set voltages to keep both activated and unactivated cells on scale.
	High side scatter with low forward scatter	May indicate that cells are lysed – check by microscopy. If cells are lysed, verify all solutions are isotonic. Avoid vortexing, especially after permeabilizing cells. If cells are activated, activation protocol may be too rigorous.
		Check samples for microbial contamination.
	Samples poorly fixed / permeabilized	Optimize fixation and permeabilization protocol to prevent cells from lysing. Avoid vortexing and high speed centrifugation.
	Old samples	Use fresh samples. Lengthy storage of samples will increase debris and dead cells, especially if samples are not fixed.

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ACKNOWLEDGEMENTS

NOTES

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