ORFLO

Two-Color Immunophenotyping With The Moxi Go II

Introduction

Leveraging antibodies against specific proteins for visualization of specific cell types has been a long-standing practice in science for decades. It's used in a variety of ways for a variety of methods, be it characterization of cells or protein abundance in tissue samples via immunofluorescence imaging[1] or identifying the cells present in a cell suspension via flow cytometry[2]. Flow cytometry can provide valuable insight into a wide variety of cell suspensions. One such example is parsing out the complex populations of different immune cells in a heterogeneous blood sample, especially when specific antibodies are strategically chosen[3]. As such, leveraging immunophenotyping in your samples is a critical step in many industry and research projects.

While not as robust in terms of cells counted or channels available for deep phenotyping when compared to a full-scale flow cytometer, the Moxi GO II provides the perfect benchtop option for basic immunophenotyping. Its dual orange/red and green channels allow you to do two color analysis of your sample on thousands of cells in seconds, and the post-run analysis is similar to your familiar flow cytometry output data. Harness the sensitivity and power of a flow cytometer right in your lab with no need for lengthy and expensive experiments sent to the core lab!

1. Im K, Mareninov S, Diaz MFP, Yong WH. An Introduction to Performing Immunofluorescence Staining. *Methods Mol Biol Clifton NJ*. 2019;1897:299-311. doi:10.1007/978-1-4939-8935-5_26

2. Finak G, Langweiler M, Jaimes M, et al. Standardizing Flow Cytometry Immunophenotyping Analysis from the Human ImmunoPhenotyping Consortium. *Sci Rep*. 2016;6(1):20686. doi:10.1038/srep20686

3. Gao J, Luo Y, Li H, et al. Deep Immunophenotyping of Human Whole Blood by Standardized Multi-parametric Flow Cytometry Analyses. *Phenomics*. 2023;3(3):309-328. doi:10.1007/s43657-022-00092-9

Meet the Moxi GO II

ORFLO's proprietary cell counting technology can count tens of thousands of cells in seconds, providing first-in-class data to you fast. Normally, accuracy is negatively impacted by acquisition time. However, by leveraging the current gold standard Coulter Principle, we bring you both with no compromises to either. All of this in a self-contained, easy-to-use, bench top-sized machine.

Microfluidics Meets Cell Counting

Don't like wasting half of your sample on figuring out whether your experiment worked correctly? Don't like spending time and money on a large-scale flow cytometry run for a simple transfection efficiency or cell viability check? Our innovative microfluidics cassettes have created a simple, effective, and self-contained way to get everything you need, all for the low, low cost of 60µL! Just insert the cassette, pipette in a small amount of your precious sample, and let the microfluidics and our proprietary cell counting technology do the rest.

Gold Standard Count Accuracy

Moxi GO II was compared to the gold standard Beckman-Coulter Z2 system, an image-based cell counter, and a hemocytometer, with respect to counts of serial dilutions of a range of particle types (beads, yeast, mammalian cells) and sizes (~4µm to ~16µm in mean diameter). The results showed that the count-to-count variability, as measured by the coefficient of variation (CV), was on the order of 20-30% for the imaging system, compared to 4% for the Moxi system.

Moxi GO II - Next Generation Dual-Channel PMT Fluorescence Cell Analyzer

With our Moxi Go II system, we have coupled our cell counting technology with the ability to detect two channels of fluorescence to revolutionize the way that cell counting is done. Our 488nm laser and Photo Multiplier Tubes (PMTs) gives the accuracy and detection capabilities of a normal flow cytometer for both a 525/45nm (Green) channel and 561nm/LP (Orange/Red) channel. This combination gives you unparalleled bench top power when working with any cell types marked with any of your favorite fluorescent cell markers!

Fluorophores

The Moxi GO II uses a 488nm laser and is equipped with a 525/40nm filter for capturing green fluorescence and a 561nm/LP filter for capturing orange/red fluorescence. Included with the Moxi GO II is an additional 646nm/LP filter that can be swapped in for the 561nm/LP filter if further red fluorophores are desired. Below is a list of known antibody conjugates that work on the Moxi GO II as well as theoretical conjugates based off of their excitation/emission spectra:

Green (525nm)
FITC
Alexa-488

Orange (561nm)
PE
RB545

Red (646nm)
PE-Cy5
RB705
RB613
PE-Texas Red
PE/Fire 640
PE-Vio 615

= known to work (tested)

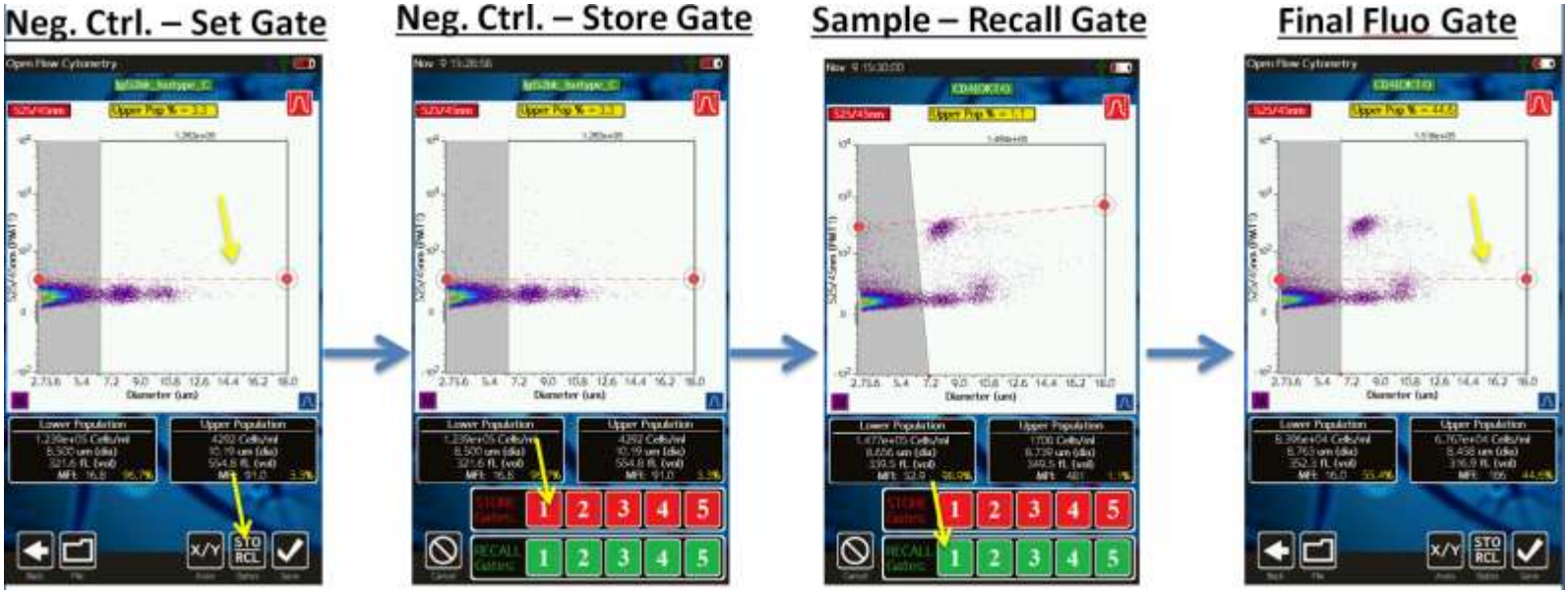
= theoretically should work (not tested)

Experiment Setup

Before performing immunoprofiling on the Moxi GO II, there are a few critical experiment design decisions to consider making sure results are accurate.

General Experiment Setup Recommendations

For optimal gating and identification of the fluorescently-positive cells, it is recommended to perform a test using an unstained sample as a negative control:



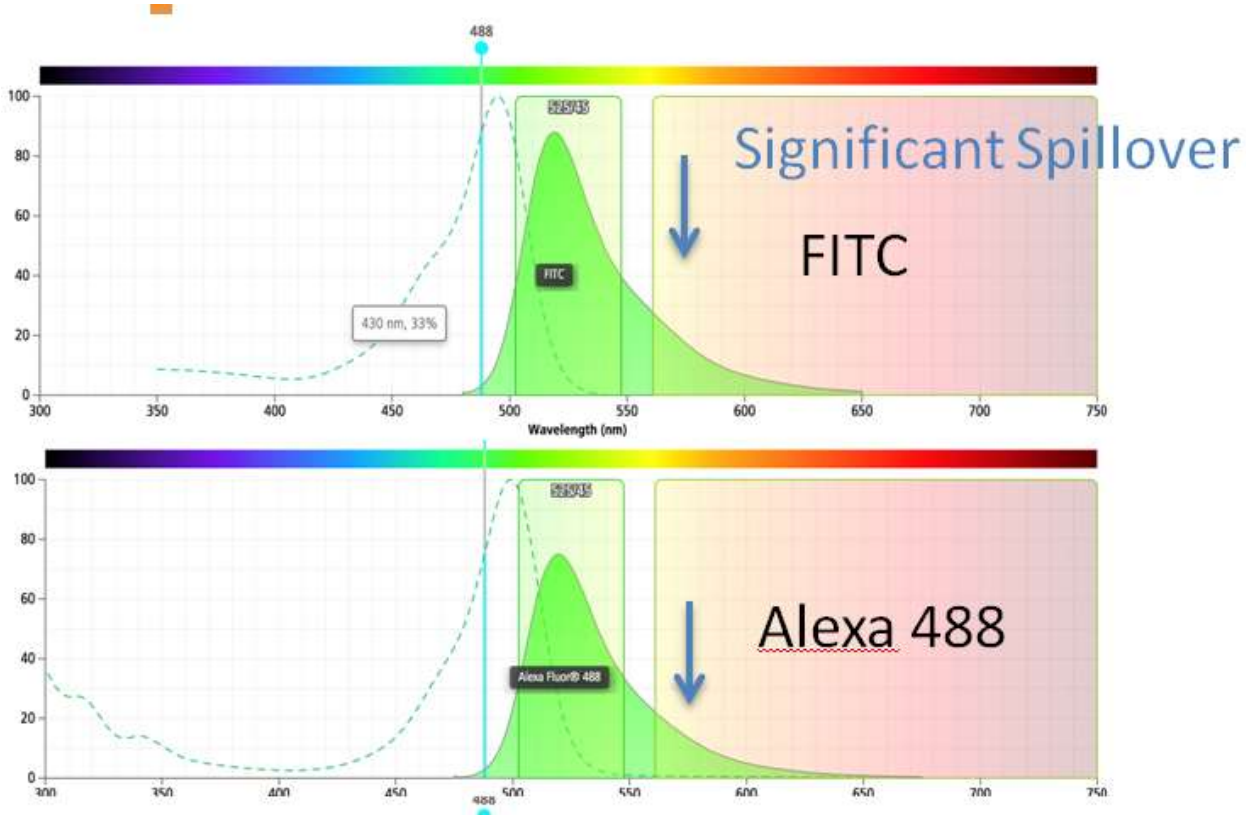
Using the negative control, the x-axis gate can be established to isolate only real cells and the y-axis gate for both the green channel (PMT1) and the orange/red channel (PMT2) can be placed just above the cell population to establish the cutoff for fluorescence. This set of gates can be stored in the instrument as a number (1-5) using the “Gates” button. When the fluorescently-positive sample is run, those gates can be recalled using the same button to ensure you are properly identifying the real fluorescently-positive cells in the sample.

Additionally, when performing immunoprofiling, it is recommended to wash the sample 1-2x in PBS before running on the instrument to minimize streaking in the results. The streaking occurs when free fluorophore floating in the sample passes through the instrument’s aperture at the same time as a cell and the subsequent signal associated with said cell is muddled by the fluorescent signal of the free-floating antibody. This can impede the proper separation of

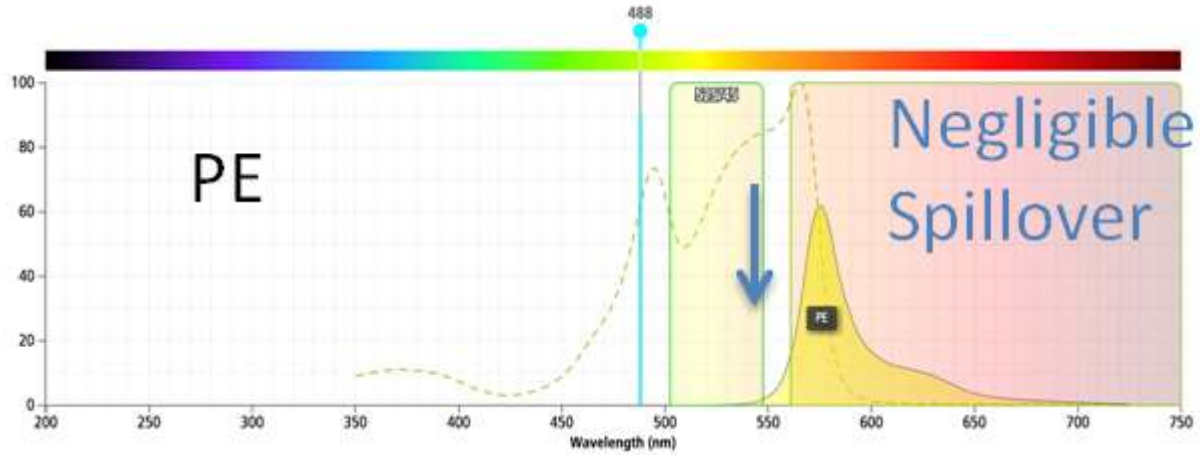
fluorescently-positive cells from fluorescently negative cells and will provide less accurate results than cells washed 1-2x to remove free unbound fluorophore.

Fluorophore Signal Spillover

If using both the 525/40nm and 561nm/LP channels, there will likely be spillover signal from the 525nm fluorescent molecule/fluorophore into the 561nm channel. Because of this, it is highly important to set up experiments to compensate for this spillover and eliminate false signal in the 561nm channel. Spillover into the 561nm/LP channel is known for the following fluorophores:



However, tested fluorophores being detected by the 561nm/LP filter have negligible spillover into the 525/40nm filter and no compensation will be required:



Untested fluorophores in the 561nm/LP and 646nm/LP filer's emission ranges are unlikely to produce spillover into the 525/40nm filter; however it is worth verifying this before performing two-color immunophenotyping with no compensation.

Performing Green Channel Spillover Compensation on a Moxi GO II

When compensation for spillover from the green channel is required, the instrument's built-in compensation feature can aid in blanking the spillover signal with a green-only fluorescent sample. It is recommended to use a sample containing the exact fluorophore that will be used for a dual-color immunophenotyping experiment, preferably on the same sample if possible. Compensation on a green-only stained sample can be done as follows:

1) In the post-run analysis screen, set the gates for the real cells and PMT1 (green).

2) Change the axes using the “Axes” button so that PMT2 (orange/red) is on the y-axis and PMT1 (green) is on the x-axis.

3) Press the “Comp” button to be taken to the compensation screen.

4) Adjust the gates such that the green-positive cells that have spillover signal are in the bottom-right quadrant labeled “Spillover Cluster.”

5) Using the “Auto Adjust” button or the up and down arrows, adjust the spillover cluster such that the events have zero signal on the y-axis. The compensation percentage can be seen in the bottom white box as well as on the y-axis

This compensation value will remain on the instrument until turned off and will persist across subsequent tests. These values can also be re-established if needed from data already acquired by loading it up as a file. As such, compensation with a green-only sample will prevent the follow-up green + orange/red dual immunophenotyping test from giving false dual-positives due to green channel spillover.

Optimal Experimental Setup to Accommodate For Spillover

When performing two-color immunophenotyping, two main things need to be taken into consideration: Are the 525/40nm and 561/LP filters being used and if so, are there going to be dual-positive cells? If both of the answers to these questions are yes, the following experimental setup is recommended:

- Run three samples: the first one with no stain, the second with just the green fluorescent channel, and the third with both green and red/orange
- Set the fluorescence cutoff gates for the green and orange channels using the negative control sample that is run first
- Run the sample with just the green fluorophore second to eliminate spillover via the instrument’s compensation.
- Using the gates established from the negative control and the compensation values established from the green-only sample, run the sample with both the green and red/orange fluorophores

This will properly identify the real double positive cells and prevent false positives from giving inaccurate results.

Of note, if the 525/40nm and 646nm/LP filters are being used, no spillover compensation is required as the signal from the green fluorophore will not be detected on the longer wavelength filter.

Additional Experimental Notes

If the two fluorescent markers being used are exclusive and are identifying separate cell types, compensation set with a green-only sample first will not be needed (though it is still recommended). In this instance, double-positive cells can be considered identical to green-positive cells as they are likely green-positive cells that have spilled over into the orange/red 561nm channel.

Immune Cell Identification

The main subset of cells that require immunophenotyping is immune cells. Whether it be for identification of how many immune cells are present in a PBMC sample or monitoring cultured T-Cells during the CAR-T process, knowing exactly which cells are present and how many are in the sample is critical. In both cases, T cells are the main cells that are identified and the Moxi GO II does an excellent job at isolating that specific cell population:

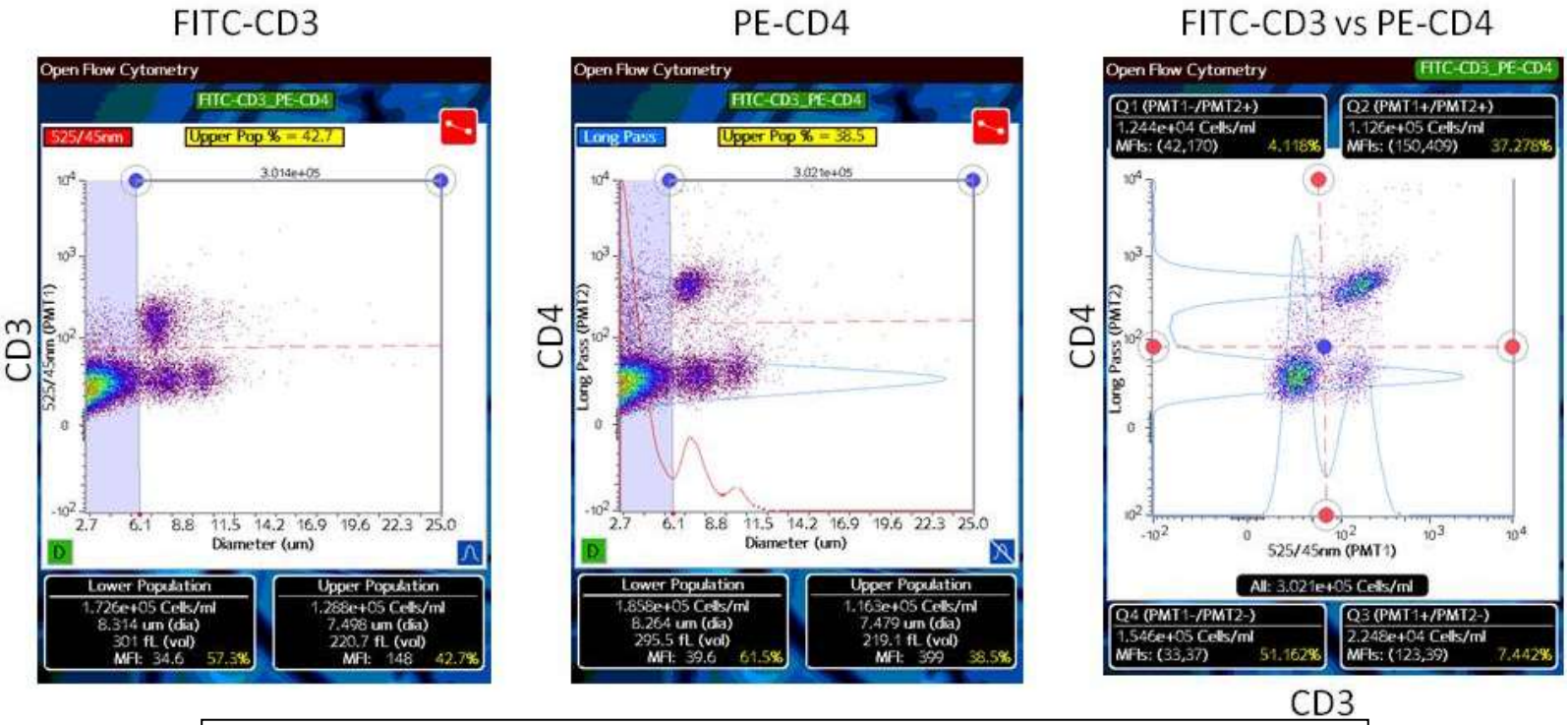
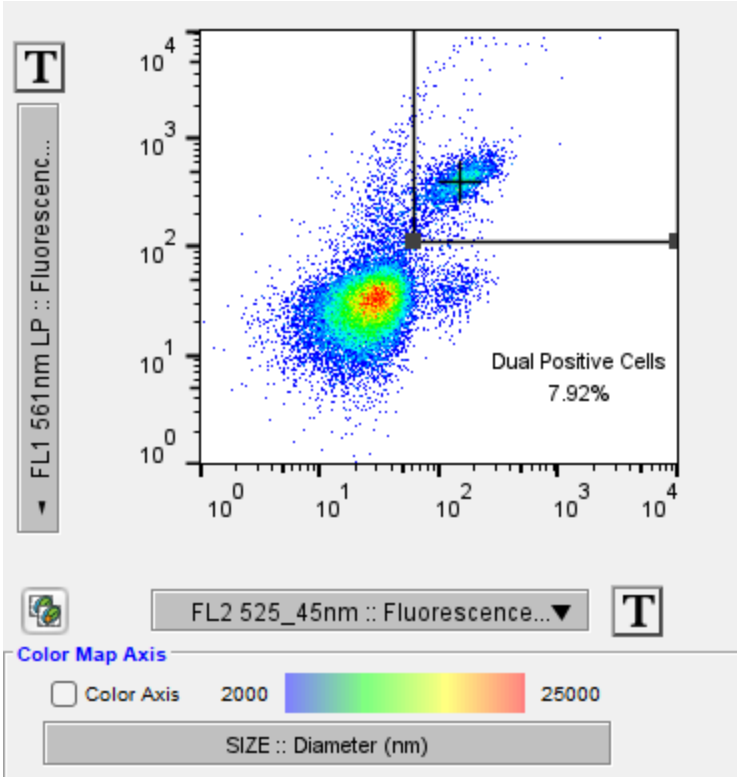


Fig. 1: Isolation and quantification of T-Cells in a PBMC sample using FITC-CD3 and PE-CD4. CD3- positive cells (left) CD4-positive cells (middle), and dual-positive CD3+CD4 cells (right) can be viewed in the post-run analysis.

This data was taken from a user's freshly isolated PBMC sample to look at the T-cell population within by using CD3 and CD4 as their identification markers. Using our intuitive post-run analysis software, it is easy to gate out debris and isolate only real cells using the particle size on the x-axis. The distinctive separation of fluorescently-positive cells on the y-axis allows for isolation of green-positive and orange/red-positive cells (**Fig. 1, left, middle**). Changing the x-axis to PMT1 (green channel) and the y-axis to PMT2 (orange/red channel) allows for easy identification of the dual-positive cell population, giving its concentration and the percentage of dual positive cells within your original sample (**Fig. 1, right**).

If further analysis is required, the .fcs files generated by the instrument can be exported to the computer and opened using a variety of third-party programs typically used for flow cytometry analysis:



Name	Statistic	#Cells
FITC-CD3_PE-CD4.FCS		36437
Dual Positive Cells	7.92%	2885

Fig. 2: Post-run analysis of FITC-CD3 and PE-CD4 labeled PBMCs utilizing FlowJo. Gating was performed to isolate the dual positive cells.

This same .fcs file used on the instrument can be easily loaded into programs such as FlowJo for more advanced analytics. The total number of events counted and the total number of dual-positive cells can be seen this way (**Fig. 2**). Additional formulas and strategies to get the most out of your data can also be performed, such as determining how many µL of the original sample are required to have 1,000,000 dual positive cells for a downstream experiment. An example of how to perform these types of analytics can be seen here.

T-Cell Activation Monitoring

For immunology researchers and biotech companies alike, monitoring the T-cell activation process for immunotherapies is extremely vital for the therapy's success. Using both channels, active T cells can be identified using both a general T-Cell marker in CD4 and an activation marker like CD25 (**Fig. 6**):

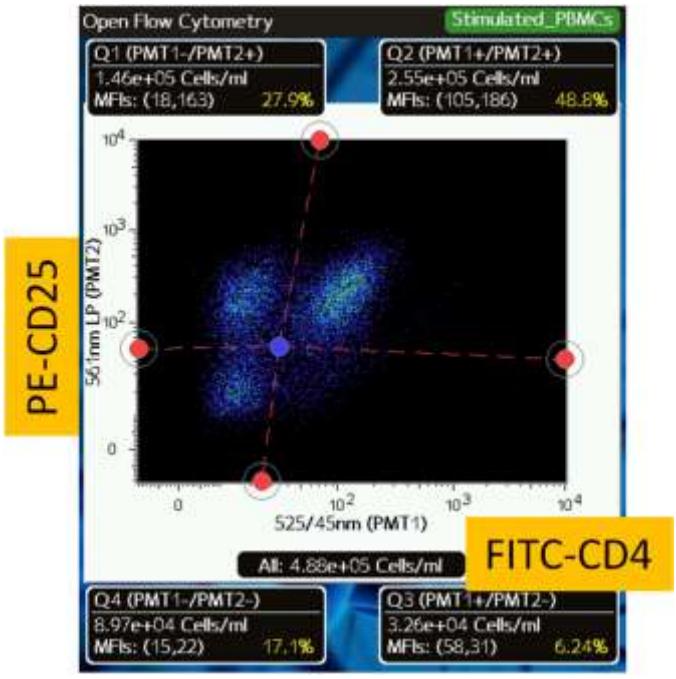


Fig. 6: Identification of active T cells using dual-color fluorescent profiling. Taken on a Moxi Go II using "Go FLOW" mode

Known Antibody List

While the majority of users have utilized the Moxi GO II for immune cell identification, there are many avenues for immunoprofiling that can be taken. Below is a list of all antibodies that have been successfully been used by users on the Moxi GO II:

Marker	Fluorophore
CD3	PE
	FITC
CD4	PE
	FITC
CD7	PE
CD8	PE
	FITC
CD11b	PE
CD19	PE
CD34	PE
CD45	PE
CD90	PE
CD105	PE
CD106	PE

This list is not comprehensive and is ever-increasing as more users try new and different antibodies on our instruments. If your favorite antibody is not present on this list, we encourage you to try it out and let us know the results!

Conclusion

For any type of work involving immunoprofiling cells, the Moxi GO II can give a bench top alternative to the flow cytometer. While not as robust in terms of the number of channels available at once or in terms of the overall number of cells counted, it is a perfect fast and easy check of your sample without the need to spend large amounts of time and money going to the flow cytometer. With the two main channels predominantly used for immunoprofiling available with the Moxi Go II, all of your favorite antibodies already being used in the lab can be simply and effectively applied without the need for re-testing and optimization.

While two-color immunophenotyping was covered here, single color options are also of course an option. If single-color immunophenotyping is done using the green channel, that leaves open the orange/red channel for additional viability measurements using a stain such as propidium iodide (PI) at the same time on the same test. While green-channel compensation will still be needed to ensure proper identification of dead cells using PI, this gives the opportunity to save time and resources by doubling up on experimental tests and getting the results all in a single run.

The number of options the Moxi GO II provides is immense, and it is highly encouraged to experiment with what possibilities exist. If you find a new and exciting way to use the instrument, please let us know!