

Monitoring Cell Viability and Other Critical Cell Attributes with Orflo Technologies' Moxi Flow System

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Monitoring cell viability can provide critical insights into the effectiveness of biological protocols and the stability of biological systems including: the efficacy of cell harvesting procedures, the toxicity of pharmacological agents (intended or incidental), the quality of cell culture conditions and protocols, and the effect of environmental insults. Recognising diminished viabilities can help preclude unnecessary investment of significant labour, time and costly reagents in downstream processing of unsustainable samples. Furthermore, visibility into the viability state of a culture empowers researchers to optimise protocols and reagent selection for maximal cell yields or, alternatively, effective killing of cells.

The definitions of cellular viability are diverse and are distinguished by the key cellular vitality attributes monitored, such as cell metabolic activity (e.g. esterase enzyme function), apoptotic markers (Annexin V), cellular reduction potential, plasma membrane potential, proliferation rate (e.g. DNA quantification techniques), mitochondrial function and plasma membrane integrity^{1,5}. However, despite the diversity in detection parameters, plasma membrane integrity, and the corresponding dye-exclusion assay, has been widely regarded as the benchmark in general cellular viability analysis. Foremost of the dye-exclusion assays is the traditional Trypan-Blue staining approach used in conjunction with manual hemocytometer counting methods. While this technique has served as a staple of virtually every biology lab, it is prone to large variability in both count and viability measures. Beyond the inherent errors that result from hemocytometer loading, statistical sampling size errors and subjective user interpretation, the viability estimation has also been shown to be highly dependent on focal plane selection² and particularly

inaccurate and prone to overestimation in samples with lower viability numbers.^{3,4} In order to address the deficiencies of the Trypan-Blue staining approach, a number of fluorometric dye-exclusions assays have been developed. These assays are based on a 100-fold or greater increase in fluorescence of membrane-impermeable fluorophores upon intercalation with DNA, thereby providing a significant distinction as compared to the slight shading differences associated with Trypan Blue. Among these fluorophores, Propidium Iodide (PI) is widely recognised as the de facto standard for both its performance and ease of use.^{3,5}

A major drawback with the use of PI and other fluorescent-based stains is that they have required the use of fluorescent imaging microscopes or flow cytometry systems. The former approach requires expensive microscopes that are equipped with fluorescent excitation sources and filter sets. And, because microscopic examination is still manual, it is time-consuming and prone to the same subjectivity and variability associated with Trypan-Blue counting



Figure 1 – Orflo Technologies Moxi Flow System and dual-use flow cassette (inset). The Moxi Flow is the World's first flow cytometer to combine the Coulter Principle for precise particle sizing and cell counts with a (single-channel) fluorescence (PMT) measurement for "Assays on Demand", including highly accurate PI-based viability, Annexin V-PE apoptosis, immunolabeling, and RFP transfection efficiency measurements.

and staining. The second method, flow cytometry, while extremely accurate, has historically required costly systems, highly-trained operators, and significant time and costs associated with running an assay (e.g., instrument warm-up times, sample-to-sample PMT and scatter adjustments, clean and shutdown procedures, etc.).

The new Orflo Technologies (**Figure 1**) Moxi Flow system addresses these deficiencies by providing highly-accurate PI-based viability flow measurements in a portable flow system that is under \$10,000, requires no warm-up time, never requires cleaning or maintenance, performs tests in under 15 seconds, and requires minimal to no training. Specifically the Moxi Flow is the World's first flow cytometer to combine the Coulter Principle for precise particle sizing and cell counts with a (single-channel)

fluorescent (PMT) measurement for truly affordable flow cytometer "Assays on Demand", including highly accurate PI-based viability measurements. What makes the Moxi Flow so versatile is its ability to produce simultaneous cell volume and counts with a single fluorescent output. This in-turn enables other applications such as apoptosis quantification, monitoring of transfection efficiency, and immunolabeling. In this technical note, we examine the performance of the Moxi Flow system as compared to other available viability estimation systems and provide examples of the extended capabilities of the system.

Methods

Cell Killing

Populations of Dead Cells were created through either heat-induced necrosis (60°C for 30 minutes), through overnight incubation in 500mM hydrogen peroxide (H₂O₂, Sigma Aldrich) containing media, or through media starvation/cell overpopulation (no feeding or passaging of cells for > 1 week). In each case, a control sample was split and maintained separately from the killed cell population under standard culture conditions (37°C, 5% CO₂).

Apoptosis

Apoptosis was induced through addition of 50uM Camptothecin (Tocris) and allowed to incubate for several (4-5) hours in a CO₂ (5%, 37°C) incubator. Cells were labelled with Annexin V-PE (BioLegend) following the manufacturers protocol.

Viability Titration Assays

For generation of controlled viability percentage samples, the killed populations of cells were mixed ratiometrically with the control ("healthy") population of cells to create healthy cell ratios of 1.0, 0.85, 0.7, 0.55, 0.4, 0.25, 0.1, and 0.0. Identical samples were then measured in parallel on the Moxi Flow system (Orflo Technologies), a Guava PCA Flow Cytometer (Merck Millipore), a Trypan-blue based imaging system (TC-10, BioRad), and on a Neubauer

Hemocytometer. Three separate measurements were made for each sample on each system with the exception of the hemocytometer in which eight, 1 mm² squares were counted per sample. Composite data curves were generated offline using custom-coded IGOR Pro (Wavemetrics) software. Linear regression fits were generated using IGOR's internal curve-fit capabilities. Error bars represent one standard deviation (n=3) and Pearson's r was used as the measure of fit quality. Initial viability values of control and killed samples were determined as the average viability output of the Moxi Flow and Guava PCA systems. These values were then used, in conjunction with the corresponding Moxi Z average counts and the mix ratio for each sample, to generate the calculated "Theoretical Viability" levels.

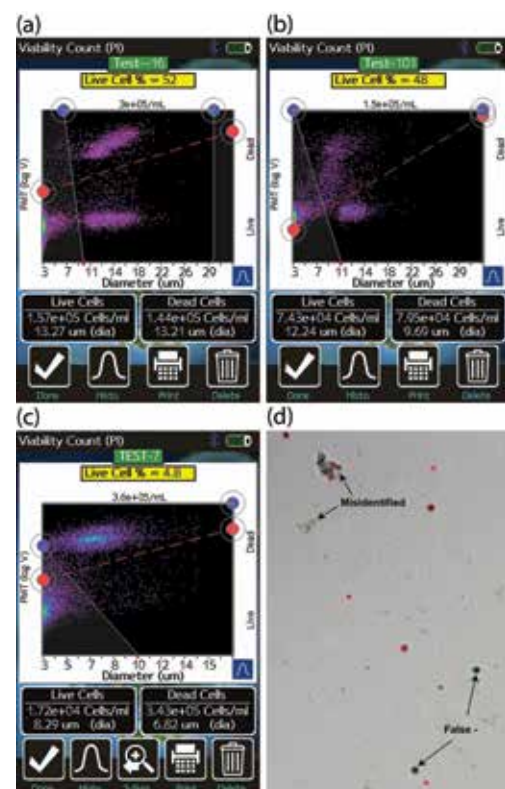


Figure 2 – Example Moxi Flow viability scatter plots for (a) heat-killed Jurkat E6-1 (b) Peroxide killed CHO-K1 cells and (c) Starved 3T3 Cells. (d) For comparison, an image of the 3T3 sample from the Trypan-Blue based imaging system is included to highlight the ambiguity in identification of dead cells (note the two false negatives on the bottom). In contrast, the PI-staining provides over two orders of magnitude in separation of live and dead signals.

Moxi Flow Cell Counts and Viability

Moxi Flow cell samples were labelled with 5µg/ml PI (Life Technologies) and allowed to incubate for a minimum of 5 minutes. Samples were tested within 20 minutes of staining. All analysis was performed on unit.

Guava PCA Counts and Viability

Guava PCA samples were prepared using the Viacount reagent (Merck Millipore) and published protocol. Briefly, 50µL of the cell sample was mixed with 450µL of Viacount reagent. The samples were allowed to incubate for five minutes and were processed within 20 minutes of staining. Analysis of the data was performed offline using the FlowJo Software Package (Treestar)

Imaging System and Hemocytometer Counts and Viability

Trypan blue samples were stained with 4% Trypan Blue solution (Life technologies) mixed at a 50:50 ratio with the cell sample. The sample was mixed 10x with pipette trituration and run immediately on the imaging system per the manufacturer's protocol. Hemocytometer counts were performed ~5 minutes following mixing. Two hemocytometer counts were performed with each hemocytometer "count" defined as of the total counts from the four corner, 1mm² squares of each of the chambers (8 total squares counted per sample).

PBMC Isolation and CD4 Labelling

Whole blood was collected from a healthy donor into heparin coated collection tubes. Human Peripheral Blood Mononuclear Cells (PBMCs) were isolated, within 1-2 hours of collection, using Ficoll-Paque Plus (GE Healthcare) following the general protocol outlined in the product instructions.⁶ CD4 receptors were labeled using a primary mouse anti-human CD4 antibody (BioLegend 300501) and PE-conjugated goat anti-mouse IgG antibody (BioLegend 405307) following the protocol posted online.⁷

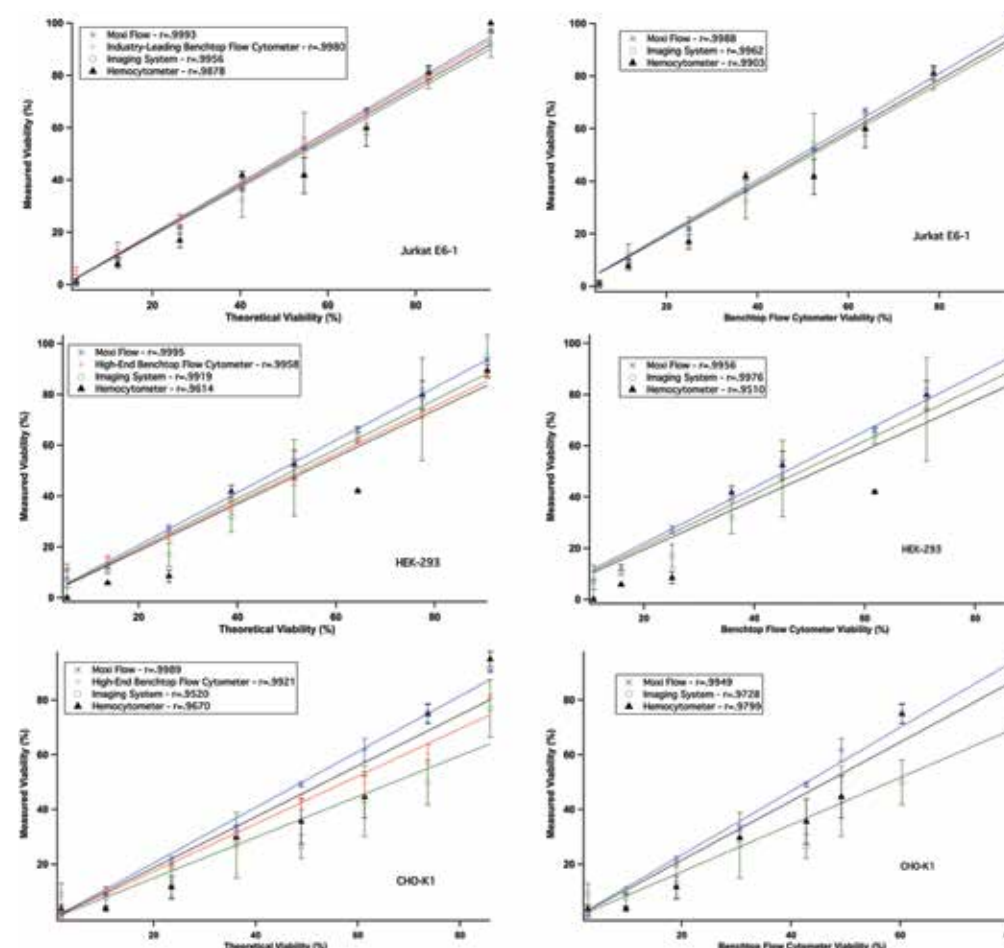


Figure 3 – (Left Column) Comparisons of Moxi Flow, Benchtop Flow Cytometer, Imaging System and Hemocytometer viabilities to the expected (calculated Theoretical) viabilities for three cell lines (Jurkat E6-1, HEK-293, and CHO-K1). The Moxi Flow consistently showed the greatest linearity. (Right column) Comparisons of the Moxi Flow, Imaging System and Hemocytometer to the Benchtop Flow Cytometer. The Moxi Flow significantly outperformed the Trypan-Blue based systems based on linear fit and variation (standard deviation) of measurements.

Results and Discussion

The Moxi Flow system provides two channels of information with respect to cell samples: 1.) Precise, quantitative volumetric sizing of particles through electric aperture-impedance (Coulter Principle) measurements and 2.) Single-channel fluorescence measurements (532nm laser, 590/40nm filter set) of cell labels or stains. **Figure 2** shows an example of the data presentation on the Moxi Flow for several different PI-stained cell samples killed through several different mechanisms (**Figure 2(a)** 60°C heat-killed Jurkat E6-1, **Figure 2(b)** H₂O₂-killed CHO-K1 cells, and **Figure 2(c)** starved/neglected 3T3 cells). The images are displayed as scatter plots with each individual point corresponding to

a PI fluorescence intensity (Y-axis, log scale) and a precise effective diameter (µm, x-axis). As can be seen from these images, despite the different morphological effects associated with the cell killing methods, the Moxi Flow is clearly able to separate out the sub-populations (clusters) of live and dead cells. Furthermore, in comparing the Moxi Flow scatter plot (**Figure 2(c)**) vs. the imaging system's trypan-blue stained image (**Figure 2(d)**), the large difference in signal separation of the two techniques becomes readily apparent. Specifically, the Moxi Flow exhibits significant (~100x, note: y-axis is log scale) separation for the dead cell cluster with respect to the baseline (see noise cluster on the bottom left of **Figure 2(c)**). In contrast, the imaging system

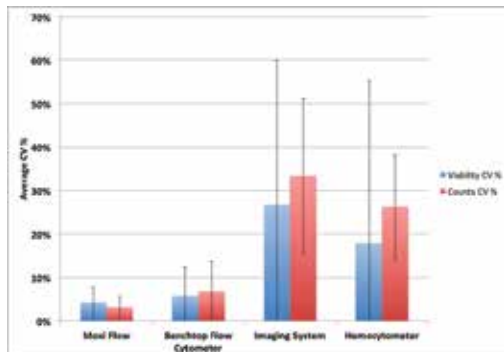


Figure 4 – Average coefficients of variation (CV) percentages for both counts and viability measurements for all systems. Error bars represent one standard deviation.

image of Trypan Blue stained cells creates challenges in interpretation of the cell shading as evidenced by the incorrect labelling of a debris/cluster (top middle of **Figure 2(d)**) and the misidentification of dead cells as live cells (**Figure 2(d)**, bottom right). Based on the measured viabilities and concentrations of the initial control and killed samples, it becomes a simple exercise to calculate the “theoretical viability” levels of ratiometrically-mixed samples. In this manner, seven theoretical viability levels were

created through control to dead cell ratios of 1.0, 0.85, 0.7, 0.55, 0.4, 0.25, 0.1, and 0.0. Data points for each sample were collected in triplicate on each of the instruments in this study and duplicate (2 wells, 8 large (1 mm²) squares) for the hemocytometer.

Figure 3 shows the resulting plots of the reported viability of each instrument vs. both the calculated, theoretical values (**Figure 3, left column**) and vs. the Guava PCA Flow System, a benchtop flow cytometer (**Figure 3, right column**). In comparing the linearity (**Figure 3, left column**, Pearson’s r values) and CV’s (**Figure 4**) for the Moxi Flow counts and viability to that of the other systems, the Moxi Flow outperforms all systems, including the high-end flow cytometer. In using the benchtop flow cytometer as a reference standard, the Moxi Flow system significantly outperformed the Trypan-Blue approaches in both linearity and variability (see **Figure 3, right column**, Pearson’s r values).

Because Moxi Flow is a true flow cytometer it can provide additional information regarding cellular

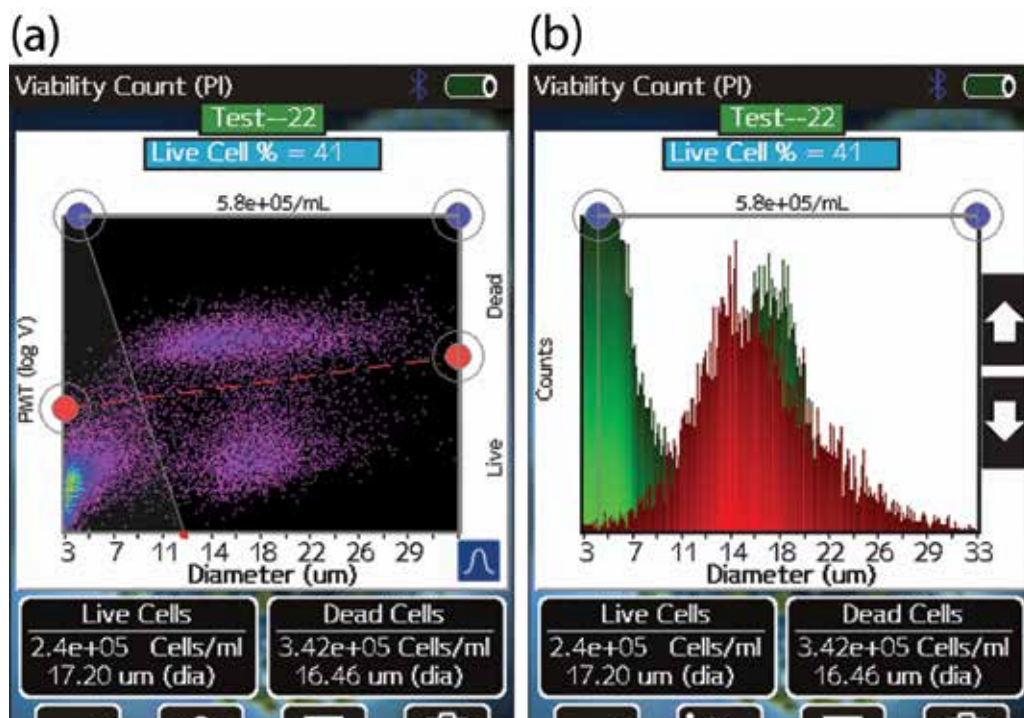


Figure 5 – Example Moxi Flow Annexin V-PE labelling of Camptothecin-treated Jurkat E6-1 cells. Both the (a) scatter plots and (b) size histogram highlight the apoptosis-induced size shift and quantitative sizing data provided by the Moxi Flow. Note that sample PI-based viability was greater than 90% (data not shown).

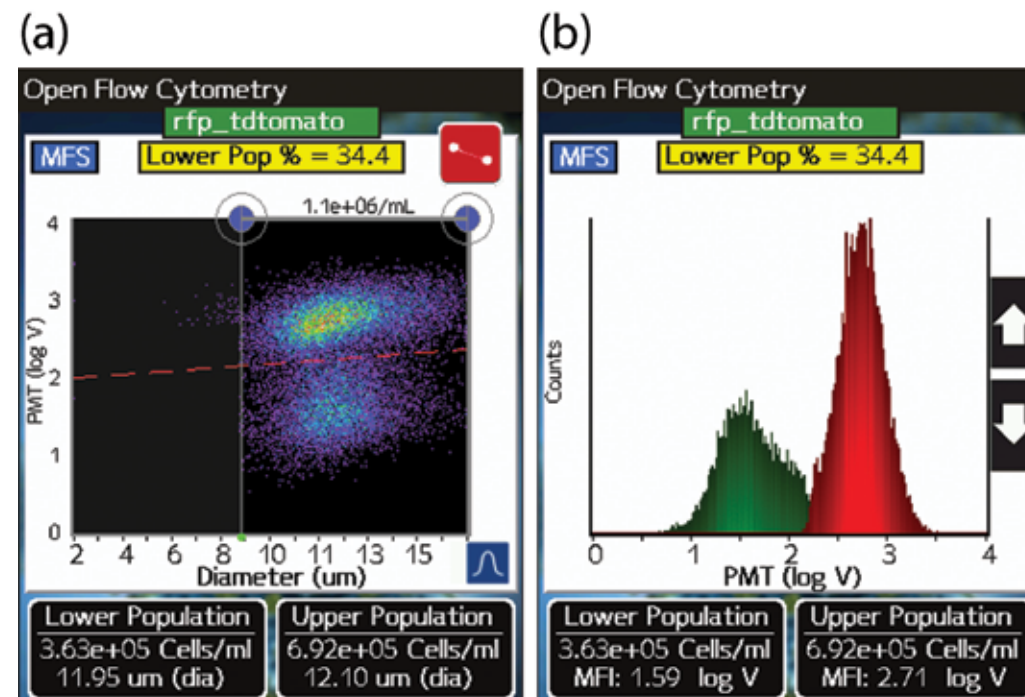


Figure 6 – Measuring RFP (TdTomato tag) transfection efficiency of a cancer cell line. In this example, the system confirmed 100% transfection with two distinct cell populations characterised by their differing levels of expression of the transfected protein.

viability (and other cell characteristics) through labelling of the cell with PE-conjugated proteins/antibodies or through transfection of cells with RFP (TdTomato) tagged proteins. **Figure 5** shows an example of Annexin V-PE labeled Jurkat cells that had been treated with 50μM Camptothecin, a known apoptosis inducer. The Moxi Flow is clearly able to resolve the Annexin V⁺ vs. Annexin V⁻ negative populations (**Figure 5 (a)**). It is notable that this particular cell population showed very high levels of viability as measured through dye-exclusion methods. This is consistent with the general knowledge of Annexin V assays in that they detect the externalisation of phosphatidylserine (PS) on the plasma membrane, an early-stage event in apoptosis. Consequently, the true “viability” of this culture would not be appropriately captured by dye-exclusion methods (e.g. Trypan Blue) but can still be identified through the Moxi Flow apoptosis assay. Furthermore, as the size histogram (**Figure 5(b)**) illustrates, the precise sizing of the particles on the Moxi Flow (via the Coulter Principle)

enables fine resolution of differences in the Annexin V⁺ vs. the Annexin V⁻ populations. This provides yet another potential insight into the health of a biological sample as has been discussed previously.⁸

Figure 6 highlights the application of the Moxi Flow to the monitoring of the transfection efficiency of a cultured cancer cell line. A commercially available red fluorescent protein (RFP, TdTomato) cloning vector was used to tag a transfected protein for subsequent fluorescent analysis on the Moxi Flow. Following transfection, a cell sample was run on the Moxi Flow using the “Open Flow Cytometry” Assay. The output, shown in **Figure 6** comprised two distinct populations of cells (divided by the red fluorescence line in the scatter plot of **Figure 6(a)** and coloured differently in the histogram of **Figure 6(b)**). As both populations of cells were clearly above the fluorescent noise (not displayed with this saved data) of the system, the Moxi Flow confirmed 100% transfection efficiency of this cell line. Furthermore, the different intensities of the clusters provided the researcher with additional

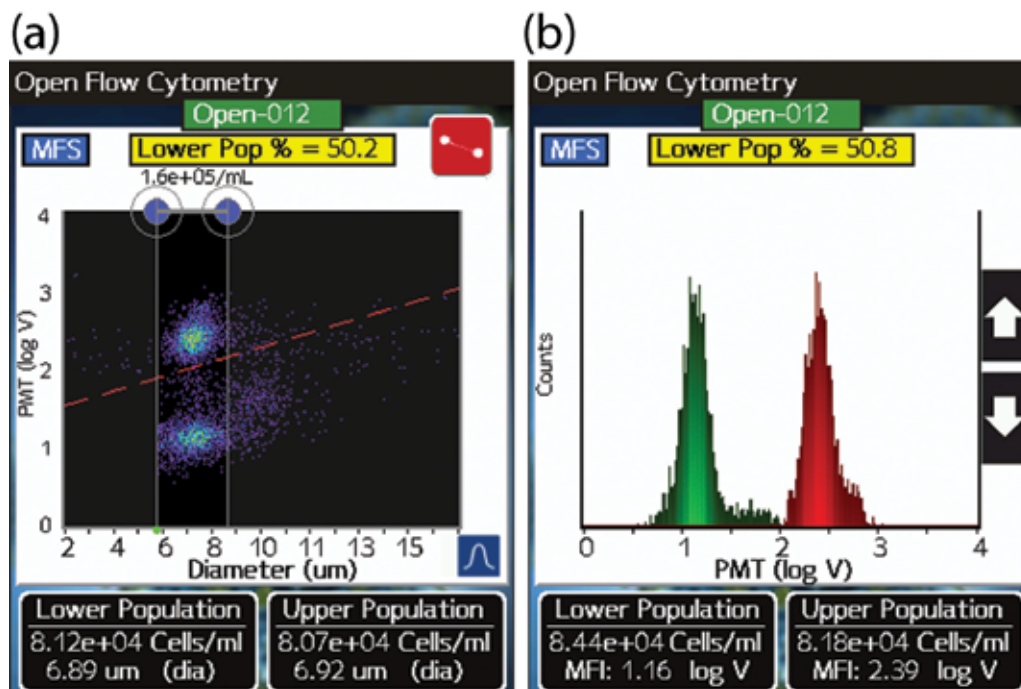


Figure 7 – Human PBMCs labelled with a secondary label for CD4 receptors. Gated lymphocyte population shows 49.8% CD4 positive cells marked by a positive fluorescence shift represented on both the (a) scatter plot and (b) fluorescence histogram.

information regarding two distinct sub-populations of cells that were characterised by their differing levels of expression of the RFP-transfected protein.

With the Moxi Flow “Open Flow Cytometry” assay, the system can additionally be leveraged to detect, and often discriminate expression levels, of innumerable proteins or other surface markers on cells. Specifically, assuming a baseline expression level of the marker and that it can be targeted with an antibody, a phycoerythrin (PE) -labelled antibody (either primary or secondary) can be used to identify the cell. One of the more ubiquitous applications of this technique is in the labelling of cluster of differentiation (CD) markers on white blood cells. **Figure 7** shows an example of a purified human PBMC population, tagged with a secondary label for the CD4 receptor. The precise sizing capabilities of the Moxi Flow allowed for the clear size-differentiation (x-axis) of the lymphocytes (**Figure 7(a)**, between the blue gate markers) from the other PMBC's (predominantly monocytes to the right of the rightmost blue marker in **Figure 7(a)**).

Then, within the gated lymphocyte population, it was easy to fluorescently distinguish and quantify the CD4+ lymphocyte population (**Figure 7(a)**, above the red dashed line and **Figure 7(b)** labelled in red on the fluorescence histogram), a key metric in HIV/AIDS monitoring.

As an overall outcome of this testing, while the benchtop flow cytometer produced results in this study that compared to those of the Moxi Flow, the Moxi Flow had two key distinguishing factors: its “Assay on Demand” capability and its ease of use. Because the Moxi Flow has implemented a dual-use disposable flow cell architecture, no samples ever touch the system itself. This eliminates a time-consuming (~10-15 minute) clean and shutdown step required by the benchtop system. Furthermore, no laser warm-up period was required for the Moxi Flow whereas the benchtop system required a 15 minute warm-up period. Finally, with a simple touch screen interface and <15second test time the actual acquisition phase proceeded significantly faster than with the benchtop flow system.

Conclusions

The Moxi Flow is the World's first flow cytometer to combine the Coulter Principle for precise particle sizing and cell counts with a (single-channel) fluorescence (PMT) measurement for “Assays on Demand”, including highly accurate PI-based viability, Annexin V-PE apoptosis, RFP (TdTomato) expression, an immunolabelling measurements. The data in this study demonstrates that the Moxi Flow system can provide significantly more accurate and comprehensive viability results than analogous imaging-based approaches. Furthermore, the performance of the Moxi Flow met or exceeded that of an industry-leading benchtop flow system. Beyond performance, the Moxi Flow distinguishes itself from traditional flow systems with its portability, low cost (\$10,000), ability to run immediately without warm-up times, never requiring cleaning or maintenance, performing tests in under 15 seconds, and requiring minimal to no training. These features should enable the Moxi Flow to become a new standard in monitoring cell viability and other cellular attributes.

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